



# Mitteilungen

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für Land- und Forstwirtschaft Berlin-Dahlem

## **Proceedings of the 1<sup>st</sup> International Symposium on Biological Control of Bacterial Plant Diseases**

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Edited by  
**Wolfgang Zeller**  
**Cornelia Ullrich**

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Cover illustration: These Proceedings are dedicated to the memory of Zoltán Klement (1926 – 2005), a pioneer in research on the Hypersensitive Response in plant-bacteria interaction. Zoltán Klement deceased a few days before this meeting he wanted to attend. This cover illustration, a natural landscape at “Cowra” near Canberra, NSW, Australia, is a water colour painting by Zoltán Klement, representing a piece of intact environment as our common world-wide aim in plant protection.

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## Preface

The necessity of biological control of bacterial plant diseases is undoubtedly reflected by the interest of more than 130 scientists from 36 different countries worldwide, who participated at the “1<sup>st</sup> International Symposium on “Biological Control of Bacterial Plant Diseases”, held from 23<sup>rd</sup> to 26<sup>th</sup> October at the Lufthansa-Centre in Seeheim/Darmstadt/Germany. The importance of this conference was highlighted by the integrating introductory overviews of the former and present Presidents of the Federal Biological Research Centre for Agriculture and Forestry (BBA), Prof. Fred Klingauf and Prof. Georg F. Backhaus, respectively, who described the past and present activities of the BBA in biological plant disease control. The aim of developing biological control methods was supported for long years of cooperation in teaching and research by scientists of the Darmstadt University of Technology, as outlined by Prof. Johann-Dietrich Wörner, President of the Technical University of Darmstadt, in his welcome address.

In the present Proceedings, manuscripts of the oral presentations and posters are combined under the key topics of the seven sessions, plus an additional chapter on biological control of phytopathogenic fungi with antagonistic bacteria. During the different sessions, there was an unanimous agreement that conventional chemical control of bacterial plant diseases is generally insufficient due to the characteristic property of bacterial infections to spread inside the plant. Although antibiotics are effective against certain bacterial diseases, their use is combined with ecological and toxicological risks and should therefore be replaced by biological methods whenever possible. Among the different bacterial diseases dealt with at the conference, special emphasis was placed on those with worldwide distribution, such as bacterial wilt of potato and tomato/*Ralstonia solanacearum*, fire blight of apple and pear trees/*Erwinia amylovora*, potato soft rot/*Erwinia carotovora* subsp., bacterial blight and canker of *Pseudomonas syringae* pathovars, bacterial blight of rice/*Xanthomonas oryzae*, crown gall of grapevine/*Agrobacterium vitis*, mushroom blotch disease/*Pseudomonas tolaasii* and various other bacterial diseases. Promising biocontrol agents such as antagonistic strains of *Pantoea agglomerans*, *Pseudomonas fluorescens* and *Bacillus subtilis* as well as non-pathogenic strains of *Agrobacterium vitis* were discussed during the four exciting days. Essential oils were reported to have high biocontrol efficacy under laboratory or in green house conditions. New and promising control agents such as the enzyme lactoperoxidase and bacteriophages and their integration in sophisticated application schemes were discussed. Further topics were enhancement of plant resistance by immunostimulants and plant growth promoting rhizobacteria (PGPR) (Chapters I, II, III, VI, VII).

The present compilation reflects the high level of both fundamental and applied research activities in the field of biocontrol of bacterial plant diseases worldwide. However, it is also obvious from the submitted contributions that three main areas and problems need more attention and support in the future:

More of basic research (Chapter I, V) should be focussed on identification of physiological, biochemical and molecular principles underlying biocontrol of bacterial plant diseases. Understanding these mechanisms will allow a more targeted development of agents with improved and reliable biocontrol activity.

The second requirement is proof of sufficient control activity also under field conditions. Although some biocontrol agents are already successfully used commercially, others still have a long way to go until they reach this stage. Increased efforts in the areas of scale-up of production, fermentation and formulation are necessary to allow more non-chemical control agents to reach the market.

A high hurdle to overcome is the legal authorisation of biological plant protection products by national and international regulatory authorities (Chapter VI). For many biocontrol agents, concepts and methods for risk analysis accepted by scientists and authorities have not been developed. Scientists working on new biological plant protection products should critically check if the agents they are developing will be acceptable by the regulatory authorities as well as by the consumer (Chapter VIII).

We are grateful to our colleagues Jeffrey B. Jones and Timur M. Momol from the University of Florida, Gainesville, who agreed to host the Second International Symposium on Biological Control of Bacterial Plant Diseases in 2008 at their institute in the U.S.A. We hope, substantial progress on the above three topics will be made by then.

We thank all participants for joining this symposium, for excellent contributions and discussions, including the joyful performances during the excursion across the colourful autumn scenery of the river Rhine area to the nearby State Research Institute of Viticulture and Horticulture in Geisenheim.

Thanks are due to the many helpers behind the stage, especially to Klaus Rudolph for initial scientific support, to Anandhakumar Jayamani for organising the Power Point presentations, to Mascha Wembacher and Gisela Zeller for their untiring struggling to prevent chaos at the desk, as well as to Wolfram Ullrich and Sandra Wright for critically reading all manuscripts; for additional corrections we also thank Tom Burr, Walter Gassmann, Jeff Jones, Klaus Naumann, Virginia Stockwell, Sherman Thomson, and Matthias Ullrich.

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These Proceedings are dedicated to the memory of Professor Zoltán Klement (1926 – 2005), a close friend and teacher to many of us and a pioneer in research on the Hypersensitive Response in plant-bacteria interactions. Zoltán Klement deceased a few days before our meeting he wanted to attend. His interesting and splendid lecture was presented by his co-worker Peter Ott. A reflection of his interests may be transmitted through the contribution of his group in these Proceedings. The cover illustration “Cowra”, kindly selected by his wife Iringo Klement out of his many paintings, will keep Zoltán Klement in warm memory of all participants.

Darmstadt, May 2006

Prof. Dr. Wolfgang Zeller,

Prof. Dr. Cornelia Ullrich

Dr. Eckhard Koch,

Dr. Jürg Huber

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**Backhaus, G.F.**

President of the Federal Biological Research Centre for Agriculture and Forestry (BBA)

## Welcome Address

Dear Ladies and Gentlemen,

I have the honour and pleasure of welcoming you to the 1st International Symposium on Biological Control of Bacterial Plant Diseases here in Darmstadt on behalf of the organizers of this symposium, which are:

- the Institute for Biological Control of the Federal Biological Research Centre for Agriculture and Forestry, Prof. Huber, Prof. Zeller and Dr. Koch.
- and the Technical University of Darmstadt, Prof. Ullrich and Prof. Scheu.

I am pleased to welcome so many colleagues from all over the world to this symposium. Prof. Zeller told me that we have participants from more than 35 countries. We appreciate your participation very much and take it as a sign of world-wide scientific interest in biological control and in particular in the aspects of biological control of bacterial plant diseases.

Tomorrow the President of Darmstadt University of Technology, Prof. Dr. Wörner, will speak to you and will introduce to you the Technical University of Darmstadt. Today it's my pleasure.

Please allow me at first a few words on the Federal Biological Research Centre for Agriculture and Forestry, short form: BBA, in particular for those, who were not able to follow Prof. Klingauf's speech last night.

The BBA is an independent superior Federal authority and a governmental research institution, subordinated to the German Ministry of Consumer Protection, Food and Agriculture. Its tasks are stipulated in the German Plant Protection Act and the Gene Technology Act. The major task of the BBA is to advise the German Federal Government and the Federal Ministry on issues of plant protection, plant health and biological safety.

The BBA's administrative tasks are closely related to research activities, which provide for the necessary up-to-date scientific and technological know-how in taking decisions. The BBA's research is aimed at sustainable, environmentally acceptable and safe protection of plants and plant products. Thus, BBA contributes to the conservation of cultivated landscapes both urban and rural, and therefore to the people's quality of life.

Most institutes of the BBA are located in Braunschweig and Berlin. The Institute for Biological Control here in Darmstadt was founded in 1948, at first as "Institute for Research and Control of the Colorado Potato Beetle". One year later, in 1949, the institute was integrated into the BBA. Nowadays the institute deals with all basic and practical aspects of biological control. Research is performed on the use of natural antagonists and their products for an environmentally friendly control of arthropod pests and of diseases in agriculture and forestry. This includes the use of parasitic and predatory arthropods and nematodes as well as micro-organisms and viruses and, of course, the substances of micro-organisms and plants.

Bacterial diseases of plants are extraordinarily difficult to manage and to control. In addition to visible disease symptoms, bacteria may become systemic in the plant vascular tissue making it impossible to eradicate the pathogen by pruning out symptomatic tissues or by applying chemicals to the plant surface. Moreover, bacteria undergo exponential growth and so bacterial diseases are explosive: by the time symptoms are recognized, the pathogen is often entrenched and well on its way in destroying the crop or is even spread by wind, rain and vectors already.

Managing bacterial diseases mostly depends on:

- host resistance (which in many cases may not be available in desirable crop varieties),
- sanitation (*e.g.*, preventing the introduction of pathogens and removing diseased plants),
- and cultural practices (*e.g.*, avoiding overhead irrigation and limiting nitrogen fertilization).

In some cases, chemical bactericides and, unfortunately only in very few cases, biological control agents are effectively integrated into the disease management program. The situation for bactericides is very difficult. Antibiotics show good control effects, however, a regular authorization for antibiotics is very unlikely in the EU because of the risks of development of cross-resistance in human pathogens. In the field of biological control of bacterial diseases the lack of effective chemicals has stimulated research.

Fire blight and the replacement of the antibiotic streptomycin, which is not allowed in most European countries, is only one example for intensive research to find alternative methods for controlling the disease. Biological control focuses primarily on suppression of the epiphytic growth phase of the pathogen on blossoms prior to infection and endophytic growth. But also growing fire blight-resistant pome fruit varieties may be regarded as an indirect measure to control fire blight, as it reduces the inoculum potential of the pathogen and thus prevents the infestation from spreading on endangered farms. The Institute for Biological Control has investigated infection of apple and pear varieties under laboratory and natural conditions, which led to preliminary suggestions on the resistance of fruit varieties against fire blight.

However, fire blight is only one dominant example. There are several other bacterial diseases in field and horticultural crops for which we urgently need biocontrol methods. For this reason we meet here to present and discuss those methods and give a state of the art in relevant research.

I would like to thank you for attending this event and presenting results. I wish you all a successful symposium, interesting discussions and a pleasant stay.

**Klingauf, F.**

Former President of the Federal Biological Research Centre for Agriculture and Forestry (BBA)

## **General Status of Biological Control - Opening and Introductory Lecture**

### **Abstract**

First, the institutions of the organizers of the First International Symposium on Biological Control of Bacterial Plant Diseases are introduced: the Department of Biology of the Darmstadt University of Technology and the Institute for Biological Control of the Federal Biological Research Centre for Agriculture and Forestry.

The first commercial development of a biocontrol measure against plant pathogenic bacteria started in the 1980s with strain K-84 of *Agrobacterium radiobacter* against crown gall of stone fruits in Australia. Despite numerous reports on the successful experimental use of further micro-organisms and other biocontrol measures, the progress in practical utilization has been very slow. In order to encourage the search for biological measures of bacterial plant pathogens the aim of the symposium is to foster an intensive exchange of ideas, results and techniques and subsequently help to overcome hurdles in their implementation.

For a long time biological control was only perceived as method to control certain singular pest insects. In the last decades the perception of this plant protection measure has changed significantly: not only pest insects but all plant pests including pathogens and abiotic factors causing plant diseases are subject of biocontrol. Special attention has been paid to biocontrol in legislation, too. As example the legal definitions of “good professional practice” and of “integrated plant protection” are outlined. Out of the spectrum of biological control methods the (periodical) application of antagonistic organisms and of natural substances inducing crop resistance offers a good alternative for control of plant pathogenic bacteria. Whereas resistance- or tolerance-inducing substances are only listed in Germany, authorization of plant protection products with micro-organisms including viruses as active substances is harmonized in the EU and laid down by Directive 91/414/EEC. For the authorization of plant protection products in the EU a detailed data package has to be submitted to the authorities. This concerns in particular biological products which usually have only a small market and perhaps cannot cover the costs for research and development of the data sets required.

### **Welcome and introduction to the institutions of the organizers of the symposium**

The First International Symposium on Biological Control of Bacterial Plant Diseases is organized by the Institute for Biological Control of the Federal Biological Research Centre for Agriculture and Forestry and the Department of Biology of the Darmstadt University of Technology. In the name of the Organizing Committee I am pleased to welcome you. Please, allow me at first to introduce to you the organizing institutions.

### **Darmstadt University of Technology**

The first predecessor of the Technical University of Darmstadt was the Higher Trade School, founded in 1836, followed by the Technical School in 1864 and the Grand Ducal Hessian Polytechnic in 1868. Heated discussions on such a poor state of higher education in the Grand Duchy of Hessen led to the foundation of the Technical High School (Technische Hochschule = TH) in the status of a university so that advanced school-leaving certificates became the basis for admission. In 1899 the TH Darmstadt was granted the right to award doctorates. The continually low number of students could be increased by the forward-looking decision to set up a Chair of Electrical Engineering, which was a novelty in academia. This paved the way for the young university to take up a leading position in Electrical Engineering, and later on in other fields of technical education. As early as 1913 a Chair of Aeronautics and Flight Mechanics was set up. A further important step forward was taken in 1924, when the General Faculty, combining until that time all the non-technical subjects, was divided into Faculties of Mathematics and

Natural Sciences, Cultural Studies and Political Science. The post-war II period was characterized by reconstruction and extension, by means of which the continually rising number of students could be integrated. Since no land was available in the city, locations at the eastern border of Darmstadt were used for new buildings, where also the Department of Biology is situated. Since the TH for more than hundred years has not only offered a wide range of subjects, but also has had the legal status of a university, the TH Darmstadt was renamed Darmstadt University of Technology (TUD) in 1997.

One of the oldest institutions of the TUD is the Botanical Garden, founded in 1814, which was re-established in 1874 at the eastern side of Darmstadt, where now also the institutes of the Biological Department are located. The Botanical Garden gave rise to the foundation of the Institute of Botany in the early seventies of the nineteenth century, followed by the foundation of additional biological institutes, at last the Institute of Microbiology in 1967. Today the Department of Biology includes the Institutes of Botany, Zoology and the Institute of Microbiology and Genetics. Today the TUD counts about 18.000 students of all disciplines, including more than 500 students in the Department of Biology ([www.tu-darmstadt.de](http://www.tu-darmstadt.de)).

### **Federal Biological Research Centre for Agriculture and Forestry (BBA)**

The Federal Biological Research Centre for Agriculture and Forestry (BBA) is an independent superior Federal authority and research institution in the operational sector of the Federal Ministry of Consumer Protection, Food and Agriculture (BMVEL). Its tasks are defined by the Plant Protection Act, the Gene Technology Act and corresponding legal regulations. It has as its main task to advise the German Federal Government and the Federal Ministry concerning issues of plant protection, plant health and biological safety. The BBA is one of the authorities responsible for the examination and authorisation of plant protection products, and is commissioned with the evaluation and registration of plant protection equipment. For the field of plant quarantine, the Department for Plant Health takes the function of an information and coordination centre for Germany according to the Directive 2000/29/EC, and it represents the Federal Republic of Germany for factual matters on issues of plant health in bodies of the European Union, the European Plant Protection Organisation (EPPO) and the International Plant Protection Convention (IPPC). As Research Centre the BBA investigates the entire field of plant protection and lays the scientific foundations for the tasks legally assigned to the BBA and for the decision making progresses particularly in the fields of agriculture and forestry, consumer protection, and environmental protection. Its tasks are to protect plants and plant products against parasitic and non-parasitic risk factors and restrictions and to avert dangers that may arise from plant protection procedures to the health of human beings, animals and the ecosystem. This includes also research on biological safety of genetically modified organisms.

The BBA dates back to 1898, when the German Reichstag decided to establish a national plant protection authority. At first, a Biological Department for Agriculture and Forestry at the Imperial Health Office was set up. In 1905 the Department became independent and moved into the newly constructed buildings in Berlin-Dahlem. Today the BBA is domiciled in Berlin and Braunschweig, operating another six institutes and outside branches, among them the Institute for Biological Control in Darmstadt (BBA 2003).

The Institute for Biological Control was founded in 1948, at first as "Institute for Research and Control of the Colorado Potato Beetle". This beetle was advertently introduced into Germany before Second World War. One year later, the institute was integrated into the Biological Research Centre. In 1953, the institute was renamed the "Institute for Colorado Beetle Research and Biological Control of Pest Insects". In 1955, the institute was renamed again, this time into "Institute for Biological Control of Pest Insects" and due to the extension of research on biological control of plant diseases, the institute was finally renamed: "Institute for Biological Control". These changes of the institution's name also reflected to some extent the development of biocontrol from efforts against certain, for the most part newly introduced pests that were difficult to control to a broadening of research on insect pests and finally on plant pathogens, too. Regarding the history of biocontrol it is interesting to note that already the first description of tasks of the Biological Department of Agriculture and Forestry of the Imperial Health Office in 1898 already emphasized the necessity of research on useful organisms of the plant and animal kingdom for use in pest control as well as for fertilization of crops. The number of established posts and jobs of the Institute for Biological Control amounts to about 35, which includes seven scientists in

permanent positions. Research is carried out in the entire field of biological control. A large portion of the institute's work deals with the development of plant protection procedures for organic farming (Zimmermann 1998).

### **Good professional practice and integrated plant protection**

For a long time, biocontrol was only perceived as method to control particular insect pests. In the last decades, the perception of this plant protection measure has changed significantly: not only pest insects but all plant pests including pathogens and abiotic factors causing plant diseases are subjects of biocontrol. Special attention has been paid to biocontrol in legislation too. According to the amended German Plant Protection Act on 1 July 1998, plant protection as a whole must be carried out by good professional practice (§ 2a), and plant protection products in particular must be applied by good professional practice as well (§ 6). Good professional practice includes considering the principles of integrated plant protection and of ground water protection. The principles of good professional practice were formulated by the Federal Ministries involved and were first published in the Federal Gazette of 21 November 1998 (BAnz Nr. 220). Good professional practice is a basic strategy in plant protection. It includes measures which are recommended by official extension services and have a sound scientific foundation. It comprises preventive cultural and other non-chemical measures of plant protection including biological control strategies (Burth and Freier 1999).

The concept of integrated plant protection dates back to the 1950s and was a first critical response to the plant protection measures practiced at that time. From the beginning of the fifties, an increasing number of publications pointed out the effects of plant protection products on biocoenosis (Klingauf and Burth 1995). The aim of integrated plant protection is to reduce damage while making the most possible use of non-chemical methods. In this respect the definition used in the European Union, which is laid down in the Council Directive 91/414/EEC adopted on 15 July 1991, defines integrated plant protection as follows: "Integrated Control: the rational application of a combination of biological, biotechnical, chemical, cultural or plant-breeding measures whereby the use of chemical plant protection products is limited to the strict minimum necessary to maintain the pest population at levels below those causing economically unacceptable damage or loss". The terms of integrated plant protection and good plant protection practice should not be considered as synonyms. The good plant protection practice is meant to effect an economical, safe and efficient use of plant protection products, in particular by adherence to special guidelines. The concept of integrated plant protection includes good practice of plant protection, but goes clearly beyond it by consciously using natural control factors and mechanisms of biological self-regulation.

More than fifty years after defining this term, the concept of integrated plant protection is given high priority, but it is still putting high demands on science, extension services and the agricultural practice. We notice a development which is leading from separate strategies in the framework of integrated pest control to complex approaches on the farm level, and thus to integrated crop production. We also notice a growing need for practicable strategies which can be handled by the farmers and which are suited to ease the natural balance. It is obvious that such strategies can only gradually be achieved, depending on the progress of knowledge and agrarian politics.

## Definition of biological plant protection

What can biological plant protection contribute to non-chemical measures in plant protection? Many pest problems have resulted from the import of insects from one region to another where natural enemies were not present. Not all of these insects are serious pests in their native habitat, presumably because they are controlled by natural biotic factors. The classical approach to biological control is the search for and introduction of exotic biotic control agents. This classical method of biocontrol has been practiced since more than 100 years. The introduction of the Australian ladybird, *Rodolia cardinalis* Mulsant, into California to control the cottony cushion scale, *Icerya purchasi* Maskell, on citrus in 1888/1889 is generally considered to mark the beginning of the practice of an effective control strategy using natural enemies. This classical approach was supplemented by various other methods of biocontrol. "Biological plant protection includes utilisation of natural enemies and also other methods in which living organisms or their products are used to combat damaging organisms. As natural methods, they should be highlighted as the fundamental methods of control that, with few exceptions, are environmentally safe and, especially when self-perpetuating, can be highly cost effective" (WAY 1986).

Methods of biological plant protection are (after WAY 1986; KLINGAUF 1990):

- Conservation and promotion of beneficials,
- Biological control by self-perpetuating natural enemies,
- Classical introduction of natural enemies,
- Use of indigenous natural enemies,
- Biological control by periodically applied natural enemies,
- Viruses, bacteria, fungi, nematodes, arthropods,
- Autocidal methods, self-perpetuating or non self-perpetuating,
- Behavioural methods,
- Host plant resistance including induced resistance,
- Methods of biological control with regard to plant pathogens,
- Conservation and promotion of beneficials.

The authorisation procedure of plant protection products in the EU includes the examination of possible side effects of the product on beneficials. This should stimulate the preferential usage of selective plant protection products, wherever possible. In the development of adequate testing methods the BBA Institute of Darmstadt played an outstanding role (Dr. S.A. Hassan and a corresponding working group of the OILB). Also the conservation or planting of hedges and stripes of wild plants can contribute to promote beneficials.

## Self-perpetuating natural enemies

The use of intentionally introduced exotic natural enemies was already mentioned as the classical method in biocontrol. According to Pimentel (1986) this approach resulted in a success rate of only 14% (including partial controls) or only 6% for complete controls. One important reason for this lack of success is that also the genetic feedback balance that evolved between parasite and host in their natural habitat was transferred to the new ecosystem. For further details it is referred to the publications of Hokkanen and Pimentel (1984).

## Periodically applied natural enemies

With respect to the subject of our symposium the periodical application of antagonistic organisms offers a good choice for control of plant pathogenic bacteria. In the invitation to the symposium it is pointed out that the first successful commercial development of biocontrol against plant pathogens started in the 1980s with strain K-84 of *Agrobacterium radiobacter* against crown gall of stone fruit in Australia. In

Europe the use of the antagonistic strain was less successful. Germany's first biological fungicide was registered in 1997. The product, Contans<sup>R</sup> WG, contains active spores of the soil fungus *Coniothyrium minitans* and is a water soluble granule for the control of *Sclerotinia spp.* in the soil. Since many years various arthropod beneficials became an important factor in integrated plant protection: the predatory mite *Phytoseiulus persimilis* against spider mites, the parasitoid *Encarsia formosa* against white flies, the egg parasitoid *Trichogramma evanescens* against corn borer and many others (Bathon 1999). Also some microorganisms are successfully used in the control of insect pests, such as *Bacillus thuringiensis* for control of some lepidopterous pests and the Colorado potato beetle (*B.t. tenebrionis*) as well as granulosis virus against codling moth. With some exceptions (i.e. *Trichogramma spp.*) the use of beneficial arthropods is mostly restricted to protected crops (e.g. glass houses etc.) for several reasons: an often higher infestation level, resistance against plant protection products, higher risk for employees, controlled climate favourable for most of the beneficials, and the higher value of the crops which allow the application of more intensive methods.

The sugar beet cyst nematode, *Heterodera schachtii*, is a good example for an integrated crop protection system which is successful and well accepted in practice. Among other measures in Germany, growing of resistant green manure crops in sugar beet/cereal rotations has produced promising results. Attack by cyst nematodes is of no economic importance in these manure crops. Resistance against the nematode was found in wild forms of oil radish, *Raphanus sativus*, and white mustard, *Sinapis alba*. These plants produce root exudates exerting a hatching stimulus for encysted juveniles of the sugar beet cyst nematodes. Nematodes hatch from the eggs and penetrate into plant roots. Whereas in susceptible plants a nutritional cell develops and serves as feeding site, in resistant plants the nutritional cell is induced, but breaks down after several days. As a consequence, only male juveniles develop and the nematode population decreases (Müller 1995).

#### **Autocidal and behavioural methods**

Autocidal methods and behavioural methods are used only against arthropod pests and will not be further discussed here. The autocidal sterile male technique was in some cases successfully applied against more or less isolated pest populations. In contrast, behavioural chemicals such as pheromones are successfully used for mating disruption in grape berry moth and some other pests.

#### **Host plant resistance and induced resistance**

Plant resistance and induced plant resistance are of great interest for biological control of bacterial pathogens of crops. Plant breeding strategies are increasingly successful in the control of crop pathogens and some insect pests. An often used method is the application of plant substances as source of natural plant protection products, which act in self-defence strategies of plants. Plant extracts act either directly on the pest or pathogen or indirectly by strengthening the host plant. This method could be referred as biological, too, if host plant resistance or the use of toxins from *Bacillus thuringiensis* (without living cells) is included in the definition. The application of natural substances in behavioural methods, such as moulting hormones and other growth disruptions, and for inducing resistance in crops or as plant protection product could be also regarded as biotechnical control rather than biological control *sensu strictu* (KRIEG and FRANZ 1989). Plant extracts acting directly can be used against harmful insects and mites, nematodes and viral, bacterial and fungal plant pathogens. One of the most important fungicide group in cereals was modelled on a substance of the fungus *Strobilurus tenacellus*. Other examples are Neem products from the Neem tree as insecticides or miticides and pyrethrum insecticides. Many research groups are working on the indirect effect, called induced resistance. Cucumbers, begonias and other plants can be protected against powdery mildew by prophylactic treatment with an extract of giant knotweed. A resistance inducing product based on the knotweed extract has been put on the market. In the Institute for Biological Control at Darmstadt research is carried out with plant extracts against plant pathogenic bacteria especially in fire-blight control.

#### **Authorization of biological plant protection products**

Resistance- or tolerance-inducing substances are only listed in Germany that means they have not to overcome the hurdles of a regular authorisation procedure. 191 such substances were listed, among them 30 of inorganic nature, 29 homoeopathics, 10 waxes, 9 algal preparations, and 13 microbials. In most

European states no listing or authorisation of parasitoids and predators is necessary. The authorisation of plant protection products in the EU is laid down by Directive 91/414/EEC (<http://europa.eu.int/eur-lex>). This Directive regulating the evaluation, distribution and use of plant protection products in the European Union was passed on 15 July 1991 by the Council of the European Union. Before an active substance can be considered for inclusion in Annex I of the Directive, companies must submit a complete data package (dossier) on both the active substance and at least one plant protection product containing that active substance. Comprehensive data and evaluations are required to allow inclusion in Annex I of the Directive. Annex II data in the Directive relate to the active substance and Annex III to the plant protection product. The requirements for micro-organisms, including viruses, are listed in Part B of the Annexes II and III, resp. Also for micro-organisms including viruses a detailed data package has to be submitted to the authorities. This is one reason for the small number of biological plant protection products authorized; moreover, selective products like biological plant protection products have, of course, only a small market and perhaps cannot cover the costs for research and development of the data sets required.

### Concluding remarks

Despite the numerous reports on the successful experimental use of micro-organisms and other biological methods, the progress in practical utilisation has been very slow. There are only a few microbial plant protection products authorised in comparison with parasitoids and predators, which need no authorisation and of which 90 specimens are listed in Germany (Bathon 1999). The world market of plant protection products altogether amounts to 30 Billion US \$, among those only 380 Million US \$ for beneficials and micro-organisms.

Especially difficult is the situation for bactericides. Antibiotics show good control effects. However, a regular authorization for antibiotics is very unlikely in the EU because of risks of development of cross-resistance in human pathogens. This opens a good chance for alternative methods including biological products. In principle there are the following ways of biocontrolling bacterial diseases of crops besides resistance breeding: the use of antagonistic micro-organisms (for periodic applications) and the use of (natural) substances inducing resistance or acting as bactericides. Already a short look into the literature shows many efforts in these directions. Just to give you one (older) example among many others: Podzucki (1989) tested 245 bacterial isolates obtained from leaves and flowers of 28 species belonging to 18 genera of wild plants. Among them 18 isolates showed antagonistic activity against *Erwinia amylovora* and *Pseudomonas syringae*. Ten of the isolates inhibited both *E. amylovora* and *P. syringae*. Also root-colonising bacteria have been found to be of relevance for biological control. Moreover there are many reports of plant substances which induce resistance in crops against bacterial and fungal plant diseases or have a bactericidal or fungicidal action. The crux is that usually these efforts do not leave the laboratory bench.

I hope that our symposium can foster an intensive exchange of ideas, results, and techniques to encourage the search for biological control measures of bacterial plant pathogens, and moreover help to overcome hurdles in putting them into practical use.

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## **I. Principles of biological control: antagonism, induced resistance, plant growth promotion (antagonistic organisms and natural compounds)**

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### **Principles in biological control of soil-borne diseases: Colonization, antagonism, plant growth promotion and induced resistance**

#### **Abstract**

Biological control of soil-borne pathogens comprises the decrease of inoculum or of the disease producing activity of a pathogen through one or more mechanisms. Interest in biological control of soil-borne plant pathogens has increased considerably in the last few decades, because it may provide control of diseases that can not or only partly be managed by other control strategies.

Recent advances in microbial and molecular techniques have significantly contributed to new insights in underlying mechanisms by which introduced bacteria function. Colonization of plant roots is an essential step for both soil-borne pathogenic and beneficial rhizobacteria. Visualization technologies using laser scanning microscopy in combination with marked strains have increased our knowledge on rhizosphere microbiology. Colonization patterns showed that rhizobacteria act as biocontrol agents or as growth promoting bacteria form microcolonies or biofilms at preferred sites of root exudation. Such microcolonies are sites for bacteria to communicate with each other (quorum sensing) and to act in a coordinated manner. Mutants defective in root colonization traits were impaired in their ability to control root diseases.

Biocontrol mechanisms by which rhizosphere bacteria may protect plants against soil-borne pathogens are multifarious. The bacteria may compete for space and nutrients in the rhizosphere. Evidence has been accumulated that antibiotics produced by rhizobacteria constitute an important factor in suppression of root diseases. The degree of disease control by rhizobacteria producing antibiotics depends on the sensitivity of the pathogen population to the antibiotics and the quantities produced. Fluorescent pseudomonads produce siderophores that sequester iron, thereby depriving the pathogen from this essential element during its deleterious activities in the rhizosphere. Mechanisms by which plant growth promoting rhizobacteria (PGPR) may stimulate plant growth are either by suppressing deleterious microorganisms or pathogens or by producing plant growth regulators (such as auxins, cytokinins and gibberellins) as well as by lowering ethylene levels in plants.

In addition, selected strains of rhizobacteria reduce diseases through activating resistance mechanisms in plants. Rhizobacteria-mediated resistance is expressed toward a broad spectrum of plant pathogens. Certain strains of rhizobacteria produce salicylic acid (SA) at the root surface and trigger the SA-dependent systemic acquired resistance (SAR)-pathway. Other rhizobacteria trigger a different signalling pathway named induced systemic resistance (ISR). ISR is dependent on the plant hormones jasmonic acid (JA) and ethylene. Simultaneous activation of both the JA/ethylene-dependent ISR pathway and the SA-dependent SAR pathway may result in enhanced levels of protection.

#### **Introduction**

Soil-borne pathogens cause economically significant diseases in cultivated crops such as damping-off, root rots, seedling blights, crown rots, foot rots, root browning and wilts. The diseases may be caused by more than 50 different fungal species, various bacterial species and viruses. The damage evoked by soil-borne pathogens is usually underestimated, since it appears below ground.

Plant diseases caused by pathogens below ground may be considered as limiting factors of plant health and securing yield quantitatively and qualitatively. Application of fungicides to control soil-borne fungal pathogens, with the exception of seed treatments, seems not to be appropriate for economical and ecological reasons (Buchenauer 1998).

Besides the use of resistant varieties which is, in general, insufficient against most of the soil-borne pathogens, application of beneficially active rhizosphere organisms has received particular attention in control of soil-borne pathogens. Contrary to application of fungicides, microorganisms are able to protect roots and other plant organs below ground against pathogens because of their ability to multiply and spread along roots and other parts of plants in the soil as living organisms.

### **Rhizosphere**

The rhizosphere comprises the zone around plant roots containing nutrients released by roots. The rhizosphere can be divided into the endorhizosphere (the root cortex), rhizoplane (the root epidermis) and the ectorhizosphere (the soil around roots, into which root exudates penetrate).

The steady release of organic nutrients such as sugars, amino acids, fatty acids, nucleotides, vitamins, plant hormones and other substances into the rhizosphere presents the matrix for growth and activity of microorganisms (Lynch 1994; Lynch and Whipps 1990). Microscopical studies indicated that bacteria are the prevailing organisms in rhizosphere colonization (Foster 1986; Kluepfel 1993). The meristematic cell regions of the root behind the root caps represent the main exudation site followed by side root regions (Rovira and Davey 1974).

Root exudation may be affected both quantitatively and qualitatively by numerous factors such as plant species, plant age, soil type, soil fertility, light, temperature and foliar treatments. Exudation may be enhanced by the activity of rhizosphere bacteria through production of plant growth hormones, vitamins, toxins, cellulytic enzymes or other compounds (Schippers et al. 1987). Also during seed germination exudates are released in considerable quantities (spermosphere).

### **Root colonization**

Colonization of plant roots is an essential step both for soil-borne pathogenic and beneficial microorganisms. Root colonization has intensively been studied with *Pseudomonas* spp., the most effective root colonizing bacteria. Electron microscopic studies as well as the use of marked strains revealed a non-uniform distribution of bacteria on the root. While some areas, such as the root tip, are almost free from bacteria, other areas may be heavily colonized. Areas intensely colonized by pseudomonads are usually junctions between epidermal root cells or sites of side roots (Chin-A-Woeng et al. 1997).

In order to simultaneously visualize different *Pseudomonas* populations in the rhizosphere in a non-invasive way mutants of *P. fluorescens* WCS365 encoding cyan, green and yellow variants of the green fluorescent protein (GFP) as well as the red fluorescent protein were constructed and tomato seedlings inoculated with the different mutants. Confocal laser microscopy studies showed that the *Pseudomonas* cells were mainly present on the plant root surface as elongated stretches at junctions between epidermal cells, deeper parts of the epidermis and on root hairs (Bloemberg et al. 1997; Dekkers et al. 2000). This method allowed that up to three differently labelled bacterial strains could be visualized simultaneously and distinguished from each other. Microcolonies consisting of mixed populations were frequently observed at the base of the root system, whereas microcolonies further toward the root tip predominantly consisted of a single population, suggesting a dynamic behaviour of microcolonies over time. For example, inoculation of tomato seedlings with two biocontrol pseudomonads, *P. fluorescens* strain WCS365 (inductor of systemic resistance, ISR) and *P. chlororaphis* strain PCL1391 (producing the antibiotic phenazine-1-carboxamide) expressing different autofluorescent proteins showed that at the older part of the root the two cell types were present in larger microcolonies of either one or both cell types. At the lower root parts microcolonies consisted predominantly of one cell type (Dekkers et al. 2000). On the middle part of the roots WCS365 cells were about five times more abundant, while root hairs were predominantly colonized by PCL1391 cells. The results indicate that the two strains of bacteria show some preference for different root sites. Mixed inoculation improved biocontrol compared to single inoculations. This may be due to synergistic effects displayed by two different mechanisms.

Numerous saprophytic *Pseudomonas* species acting as potential biocontrol agents (de Weger et al. 1996) or as growth promoting bacteria (Fukui et al. 1994) have been observed to form microcolonies or aggregates in the rhizosphere and phyllosphere. Rhizobacteria occur primarily as microcolonies at sites of root exudation (Rovira and Campbell 1974). Microcolony formation on plant surfaces can be considered as a kind of biofilm formation. Biofilms are assemblages of microorganisms adhering to each other and embedded in a matrix of exopolymers. Microcolonies create growth conditions different from those of the ambient environment. Such microcolonies are ideal places for bacteria to communicate with each other, e.g. quorum sensing (QS). QS describes mechanisms of bacterial cell-to-cell communication, and certain traits are only expressed when bacteria are assembled together in microcolonies. This allows them to act in a coordinated manner. Thus individual bacterial cells benefit from cooperative group behaviour to survive, compete and persist in nature or to colonize a particular host. QS involves the exchange of low molecular weight diffusible signal molecules between members of a localized population. Induction of the expression of certain beneficial genes in microcolonies leads to the accumulation of diffusible molecules such as homoserine lactones (AHLs) (Davey and O'Toole 2000; von Bodmann et al. 2003).

Biocontrol mechanisms by *Pseudomonas* strains are, in general, also based on secreted bioactive factors that contribute to ecological fitness by competing with the resident microflora. Inadequate colonization leads to decreased biocontrol activity (Schippers et al. 1987). The availability of a series of different colonization genes made it possible to test whether colonization is required for biocontrol (Chin-A-Woeng et al. 2000). The parental strain PCL1391 of *P. chlororaphis* controls tomato foot and root rot caused by *F. oxysporum* f.sp. *radicis-lycopersici* by secreting of phenazine-1-carboxamide (PCN). Different mutants of this strain defective in root colonization traits were constructed. All mutants (non-motile, auxotrophic for phenylalanine or containing a tetracycline cassette in the *sss*-gene) were impaired in root tip colonization and in their ability to control tomato foot and root rot, although the mutants were still able to produce the extracellular metabolites PCN, HCN, chitinase and protease.

The mechanism of quorum sensing is based on intracellular requirement of membrane permeable N-acyl-homoserine lactones (N-AHLs) as auto-inducers and transcription factors (Fuqua et al. 1994). *Pseudomonas aureofaciens* strain 30-84 has been used as seed treatment to protect wheat from take-all-disease (Parsek et al. 1997). This strain reduced the severity of take-all by producing the phenazine antibiotics phenazine-1-carboxylic acid and 2-hydroxy-phenazine (Pierson 1996; Pierson and Thomashow 1992). Phenazine production also contributed substantially to the rhizosphere competence of *P. aureofaciens*. Loss of phenazine production resulted in a 10<sup>4</sup>-fold decrease in strain 30-84 populations on wheat roots in the presence of other microorganisms. The *phzFABCD* operon is responsible for the biosynthesis of the phenazine antibiotics by strain 30-84 (Pierson et al. 1995). Expression of *phzFABCD* is regulated by the LuxRI LuxI homologs PhzR and PhzI (Wood and Pierson 1996). PhzI encodes a hexanoyl-homoserine lactone (HHL) synthase. The rationale for the evolution of AHL-mediated regulation of phenazine gene expression in the root-colonizing bacterium *P. aureofaciens* strains 30-84 is even more interesting. Phenazine antibiotics inhibit a wide range of bacteria and fungi *in vitro* (Gurusiddaiah et al. 1986), and their production is necessary for the persistence of strain 30-84 in a competitive rhizosphere (Mazzola et al. 1992). They appear to play an important role in maintaining the bacterial population either by inhibiting the growth of competitors and/or by serving an additional as yet undetermined physiological function. AHL-mediated regulation may ensure that phenazines are produced only when the bacterial population reaches a high cell density after initial colonization and growth has occurred. Alternatively, because strain 30-84 is responsive to signals produced by other root colonizing bacteria, AHL-mediated gene regulation may trigger phenazine gene expression in the presence of microorganisms.

Genetic engineering has shown that colonization ability of wild type *Pseudomonas* strains can be improved. Introduction of multiple copies of an *sss*-containing fragment from WCS365 into a poor colonizer, *P. fluorescens* WCS307, and into a good colonizer, *P. fluorescens* F 113, increased the competitive tomato root tip colonization ability of the latter strains 16-40 fold and 8-16 fold, respectively (Dekkers et al. 2000).

Thus, development of new techniques can dramatically influence our knowledge of rhizosphere microbiology. Visualization technology, using confocal laser scanning microscopy (CLSM) in combination with various forms of GFP, will allow detailed studies on interactions between various

organisms as well as temporal and spatial aspects of gene expression. Functional genomics (including proteomics and metabolomics) will allow identification of all genes expressed in the rhizosphere. The use of promoters that are specifically expressed in the rhizosphere allows engineering of microorganisms for beneficial purposes with minimal loss of energy. Finally, the interactions between bacteria and fungi in the rhizosphere are an exciting new field of research.

### Siderophores

Specific root-colonizing fluorescent pseudomonads and other beneficial microorganisms are capable of enhancing plant growth and of controlling certain soil-borne pathogens by producing siderophores under iron-limiting conditions. Siderophores represent low-molecular weight (500-100 Da) iron(III)-transport compounds, which selectively complex iron(III) with very high affinity. Their function is to supply iron to the producing organism (Hider 1984; Neilands 1981; Neilands 1982). A yellow-green fluorescent siderophore, designated pseudobactin, was isolated from iron-limiting cultures of strain B10 of a fluorescent *Pseudomonas* species. The siderophore exhibited antibiotic activity against *Erwinia carotovora in vitro* (Kloepper et al. 1980a). Treatment of conducive soil with strain B10 or its siderophore pseudobactin reduced take-all disease in barley, caused by *Gaeumannomyces graminis* var. *tritici* (Kloepper et al. 1980b). Inoculation of flax seeds or dipping of flax transplants in a suspension of strain B10 increased survival of flax plants in conducive soils infested with the flax wilt pathogen, *Fusarium oxysporum* f.sp. *lini*. The ability of strain B10 or pseudobactin to convert pathogen-conducive soils into pathogen-suppressive soils suggests that soil suppressiveness is partly associated with production of siderophores which sequester iron(III) and make it unavailable to root pathogens (Kloepper et al. 1980b).

The production of siderophores by other specific fluorescent pseudomonads has also been implicated in the suppression of take-all of wheat and root rot of wheat caused by several *Pythium* species (Becker and Cook 1984). Mutant strains defective in siderophore production did not protect wheat although they colonized roots (Becker and Cook, 1984; Weller et al. 1985). *Alcaligenes* sp. strain MFA1 also appeared to suppress the incidence of Fusarium-wilt of carnation in part by producing siderophores under iron-limiting conditions. The siderophore deprives the carnation wilt pathogen of essential iron(III) and also inhibits germination of chlamydospores, which might result in reduced colonization and infection of carnation roots by *F. oxysporum* f.sp. *dianthi* (Yuen and Schroth 1986).

Root-colonizing beneficial fluorescent pseudomonads producing siderophores under iron-limiting conditions may also partly enhance plant growth by reducing the population of deleterious rhizobacteria. Deleterious rhizobacteria represent a major component of bacterial microflora of roots of numerous plants which may limit plant growth and crop yield without causing root diseases (Gross and Cody 1985; Leisinger and Margraf 1979). Studies revealed that beneficial strains inhibited growth of deleterious strains primarily by depriving iron and deleterious strains were not able to use siderophores from the beneficial strains (Buyer and Leong 1986). On the other hand, deleterious strains resistant to the beneficial strains are probably capable of utilizing the siderophores of the beneficial strains for iron transport. Furthermore, populations of beneficial and deleterious strains which cannot use each other's siderophores may compete for iron and factors involved in highest competition for iron by siderophores, and iron uptake may determine which species predominate under an iron-limiting environment (Neilands 1982; Bailey and Taub 1980).

### Production of antibiotics

Beside siderophores, rhizosphere bacteria produce antibiotics, enzymes and volatiles, which may play important roles in control of soil-borne plant pathogens (Weller 1988; Whipps 1997). Antibiotics produced by bacterial biocontrol agents comprise chemically heterogeneous groups of organic low molecular weight compounds (Raaijmakers et al. 2002; Haas and Keel 2003); for example antibiotics produced by *Pseudomonas* species include: phenazines, phloroglucinols (mono- and diacetylphloroglucinol), oomycin A, pyoluteorin, pyrrolnitrin, 2,3-de-epoxy-2,3-didehydro-rhizoxin, viscosinamide, butyrolactones, and butylbenzene-sulphonamide. *Bacillus cereus* produces, e.g., kanosamine and zwittermycin A, and *Pantoea agglomerans* produces pantocin A and B. Many of the antibiotics produced display a broad spectrum of activity. For instance, pyrrolnitrin exhibits a wide range of activity against fungal pathogens including *Basidiomycetes*, *Deuteromycetes* and *Ascomycetes*

(Howell and Stipanovic 1979; Keel et al. 1992; Kalbe et al. 1996; Burkhead et al. 1994; Ligon et al. 2000) as well as against several Gram-positive bacteria (e.g. *Streptomyces* spp.) (El-Banna and Winkelmann 1998). Also diacetylphloroglucinol (DAPG) displays a broad spectrum of activity against fungal and bacterial pathogens and nematodes. Zwittermycin A affects growth and activity of a wide range of plant pathogenic fungi, in particular *Phytophthora* and *Pythium* species (Silo-Suh et al. 1994, 1998). In addition, *P. fluorescens*-strains (CHA0 and Pf5) (Keel et al. 1996; Bender et al. 1999) and *B. cereus* strain UW85 (Handelsman and Stabb 1996) produce several antibiotics differing in their activity against plant pathogens, suggesting that certain biocontrol agents may suppress specific or various plant pathogens. Recent advances in microbiological and molecular techniques have significantly contributed to new insight in underlying mechanisms by which rhizobacteria function. Both the structural and regulatory genes involved in producing the antibiotics have been identified and characterized. Such studies have been carried out for phloroglucines, phenazines, pyoluteorin, pyrrolnitrin and HCN.

Involvement of antibiotics in biological control of plant pathogens has been documented in numerous studies. Inactivation of antibiotic production by mutagenesis revealed diminished biological control of plant diseases by the mutants compared to the wild type strains. Complemented mutants with restored phenotypes showed retrieved biocontrol activity. Studies carried out for phenazines, DAPG, pyrrolnitrin, pyoluteorin and HCN demonstrated that antibiotics produced by *Pseudomonas* species and *Burkholderia cepacia* play an important role in biocontrol activity (Thomashow and Weller 1988; Keel et al. 1990; Kang et al. 1998; Silo-Suh et al. 1994).

Introduction or modification of antibiotic biosynthetic or regulatory genes enhanced antibiotic production. For example, increased production of DAPG and pyoluteorin in *P. fluorescens* strain CHA0 improved the control of *Pythium* root rot in cucumber (Maurhofer et al. 1992). Introduction of multiple copies of the regulatory *gacA*-gene into *P. fluorescens* strain BL915 enhanced the pyrrolnitrin production 2.5-fold.

A 6-kb fragment from DAPG-producing *P. fluorescens* strain F113 was introduced in a DAPG-nonproducing strain M114. The resulting mutants producing DAPG were markedly more effective in controlling *P. ultimum* on sugar beet than the parental strain (Fenton et al. 1992). In many more studies the role and function of antibiotics in biological control of plant pathogens has been confirmed. These investigations also demonstrate that knowledge on genetics, regulation and biochemistry of antibiotic biosynthesis provide effective tools to improve the efficiency of bacterial control agents.

DAPG-producing fluorescent pseudomonads have been isolated from suppressive soils at densities of  $10^5$ - $10^6$  cfu/g root tissue (Raaijmakers et al. 1997), representing about 0.1-1 % of the total bacterial rhizosphere population growing in microcolonies (Bonsall et al. 1997; Notz et al. 2002). A biocontrol strain of *P. aureofaciens* produces 2-3 mg phenazine-carboxylic acid (PCA)/ml inside the colonies on nutrient agar (Séveno et al. 2001). Similar concentrations inside microcolonies have been calculated for DAPG produced by *P. fluorescens* strain CHA0 on malt agar (Schnider et al. 1995). It has been suggested that the antibiotic concentration inside a microcolony might be sufficient to prevent invasion of sensitive rhizosphere microorganisms.

Under natural conditions antibiotic producing biocontrol strains only occupy a small percentage of the root surface. Suppression of rhizosphere pathogens may in general not result in killing the pathogens, but it may be assumed that subinhibitory concentrations may rather cause physiological effects on target organisms. It has been shown that subinhibitory levels of quinoline and macrolide antibiotics interfere in cell to cell signalling and production of virulent factors. Our knowledge on modes of action of the different antibiotics produced by beneficial rhizobacteria at inhibitory and subinhibitory concentrations in target organisms is still insufficient. For example, antibiotics produced by *P. fluorescens* strain CHA0 seemed to suppress production of lytic enzymes by myxobacteria and protect the producer from lysis (Bull et al. 2002).

Antibiotics produced by rhizobacteria might also function in inducing resistance in host plants. An endophytically colonizing *P. fluorescens* strain deposited DAPG crystals in and around the roots of tomato and it was assumed that the DAPG deposits protect the plant against wilt disease possibly also by induction of systemic resistance (Aino et al. 1997).

It has been shown that several abiotic and biotic factors (e.g. mineral content, oxygen tension, osmotic conditions, phosphate, carbon and nitrogen sources as well as fungal, bacterial and plant metabolites) can influence production of antibiotics by *Pseudomonas* spp. *in vitro* (Duffy and Defago 1999; Gaballa et al. 1997; Nakata et al. 1999). For example, fusaric acid, an antibiotic and mycotoxin produced by *Fusarium oxysporum*, suppresses the production of the antifungal compound DAPG by *P. fluorescens* CHA0 without affecting growth and rhizosphere colonization of wheat roots (Notz et al. 2002), showing that production of antibiotics may be affected by secondary products produced by other competitors in the rhizosphere.

### **Bacteriocins**

Bacteriocins may be defined as antibacterial substances that display a specific inhibitory effect against organisms closely related to the producers (Vidaver 1983). The chemical nature of bacteriocins constitutes of low or high molecular weight proteins or low molecular weight nucleotides. Thus, the specificity and chemical structure of bacteriocins differ from the low molecular weight broad spectrum antibiotics.

Production of bacteriocins has been demonstrated for both rhizosphere (e.g. *Pseudomonas solanacearum*, *Erwinia carotovora* ssp. *carotovora*, *Agrobacterium radiobacter*) and phyllosphere bacteria (e.g. *Pseudomonas syringae*, *Erwinia herbicola*) where they may play a role in biocontrol. Numerous strains of *E. carotovora* ssp. *carotovora* produce high molecular weight bacteriocins (carotovoricins) *in vitro* resembling defective phage particles. Axelrod et al. (1988) determined a close correlation between production of these bacteriocins *in vitro* and inhibition of competing *Erwinia* strains in potato wound infections. The closely related *E. carotovora* ssp. *tetravascularum* (pathogen of sugar beet) produces a bacteriocin exhibiting inhibitory activity to *E. carotovora* ssp. *carotovora* and *E. amylovora* (Axelrod et al. 1988). The best known example for practical use of a bacteriocin in biological control is the low molecular weight compound agrocin 84 produced by the non pathogenic soil organism *A. radiobacter* strain 84. This strain has been used since 1973 in Australia to control crown gall in stone fruits and roses (Kerr and Htay 1973). The development of this biological control system represented the first use of a specific microorganism to control a plant pathogen in soil and the first use of a bacterium to control any plant disease. Agrocin 84 produced by a plasmid of strain 84 of *A. radiobacter* represents an adenine nucleoside and specifically inhibits DNA synthesis in *A. tumefaciens*. The production of agrocin 84 might be involved in crown gall control. For example, transfer of the plasmid determining agrocin production to a non-bacteriocinogenic strain was insufficient compared to the effectiveness of strain 84 (Ellis et al. 1979), suggesting that additional factors are necessary for high biocontrol activity. It has been shown that effectiveness of the biocontrol agent *A. radiobacter* strain 84 against *A. tumefaciens* not only depends on the production of agrocin 84 but also on site competition with the pathogen including nutrient requirements, growth rate parameters, chemotaxis and general motility. Using bacteriocin-producing bacteria as biocontrol agents has the advantage that the producer strain and related target strain occupy similar ecological niches.

### **Systemic resistance induced by rhizobacteria**

Induced resistance involves the activation of latent resistance mechanisms which are expressed after subsequent challenge inoculation with a pathogen. Induced resistance may be triggered by avirulent pathogens, incompatible races of pathogens, non-pathogens and certain chemicals. Generally, resistance is not only induced locally (localized induced resistance, LIR), but also in plant parts separated spatially from the inducing agent (systemic induced resistance, SIR) (Sticher et al. 1997; van Loon et al. 1998). Systemic resistance may be induced by two different signalling pathways: the pathogen- and the rhizobacteria-activated resistance.

The pathogen-induced resistance results of limited infection by a pathogen, especially when the plant develops hypersensitive reaction. This type of resistance is designated systemic acquired resistance (SAR). SAR is effective against a broad spectrum of plant pathogens including viruses, bacteria and fungi in mono- and dicotyledons. SAR is characterized by an accumulation of salicylic acid (SA) and pathogenesis related proteins (PRs). SA is accumulated both locally and at a lower extent systemically. The long distance signal for systemic induction is probably a lipid transport protein (Maldonado et al. 2002). The level of SAR expression may be affected by ethylene and jasmonic acid suggesting that

induction and expression of SAR might be regulated through interference of several signalling chemicals (van Wees et al. 2000).

Induced systemic resistance is triggered by certain rhizobacteria which cause no visible symptoms in host plants and generally enhance plant growth. ISR triggered by rhizobacteria is also expressed by reduced disease incidence and retarded disease severity. While SAR has been shown to be active in numerous plant species, studies on ISR have been restricted so far to a few plant species. Colonization of *Arabidopsis* roots by the rhizobacterium *P. fluorescens* WCS4175 induced ISR against different pathogens, for example *P. syringae* pv. *tomato*, *X. campestris* pv. *amoracia*, *F. oxysporum* f. sp. *raphani*, and *P. parasitica*. Interestingly, no ISR has been reported in monocotyledons (Pieterse et al. 1998). In general, resistance is induced only when rhizobacteria exceed a threshold population. For example, at least  $10^5$  cfu g<sup>-1</sup> root of *P. fluorescens* WCS375 and WCS417 were required for induction of ISR. At lower levels of rhizosphere colonization by WCS374 and WCS417 strains no systemic resistance against diseases was induced (Raaijmakers et al. 1995). While SAR requires SA accumulation in plants (Sticher et al. 1997), ISR is dependent on ethylene and jasmonic acid (Pieterse et al. 1998). ISR expressing plants are primed to express JA and/or ethylene dependent defense reactions faster or to a higher level after infection by a challenging pathogen.

Several bacterial determinants seem to be involved in induced resistance (van Loon et al. 1998). The outer membrane lipopolysaccharide (LPS) acts as a constituent of resistance induction in carnation and radish. The structure of the O-antigenic side chain of the rhizobacteria WCS374 determined the induction of systemic resistance both when applied to roots or to cotyledons of radish. Under iron-ample conditions the O-antigenic side chain (OA) of LPS was the main component of ISR, however, under iron-limiting conditions, OA<sup>-</sup>mutants of *P. fluorescens* WCS374 and WCS417 were still capable of inducing resistance in radish against *Fusarium* wilt (Leeman et al. 1995). The results indicate that more than one determinant may be active in triggering ISR in plants. Under iron deficiency, *P. fluorescens* WCS374 and WCS417 produced pseudobactin siderophores. The siderophore isolated from WCS374 induced resistance to the same level as the bacterial LPJ. However, a pseudobactin mutant of WCS374 proved to be as effective as the parental strain in disease reduction. Further studies revealed that possibly the iron-regulated ISR by WCS374 was mediated by the SA-containing siderophore pseudomonine (Mercado-Blanco et al. 2001). The root-colonizing *P. aeruginosa*-strain 7NSK2, which induces resistance in tobacco, bean and tomato, acts probably not by SA production but rather by production of pyochelin and pyocyanin (Andenaert et al. 2002).

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## **Basal resistance of plants to bacterial infection as a possible means for biological control**

### **The role of basal (innate) resistance in plant pathology**

Billions of micro-organisms including saprophytes and pathogens are to be found on the surface of the human body without causing illness. However, when an organism has a weak constitution or the immune system has collapsed many micro-organisms invade the body and cause illness or death. This phenomenon can be observed in everyday life such as for example in hospital epidemics, when ageing weakened patients are attacked by so-called opportunistic pathogens. These organisms, mostly saprophytes multiply profusely only in the bodies of weakened patients. The nurses and doctors remain immune.

In plants, the situation is similar. Numerous saprophytes and pathogens land and live on plant surfaces, with a good chance of entry into the plant tissue through stomata and tissue injury. By utilising nutrients from plants they try to multiply but it rarely escalates to serious damage.

However, when the temperature drops below 10 °C the non-specific immune response is delayed or does not develop, enabling pathogens, like *P. syringae* pv. *syringae* to cause serious disease in many plant species. At elevated temperatures, on the other hand, some other bacterial pathogens become unable to cause the hypersensitive response (HR, a blend of strong resistance and cell death), the plant remains immune, which is an example of symptomless non-host resistance. The saprophyte *Pseudomonas fluorescens* multiplies well in the intercellular washing fluid (IWF) obtained by centrifugation but not in living plant tissue.

The question is why? What kind of mechanisms can inhibit bacterial attack in healthy living organisms? In human pathology this defence mechanism is known as innate immunity. In the plant kingdom at present there is no consensus in the literature on how to call these non-specific, general mechanisms. There are many synonyms, such as general or sometimes innate immunity of plants, induced resistance etc. We will refer to it as basal resistance (BR), explained in the following chapters.

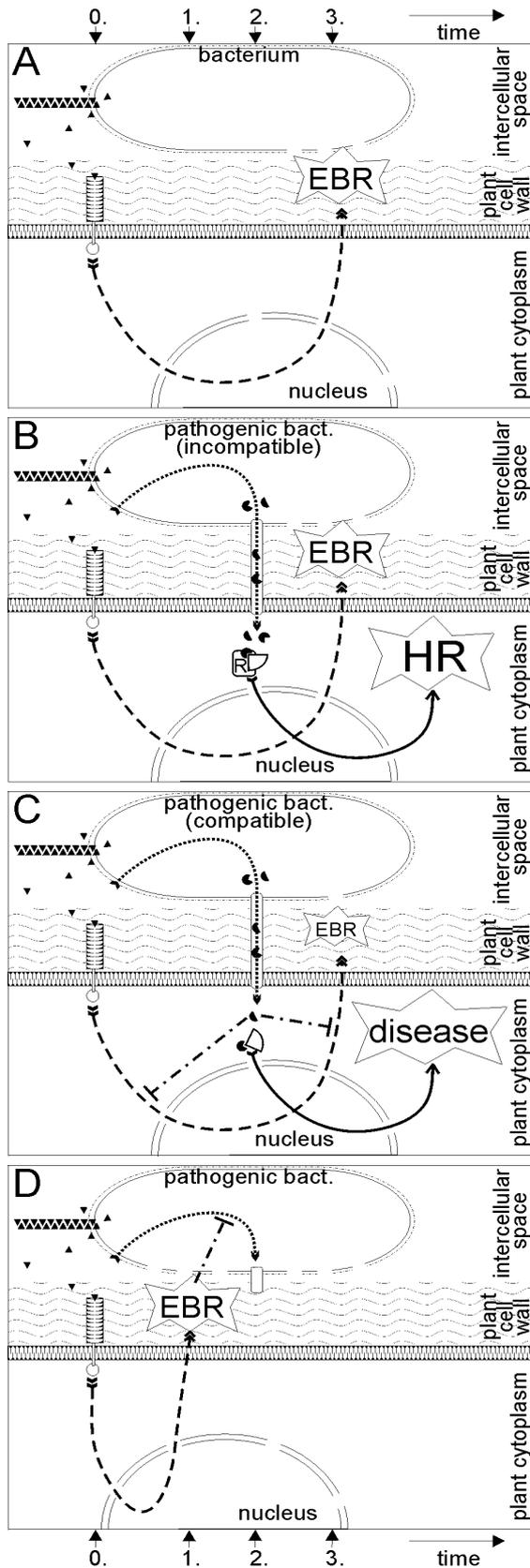
Here, we attempt to describe and discuss the events connected with BR in a temporal order, along with a model developed by our group, supervised by our late professor, Zoltán Klement.

### **Pre-infection state**

The plant surveillance system keeps receptors and downstream components for recognition of non-self constantly expressed and alert. The earliest responses to bacteria, measured as transcription induced within a couple of minutes (G.J. Varga unpublished), can be explained this way. In Fig. 1 we follow the main stages of BR development and its relation with pathogenic and non-pathogenic interactions.

### **Receptor activation by general elicitors**

Bacteria, including saprophytes and pathogens, after penetration into the intercellular spaces attach to the plant cell surface, and unavoidably present their constitutive molecular patterns to be recognised as non-self by plant cells. It is important to see that these 'general elicitors' are the very first signs of bacterial presence. In human pathology these common molecules are called 'pathogen associated molecular patterns' (PAMPs) that initiate innate immunity. There are several potential general elicitors, such as lipopolysaccharides (LPS, Graham et al. 1977; Newman et al. 2002), LPS-protein complexes (Mazzucchi et al. 1979), cold shock protein (CPS, Felix and Boller 2003), elongation factor Tu (Kunze et al. 2004) and a conserved motif of the flagellar protein flagellin (Zipfel et al. 2004).



**Fig. 1** The role of EBR in different types of plant-bacterial interactions. When a bacterial cell enters the intercellular spaces of plants different defence response pathways are induced in the plant and bacterial cells depending on the type of bacterium (A-D). The time course, from left to right, is relevant for arrowed pathways. Below and above the figure, upward arrows indicate ordered key time points.

- A)** Non-pathogenic bacteria without TTSS and effectors induce only BR and plants remain symptomless. Bacteria themselves lack specific responses. Plant receptors (here, Fls2 is shown spanning the plant cell membrane) activation by general elicitors at time zero (flagellin shown as filled triangles), signal transduction and development of EBR (only transcriptional pathways are shown with double headed arrow and dashed line) occurs and has an antibacterial effect at usu. 2-6 hpi (time 3). EBR inflicts damage (indicated by gaps in bacterial cell wall), interfering with bacterial metabolism and multiplication.
- B)** For pathogens, time is needed to build TTSS and for the translocation of effectors (filled symbols and pointed line starting at time zero, the same time point as for the plant recognition event). This time (1-3 hpi) is frequently shorter (ending at time 2) than the time needed for EBR (time 3). Binding of some effectors (filled 3/4 circles) of incompatible bacteria to a target protein (empty 1/4 circle) activates resistance protein (R)-mediated HR (solid line starting at time 2). The strong phenotype of HR masks the more subtle effects of EBR.
- C)** Compatible bacteria have more means to cope with the host. They are faster with translocation and can tolerate more of host defence than incompatible ones. They are still susceptible to EBR, and translocate effectors with EBR-suppressing activity (filled half circles and dashed-pointed line). The result is weaker EBR (smaller character type). Other effectors may block plant cell death. With effectors finding the respective targets at time 2, these bacteria can establish favourable growth conditions and cause disease (solid line).
- D)** Under certain conditions (recent encounter with general elicitors, higher temperature to speed up metabolism) EBR may be set up earlier (time 1) than completion of bacterial responses (time 2). The general antibacterial efficacy of EBR may delay or inhibit bacterial pathogenesis (dashed line), resulting in aborted effector synthesis/translocation (shown as lack of effectors and shorter TTSS pilus).

We set out to find general elicitor(s)/PAMP(s) of non-specific early BR (EBR, Klement and Burgyán 1978; Burgyán and Klement 1979) in tobacco, using the HR inhibition biotest and chitinase markers (see later). We used known bacterial elicitors of plant responses reported to interfere with bacterial growth. Flagellin was very similar to heat-killed bacteria in most aspects of EBR (G.J. Varga unpublished). The recently discovered general elicitor, CSP, (here data on induced resistance are lacking) led to a later/weaker BR at 24 hours post inoculation (hpi). LPS was even slower, with significant effect only at 48 hpi. Peptidoglycan proved the least potent general elicitor (G.J. Varga unpublished). Thus, only flagellin from the elicitors investigated so far seemed to be compatible with EBR, at least in tobacco. Although other bacterial components are likely to have a role (some are now under study), this finding is an important step forward that obviates the use of exceedingly complex elicitors (bacterial cells), enabling more mechanistic and traceable studies in the future.

### **Signal transduction**

From the many putative receptors only Fls2, the receptor for flagellin is known (Zipfel et al. 2004). The flagellin-engaged Fls2 activates hundreds of target genes (Navarro et al. 2004) through the signal transduction pathway involving a mitogen activated protein kinase (MAPK) cascade (Asai et al. 2002). PAMP receptors used by animals, the so-called TLRs, bear a very similar domain structure to that of Fls2, however, many TLRs require factors additional to their respective PAMP (e.g. Numberger and Brunner 2002)

We found genes with a potential role in transcriptional reorganisation, members of signalling pathways (e.g. kinases, phosphatases and transcription factors), to be down- or up-regulated in gene array and real-time reverse transcription polymerase chain reaction (RT-PCR) assays. Intriguingly, the best known signal molecules for coordination of plant resistance responses, salicylic acid, methyl jasmonate and ethylene did not have a significant effect (Bozsó and Szatmári unpublished).

### **Development of EBR**

One of the first measurable consequences of this recognition on invader bacteria is EBR. EBR is a local, symptomless response of plants to exploit general elicitors for first-line defence purposes. EBR may reach a state to interfere with bacterial metabolism within 2-6 h of post-inoculation (hpi). This can result in inhibition of bacterial growth/survival and/or of expression of the so-called *hrp* genes (Bozsó et al. 1999; Ott 2002). It is well known that *hrp* genes code for the type III secretion system (TTSS), crucial for inducing both pathogenesis and HR. Using TTSS as a molecular syringe, pathogenic bacteria translocate/inject proteins, called effectors, directly into the plant cell cytoplasm. These effectors are the tools of host cell manipulation, i.e. pathogenesis (e.g. Jin et al. 2001; Schechter et al. 2004). Thus, EBR action can eventually culminate in the lack of HR during incompatible interactions or in a delay of disease progress during compatible (Ott et al. 2006) or opportunistic interactions. Of note, we use HR inhibition as a quick test for EBR activity (Klement et al. 2003).

### **Significance of earliness**

Quickness of EBR is its most important appeal for practical (biocontrol) reasons as well as for its possible role in nature. The natural role is obvious: when basic bacterial pathogenesis is prevented, invading pathogens do not threaten plants anymore as no intracellular virulence factors can be delivered into the plant cells. The pathogens are forced to suffer the fate of saprophytes or pathogenesis-mutants: eventually succumb to the adverse conditions set by BR. EBR is about time-racing with the pathogen, since the bacterium also needs time (1-3 hours, depending on the bacterium) to attach to the plant and build its own weapon, the TTSS.

### **Influence of temperature**

Ambient temperature is discriminatory to the above adaptive processes by the plant and bacterium: at 30 °C EBR development is more rapid than at 20 °C while the time for bacteria to prepare injection barely changes in this range (Klement et al. 1999). Keeping with this tendency, neither EBR nor HR was detectable for at least 2-3 days post inoculation in the cold (5 °C). This may enable certain psychrophile

pathogens to colonise plants (Besenyei et al. 2005) and lead to serious 'cold-weather' diseases, such as those by the opportunistic pathogen *P. syringae* pv. *syringae*.

### **Effector mechanisms and molecules marking EBR**

It is not clear at present what processes are responsible for the EBR-related effect. They may be sufficient but less mature parts of late BR (see chapters below) and/or may be transient ones. Classical approaches on plant metabolism during BR, such as ultrastructural studies and measurements of pro-oxidative changes provided some information. For example, no specific symptom, not even papilla formation (see chapter at late BR) could be attributed to EBR (Ott et al. 1998). On the other hand, a peroxidase- and H<sub>2</sub>O<sub>2</sub>-dependent tissue staining was described as correlating well with EBR (Bozsó et al. 2005). To find more inclusive and basic information, new experiments started at two levels, gene transcription and protein expression. So far we collected data on genes and proteins that mark the development of EBR. From these a subset will be chosen for functional studies. Choosing the good ones for engineering is not going to be easy, as EBR emerges as an encyclopaedic physiological system, exemplified below.

### **Changes in gene transcription**

Using the most comprehensive gene oligonucleotide array available in *Medicago truncatula* and RT-PCR with tobacco samples has shown that about 12% of the genes were either up- or down regulated after application of complex EBR elicitors (pathogenesis mutant bacteria). Strong activation of secondary metabolism, detoxification genes (e.g. glutathione S-transferase, epoxide hydrolase), cell wall fortification (lignin synthesis, proline or glycine-rich proteins), defence-related genes (PR (pathogenesis-related) proteins, various elicitor-inducible genes) and several with unknown functions were revealed. Thus, a large portion of changes can be associated directly and indirectly with pathogen resistance. As an example of puzzling complexity, genes of different isoenzymes (e.g. peroxidases, Bozsó et al. 2002) with the same or similar predicted function were activated or repressed, warning us that basics await still to be learned. Activation of several genes increases 1-3 hpi and following a peak at 6-12 hpi the transcription level decreases nearly to the basal level. This temporal pattern is correlated well with the activation and termination of EBR.

### **EBR-associated apoplastic proteins**

Plant pathogenic bacteria do not enter living plant cells. Consequently, plant molecules effective in resistance transverse the apoplast. Non-denaturing electrophoresis of apoplastic proteins from general elicitor-treated and control tobacco plants led to the identification of two novel chitinases as markers of EBR. BR chitinases were transcriptionally induced, and unlike for PR proteins, environmental stresses or major defence hormones seemed to have minor or no influence. Interestingly, expression of chitinases and efficacy of EBR are quantitatively correlated (Ott et al. 2006). In pepper a 22 kDa chitinase and a 32 kDa peroxidase, both of apoplastic origin, can also be of use to mark the BR. Neither of these markers is entirely qualitative, as they are constitutively expressed at a low level (E. Besenyei unpublished). Roles of EBR-related chitinases against bacteria are being elucidated.

### **Progress into late BR**

Development of EBR continues beyond the early times, some effects peaking around 12-16 hpi, some do later, about 48 hpi (Klement et al. 2003). This transition could be seen with proteomic approach (Ott et al. 2006) and traced in more detail with molecular genetic studies (Szatmári et al. unpublished; Bozsó et al. 2005). Actually, this later stage of BR had been known for much longer than the early one, perhaps because its effects are more pronounced against compatible pathogens, the most obvious threats to plants. The consequences of late BR were studied in classical induced resistance experiments where a pretreatment with general elicitors (heat-killed cells or different cell constituents) was followed by an inoculation with a pathogen 1-2 days later. Thus, there was an artificial gap between resistance induction and challenge infection, to give the plant more time to react and reach a strong level of protection.

These studies provided basic knowledge. The first work with bacteria (Lovrekovich and Farkas 1965), based on a simple and revolutionary leaf injection technique to artificially introduce bacteria into leaf

intercellular spaces (Klement 1963) showed that heat-killed cells of a range of bacterial species elicited the same kind of symptomless resistance, suggesting the presence of general elicitors. As the resistance induced was ineffective against the bacterial toxin, its effect against the active stage of the living pathogen was inferred. Late BR also protected a plant tissue from undergoing the HR induced by incompatible bacteria (Lozano et al. 1970; Novacky et al. 1973). Studies on the effects of BR on bacteria had shown repeatedly that BR interferes with bacterial multiplication, especially in the first 6-8 hours of challenge (Lozano et al. 1970; Sequeira and Hill 1974). The intercellular washing fluid (IWF) from plants exhibiting BR interfered with bacterial division (Rathmell and Sequeira 1975). We mention two other phenomena, possibly connected with BR: immobilisation of non-pathogenic bacteria, but not compatible ones, to the plant cell wall and the highly localised apposition of material, e.g. callose, inside cell wall and outside the cytoplasmic membrane, called papilla (Sequeira et al. 1977)

### The role of HR and BR in the practical plant breeding

With the aid of HR it has become possible to produce resistant cultivars, however the resistance was short-lived because of the appearance of new pathological biotypes. It seems that it is more promising to apply the BR which ensures non-specific resistance over a wide spectrum of microbes. EBR quickness can anticipate the real threat, the invasion of pathogenic effectors, so we should learn how certain plants, e.g. tobacco, are able to respond to general elicitors so quickly, by dissecting the most direct pathways to plant factors effecting this type of resistance.

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## Biological control of plant diseases by application of natural, epiphytic living non-pathogenic antagonists to the plant phyllosphere

Only few details are known about the influence of non-pathogenic microorganisms in the phyllosphere of a host plant, in particular the action of non-pathogenic bacteria, which improve the growth or the defense of plants against pathogenic bacteria and fungi. In general, the phyllosphere of plants is a biocoenosis of different non-pathogenic microorganisms, such as bacteria and fungi including yeasts that colonize the host plant without causing significant morphological changes in the appearance of the plant (Beattie and Lindow 1995; Burr et al. 1996). Some of these organisms have the potential to act as antagonists against some plant pathogens, such as fungi. Despite numerous investigations and practical approaches, the biological control of plant diseases by antagonist treatment as natural replacement of fungicides in the phyllosphere is only marginally understood (Barbosa 1998; Elad et al. 2001; Boland and Kuykendall 1997), and this limited knowledge explains failures of alternative methods using natural antagonists instead of excess fungicides (Myers 2000). Although the biological control of pathogenic microorganisms has a great potential to reduce or even to avoid the chemical treatment of crop plants with pesticides, due to the performance, also the acceptance of this alternative approach is still low.

In a previous study it has been shown that the non-pathogenic bacterium *Pseudomonas fluorescens* Bk3 can suppress the conidial germination of the pathogen *Venturia inaequalis* (Kucheryava et al. 1999) and reduce the *in vitro* mycelium growth of *V. inaequalis* (Singh et al. 2004).

Application of the non-pathogenic bacterium *P. fluorescens* Bk3 to the phyllosphere of *Malus domestica* cv. Holsteiner Cox caused the induction of a number of pathogenesis-related (PR) proteins as found in the intercellular washing fluid which was obtained from the apoplast of the leaves. Sequencing of the induced proteins by ESI-Q-ToF mass spectrometry and homology search identified these additional proteins as PR proteins like  $\beta$ -1,3-glucanase, thaumatin-like protein, chitinase and hevein-like protein. In addition we found the decline of a non-specific lipid transfer protein to a non-detectable level within five days.

To confirm these findings a suppressive subtractive hybridization with total RNA from leaves before and after inoculation of *P. fluorescens* Bk3 to the leaves of the host plant was performed and revealed an increased expression level of many PR and stress related genes e.g. ribonuclease-like PR10b, germin-like protein, proteasome subunit alpha type 4, endochitinase class III, heatshock protein 90 and glutathions-S-transferase.

Thus, the present results underline the hypothesis that bacterial antagonists in the phyllosphere of plants, causing no visible morphological alterations of the plant, can initiate the transcriptional activation of plant defence genes. The induction of such transcripts leading to synthesis of PR proteins, which in part are transported into the apoplast, can help to prepare the plant against a pathogen attack.

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## **Studies on induced resistance against common blight of bean caused by *Xanthomonas campestris* pv. *phaseoli***

### **Abstract**

The plant defence activator acibenzolar-S-methyl was assayed for its ability to induce systemic acquired resistance (SAR) against common blight of bean caused by *Xanthomonas campestris* pv. *Phaseoli* (*Xcp*) In greenhouse conditions the bean variety “Red Kidney“ treated with Bion resulted in a marked induction of SAR against bacterial common blight disease. A high decrease of the disease rate of 68% was correlated with a reduction of the bacterial multiplication up to 69.1%. The protection of bean plants was constantly associated with the activation of phenolic compounds. Increased peroxidase activity was induced systemically in leaves and was sustained for at least 8 days.

### **Introduction**

The common bean (*Phaseolus vulgaris* L.) is one of the most important food legumes for direct human consumption in the world (Schwartz and Pastor-Corrales 1989). In Egypt, bean is obviously exposed to a wide range of climatic conditions and several diseases (Lienert and Schwartz 1993), e.g., common blight caused by *Xcp*. It is considered as the most widely distributed and serious disease of bean in warmer areas (Harby 2000).

Disease control is difficult because of the lack of commercially acceptable resistant bean cultivars. One of the potential methods of reducing the severity of disease is the induction of plant resistance against the pathogen. Certain chemicals, such as salicylic acid and 2,6-dichloroisonicotinic acid, potassium salts and amino butyric acid were reported to induce SAR in plants (Oostendorp et al. 2001).

Bion has been developed as a potent inducer of SAR which does not have antimicrobial properties, but instead it increases crop resistance to diseases by activating the SAR signal transduction pathway in several plant species. Induction of SAR by Bion was reported in several host plants against bacterial pathogens (Siegrist et al. 1997; Brisset et al. 2000; Colson-Hanks et al. 2000; Abo-Elyousr et al. 2004).

In this study, the effect of induced resistance of Bion was investigated on the bean variety “Red Kidney” and also its relation to potential changes of peroxidase activity as biochemical marker for SAR against infection with *Xcp*.

### **Material and methods**

**Bacterial isolates and inoculation:** Virulent isolates of *Xcp* were obtained from the stock cultures of Plant Pathology Dept., Faculty of Agriculture, University of Assiut, and were used in all experiments. The inoculum was prepared from early log-phase cells by growing the bacteria in nutrient yeast extract broth in 25 ml sterile tubes, incubated at 25 °C on an orbital shaker at 200 rpm for 24 h. Bacteria were subsequently pelleted by centrifugation at 15000 rpm for 5 min and washed in sterile distilled water. Their concentration was adjusted to 10<sup>8</sup> colony forming units (cfu) by dilution to give the required suspensions at an OD of 660 nm of 0.2. The middle leaf vein was injected with 0.1 ml bacterial suspension (Klement et al. 1990). Control plants were treated similarly with bacteria-free solution of 0.1% saline. Inoculated plants were covered with polyethylene bags for 48 h at 25-27 °C in the greenhouse and daily examined for disease development.

**Plant material:** The greenhouse grown bean variety “Red Kidney” was used for all experiments. Plants were grown in 20 cm diameter pots in a soil mix containing sand, and slow-release fertilizer NPK, at 25 ±5°C with 68-80% RH, light at 5000-14000 lux, and were watered when necessary.

Application of Bion: Bion (50% active ingredient in wettable powder formulation) was dissolved in distilled water. Aqueous solution of Bion was prepared at 0.05 g/100 ml water and sprayed onto whole plants.

To determine the most efficient induction interval time conferred by Bion, bean plants were assigned in equal numbers to five treatment groups. Plants in the first four groups received treatment with Bion and were inoculated with a bacterial suspension at 1, 2, 3, or 4 days after treatment. Plants in the fifth group served as control and were treated with water, then inoculated with the pathogen at the same time intervals. The level of induced resistance in plants against the disease was evaluated at 4, 7, 10 and 14 days after inoculation.

Disease index: Disease index was determined 30 days after inoculation, according to Louws et al. (2001). Percentage of protection was calculated for each treatment (Godard et al. 1999).

Effect of Bion on bacterial multiplication *in vivo*: Cfus were recovered from inoculated bean plants, treated with either Bion or water 3 days before inoculation, by removing 5-mm-diameter leaf discs aseptically from the region of inoculation. Excised discs were homogenized in 1 ml of sterile 0.06% NaCl solution, and were serially diluted. Aliquots of alternate dilutions (0.1 ml) were plated onto King's medium (KB) agar plates. Plates were incubated at 26 °C for 48 h, and emerging colonies were counted on all dilution plates showing bacterial growth. Each dilution from each leaf disc was duplicated.

Preparation of crude enzyme: Bean plants were assigned in equal numbers to four treatment groups. Plants in the first group were treated with Bion and inoculated with the bacterial suspension 2 days after treatment. Plants in the second group were treated with water and inoculated with the bacterial suspension 2 days after treatment (control). Plants in the third group were treated with water but not inoculated with bacteria; plants in the fourth group were treated with Bion but not inoculated with bacteria. Samples for enzyme extractions from all treatments (inoculated or not) were separately harvested 2, 4, 6 and 8 days after inoculation, weighed and immersed in liquid N<sub>2</sub>. The frozen leaf segments were homogenized in an ice-cooled mortar, using (1:5 w/v) 50 mM potassium phosphate buffer pH 7.0, containing 1 M NaCl, 1% polyvinylpyrrolidone, 1 mM ethylene diamine tetra-acetic acid (EDTA) and 10 mM β-mercaptoethanol. Thereafter, the homogenates were centrifuged at 15000 rpm for 20 min at 4°C and finally the supernatants (crude enzyme extract) were collected. The protein content in the extracts was determined according to Bradford (1976) using the Coomassie<sup>®</sup>-Protein assay reagent.

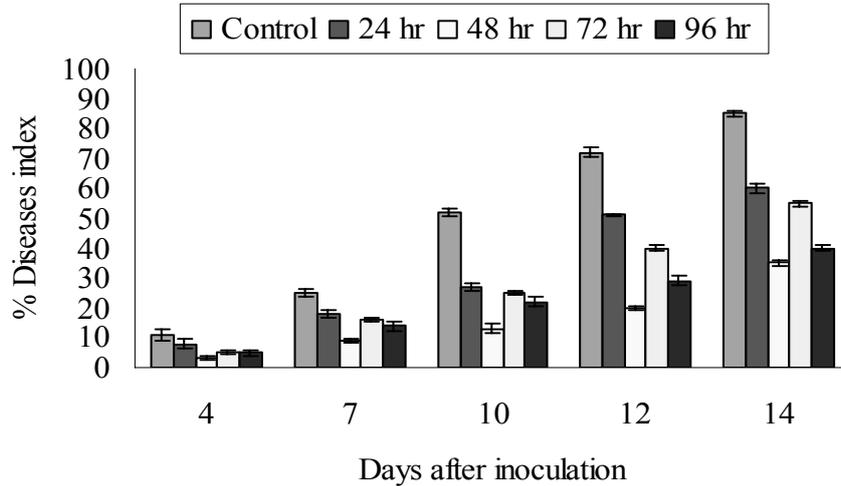
Enzyme assay: Peroxidase activity was determined according to Putter (1974), with guaiacol as a common substrate for peroxidase. The reaction mixture was: 0.2 ml supernatant, 1 ml 0.1 M Na-acetate-buffer pH 5.2, 0.2 ml 1% guaiacol and 0.2 ml 1% H<sub>2</sub>O<sub>2</sub>. The mixture was incubated at 25 °C for 5 min and then measured at 436 nm. Extraction buffer was used as blank. The enzyme activity was calculated according to the change in absorbance and was expressed as enzyme unit/mg protein.

Estimation of total phenol content: Total phenol content was determined according to Rapp and Ziegler (1973): Bean leaves were immersed in liquid N<sub>2</sub>, homogenized in 80% methanol (1g plant material in 10 ml) and stored at -20 °C. Later, the homogenate was centrifuged at 15000 g for 30 min at 4 °C. The pellet was discarded. After addition of ascorbic acid (0.1 g/5 ml) the homogenates were evaporated in a rotary evaporator at 65 °C 3 times for 5 min. The residues were dissolved in 5 ml 80% methanol. For the determination, 0.02 ml methanol extract was incubated for 1h with 0.5 ml Folin Ciocalteu reagent, 0.75 ml of Na<sub>2</sub>CO<sub>3</sub> solution (20%) and 8 ml water. Total phenol content was assayed spectrophotometrically at 767 nm with gallic acid (0-5 mg) as a standard. Methanol was used as blank. Total phenol was estimated as mg gallic acid/g plant material

Experimental design and statistical analyses: All experiments were arranged in a completely randomized split-plot design and repeated twice with 3 replicates, each replicate consisting of four pots. All data were subjected to statistical analysis and means were compared using the L.S.D. test according to Gomez and Gomez (1984).

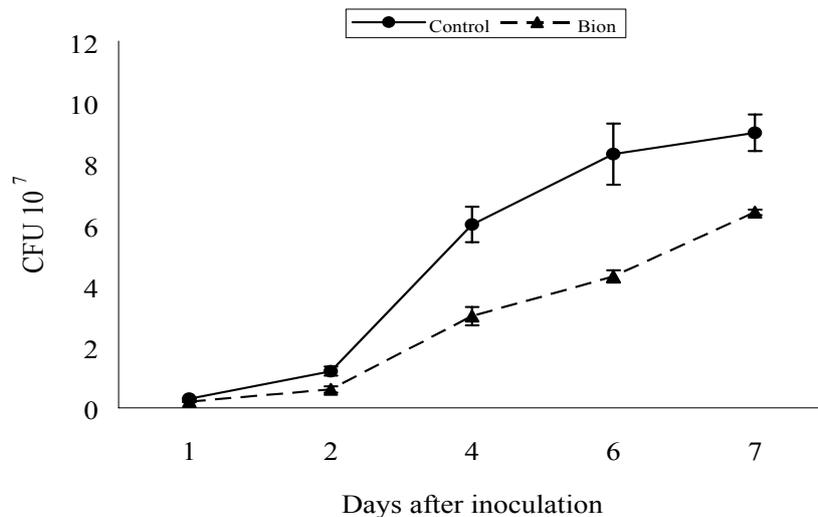
**Results**

The effect of Bion on disease resistance: Results in Figure 1. indicate that bean plants treated with Bion significantly reduced common blight disease incidence compared with controls at all tested interval times. The greatest disease suppression was achieved by Bion treatment 2 days before inoculation. The disease index was reduced by 75% in Bion treated bean plants 10 days after inoculation, which was maintained at the same level until 14 days.



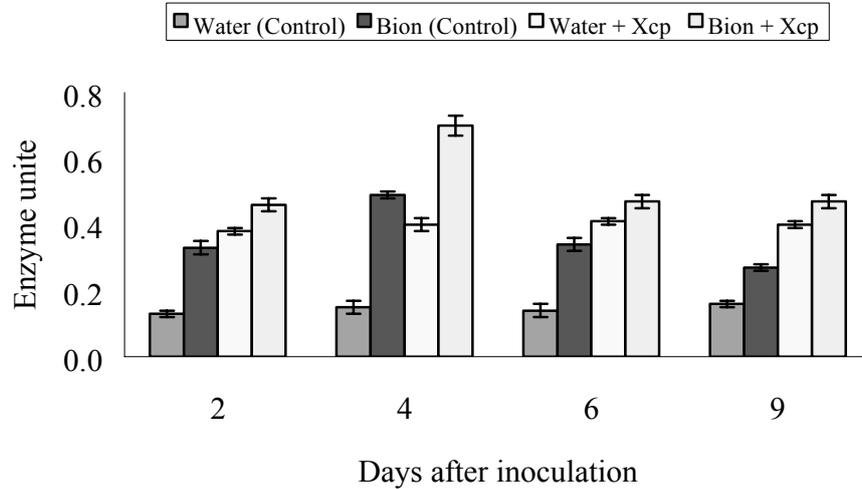
**Fig. 1** Time dependence of the effect of Bion pre-treatment on the severity of common blight disease caused by *Xanthomonas campestris* pv. *phaseoli*. Error bars indicate the standard error

Bacterial multiplication in plants: Results in Figure 2. show that the multiplication rate of *Xcp* was lower in Bion treated than in control plants. This inhibitory effect was first observed 2 days after inoculation monitored until 7 days. The bacterial population was reduced approximately 30, 50, 48 and 28 fold by Bion treatments, compared with control plants, at 2, 4, 6 and 7 days, respectively. Since the lowest disease indices were recorded at a time interval 2 days between treatment and inoculation, this interval was used in order to determine the bacterial growth and enzyme levels.



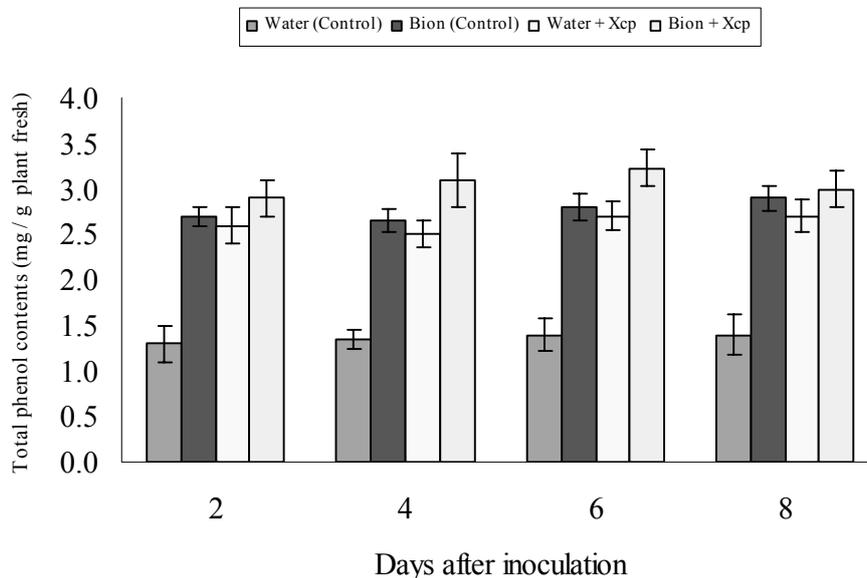
**Fig. 2** Effect of Bion treatment on the number of *Xanthomonas campestris* pv. *phaseoli* in bean leaves. Error bars indicate the standard error

Effect of Bion treatment on peroxidase activity: Results in Figure 3 show that in bean plants treated with Bion, the expression of induced resistance was associated with enhanced peroxidase activity. Plants treated with Bion and inoculation for 2 days with *Xcp* had significantly higher level of PO activity than the three other treatments. Results also indicate that plants treated by Bion alone or without inoculation also exhibited higher increase in PO activity than control plants.



**Fig. 3** Effect of Bion treatment on the induction of peroxidase (PO) activity in bean leaves. Error bars indicate standard error

Total phenol content: Plants treated with Bion had significantly higher levels of phenol content after 2 days from inoculation with *Xcp* than the three other treatments. Results also indicate that treatment with Bion alone without inoculation also increases the phenol content in comparison to control plants (Figure 4).



**Fig. 4** Effect of Bion treatment on the phenol content of bean leaves. Error bars indicate standard error

### Discussion

This study assessed the effect of Bion on bean common blight disease development caused by *Xcp*. Results confirm that application of Bion induces resistance in bean plants, similar to findings against potential fungal, bacterial and viral disease agents (Louws et al. 2001; Buonauro et al. 2002; Anfoka,

2000). Resistance mediated by SAR agents requires an interval before challenge with a pathogen. In most cases this interval was reported to be between 1 to 7 days. In the current study, the best protection against *Xcp* was obtained when Bion was applied 2 days before inoculation. A later challenge with the pathogen did not result in higher disease protection. Such result is comparable to findings by Godard et al. (1999) and Lopez and Lucas (2002) in the cauliflower and cashew pathosystems, respectively.

According to Siegrist et al. (1997), a minimum interval period of 96 h was necessary for Bion or 2,6-dichloroisonicotinic acid (INA) to induce resistance in bean leaves against fungal and bacterial pathogens. (Ishii et al. 1999) reported that Bion induced resistance in cucumber against anthracnose and scab when applied only 3 h prior to inoculation with the pathogens. In cocoa, however, longer interval periods were needed for the development of resistance against *Verticillium* wilt and witches' broom disease with 15 to 30 days prior to inoculation, respectively (Resende et al. 2002).

In greenhouse experiments, the severity of the development of common blight symptoms in bean plants after application of resistance inducers was recorded. For all tested periods after treatment with Bion, the disease index decreased considerably more than in the control plants. These results agree with those reported by Louws et al. (2001), Buonaurio et al. (2002) and Baysal et al. (2003).

*Xcp* populations in treated bean leaves with Bion were considerably lower than in untreated. Reduced bacterial growth was also reported in bean, tobacco, tomato and pepper plants treated with Bion (Cole 1999; Buonaurio et al. 2002; Baysal et al. 2003). This effect may be due to an indirect effect on decreasing nutrients for bacterial growth. A low nutrient concentration in the intercellular space can be a limiting factor for growth of pathogens (Goodman et al. 1986).

Biochemical markers of SAR have not been previously reported in bean plants. We arbitrarily chose peroxidase (PO) and phenol content, whose role in SAR has been demonstrated in a number of plant species. PO participates in cell-wall lignin biosynthesis and the cross-linking of cell wall proteins (Brisset et al. 2000). They are usually related to local defense responses but they have been associated with SAR in several plants such as tobacco (Ye et al. 1989), potato (Chai and Doke 1987) and tomato (Baysal et al. 2003). In bean plants, the present results show a significant accumulation of the enzyme in treated plants and also in treated and inoculated plants. It is known that peroxidase plays an important role in plant resistance against leaf spot causing bacteria (Rudolph 1970). Also Zeller (1985) suggested that the increase in peroxidase activity can be involved in the formation of lignin that inhibits the spread of pathogens in xylem.

Bion-treated bean leaves showed higher phenol content than untreated leaves. These results are in agreement with those reported by Evrenosoğlu et al. (1999) and Zehnder et al. (2001). Accumulation of phenolic compounds at the infection site has been correlated with the restriction of pathogen development since such compounds are toxic for pathogens. Also, the resistance may be increased by pH change of the plant cell cytoplasm due to the increase in phenolic acid concentration, resulting in inhibition of pathogen development (Ojalvo et al. 1987).

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## Potential use of essential oils for plant bacterial disease control

### Abstract

The main ( $\geq 0.7\%$ ) monoterpene and sesquiterpene components of cumin, caraway, coriander and wild fennel essential oils were assayed *in vitro* for antibacterial activity towards two laboratory and 26 phytopathogenic and mycopathogenic bacterial species. Among oxygenated monoterpenes a considerable antibacterial activity was shown by phenol and alcohol oil components such as carvacrol, eugenol, dihydrocarveol, geraniol, nerol and linalool. A lower activity was observed in the case of ketones, aldehydes, ethers and esters as well as non-oxygenated monoterpenes. Some of the latter substances did not inhibit, also when assayed at high concentration, the growth of some of the target bacterial species. The sesquiterpene caryophyllene did not show any bactericidal activity even when 10,000  $\mu\text{g}$  of the substance were used. Preliminary results showed that eugenol, applied to bean seeds, artificially contaminated with *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* (*Xcpcf*), significantly reduced the bacterial population. At the assay concentrations only a limited effect on seed germination was observed.

**Keywords:** Bactericides, phytopathogenic bacteria, mycopathogenic bacteria, essential oil components, monoterpenes, sesquiterpenes, bean seed sanitation.

### Introduction

The control of bacterial diseases of plants is a considerable problem in the agricultural practice because of the limited availability of bactericides and of the ability of a large number of phytopathogenic bacteria to spread, even at long distances, by contaminated and/or infected seeds (Claflin 2003). Besides the antibiotic and copper compounds no other bactericides are available for bacterial plant disease control. Antibiotics are actually forbidden in the agricultural practices in many countries mainly for the possible selection of resistant strains (McManus et al. 2002) and the consequent horizontal transfer of this property to other bacteria either in the phytosphere (i.e. saprotrophs, phytopathogenic bacteria, etc) or in other environmental niches (i.e. human and animal pathogens, etc). The use of copper compounds, because of their general toxicity and mainly for the impact on the environment, is on the way to be restricted and controlled in the European Union (EU rule n° 473/2002).

The above consideration prompts the need for the development of new bactericides and/or alternative methods for the control of plant bacterial diseases to be used in integrated crop management as well as in bio-organic agriculture. Several studies have pointed out the possibility to use the essential oils and/or their components in medical and plant pathology as well as in the food industry for the control of micro-organisms pathogenic to consumers and/or responsible for food spoilage. However, most of the studies are mainly limited to the assessment of the antibacterial activity *in vitro* and the exploitation of essential oils for the control of plant pathogenic bacteria is still in its infancy.

Our recent studies showed the antibacterial activities of essential oils of coriander, wild fennel, caraway and cumin towards two laboratory and 29 phytopathogenic bacterial species as well as responsible of cultivated mushroom diseases (Lo Cantore et al. 2004; Iacobellis et al. 2005). A significant antibacterial activity was shown against Gram-positive and Gram-negative bacteria belonging to *Clavibacter*, *Curtobacterium*, *Rhodococcus*, *Pseudomonas*, *Xanthomonas*, *Ralstonia*, *Erwinia* and *Agrobacterium* genera, responsible for several plant and mushroom diseases. A much weaker effect was observed for the wild fennel oil (Lo Cantore et al. 2004). Furthermore, the above studies showed the complex composition of the above four essential oils (Lo Cantore et al. 2004; Iacobellis et al. 2005). However, how the components account for the observed activities is still unknown.

The objective of this study was to evaluate the antibacterial activity *in vitro* of the main components of essential oils towards bacteria responsible for diseases on plants and cultivated mushrooms and to evaluate the possible use of the most active compounds for seed sanitation.

### Materials and methods

**Chemicals:** The oxygenated monoterpenes carvacrol, eugenol, dihydrocarveol, geraniol, nerol, linalool, carvone, fenchone, canphor, t-dihydrocarvone, cuminaldehyde, anethole, geranylacetate,  $\alpha$ -pinene,  $\beta$ -pinene, limonene, p-cymene,  $\gamma$ -terpinene, the sesquiterpene caryophyllene, cycloheximide and the antibiotics rifampicin and tetracycline were obtained from Sigma-Aldrich (Milan, Italy).

**Bacterial cultures:**

One to three strains of the 28 bacterial species and/or pathovars reported in Table 1 were used. Subcultures were obtained by growing bacteria for 48 - 72 h on the medium B of King (KB; King et al. 1954) in the case of pseudomonads and on WA (Koike 1965) for the other bacteria.

**Disc diffusion assay:** Ten  $\mu$ l 1:1 serial dilutions in methanol of stock solutions of essential oil components and of 1.6 mg/ml of rifampicin were added to 6 mm diameter sterile blank disks previously deposited onto the surface of Petri plates, containing 10 ml of KB or WA (0.7% agar) depending on the bacterial species. Aliquots of target bacteria suspensions were added to the above media, maintained at 45°C, to obtain a final population of about  $10^7$  cfu/ml. After 48 h incubation at 25°C the Minimal Inhibitory Quantity (MIQ), expressed in  $\mu$ g, which causes an apparent inhibition zone around the 6 mm diameter disks, was recorded. The assays were performed twice with three replicates.

**Bean seed treatments:** Nine groups of 300 bean seeds were treated for 3 min with 1% sodium hypochlorite, washed three times with sterile distilled water and then dried under an air flow at room temperature for 20 min. Subsequently seeds were immersed for 20 min in an about  $10^8$  cfu/ml bacterial suspension of a natural mutant, resistant to rifampicin, of *Xcpf* strain ICMP239 and then dried as above. Then each group was immersed for 20 min at room temperature in eugenol emulsions (1, 2, 4 and 8 mg/ml) and tetracycline solutions (50, 100 and 200  $\mu$ g/ml) in sterile distilled water with 0.01% Tween 20. Bean seeds not treated or treated with sterile distilled water with 0.01% Tween 20 were used as controls.

After treatment each seed group was dried and divided in three subgroups of 100 seeds each. Then seeds were put in 500 ml Erlenmeyer flasks containing 100 ml of sterile distilled water with Tween 20 (0.01%) under agitation for 1 h at 25°C. Aliquots of 100  $\mu$ l of decimal dilutions of bacterial suspension were streaked on KB Petri plates with rifampicin (100 ppm) and cycloheximide (100 ppm). After 5 d incubation at 25°C the density of the bacterial population was determined. Treatments were performed twice with three replicates.

**Determination of seed germination:** Groups of 100 bean seeds were treated as described above and then for germination put on filter paper soaked with distilled sterile water in sterile trays. After 24, 48 and 72 hours incubation at 25°C the number of the germinated seeds was determined. The germination rate was expressed in percentage. The assays were performed twice with three replicates.

### Results

**Disc diffusion assay:** Most of the essential oil components inhibited the growth of the majority of the bacteria (Gram + and Gram -) used in this study, although the MIQ was different among the substances depending on the target bacterial species and/or pathovars. Among oxygenated monoterpenes phenols and alcohols showed the higher bactericidal activity inhibiting the growth of all bacterial strains, whereas a lower activity was found of ketones, aldehydes, ethers and esters, which inhibited the growth of only a part of the target bacteria. The non-oxygenated monoterpenes had lower activity and only against a limited number of the target bacteria (Table). The sesquiterpene caryophyllene had no bactericidal activity even when 10,000  $\mu$ g of the substance were used (Table 1). The MIQ of the antibiotic rifampicin, determined against a representative number of the bacterial species and/or pathovars reported in Table 1, was between 1 and 4  $\mu$ g for strains of the fluorescent pseudomonads and less than 1  $\mu$ g for

strains of *Xcp* and the Gram-positive *Clavibacter michiganensis* subspecies and *Curtobacterium flaccumfaciens* pathovars.

**Table** Minimal Inhibitory Quantity (MIQ) ( $\mu\text{g}$ ) of essential oil components on selected Gram-positive and Gram-negative phytopathogenic and mycopathogenic bacteria

Bacteria	N° of strains	MIQ ( $\mu\text{g}$ ) <sup>a</sup>							
		Oxygenated monoterpenes						non-oxygenated monoterpenes	Sesqui-terpene
		Phenols	Alcohols	Ketones	Aldehyde	Ether	Ester		
<i>Escherichia coli</i>	1	78÷312	78÷312	625÷NA	NA	NA	NA	5,000÷NA	NA
<i>Pseudomonas syringae</i> pathovars (n. 9)	19	19÷1,25	39÷468	234÷NA	625÷NA	1,25÷NA	2,500÷NA	1,250÷NA	NA
<i>Pseudomonas</i> spp. (n. 6)	7	19÷625	39÷625	312÷NA	625÷NA	5,00÷NA	NA	2,500÷NA	NA
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i>	1	39÷312	39÷156	312÷2,50	125	1,25	NA	2,500÷NA	NA
<i>Agrobacterium tumefaciens</i>	1	39÷312	39÷312	312÷NA	NA	1,25	NA	5,000÷NA	NA
<i>Burkholderia gladioli</i> pv. <i>agaricicola</i>	1	39÷312	39÷312	1,25÷NA	NA	5,00	NA	2,500÷NA	NA
<i>Ralstonia solanacearum</i>	1	39÷312	156÷312	625÷5,00	5,00	1,25	5,000	2,500÷NA	NA
<i>Xanthomonas campestris</i> pathovars (n. 4)	10	19÷312	39÷260	520÷3,33	1,25÷2,50	2,50÷NA	5,00÷NA	1,250÷NA	NA
<i>Curtobacterium flaccum-faciens</i> pathovars (n. 2)	2	58÷781	78÷468	937÷3,75	937	NA	2500	5,000÷NA	NA
<i>Clavibacterium michiganensis</i> ssp. <i>michiganensis</i>	2	39÷468	234÷625	625÷3,75	937	NA	5000	2,500÷NA	NA
<i>Bacillus megaterium</i>	1	19÷312	39÷312	625÷5,00	2,500	NA	5000	2,500÷NA	NA

<sup>a</sup> MIQ, average quantity needed for the bacterial growth inhibition. NA, the deposition of 10,000  $\mu\text{g}$  of component essential oils on sterile blank disks did not lead to an inhibition zone

**Bean seed treatments:** Treatment of bean seeds with eugenol caused a significant reduction of the bacterial population on seeds contaminated (about  $2.7 \times 10^6$  cfu/seed) with a natural mutant resistant to the antibiotic rifampicin of *Xcpf* ICMP239. In particular, treatment with 1, 2, 4 and 8 mg/ml eugenol caused a significant reduction of the bacterial population which resulted in  $2.5 \times 10^5$ ,  $1.6 \times 10^5$ ,  $2.2 \times 10^4$  and  $5.2 \times 10^3$  cfu/seed, respectively. The treatment with sterile distilled water with 0.01% Tween 20 caused a limited reduction to  $5.4 \times 10^5$  cfu/seed. Treatment with the antibiotic tetracycline was less effective in the reduction of the bacterial population on bean seeds. In particular, the bacterial populations after treatment with tetracycline solutions containing 50, 100 and 200  $\mu\text{g}/\text{ml}$  were, respectively,  $1.8 \times 10^5$ ,  $1.6 \times 10^5$  and  $1.2 \times 10^5$  cfu/seed, which were not different from those obtained after seed treatment with sterile distilled water with Tween 20 (0.01%).

Treatment of bean seeds with different concentrations of eugenol caused a reduction of the germination but the effect was statistically different from the control only when the concentration of eugenol was equal to or higher than 4 mg/ml. In particular, the germination rate of bean seeds after the treatment with 1 mg/ml of eugenol was similar to that observed in the case of the control treatments; a limited reduction of the seed germination (2-3%) was obtained after the treatment with 2 mg/ml of eugenol. A germination

reduction of 7% and 16% was observed after bean seed treatment with 4 and 8 mg/ml of eugenol, respectively.

## Discussion

Data reported in this paper indicate that the antibacterial activity of components of essential oils is different and is correlated with their chemical structures. Oxygenated monoterpenes containing phenol and alcohol functions were more active than those containing aldehyde, ketone, ether and ester functions as well as of non-oxygenated monoterpenes and sesquiterpenes. It is well known that the presence of alcoholic or ketonic groups and double bonds improve the biological activity of these molecules (Griffin et al. 1999). In particular, the antibacterial activity appears mainly correlated with the amphipathic nature of the considered substances which is in accord with the already known effect of the essential oils on cell membrane functions (Uribe et al. 1985; Sikkema et al. 1994; Ultee et al. 1999; Lambert et al. 2001). However, it is not excluded that under the conditions of the agar diffusion assay the different solubility of the substance in the aqueous media such as the agar layer may be important. Further studies for the development of alternative antimicrobial assays may be useful. The above results indicate that the antibacterial activity of the essential oils may depend on the relative concentration of the active components. However, possible chemical interaction among the components is not excluded with synergic and/or antagonistic effects. Studies aimed to assess the antibacterial activity of pure components in different combination are necessary.

The MIQs of the essential oils were higher when compared to those found with the antibiotic rifampicin on the same target bacteria. The MIQs of phenol and alcohol derivatives, and aldehydes, ethers, ketones, esters, and non-oxygenated monoterpenes were 10 to 150 and about 1,000 fold higher than those caused by the antibiotic, respectively. The different specific activity caused by the more active essential oils is not surprising since the antibiotic and the substances in consideration have different mechanisms of action. Rifampicin inhibits the RNA-polymerase biosynthesis and hence protein biosynthesis (Buss et al. 1978), while the essential oil components interfere with the cell membrane functions (Uribe et al. 1985; Sikkema et al. 1994; Ultee et al. 1999; Lambert et al. 2001).

The application of eugenol to bean seeds, artificially contaminated with a strain of *Xcpf*, strongly reduced the bacterial population on the seeds. The application of 4 and 8 mg/ml eugenol caused an about 100 to 1,000 fold reduction of the pathogen population per seed. However, the seed contamination used in this study is too high and the real efficacy of the method here reported needs to be determined on bean seeds contaminated by a lower number of bacteria similar to natural contamination. It is not excluded that in these conditions more efficient seed sanitation may be obtained.

The statistical reduction of bean seed germination after treatment with higher concentrations of eugenol is not surprising since this feature of essential oils was already reported (Asplund 1968; Reynolds, 1987; Oosterhaven et al. 1995). However, the small effect on bean seed germination, mainly observed at high concentrations, indicates the possible amelioration of the sanitation method either by the modulation of concentration of the solution, exposure time, as well as the way of application of the substances to seeds.

In conclusion, essential oils and pure components appear to be good bactericides alternative to antibiotics, although the specific activity of the former are higher, for the control of bacterial diseases of plants. Of particular interest is the possible use for seed sanitation of the above substances, as demonstrated in this study.

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## **Rhizobacteria-mediated induced systemic resistance against various plant pathogens and its mechanisms of action**

### **Abstract**

Treatment with *Bacillus amyloliquefaciens* strain EXTN-1 showed a broad disease-controlling spectrum to the plant diseases caused by viral, bacterial, and fungal pathogens resulting in plant growth promotion. When *B. amyloliquefaciens* strain EXTN-1 was drenched to lettuce, grown in a hydroponic system, the population of *B. amyloliquefaciens* strain EXTN-1 remained similar or increased in the rhizosphere, while its population gradually decreased to one tenth in the hydroponic solution within 4 weeks after treatment, compared with the initial population. Treatment with EXTN-1 enhanced the oxidative burst in early stages and induced the expression of resistance genes PR-1a and PDF1.2. The mechanism involved in induced systemic resistance by EXTN-1 was revealed as simultaneous activation of salicylic acid (SA) and jasmonic acid (JA) or ethylene metabolic pathways; pre-treatment with EXTN-1 reduced germination and appressorium formation of conidia of *Colletotrichum orbiculare* on leaves of cucumber and increased callus formation. Furthermore, treatment with EXTN-1 inhibited the bacterial wilt on tomato caused by *Ralstonia solanacearum* for 4 weeks after treatment. EXTN-1 increased the plant height of three barley varieties and shortened heading stage of two varieties compared with non-treated controls. Taking together the above results, *B. amyloliquefaciens* strain EXTN-1 can be considered as a promising agent for practical application.

### **Introduction**

Rhizobacteria are present in large numbers in the rhizosphere, where plant exudates and lysates provide nutrients. Certain indigenous strains of rhizobacteria are referred to as plant growth-promoting rhizobacteria (PGPR), because they can enhance the health and productivity of crop plants (Kloepper et al. 1980). Therefore, PGPR are one type of soil-living bacteria that have many beneficial effects on crops.

By pre-inoculation with PGPR plants can also activate defence mechanisms to protect themselves against attack of various pathogens. In such plants, various products and mechanisms are activated, which are encoded by host defence genes, e.g., those for cell wall modifications such as lignification, accumulation of secondary metabolites, such as phytoalexins, phenolics or primary gene products, PR proteins and other defence-related proteins (Collinge et al. 1994). This induced systemic resistance (ISR) mimics the original resistance regulated by dominant single *R* genes and exhibits a broad application spectrum, including fungal, bacterial, viral, and nematode diseases. ISR-mediated plant protection is generally induced by colonization of the root with biocontrol rhizobacteria (van Loon et al. 1998). A series of elegant studies to elucidate the mechanisms of ISR have been continuously carried out using a strain of *Pseudomonas fluorescens* to colonize *Arabidopsis* roots (Knoester et al. 1998; Pieterse et al. 1996; Ton et al. 1999).

In previous reports we showed some biological properties of the selected rhizobacterium *B. amyloliquefaciens* (strain EXTN-1) with respect to ISR-mediated cucumber anthracnose disease controlling activity and expression of defence-related genes including *PR-1a* (Jeun et al. 2001; Park et al. 2001). The characterization of expression patterns of defence-related genes in a plant that is preconditioned with systemic resistance responses could confer direct evidence towards understanding the protective changes induced by *B. amyloliquefaciens*. Various beneficial effects on plants were produced by *B. amyloliquefaciens*; the mechanisms for these effects were proposed as well as their interactions (Park et al. 2001; Ahn et al. 2002). The purpose of this study was to determine whether *B. amyloliquefaciens* has a similar effect on various crops in the field.

## Material and methods

**Bacterial isolates and plant source:** *B. amyloliquefaciens* (strain EXTN-1) used in these experiments was demonstrated to induce systemic resistance for control of multiple pathogens (Park et al. 2001). *Erwinia carotovora* SCC1 was used for challenge inoculation on *Nicotiana tabacum* cv. Xanthi-nc. All bacterial strains were maintained at -80°C in tryptic soy broth amended with 20% glycerol. For detection of the mechanisms of action the following genes and mutants were investigated: resistance genes *PR-1a* and *PDF1.2* of *Arabidopsis thaliana* (Columbia wild type Col-O), and the *A. thaliana* mutants encoding salicylate hydrolase *nahG*, ethylene insensitivity *etr1*, and jasmonate responsiveness *jar1*.

**Oxidative burst:** The autophotogram was determined by using chemiluminescence-triggered luminol and X-ray film. Cucumber stem slices were inoculated with 20 µl bacterial cell suspension and then 20 µl luminol (100 µl). An X-ray film was exposed on the slices for 24 h after inoculation. One mM verapamil, 100 µM DPI, 2 mM Tiron and 100 units of catalase were applied as internal inhibitors for detection of the oxidative burst.

**Fluorescence microscopical observations:** For microscopical observation, the infected leaves were fixed with 2% glutaraldehyde 3 days after the challenge inoculation. For staining of callose and fungal structures the leaf disks were treated with 0.005% aniline blue and 0.02% Uvitex 2B, respectively.

**Preparation of bacterial endospore exudates:** *B. amyloliquefaciens* (strain EXTN-1) was grown on TSB at 28 °C for isolation of endospores. The endospores were harvested, washed with distilled water by stepwise centrifugation after 20 days, adjusted to 10<sup>10</sup> cfu/ml and autoclaved at 121 °C for 15 min. The supernatant of EXTN-1 was isolated by centrifugation at 8000g for 5 min at 4 °C.

**RNA hybridization analysis:** Total RNA isolated by the phenol/SDS method (Shirzadegan et al. 1991) was size-separated on formaldehyde-1% agarose gels and transferred to a nylon membrane (Hybond-N, Amersham, USA). The blots were hybridized with PR- and PDF 1.2 probes that had been labeled with the DIG (Digoxigenin) DNA Labeling Kit (Roche Diagnostic) 2 days after challenge inoculation.

**Protection of tobacco plants by infiltration of the exudates:** 100 µl of isolated exudates (10<sup>-1X</sup> to 10<sup>-4X</sup>) and distilled water as control treatment was infiltrated into the first bottom leaf of tobacco plants at 20 day-old-seedlings. Five days later, a bacterial suspension (2.0 x 10<sup>7</sup> cfu/ml) of *E. carotovora* in 0.02% Silwet L-77 was applied on the plants. Plants were then placed in a humid chamber (RH 100%) for 24 h prior to placing on a greenhouse bench. Disease severity per plant was recorded.

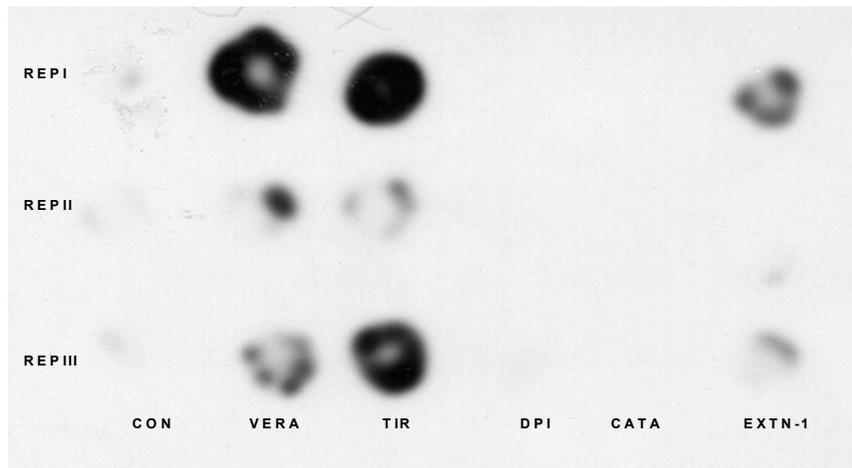
**Plant growth promotion of lettuce and rhizosphere colonization:** Lettuce seeds (*Lactuca sativa* cv. Jukchima) were sown on seed bed plug after surface disinfection and 14-day-old seedlings were transplanted in hydroponic systems, controlled by a circulation system. *B. amyloliquefaciens* strain EXTN-1 was grown overnight on TSA at 28 C° as described by Park and Kloepper (2000). The bacterial suspension was adjusted to 10<sup>6</sup> cfu/ml of hydroponic solution. For the examination of the bacterial population, plant roots and the hydroponic solution were sampled at 7 day intervals up to 4 weeks after inoculation.

**Plant growth promotion of barley:** Three kinds of main varieties of barley seeds (*Hordeum vulgare* cv. Jinyang, Bunong, Olbori) were soaked in the bacterial suspension of *B. amyloliquefaciens* strain EXTN-1 (10<sup>6</sup> cfu/ml) in 0.1% sodium alginate solution for 2 h before planting, with 3 treatments per variety and with 3 replicates each. The experiments started in early November and ended in late June in both years (2002-2003).

**Tomato plant protection against *Ralstonia solanacearum*:** Four *Bacillus* strains, EXTN-1, *B. subtilis* strain 816-6, *B. pumilus* strain 228-7, *Bacillus* sp. 111-3, and *Paenibacillus polymyxa* strain H32-5 were used in this study. The strains were identified by analysis of their fatty acid pattern using gas chromatography. All strains were screened and selected for antimicrobial activity against *Ralstonia solanacearum*. Bacterial strains were kept at -80°C in TSB with 20% glycerol. To investigate the suppressive effect of EXTN-1 on bacterial wilt, the suspension of *R. solanacearum* was drenched on the root of 20-day-old tomato plants (*Lycopersicon esculentum* cv. Coco) at 7 days after treatment of four bacterial suspensions including strain EXTN-1 (10<sup>6</sup>cfu/ml) in the hydroponic system. All plants were grown in a hydroponic perlite system using circulating chambers.

## Results and discussion

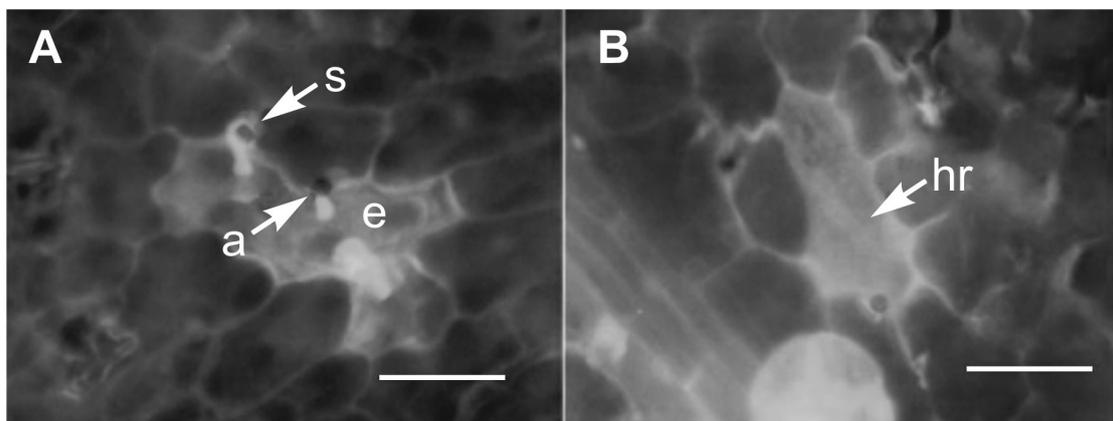
**Oxidative burst:** An oxidative burst was observed when plants were treated with EXTN-1 and 0.5 mM salicylic acid without AOS (active oxygen species) scavenger, but not in water controls (Figure 1). The EXTN-1-induced oxidative burst was not inhibited by treatment with verapamil, a modulator of calcium channels, which are anchored in the plasma membrane, and not by the membrane-permeable radical scavenger, Tiron; in contrast, the NADPH oxidase inhibitor, dibenziodolium (DPI), nearly completely inhibited, as well as the superoxide scavenger, catalase. These results indicate that EXTN-1 triggers defence systems *via* an NADPH oxidase-dependent oxidative burst rather than *via* Ca<sup>2+</sup> influx through plasma membrane-anchored calcium gates.



**Fig. 1** Induction of oxidative burst in EXTN-1 treated cucumber tissues. CON, control; VERA, verapamil; TIR, tiron; DPI, dibenziodolium; CATA, catalase

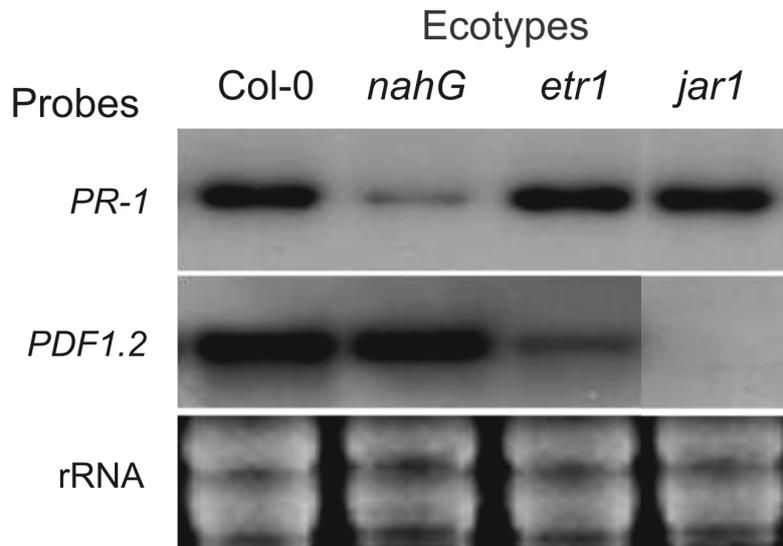
A radical scavenger, tiron may trap O<sub>2</sub><sup>-</sup> and OH<sup>-</sup>, but not H<sub>2</sub>O<sub>2</sub> suggesting that systemic oxidative burst by EXTN-1 seems to be due to hydrogen peroxide (Figure 1).

**Fluorescence microscopical observation:** Histological studies showed that the rate of appressorium formation was not decreased in the leaves of EXTN-1 pre-inoculated plants. However, callose formation was generally enhanced in the leaves of EXTN-1 treated plants compared to the control (data not shown). Furthermore, a hypersensitive reaction (HR) was frequently observed in epidermal cells of the leaves expressing ISR (Figure 2B). Histological observations suggest that ISR may be expressed in epidermal cells showing intensive callose formation and HR against fungal invasion (Figure 2).



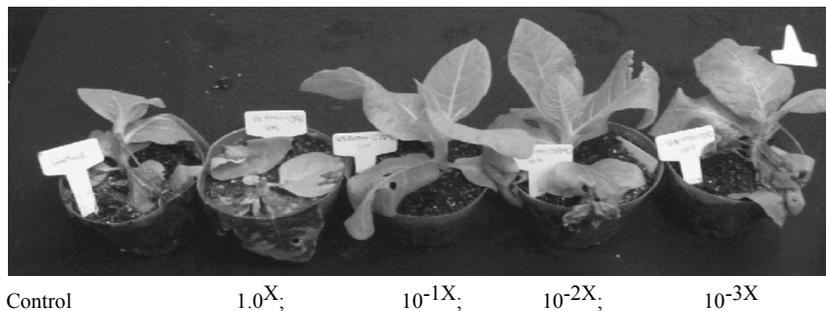
**Fig. 2** Penetration sites on the leaves of cucumber plants inoculated with *Colletotrichum orbiculare* 3 days later. (A) Non-treated control leaf and (B) pre-inoculated leaf with EXTN-1. All bars = 20 μm. a, appressorium; e, epidermal cell; hr, hypersensitive reaction; s, spore

RNA hybridization analysis: *B. amyloliquefaciens* strain EXTN-1 triggers both *PR-1* and *PDF1.2* in *A. thaliana* (Col-O wild type). The *nahG* mutant did not express the activation of *PR-1* by bacteria upon root inoculation; however, *etr1* and *jar1* mutants resulted in strong expression of *PR-1*. In contrast, the *nahG* mutant resulted in low level of *PDF1.2* and did not affect *PR-1* expression by treatment with EXTN-1 (Figure 3). Whereas SAR requires accumulation of SA in plants, ISR is dependent on intact plant responses to JA and ethylene. JA and ethylene are produced together with SA during pathogen-induced necrotizing reactions giving rise to SAR, but, in contrast to SA, they are not involved in the establishment of SAR (Pieterse et al. 1998). When these different signal transduction pathways are triggered simultaneously in *A. thaliana*, disease suppression is enhanced (van Wees et al. 1999, 2000). EXTN-1 could induce systemic resistance based on both signal transduction pathways. When the endospore exudate from EXTN-1 was infiltrated in *A. thaliana* (Col-O wild type), *PDF1.2* and *PR-1* were expressed simultaneously (Figure 3).



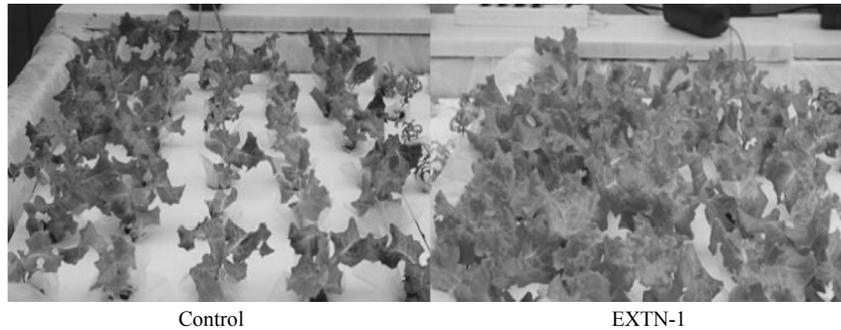
**Fig. 3** Activation of *Arabidopsis PR-1* and *PDF1.2* in response to pretreatment with EXTN-1 bacteria (*nahG*: SA deficient; *etr1*: ethylene insensitive; *jar1*: affected in JA response; col-0: wild type *Arabidopsis*).

Protection of tobacco plants by infiltration with EXTN-1: In greenhouse tests, endospore exudates significantly suppressed the development of soft rot of tobacco plants, caused by *Erwinia carotovora*. The maximum control value was recorded by infiltration of  $10^{-1X}$  to  $10^{-2X}$  treatments. Water and 1X treatment of endospore exudates did not induce systemic resistance (Figure 4). When endospore exudates of EXTN-1 were infiltrated in *Arabidopsis*, *PR-1* and *PDF1.2* genes were expressed and systemic resistance against *E. carotovora* was induced (data not shown). This result suggests that EXTN-1 triggered unknown bacterial components from endospores of EXTN-1.

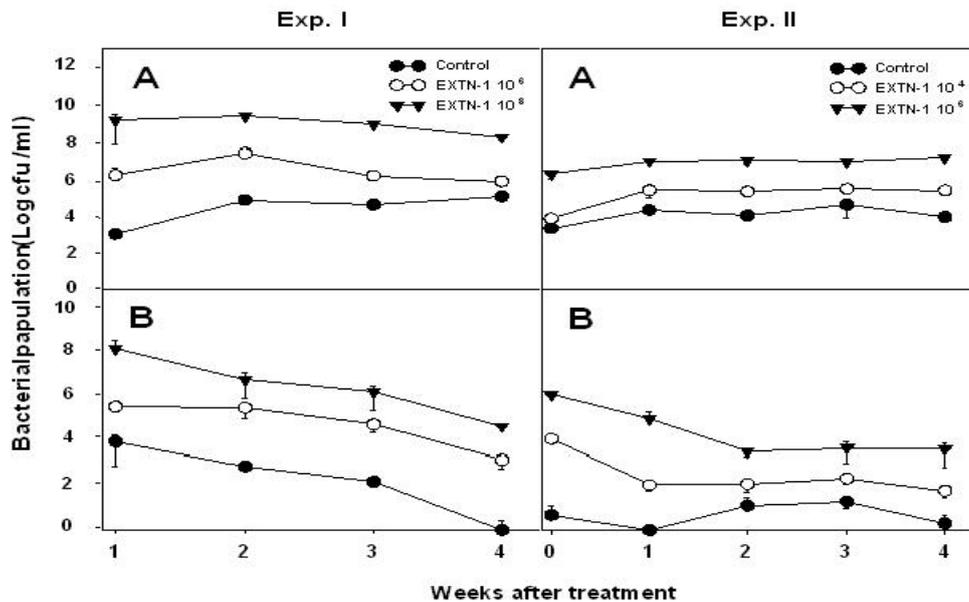


**Fig. 4** Suppression of soft rot disease caused by *Erwinia carotovora* SCC1 in tobacco by treatment with bacterial endospore exudate of *Bacillus amyloliquefaciens* EXTN-1.

Plant growth promotion of lettuce and rhizosphere colonization: Lettuce plants treated with EXTN-1 showed considerable growth promotion compared to the untreated controls (Figure 5). When EXTN-1 was drenched to lettuce grown in hydroponic systems, the population of EXTN-1 was similar or increased in the rhizosphere compared to the initial population, while the population gradually decreased to one tenth in the hydroponic solution 4 weeks after treatment (Figure 6). In another experiment, we found that induced systemic resistance and plant growth promotion activity of EXTN-1 were stronger in cool than in the summer season.



**Fig. 5** Plant growth promotion of lettuce by treatment with *B. amyloliquefaciens* strain EXTN-1 in a hydroponic system.

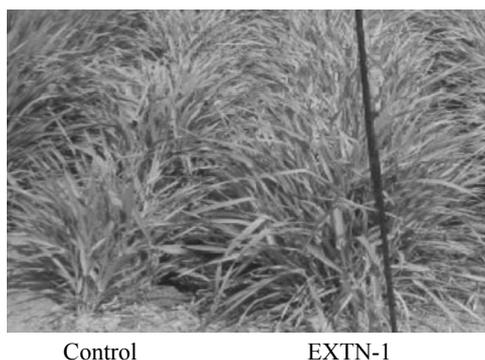


**Fig. 6** Change of the population density of *B. amyloliquefaciens* EXTN-1 in the rhizosphere of lettuce and in hydroponic solution (A: root colonization, B: hydroponic solution).

Plant growth promotion of barley: Treatment by EXTN-1 increased plant height of three barley varieties and shortened the heading stage of two varieties, except Jinyang, compared with the non-treated control (Table 1 and Figure 7).

**Tab. 1** Plant growth promotion of barley by seed treatment with *B. amyloliquefaciens* strain EXTN-1 in the field

Treatment	Plant height (cm)			No. of heading / 50plants		
	Jinyang	Bunong	Olbori	Jinyang	Bunong	Olbori
Control	70.8	65.2	73.8	0	1.7	2.1
EXTN-1	74.5*	72.1*	79.2*	0	11.4*	3.2
EPS	62.4*	72.1	81.7*	0	21.3*	3.2
LSD ( $p=0.05$ )	3.4	5.4	3.1	0	3.9	2.3



**Fig. 7** Plant growth promotion of barley by seed treatment with *B. amyloliquefaciens* strain EXTN-1 in the field (var Bunong)

Tomato plant protection against *Ralstonia solanacearum*: Bacterial wilt of tomato caused by *R. solanacearum* was significantly suppressed by treatment with EXTN-1 up to 3 weeks in a hydroponic system (Table 2). In plastic green house, treatment with EXTN-1 resulted in control of *R. solanacearum* on tomato plants until one month with application only to seedlings and decreased the incidence of powdery mildew and bacterial wilt.

**Tab. 2** Tomato plant protection against *R. solanacearum* causing bacterial wilt by rhizobacterium *B. amyloliquefaciens* EXTN-1 in hydroponics

Treatment	Diseased plant (%)				
	Trial I			Trial II	
	12 DAT	20 DAT	30 DAT	12 DAT	20 DAT
816-6	0.0 b	15.0 d	80.0 b	8.0 a	16.7 a
228-7	0.0 b	50.0 b	75.0 b	0.0 b	0.0 d
111-3	0.0 b	5.0 e	90.0 a	0.0 b	14.7 b
H32-5	0.0 b	25.0 c	80.0 b	8.3 a	8.3 c
EXTN-1	0.0 b	15.0 d	65.0 c	0.0 b	0.0 d
Control	15.0 a	85.0 a	95.0 a	8.3 a	16.7 a

In conclusion, *B. amyloliquefaciens* strain EXTN-1, as tested in the present experiments, can be used for plant growth promotion and improving product quality of vegetables as well as inducing systemic resistance in vegetables to plant pathogens. Furthermore, treatment with EXTN-1 reduced the incidence of tomato blossom rot caused by calcium deficiency in the high temperature season, which indicates that application of EXTN-1 improves the physiological status of plants.

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## Induction of oxidative burst in tomato leaves treated with unsaturated fatty acids of turtle oil (*Caretta caretta*) against *Pseudomonas syringae* pv. *tomato*

### Abstract

Tomato bacterial speck disease caused by the bacterium *Pseudomonas syringae* pv. *tomato* (*Pst*) is effectively controlled on tomato (*Lycopersicon esculentum* Mill.) by foliar spray of unsaturated fatty acids. Effecting induced resistance by different unsaturated fatty acids (UFAs) was investigated with three-week old plants. The plants were inoculated with 10<sup>8</sup> cfu/ml bacterial suspension and bacterial growth was evaluated 48 h post inoculation. Resistance induced by UFAs showed a suppressive effect against *Pst*, and was correlated with rapid resistance response of treated leaves challenged with pathogens by day 1 after treatment. The bacterial multiplication significantly decreased in decosohexaenoic acid and eicosapentaenoic acid treated plants by 2 days post inoculation. In order to understand the cause of the suppressive effect of UFAs, H<sub>2</sub>O<sub>2</sub> generation and NADPH oxidase activity were investigated in the early phase of inoculation. In these studies, the plants, sprayed with linoleic acid, decosohexaenoic acid and eicosapentaenoic acid, accumulated H<sub>2</sub>O<sub>2</sub> and had elevated activity of NADP(H) oxidase. However, oleic acid failed to show higher NADP(H) oxidase activity, while it increased by linoleic acid, decosohexaenoic acid and eicosapentaenoic acid treatment. These findings indicate the lack of suppressive effect of exogenous oleic acid application in plant defence pathways. No considerable changes were observed in water-treated plants. The findings suggest that linoleic acid, decosohexaenoic acid and eicosapentaenoic acid activate plant defence mechanisms, and the treatments with UFAs lead to induction of active oxygen species, acting as mediators of plant immunity against the bacterial pathogen.

### Introduction

*Pst* is the causal agent of bacterial speck disease in tomato. This disease is of moderate economic importance to tomato production under greenhouse or field conditions and is disseminated primarily by water. It has the potential to rapidly move through plug greenhouses; thus, infested seedlings could become an important inoculum source for field epiphytes. The frequent use of copper compounds leads to resistant bacterial strains making the control of the disease more difficult. In addition, copper contributes to environmental and water pollution. Therefore alternative and efficient control methods should be improved, particularly in greenhouse crops in southern Turkey.

Recent studies on defence signalling pathways revealed that induced plant defence against microbial pathogens is regulated by a network of interacting signalling pathways in which plant signal molecules, such as salicylic acid, jasmonic acid (JA), and ethylene, play a dominant role (Feys and Parker 2000). However, fatty acids and oligosaccharides (Kobayashi et al. 1993; Sticher et al. 1997) are also known to be effective inducers of plant resistance to various diseases.

Plants have developed a complex protection system to cope with pathogen attack. Active oxygen species (AOS) and enzymatic systems govern their metabolism. H<sub>2</sub>O<sub>2</sub> plays an important role in pathogenesis (Baker et al. 1995). Rapid generation of AOS is considered to be an important component of the resistance response of plants to pathogen challenge. AOS intermediates can serve as direct protective agents by their toxicity or by their ability to prevent pathogen ingress by enhancing the cross-linking of the cell wall (Baker and Orlandi 1995). A burst of AOS is known to be involved in local cell death as part of plant defence against pathogens. The hypersensitive response is characterised by the rapid production of active oxygen species referred to as the oxidative burst, which prevents further spread of

the pathogen, and by programmed cell death. NADPH oxidase, which catalyses the generation of O<sub>2</sub><sup>-</sup>, is responsible for the oxidative burst (Slusarenko 1996).

In recent years, a new approach using oils for controlling destructive plant pathogens has been developed and an emulsion of fish oil stimulated the host defence against some bacterial diseases (Abbasi et al. 2003). In other studies fatty acids were suggested as signal molecules involved in activation of defence responses in plants and its relation with other signalling compounds such as jasmonic acid were described (Itoh et al. 2002). On the other hand, sea turtle oil is well known among Mediterranean fishermen as an effective cure for wounds and our previous study indicated that turtle oil treatment resulted in lower bacterial growth, related to the increase of LPOX and peroxidase activity in tomato against *Clavibacter michiganensis* ssp. *michiganensis*. UFA ingredients of turtle oil were investigated with GC analysis, and docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid (LA) and oleic acid (OA) were also determined (Baysal et al. 2005). In higher plants, lipoxygenase reacts with either linoleic acid or linolenic acid and forms hydroperoxides. These products are involved in plant defence against pathogens and insects (Rusterucci et al. 1999), wound response, senescence, and development (Hildebrand 1989).

Up to now there is no information regarding the role of active oxygen species in plants treated with UFA components of turtle oil against bacterial pathogens (*Pst*). In addition to our previous study, in this paper H<sub>2</sub>O<sub>2</sub> generation and changes in NADPH oxidase activity were followed in treated plants, compared to uninoculated and *Pst* inoculated plants.

### **Material and methods**

Plant material: Greenhouse-grown ten-week-old tomato seedlings (Ikram) with four fully expanded leaves were used for all experiments. Plants were grown in pots in a soil mix containing sand, perlite, and peat compost, in the greenhouse at 25 ± 5°C with 68 - 80% RH. The soil mix also contained a slow-release fertilizer (14-12-14, N-P-K). Natural light was supplemented by a single 1000-watt sodium vapour lamp during a 16 h photoperiod.

Bacterial strain and inoculation: The two youngest leaves of the seedlings were sprayed and inoculated with a *Pst* suspension of 10<sup>8</sup> cfu/ml as described by Scarponi et al. (2001). Before 24 h inoculation the two reciprocal plant leaves were uniformly sprayed with an aqueous solution of turtle oil and five unsaturated fatty acids. Turtle oil (1 ml turtle oil dissolved in chloroform at 4:1v/v) was diluted in 100 ml of water and sprayed (ca. 200 µl turtle oil per leaf). Control plants were sprayed with the solution water/chloroform (4:1 v/v, ca. 200 µl per leaf). The seedlings were covered with plastic bags after the treatments. The treated leaves were inoculated with the bacterial suspension according to Scarponi et al. (2001) one day after treatments.

Effect of turtle oil and UFAs on bacterial growth in planta: Bacterial colony forming units (cfu) were recovered from *Pst*-inoculated tissues, treated with either turtle oil or water 24 h before inoculation, by removing 5 mm-diameter leaf discs aseptically from the region of inoculation. Excised discs were homogenized in 1 ml of sterile 0.06 % NaCl solution, diluted serially in 10-fold dilutions. Aliquots of alternate dilutions were plated on NYA agar plates, containing appropriate antibiotics. Plates were incubated at 26°C for 48 h, and emerging colonies were counted on all dilution plates showing bacterial growth. Young leaves were sampled from seedlings for physiological assays.

Physiological studies: The first group consisted of uninoculated plants and seedlings were sprayed with four unsaturated fatty acids: docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid (LA) and oleic acid (OA), obtained from Sigma-Aldrich chemicals. 5 mg/ml fatty acid were sonicated in 100 ml of distilled water and sprayed on plants. In a second group, the plants were treated with fatty acids or water and inoculated with the pathogens 24 h post treatment. From inoculated leaves, tissues were taken at the actual site of inoculation with *Pst*. From control plants, tissues were taken from sites similar to inoculated leaves. Preparation of samples for the physiological assays and samples for enzyme extraction from all treatments (inoculated or not) were separately taken 4, 8, 12, 24, 48 and 72 h after treatment. Thereafter, the supernatant (crude enzyme extract) was collected into 1.5 ml portions. Protein concentrations were determined by the method of Bradford (1976) using BSA as standard. The extract

was obtained from two different lots of leaf samples (1 g fresh weight each) for each treatment. All assays were spectrophotometrically performed at 25°C.

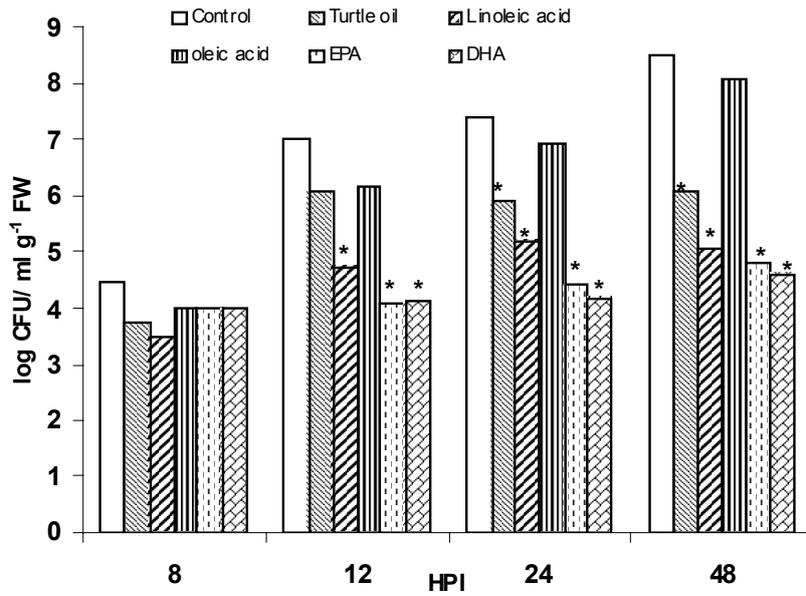
Assay of hydrogen peroxide concentration: The concentration of H<sub>2</sub>O<sub>2</sub> in the leaves was determined according to a modified method of Baker et al. (1995). Leaves were ground in 5% TCA (2.5 ml per 0.5 g leaf tissue) with 50 mg active charcoal at 0°C and centrifuged for 10 min at 15000g. Supernatant was collected, neutralised with 4 N KOH to pH 3.6 and used for H<sub>2</sub>O<sub>2</sub> assay. The reaction mixture contained 200 µl of leaf extract, 100 µl of 3.4 mM 3-methylbenzothiazoline hydrazone. The reaction was initiated by adding 500 µl of horseradish peroxidase solution (90 U 100 ml<sup>-1</sup>) in 0.2 M sodium acetate (pH 3.6). Two minutes later 1400 µl of 1 N HCl was added. The extinction at 630 nm was read after 15 min.

NADP(H) oxidase assay: NADPH oxidase activity in plants was evaluated by measuring the superoxide dismutase-inhibited reduction of NBT (van Gestelen et al. 1997). The reaction mixture consisted of 50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, and 0.1 mM NBT. After 5 min pre-incubation at 25 °C, the reaction was initiated by the addition of 0.1 mM NADPH. The reduction of NBT was measured as the change of extinction at 560 nm. The activity was expressed as nmol min<sup>-1</sup> mg<sup>-1</sup> protein using extinction coefficient of 12.8 mM<sup>-1</sup>cm<sup>-1</sup>.

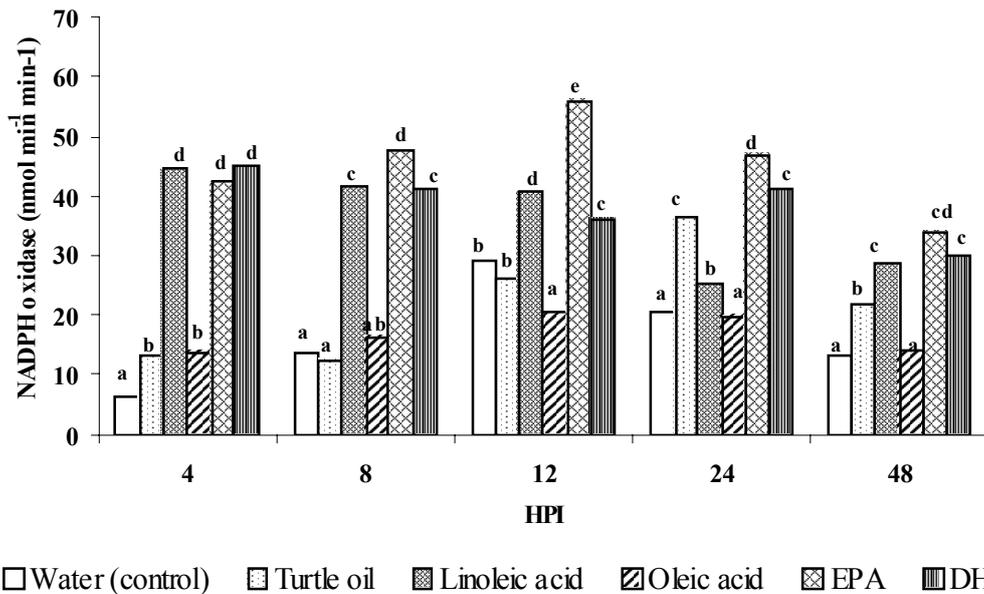
## Results and discussion

Turtle oil and its UFAs induced substantial levels of oxidative burst and enhanced disease resistance in tomato seedlings. Interestingly, this effect of turtle oil and its UFAs have not been shown in tomato plants against any plant pathogens, except for our previous studies (Baysal et al. 2005). The oxidative burst in plants was induced against *Pst* when turtle oil and its UFAs were applied 1 day before inoculation. In the present study, turtle oil and fatty acids induced substantial levels of resistance in tomato plants against *Pst*, and suppressed bacterial growth *in planta* (Figure 1). Significant changes were found in the enzymatic activities related to oxidative burst in plants expressing induced resistance following treatment with turtle oil and UFAs. The signal released by the pathogen may play an important role in activation of resistance if unsaturated fatty acids are applied as an inducer. A remarkable increase of H<sub>2</sub>O<sub>2</sub> occurred in inoculated UFA-treated plants, compared to inoculated water-treated plants, which can be compared with the findings of Milosevic and Slusarenko (1996) on disease resistance reactions. The results indicate that the activation of resistance is stronger if plants are challenged with a pathogen (Figures 2, 3). AOS, produced via an oxidative burst, are under the control of enzymes such as NADPH oxidase and peroxidases (POXs) (Wojtaszek 1997). The here obtained results show that *Pst* are sensitive to H<sub>2</sub>O<sub>2</sub> and they apparently confirm the role of hydrogen peroxide in inhibition of bacterial multiplication in plant tissue. The higher level of hydrogen peroxide generation in tomato leaves (Figure 3) of UFA treated plants is likely to be much more harmful to the bacteria than to the plant tissues; they restrict pathogen multiplication. Therefore the increase of peroxidase activity, which was shown in our previous study (Baysal et al. 2005) on tomato plants, treated with turtle oil, and NADPH oxidase activity (Figure 2) indicate that these activities are involved in the regulation of the level of H<sub>2</sub>O<sub>2</sub> in plant tissues (Wojtaszek 1997). Peroxidases can decompose and produce hydrogen peroxide. The locally accumulated H<sub>2</sub>O<sub>2</sub> may directly damage the bacteria (Brown et al. 1998). In our study, the increase in H<sub>2</sub>O<sub>2</sub> concentration was found to be significantly higher in the leaves of UFA-treated plants and showed a close relationship with the induction of resistance in UFA-treated plants. Therefore, the lower number of bacteria on UFA-treated plants can be assumed to result from the effect of UFA on tomato plants. However, further molecular studies are also necessary and are carried out in our lab.

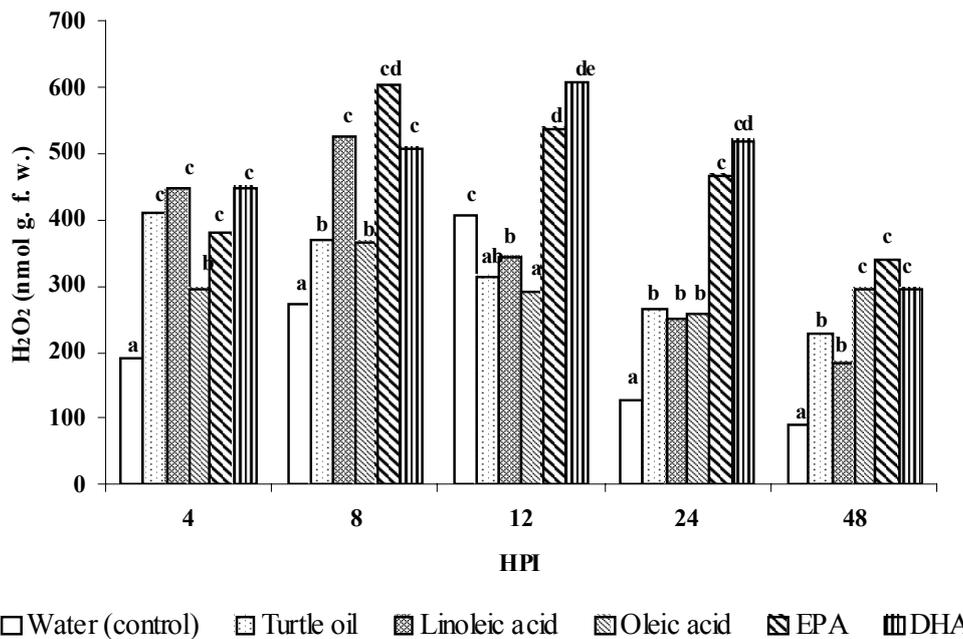
In conclusion, the present study indicates that the linoleic acids EPA and DHA are able to trigger active oxygen species that can act as mediators of plant immunity, thus new non-pesticidal plant protection strategies could be developed. Therefore, the protective effect of the linoleic acids, EPA, and DHA could be used as means of controlling the severity of bacterial speck on tomato.



**Fig. 1** The effect of UFAs on bacterial growth of *Pst.* One day after treatment with UFAs or water, the tomato seedlings were inoculated with *Pst.* The asterisks on bars represent significantly different values, compared to the control ( $P < 0.05$ ).



**Fig. 2** The effect of UFAs on NADPH oxidase. One day after treatment with UFAs or water, the tomato seedlings were inoculated with *Pst.* Data are mean values of three leaf discs.



**Fig. 3** The effect of UFAs on H<sub>2</sub>O<sub>2</sub> generation. One day after treatment with UFAs or water, the tomato seedlings were inoculated with *Pst*. Data are mean values of three leaf discs. Inhibitory effect of BABA treatment on bacterial growth was significantly different after inoculation according to Student's two-sample *t*-test ( $P < 0.05$ )

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## **An early oxidative burst in apple rootstocks treated with DL-β-amino butyric acid (BABA) against fire blight (*Erwinia amylovora*)**

### **Abstract**

Systemic acquired resistance (SAR) was induced by pre-treatment with the chemical inducer DL-β-amino butyric acid against fire blight disease caused by *Erwinia amylovora* (Ea 7/74). The plants were inoculated with 10<sup>8</sup> cfu/ml bacterial suspension, and disease development was evaluated up to 14 days post inoculation. Although *in vitro* growth of bacteria was not affected by DL-β-amino butyric acid treatment, its pre-inoculation application (500 µg/ml) significantly reduced disease severity and bacterial population. DL-β-amino butyric acid treated plants showed significantly higher H<sub>2</sub>O<sub>2</sub> generation, compared to untreated plants. The findings indicate that pre-treatment with the chemical inducer DL-β-amino butyric acid activated H<sub>2</sub>O<sub>2</sub> generation *in planta* more strongly when the plants were challenged with the pathogen; this may be associated with induction of plant resistance to bacterial pathogens and an effect of BABA in modulation of pathogen defence pathways.

### **Introduction**

Fire blight is one of the most serious diseases affecting apple and pear trees as well as other *Rosaceae* plants (van der Zwet and Beer 1992). Fire blight attacks all aboveground organs of the host plants often leading to their death. Chemical control of the disease relies upon the use of antibiotics (such as streptomycin) and copper compounds which prevent bacterial multiplication and further infection. Unfortunately, the antibiotics lead to the selection of resistant bacterial populations and therefore their use is strictly limited or even forbidden in several countries. In addition, none of these chemicals is systemic, and to be effective, they have to be applied on the whole plant surface before the pathogen enters the plant tissues. The induction of plant resistance may be one of the potential methods of reducing the severity of disease caused by the pathogen.

Pre-treatment of susceptible plants with avirulent pathogens (biotic inducer) can enhance resistance to subsequent attack not only at the site of treatment, but also in tissues distant from the initial infection sites. Typically, this inducible resistance system known as systemic acquired resistance (SAR) is effective against diverse pathogens including viruses, bacteria and fungi (Ryals et al. 1996). SAR is characterized by a reduction in the number and severity of lesions following challenge inoculation with a normally virulent pathogen. In addition to biotic inducers, certain chemicals with no direct anti-microbial effect can also induce SAR in plants. Natural products such as salicylic acid (SA) and synthetic chemical compounds, such as 2,6-dichloroisonicotinic acid (INA), may serve as good alternatives to classical pesticides depending on their efficacy. Both SA and INA, however, are not tolerated by some plants (Ryals et al. 1999). Potassium salts, acibenzolar-S-methyl, and amino butyric acid (BABA) were reported to induce SAR in plants (Cohen et al. 1999; Narusaka et al. 1999; Oostendorp et al. 2001; Baysal et al. 2003; Baysal and Zeller 2004; Baysal et al. 2005). In our previous study (Baysal et al. 2005) BABA and the effect of its different doses were tested against bacterial canker disease in tomato and the suppressive effect was found to be 52%. The aim of the present study was to test BABA for its ability to induce resistance in apple rootstocks against Ea.

## Material and methods

**Plant material:** Young, greenhouse-grown apple rootstocks (M9 Bursa, Turkey) were used for all experiments. These rootstocks are highly susceptible to fire blight caused by *E. amylovora*. Plants were grown in 10 cm pots in a soil mix containing sand, perlite, and peat compost under greenhouse conditions at  $25 \pm 5$  °C with 68-80% RH. The soil mix also contained a slow-release fertilizer (14-12-14 N-P-K). Plants were watered daily. The plants were used 4 weeks after planting (young shoots were 10-12 cm long with 6-8 leaves per shoot). This environment was maintained during the entire period of the experiment.

**Bacterial strain and inoculation:** The bacterial strain of *E. amylovora* (Ea7/74) was obtained from the Federal Biological Research Centre, Germany (BBA). Inoculum suspension was prepared from early log-phase cells, which were obtained by growing the bacterial strain in nutrient broth in 25 ml sterile tubes and incubated at 27 °C on an orbital shaker at 200 rpm for 24 h. Bacteria were subsequently pelleted by centrifugation (twice, each at 3500 g for 5 min) and washed in sterile distilled water (SDW). Their concentration was adjusted to  $10^8$  cfu/ml by dilution to give an OD<sub>660</sub> of 0.1. The two youngest leaves of the seedlings were cut at the tip and inoculated by dipping into a suspension of  $10^8$  cfu/ml, as described by Baysal and Zeller (2004).

**Application of BABA:** BABA was dissolved in distilled water to obtain a concentration of 500 µg/ml BABA, and was sprayed (ca. 200 µl per seedling) on whole rootstocks. After the treatment, the rootstocks were maintained in a greenhouse as described above. Application on M9 rootstocks before inoculation control plants were sprayed with water at the same intervals. Plants of the first group were treated with BABA alone, the second group with BABA and inoculated with a bacterial suspension 3 days after treatment. The plants of the control group were treated with water as described. The level of the resistance induced in apple seedlings against *Ea* was evaluated at 4, 7, 10 and 14 days after inoculation (dai) by using a 0-10 arbitrary scale (Baysal and Zeller 2004). A mean disease severity index (DSI) was calculated from each treatment by summing the score of the 60 plants (three replicates of 20 plants for each treatment), and expressing the value as percentage according to Anfoka (2000). Mean percentage protection for each treatment was calculated as previously described (Godard et al. 1999):  $[(DI_w \times DI_t) / DI_w] \times 100$  where  $DI_t$  is the mean DI of the treatment and  $DI_w$  the mean DI of the water control.

**Effect of BABA on bacterial growth in planta:** Bacterial colony forming units (cfu) were recovered from inoculated tissues, treated with either BABA or water 3 days before inoculation, by removing inoculated shoot tips (1g plant material) which were homogenized in 0.06% NaCl solution (1:1), diluted serially from  $10^{-1}$  to  $10^{-6}$  and plated on the modified Miller-Schroth medium (Brulez and Zeller 1981). Aliquots of alternate dilutions were plated on NYA agar plates. Plates were incubated at 27 °C for 48 h, and emerging colonies counted on all dilution plates showing bacterial growth. Bacterial numbers *in planta* were calculated for each of the dilution plates, and a mean value was obtained from replicates. Each dilution from each leaf disc was duplicated. Results presented are means of two separate experiments in which three leaf discs from each treatment were homogenized.

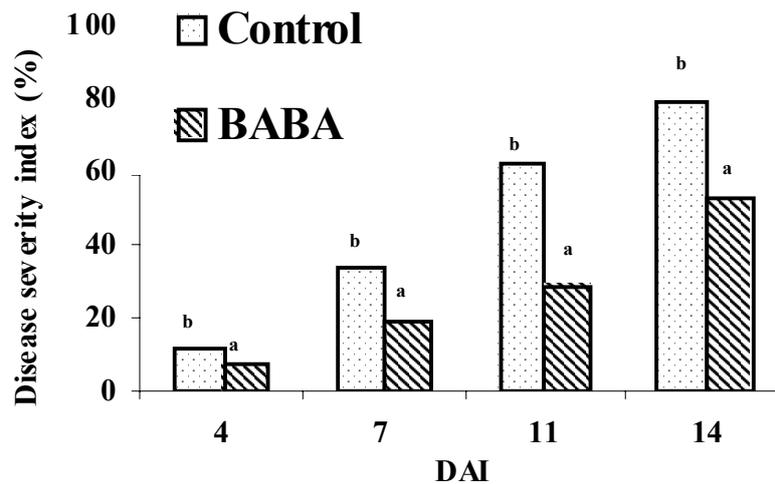
**Assay of hydrogen peroxide concentration:** The concentration of H<sub>2</sub>O<sub>2</sub> in the leaves was determined according to a modified method of Capaldi and Taylor (1983). Leaves were ground in 5% TCA (2.5 ml per 0.5 g leaves tissues) with 50 mg active charcoal at 0°C and centrifuged for 10 min at 15000g. The supernatant was collected, adjusted with 4 N KOH to pH 3.6 and used for the H<sub>2</sub>O<sub>2</sub> assay. The reaction mixture contained 200 µl of leaf extract, 100 µl of 3.4 mM 3-methylbenzothiazoline hydrazone. The reaction was initiated by adding 500 µl of horseradish peroxidase solution (90 U 100 ml<sup>-1</sup>) in 0.2 M sodium acetate (pH 3.6). Two minutes later 1400 µl of 1 N HCl was added. The Extinction at 630 nm was read after 15 min.

**Experimental design and statistical analyses:** The experiment was arranged in a completely randomized split-plot design with three replicates of 20 plants per treatment. Data obtained on various numbers of days after inoculation, and which usually included typical disease development, are presented. All experiments were repeated at least twice. Standard analyses of variance (ANOVA) were carried out by using the SPSS Statistical computer software program (Version 10). ANOVA was performed to analyze

the data, and the significance of differences among treatments was determined according to Duncan's Multiple Range Test ( $P < 0.05$ ).

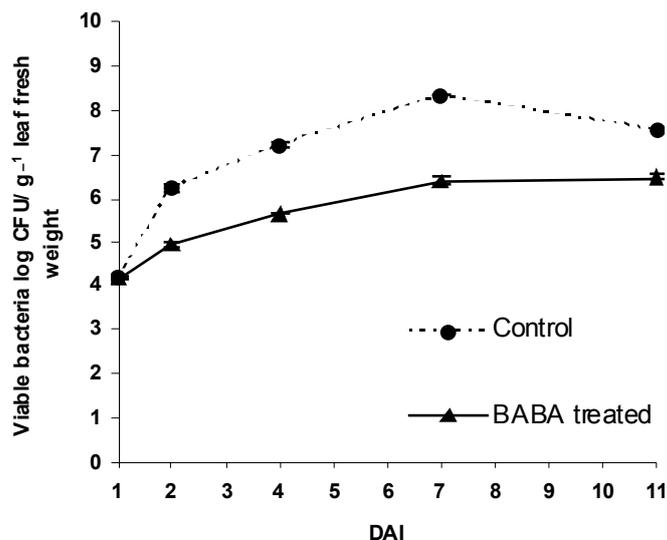
## Results

**The effect of BABA on disease resistance:** Resistance induced in apple rootstocks by BABA is shown in Fig. 1. Initial symptoms appeared on control plants as small marginal wilting 4 days after inoculation. The mean disease severity index (DSI) in these plants was 11%. The progress of the disease in control plants increased with time and by 14 dai most of the plant leaves developed severe symptoms. The mean DSI in these plants reached 80%. After application of BABA, a remarkable reduction in the disease severity index occurred (Figure 1). The time between initial treatment with BABA and subsequent inoculation with Ea7/74 significantly affected the efficacy of the induced resistance. Although all interval times significantly reduced the disease index, the greatest disease suppression was caused by BABA treatment 3 days before inoculation (Figure 1). The resistance induced by the BABA treatment was already evident 4 dai and lasted for the entire experimental period (until 14 dai). Untreated plants showed a significantly faster disease development during this period. The disease index was reduced in BABA-treated rootstocks 7 dai, and this was maintained at the same level until 14 dai. Disease indices of control seedlings were 80% whereas those of BABA-treated seedlings were only 53% at 14 dai. Since the highest induced resistance was observed at a time interval of 3 days between treatment and inoculation, this interval was taken into consideration in order to determine the bacterial growth and analysis of H<sub>2</sub>O<sub>2</sub> generation.



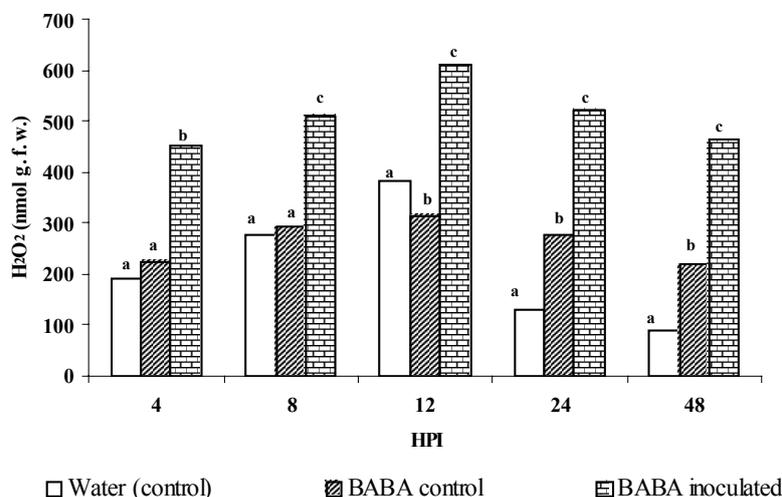
**Fig. 1** Effect of BABA on the severity of the disease symptoms caused by *E. amylovora* 7/74. After treatment with different plant activators or water (control), rootstocks were inoculated 72 h later with the Ea 7/74. Inoculated leaves were scored at 4, 7, 11 and 14 days after inoculation (dai) using the 0-10 scale as described in Material and methods section. A mean disease severity index (DSI%) was calculated from each treatment by summing the score of the 30 plants (two replicates of 15 plants per treatment), and expressing the value as a percentage according to Anfoka (2000). Data are presented as the mean of the two independent experiments. Bars with the same patterns which have the different letters are significantly different according to Duncan's Multiple Range Test ( $P < 0.05$ ).

**Bacterial multiplication *in planta*:** The growth of Ea was markedly reduced in BABA-treated rootstocks, compared to the water-treated control (Figure 2). This inhibitory effect was first observed 4 dai and monitored until 11 dai. The bacterial population was reduced by BABA, compared to control plants at 4, 7 and 11 dai, respectively (Figure 2).



**Fig. 2** Effect of BABA on bacterial growth of *E. amylovora* in apple rootstocks. Three days after treatment with BABA or water, rootstocks were inoculated with Ea7/74. Data are means values for three leaf discs, and the bars represent standard deviations. Inhibitory effect of BABA treatment on bacterial growth was significantly different after inoculation according to Student's two-sample t-test ( $P < 0.05$ ).

Assay of hydrogen peroxide concentration: The data showed that BABA leads to increasing H<sub>2</sub>O<sub>2</sub> generation in plants when the plants were challenged with pathogens. Although the plants, when sprayed with BABA alone, showed no significant increase in H<sub>2</sub>O<sub>2</sub> generation up to 24 h post inoculation, BABA-treated and inoculated plants showed significantly higher H<sub>2</sub>O<sub>2</sub> generation up to 48 h post inoculation (Figure 3).



**Fig. 3** H<sub>2</sub>O<sub>2</sub> concentration (nmol g<sup>-1</sup> FW) in BABA-treated and water-treated apple rootstocks inoculated with Ea over 48 h. Leaves were treated with BABA at 72 h before inoculation. For controls, leaves were sprayed with water at 72 h before inoculation. The values ( $\pm$  standard deviations) of 4 different samples with the same letters represent values that are not significantly different according to Duncan's Multiple Range Test ( $P < 0.05$ )

### Discussion

The current study assessed the effect of the plant activator BABA on disease development caused by Ea. Results confirm that BABA induces resistance in apple rootstocks. Apart from this study, amino butyric acid has been described in tomato against fungal pathogens (Cohen et al. 1994). For the development of

resistance plants need an interval period before being challenged with a pathogen. In most cases this interval was reported to be between 1 and 7 days. In plants, the increased production of both the superoxide radical and H<sub>2</sub>O<sub>2</sub> is a common feature of defence responses to challenge by avirulent pathogens and elicitors (Lamb and Dixon 1997). It may be hypothesised that a low nutrient concentration or/and accumulation of antimicrobial compounds in the intercellular space of treated leaves of apple rootstock tissues, where bacteria grow, or cell wall alterations such as physiological barriers in the xylem tissues may be a limiting factor for bacterial growth. BABA dependent resistance does not appear to be due to an antimicrobial effect of the compound, BABA seems to be a useful tool for induced resistance studies in apple as observed in other plant species. Biocontrol organisms, improved varieties and BABA will provide the farmer with a new option for disease control. We believe that the future use of BABA will have a profound impact combined with lower doses of conventional copper compounds, on the control of fire blight disease.

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## **Antagonistic coccus type bacteria killing a coryneform type bacterium, the pathogen of *Agave tequilana* Weber var. *azul***

### **Abstract**

A coccal bacterium causes the death of the coryneform type bacterium, pathogen of *Agave*, in a short time in culture. This form of antagonism may function in nature as a biological control system, without being noticed or well appreciated until now. No literature is available yet about this case. This is the first report of this kind concerning *Agave*, its pathogen and an antagonist of this pathogen.

### **Introduction**

It is well known that many antagonistic bacteria have been found in nature that affect other bacteria, using different modes of attack, in order to subsist and multiply. Some examples can be cited. Stolp and Petzold in 1962 in Berlin isolated a new kind of bacterium, now known as *bdellovibrios* (6,7). Many bacteria produce antagonistic proteinaceous substances in nature that are lethal in very short time to other strains of bacteria and these are called bacteriocins. The first record of these substances and their use against plant pathogenic bacteria was in 1954 by Okabe, when bacteriocinogenic strains of Gram-negative bacteria, the known *Pseudomonas solanacearum* (now *Ralstonia solanacearum*), was used for identification of pathogenic groups of the same bacterium (2). In 1976, Echandi published an article (1), reporting that a Gram-positive bacterium, namely *Corynebacterium michiganense* (now *Clavibacter michiganensis* subsp. *michiganensis*) can also produce bacteriocin against the same genus. A notable discovery took place when Kerr and Htay (4) and later Kerr (3) informed about a practical way of using *Agrobacterium radiobacter* as a producer of Agrocin 84 in order to biologically control *A. tumefaciens* by this bacteriocin. The present work informs about a Gram-positive coryneform type bacterium which is pathogenic on *Agave* and which was killed on King's B medium (5) in a very short time by another Gram-positive bacterium which was not suspected to be present and which was of a coccus type.

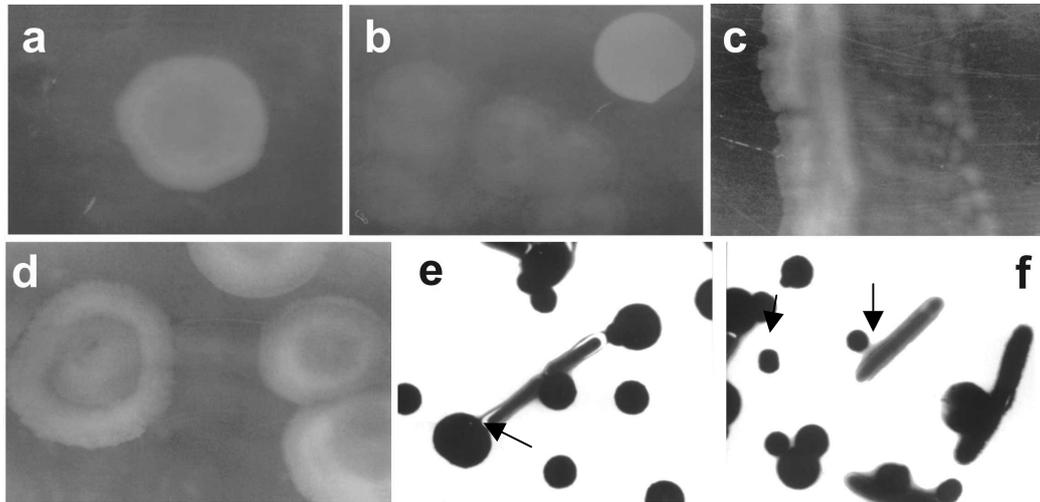
### **Materials and methods**

A coryneform, Gram-positive bacterium was re-isolated from diseased tissues of *Agave*, transferred to King's B medium (5), and placed for 24 hours in an incubator at 28 °C. This isolate has been known previously to be pathogenic on *Agave*. However, in our case after this period, the formed colonies disappeared, leaving a "ghost" type of colonies on the plate. Gram stain was performed on these rare colonies. Apart from this, electron microscopy work was done to see more details and confirms the presence of another bacterium.

### **Results and discussion**

The colonies of the coryneform bacterium, after being attacked by the coccus type bacterium, did not grow and the bacteria could not be recovered after transfer to the same new medium. The normal, unaffected type of coryneform colonies grew after transfer to a new medium. The coccus type bacterium never produced any colonies when transferred together with the coryneform type bacterium to another plate, even, after trying to separate the two bacteria using the normal streak method, in order to obtain separate colonies. When a coryneform type colony was partially affected, after transfer it first grew slightly, but then disappeared in a short time, because of the activity of the other co-transferred bacterium. The infestation of the colonies usually started in the center of a developing round colony. After viewing the electron micrographs, it can be suggested that the coccus type bacteria attach to the coryneform bacteria or are in a very close proximity as observed, causing contraction of the cytoplasm of the attacked cells and probably their subsequent death. Attachment of one or several of the coccal type cells to one coryneform bacterium was observed. Attachment can be at the center of the cell, but also at

the poles. The coccid bacteria never produced colonies on King's B medium and possibly in nature their specific *modus vivendi* is only on the coryneform bacterium as a nutritional source, because it was also observed that a contaminating white colony of an unknown bacterium on the plate was unaffected after placing the coccid bacteria in contact with it. All this may mean that the attacking coccid bacterium may be specific and appears to be confirmed, because both bacteria are Gram-positive. The short time in which the coryneform type bacterium dies, suggests that a bacteriocin-like substance could be involved. However this needs to be verified. If this process functions in nature it could be a possible biological control method.



**Figure** Coryneform pathogen of *Agave* killed by coccal bacteria. **a** Normal coryneform bacterial colony after 24 h of growth. **b** Coryneform bacteria (left) not yet attacked by coccal bacteria (right). **c** Coryneform bacterial colonies (right) affected by the coccal bacteria; bacteria on the left are still living. **d** Coryneform bacteria are affected by the coccal bacteria in the center of the colonies. **e** Coccal bacteria affect coryneform bacteria, inducing contraction of their cytoplasm (arrow). **f** Direct contact is necessary (arrows). (EM micrographs, x 1,500)

### Acknowledgement

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## ***In vitro* bactericidal activity of essential oils from *Hyptis suaveolens* (L.) Poit and *Coleus amboinicus* (Lour)**

### **Abstract**

In the last years the study of fungicidal and bactericidal properties of plant extracts has extended towards phytopathogens in search of alternative solutions for their control. Essential oil extracts of Wild oregano (*Hyptis suaveolens* (L.), Poit) and French oregano (*Coleus amboinicus* Lour) were tested for determining their bactericidal activity against isolates of *Xanthomonas axonopodis* pv. *vesicatoria*, *Xanthomonas campestris* pv. *campestris*, *Xanthomonas axonopodis* pv. *malvacearum*, *Xanthomonas axonopodis* pv. *manihotis*, *Xanthomonas* sp., *Erwinia carotovora* subsp. *carotovora*, *Erwinia chrysanthemi*, *Burkholderia glumae*, *Acidovorax avenae* and *Pseudomonas syringae* pv. *tomato*. Impregnated disks with essential oil were placed onto the surface of dishes with nutrient agar, previously inoculated with a bacterial suspension. Three replicates for each variant were carried out. The inhibition area determined the bactericidal effect after a 24 hours incubation period. All bacterial isolates resulted to be sensitive to the extracts of the two oregano species at 1%, with inhibition areas between 14.3 – 28.0 mm for the Wild oregano, and of 14.3 – 25.6 mm for the French oregano. This latter species extract at 0.5% showed a bactericidal effect against all tested bacterial isolates. Eleven isolates were sensitive to the extracts at 0.25% with inhibition areas between 4.0 – 13.0 mm. These results encouraged us to continue the study of the effectiveness of plant extracts for the control of these bacterial species in their host plants.

### **Introduction**

Plants naturally synthesize a great quantity of chemical substances. Their combination results in various natural bioactive products whose therapeutic effect has been recognized since ancient time (Rodríguez 1996).

In the last years, the study of fungicidal and bactericidal properties of plant extracts has extended towards phytopathogens in search of alternative solutions for their control. Many species as: *Matricaria recutita*, *Chamaemelia nobilis*, *Ruta graveolens*, *Ruta chalepensis*, *Jacaranda acutifolia*, *Aloe vera*, *Zingiber chrysanthemi* and *Bixa orellana* have demonstrated *in vitro* bactericidal effects against several species of phytopathogenic bacteria (Csizinszky et al. 1993; García and Rodríguez 1994; Martínez de Carrillo and Colmenares 1999).

Oregano belongs to the *Labiatae* family and it is known as an excellent anti-bactericidal agent. Its essential oil is highly active against a wide range of bacteria known as pathogenic to man ([http://www The Wolfe Clinic-Oil of Oregano](http://www.TheWolfeClinic-OilofOregano)).

In Cuba, the warm and humid climate favors the cultivation of Wild oregano (*H. suaveolens* (L.), Poit.) and French oregano (*C. amboinicus* Lour.), which are extended through the whole country (Roig, 1974). Particularly French oregano is cultivated in the urban and peri-urban agriculture.

Essential oil extracts of *Hyptis suaveolens* and *Coleus amboinicus* were tested *in vitro* for determining their activity against bacterial isolates from different host plants.

### **Materials and methods**

Leaves from oregano plants were air dried for six days before lab-distillation. The oil was obtained by hydrodistillation for 4 h in a clevenger-type apparatus and were tested against isolates of *Xanthomonas axonopodis* pv. *vesicatoria*, *Xanthomonas campestris* pv. *campestris*, *Xanthomonas axonopodis* pv. *malvacearum*, *Xanthomonas axonopodis* pv. *manihotis*, *Xanthomonas* sp., *Erwinia carotovora* subsp. *carotovora*, *Erwinia chrysanthemi*, *Burkholderia glumae*, *Acidovorax avenae* and *Pseudomonas syringae* pv. *tomato*. The disks of 12 mm diameter, soaked with the essential oils, were placed onto the surface of

the dishes with nutrient agar, previously inoculated with a bacterial suspension. Three replicates for each variant were carried out. The bactericidal effect was determined by the inhibition area after 24 hours incubation period. Three concentrations (1%, 0.5% and 0.25%) the French oregano extract were used, and only the 1% extract from Wild oregano. Paper disks impregnated with petroleum ether of and with distilled sterile water were included as control.

## Results and discussion

All bacterial isolates turned out to be sensitive to the extracts of the two oregano species at 1%, with inhibition areas between 14.3 – 28.0 mm for the Wild oregano, and of 14.3 – 25.6 mm for the French oregano. The extract of this latter species at 0.5% showed a bactericidal effect against all bacterial isolates tested. Eleven isolates belonging to *Xanthomonas axonopodis* pv. *vesicatoria*, *Pseudomonas syringae* pv. *tomato*, *X. axonopodis* pv. *malvacearum* and *X. axonopodis* pv. *manihotis* were sensitive to the extracts at 0.25% with inhibition areas between 4.0 – 13.0 mm (Table 1).

Scortichini and Rossi (1993) demonstrated the effect of oregano essential oil in a concentration of 900 mg/l against *Erwinia amylovora*. Recently, Daferera et al. (2003) determined the effectiveness of several essential oils, including oregano (*Origanum vulgare*), against *Clavibacter michiganensis* subsp. *michiganensis*, bacterial cancer in tomato. The latter was completely inhibited by a concentration of 100 mg/ml.

Oregano, an aromatic plant, cultivated in many parts of the world, is greatly demanded in the pharmaceutical sector and other industries (<http://www.infoagro.com> 2003). More than 40 species exist, but not all have the same concentration of phenolic compounds, and they do not express the same mode of activity (<http://www> Antibiotics in agriculture). As García Salmán et al. (1996) point out the volatile compounds are required due to their great antioxidant, fungicide and bactericide properties.

The results of this study show that French oregano extract is effective *in vitro* against all the bacterial species at concentrations of 1 – 0.5%, and against some isolates of these bacteria, even at the lower concentration of 0.25%.

Copper fungicides are used in the control of bacterial diseases in plants, but the emergence of resistant isolates has been demonstrated (Ritchie and Dittapongpith 1991; Sahin and Miller 1997; Carrillo- Fasio et al. 2001), thus new drugs are required. On the other hand, species such as *X.s axonopodis* pv. *vesicatoria*, *P. syringae* pv. *tomato*, *X. campestris* pv. *campestris* and *Xanthomonas* sp., affect horticultural crops (tomato, cabbage and onion), also cultivated in the urban and peri-urban agriculture, where biological products are required for pest control. Considering the *in vitro* promising results *H. suaveolens* and *C. amboinicus* extracts will be a biological alternative for the control of these bacteria. Extended studies are required at the level of the host plants.

**Table** *In vitro* bactericidal activity of *Hyptis suaveolens* and *Coleus amboinicus* essential oils. Diameter of inhibition area

Bacterial species/host plant	Strains	Wild origanum 1%	French origanum 1%	French origanum 0.5%	French origanum 0.25%
<i>X. vesicatoria</i> / tomato	Xv-1	19.7	20.2	13.3	8.3
	Xv-20	19.9	17.19	14.3	13.0
<i>X. campestris</i> / cabbages	Xc-5	22.6	14.3	12.6	0
	AH2	15.0	14.3	13.3	5.0
	X-32	17.0	14.6	14.3	0
	952	21.6	22.0	15.3	0
<i>Xanthomonas</i> sp. / onion and garlic	C-1	21.3	20.0	13.6	0
	Ceb-2 <sup>a</sup>	25.0	24.0	14.3	4.3
	Ceb-2 <sup>p</sup>	24.3	22.9	12.3	0
<i>X. malvacearum</i> / cotton	X-malv	20.3	17.0	14.0	0
<i>X. manihotis</i> / cassava	Xm	-	23.6	16.3	0
	Cub 3 t	20.3	17.0	-	-
<i>Burkholderia glumae</i> / rice	Cub 2 a	25.0	13.3	12.6	12.0
	Par 6p	25.0	23.3	14.3	0
<i>Acidovorax avenae</i> / rice	Par 7	26.6	19.6	13.3	4.0

Bacterial species/host plant	Strains	Wild origanum 1%	French origanum 1%	French origanum 0.5%	French origanum 0.25%
<i>E. chrysanthemi</i> / potato	As 2p	23.3	23.3	13.3	0
	San 15	22.0	25.6	14.6	8.6
	Gui 71	20.6	20.6	13.6	11.6
	Alq 41	22.3	22.0	14.0	0
	GNES 81	28.0	22.3	14.3	0
<i>E. carotovora</i> / potato	MNA 611	19.3	15.3	15.0	5.0
	Art 41	24.6	16.0	15.0	8.3
	GNES 3	21.6	18.0	13.6	13.0
<i>P. tomato</i> / tomato	Pt-1	20.9	18.5	15.9	0
	Pt-3	21.4	19.2	17.2	0
	1662	20.9	18.9	16.5	0

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## Effectivity of essential oils against pectinolytic *Erwinia chrysanthemi* and *Pseudomonas marginalis*

### Abstract

Soft rot bacteria are pathogenic against many plant species. They cause high economic losses on vegetable and flower plants in glasshouses and fields. Chemical preparations have been used against soft rot bacteria, however resistance development in bacterial populations decreases their effectivity, and therefore new chemical compounds have been considered. In our experiments, 33 essential oils from different plants were examined for their inhibitory effect against pectinolytic erwinias and pseudomonads. The antimicrobial activity tests were conducted *in vitro*. The inhibitory zones were measured on agar plates where extracts were dropped. Streptomycin and erythromycin were used as controls. Against *Erwinia chrysanthemi* weak inhibitory activity (but higher than erythromycin) was found in essential oils from *Eugenia caryophyllata*, *Lavandula angustifolia*, *L. latifolia*, *Melaleuca quinquenervia*, *Mellisa officinalis*, *Mentha pulegium*, *Origanum majorana*, *Pelargonium graveolens*, *P. roseum*, *Rosmarinus officinalis*, *Salvia officinalis*, *Thuja occidentalis* and *Thymus matschiana* (zones 5.1-10.0 mm); medium inhibitory efficiency was shown in essential oils from *Artemisia absinthium* and *Ocimum basilicum* (zones 10.1-15.0 mm); strong inhibitory activity was found with essential oils from *Origanum compactum*, *O. vulgare* and *Thymus vulgaris* (zones 15.1-20.0 mm). *Pseudomonas marginalis* pv. *marginalis* weak inhibitory activity (but higher than streptomycin) was found with essential oils from *Citrus aurantifolia*, *Lavandula latifolia*, *Mentha quinquenervia*, *M. spicata*, *Melissa officinalis*, *Origanum majorana*, *Pelargonium roseum*, *P. graveolens*, *Rosmarinus officinalis* and *Thymus mastichina* (zones 5.1-10.0 mm); medium inhibitory efficiency was shown with essential oils from *Ocimum basilicum*, *Tagetes bipinata* and *Tsuga canadensis* (zones 10.1-15.0 mm); strong inhibitory activity had essential oils from *Origanum compactum*, *O. vulgare* and *Thymus vulgaris* (zones 15.1-20.0 mm).

**Key words:** *Erwinia chrysanthemi*, *Pseudomonas marginalis* pv. *marginalis*, antimicrobial activity, essential oils.

### Introduction

Soft rot bacteria such as *Erwinia chrysanthemi* Burkholder et al. (*Ech*) and *Pseudomonas marginalis* pv. *marginalis* (Brown) Stevens (*Pmm*) are pathogenic against many plant species (Dickey 1981; Schaad et al. 2001). They cause high economical losses on vegetables, flowers and ornamental crops planted in glasshouses and fields (Smith et al. 1977). Plants with symptoms are refused on the market and become unmerchantable. Bacteria do not only infect succulent plant organs such as roots, tubers, stems and leaves, but they colonize also vascular tissues and thus can spread in cuttings from plants (Smith et al. 1997). They disseminate under humid (*Pmm*) and warm (*Ech*) conditions depending upon the species, cultivars and culture conditions. Irrigation of plants and direct contacts via wounds arising during the care and cultivation increases transmission of soft rot pathogens. Copper preparations against soft rot bacteria are sometimes phytotoxic and their efficiency is not sufficient. Antibiotics such as streptomycin and erythromycin are not allowed to be used for plant protection in the majority of EU countries, because resistance to them could occur in bacterial populations (Iacobellis et al. 2005). Therefore new compounds are required to be available on the market. Combination of plant extracts or etheric oils from plants with copper and other chemical compounds can increase their effectiveness (Zeller 2005). The chemistry of oils from plants including monoterpenes, sesquiterpenes and phenols is well documented (Guenther 1972-98; Bruneton 1999). Some essential oils proved strong antifungal (Wang et al. 2005), insecticide (Isman 2000; Çalmaşur et al. 2005; Pavela 2005), and antibacterial activity (Burt 2004; El-Kamali et al.

2005). The aim of this study was to find potential effectivity of essential oils against pectinolytic *E. chrysanthemi* and *P. marginalis*.

### Material and methods

In our experiments thirty-three essential oils obtained from different plants were examined for inhibitory effect against *Ech* and *Pmm* (Table). The antimicrobial activity tests were conducted *in vitro* on agar plates inoculated by the target bacterium. Reference cultures of *Ech* CCM 989 and *Pmm* BCCM/LMG 2210 were grown on solid nutrient meat-peptone medium and used as 2-day cultures in concentration corresponding to  $OD_{620} \approx 0.5$  in all tests. The crude extracts were dropped on the agar surface in a dosage of 1  $\mu$ l per 6 grounds. After the treatment, the Petri dishes were incubated in a thermostat at 26 °C for three days, and then evaluated. The average of inhibitory zones was measured and the average of the obtained values was calculated. Efficiency of the essential compounds was directly proportional to the size of the inhibitory zone. Streptomycin and erythromycin in 0.02 – 0.2 % concentrations were used as controls (Kokošková 1992). The effectiveness of essential oils was evaluated according to the three degrees: effectivity weak (zones 5.1-10.0 mm); medium (zones 10.1-15.0 mm); strong (zones 15.1-20.0 mm).

### Results and discussion

Of all essential oils examined in this study only *Origanum vulgare*, *O. compactum* and *Thymus vulgaris* (zones 15.1-20.0 mm) appeared to be most efficient in bacteriostasis and as bactericides against *Ech* and *Pmm*. Similar results were reported also by other authors against other bacteria (Charai et al. 1996; Sivropoulou et al. 1996; Iacobellis et al. 2005). (Figure).

Intermediate inhibitory activity against *Ech* was found by essential oils from *Artemisia absinthium* and *Ocimum basilicum* (zones 10.1-15.0 mm); weak inhibitory activity against *Ech* was found by essential oils from *Eugenia caryophyllata*, *Lavandula angustifolia*, *L. latifolia*, *Melaleuca quinquenervia*, *Mellisa officinalis*, *Mentha pulegium*, *Origanum majorana*, *Pelargonium graveolens*, *P. roseum*, *Rosmarinus officinalis*, *Salvia officinalis*, *Thuja occidentalis* and *Thymus matschiana* (zones 5.1-10.0 mm). (Figure).

A weak inhibitory activity (but stronger than streptomycin) against *Pmm* was found with essential oils from *Citrus aurantifolia*, *Lavandula latifolia*, *Mentha quinquenervia*, *M. spicata*, *Melissa officinalis*, *Origanum majorana*, *Pelargonium roseum*, *P. graveolens*, *Rosmarinus officinalis* and *Thymus mastichina* (zones 5.1-10.0 mm), a medium inhibitory effectivity was found with essential oils from *Ocimum basilicum*, *Tagetes bipinnata* and *Tsuga canadensis* (zones 10.1-15.0 mm). (Figure).

Streptomycin and erythromycin at a concentration of 0.02 % were not sufficiently effective; therefore both antibiotics were used at a concentration of 0.2% for both bacteria.

Essential oils from some plant species (e.g. *Origanum* sp. and *Thymus* sp.) are rich in phenolic compounds, which are believed to be responsible for the marked antimicrobial activity (Beuchat and Conner 1984; Zaika and Kissinger 1981). The phenolic compounds dissolve within the bacterial membrane and thus penetrate inside the cell, where they interact with the cell metabolism (Judis 1963; Juven et al. 1972).

The weak antimicrobial efficiency of *Lavandula latifolia*, *Melaleuca quinquenervia*, *Mentha spicata*, and *Tagetes bipinnata* essential oils tested in this study is in contrast with results obtained by other authors (Mazzanti et al. 1998). Essential oils of mint caused a limited decrease in bacterial growth rates, as reported by other authors (Sivropoulou et al. 1995). However, comparison of the data obtained in this study with previously published results is not easy, considering that the composition of plant oils and extracts vary according to environmental conditions and plant species.

Essential oils obtained by supercritical CO<sub>2</sub> extraction and alcoholic Soxhlett extraction have promising potentials for incorporation into various pesticide products for which antimicrobial and insecticidal effects are desired. The extracts examined might have a role in preservation against bacteria in greenhouses, especially SFE extracts, which are able to suppress the bacterial growth. Further experiments are required to establish the real application of oils extracted with supercritical CO<sub>2</sub> against plant pathogenic bacteria.

The work was supported by the Ministry of Agriculture of CR, project No. 320/5305 and project No. MZE0002700603.

**Table** Plant material used in the experiments

<b>Oil</b>	<b>Plants</b>	<b>Family</b>	<b>Origin</b>	<b>Part</b>
Amyris	<i>Amyris balsamifera</i>	Rutaceae	Haiti	Wood
Basil	<i>Ocimum basilicum</i>	Lamiaceae	Egypt	Herb
Bergamot	<i>Mentha citrata</i>	Lamiaceae	USA	Herb
Calamus	<i>Acorus calamus</i>	Araceae	Nepal	Root
Catnip	<i>Nepeta cataria</i>	Lamiaceae	Canada	Flowg tops
Cedarleaf	<i>Thuja occidentalis</i>	Cupressaceae	Canada	Leaf/twig
Cedarwood	<i>Juniperus virginiana</i>	Cupressaceae	USA	Wood
Clary Sage	<i>Salvia sclarea</i>	Lamiaceae	USA	Flower/leaf
Clove bud	<i>Eugenia caryophyllata</i>	Myrtaceae	Madagascar	Bud
Cornmint	<i>Mentha arvensis</i>	Lamiaceae	India	Herb
Fir Needle	<i>Abies sibirica</i>	Pinaceae	Russia	Needle
Geranium	<i>Pelargonium graveolens</i>	Geraniaceae	Reunion	Leaf
Geranium	<i>Pelargonium roseum</i>	Geraniaceae	Madagascar	Leaf
Juniperberry	<i>Juniperus communis</i>	Cupressaceae	Croatia	Berry
Lavender a.	<i>Lavandula angustifolia</i>	Lamiaceae	France	Flower
Lavender l.	<i>Lavandula latifolia</i>	Lamiaceae	France	Flower
Lemon	<i>Citrus limonum</i>	Rutaceae	Italy	Peel
Lime	<i>Citrus aurantifolia</i>	Rutaceae	Mexico	Peel
Marjoram	<i>Thymus mastichina</i>	Lamiaceae	Spain	Flower/leaf
Marjoram, Sweet	<i>Origanum majorana</i>	Lamiaceae	Egypt	Flower/leaf
Melissa "rectified"	<i>Melissa officinalis</i>	Lamiaceae	Spain	Flower/leaf
Niaouli MQV	<i>Melaleuca quinquenervia</i>	Myrtaceae	Madagascar	Leaf
Oregano	<i>Origanum compactum</i>	Lamiaceae	Morocco	Herb
Oregano	<i>Origanum vulgare</i>	Lamiaceae	Greece	Herb
Patchouli	<i>Pogostemon cablin</i>	Lamiaceae	Indonesia	Leaf
Pennyroyal	<i>Mentha pulegium</i>	Lamiaceae	USA	Leaf
Rosemary	<i>Rosmarinus officinalis</i>	Lamiaceae	Morocco	Leaf
Sage	<i>Salvia officinalis</i>	Lamiaceae	Greece	Leaf
Spearmint	<i>Mentha spicata</i>	Lamiaceae	China	Flowg. herb
Spruce	<i>Tsuga canadensis</i>	Pinaceae	Canada	Needle
Tagetes	<i>Tagetes bipinnata</i>	Asteraceae	Madagascar	Flower
Thyme	<i>Thymus vulgaris</i>	Lamiaceae	Spain	Herb
Wormwood	<i>Artemisia absinthium</i>	Asteraceae	USA	Flowrg tops



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## Effectivity of essential oils against *Xanthomonas hortorum* pv. *pelargonii*, the causal agent of bacterial blight on geranium

### Abstract

*Xanthomonas hortorum* pv. *pelargonii* (Brown), Vauterin et al. (*Xhp*) is an important plant pathogenic bacterium causing leaf spot and stem rot of plants resulting in serious losses in areas where geraniums (*Pelargonium* spp.) are cultivated. The pathogen is transmitted by seeds and disseminated by infected nursery plants and cuttings, where it latently persists. At present, a sufficient choice of chemicals, effective against *Xhp*, is not available on the market. In our experiments, 33 essential oils, obtained from various plants, were tested for antimicrobial effectivity against *Xhp*. The screening was performed *in vitro* on agar plates inoculated with *Xhp*, and the average of the inhibitory zones was measured. Streptomycin and erythromycin at 0.02 – 0.2 % concentrations were used as controls (zones 2.7 - 7.2 mm). Weak inhibitory activity against *Xhp* was recorded with essential oils from *Lavandula latifolia*, *Melaleuca quinquenervia*, *Mentha spicata*, *Tagetes bipinata* and *Zingiber officinale* (zones 7-10 mm); medium inhibitory activity was found in essential oils from *Citrus aurantifolia*, *Eugenia caryophyllata*, *Lavandula angustifolia*, *Melissa officinalis*, *Mentha arvensis*, *M. citrata*, *M. pulegium*, *Nepeta cataria*, *Origanum majorana*, *Rosmarinus officinalis*, *Thuja occidentalis* and *Thymus mastichina* (zones 11 - 20 mm); strong inhibitory effectivity was found in essential oils from *Artemisia absinthium*, *Ocimum basilicum* and *Salvia officinalis* (zones 21 - 30 mm) and finally, essential oils from *Origanum compactum* and *Thymus vulgaris* (zones 31 - 50 mm) had the strongest inhibitory activity.

**Key words:** *Xanthomonas hortorum* pv. *pelargonii*, antimicrobial activity, essential oils

### Introduction

*Xanthomonas hortorum* pv. *pelargonii* (Brown) Vauterin et al. (*Xhp*) is an important plant pathogenic bacterium causing wilting, leaf spot and stem rot of plants resulting in serious losses in areas where geraniums (*Pelargonium* spp.) are cultivated. Under warm and humid conditions, losses may be as high as 100 % depending on the cultivar and culture conditions (Taylor et al. 1996). The pathogen is transmitted by seeds and disseminated by infected nursery plants and cuttings, where it latently persists. Irrigation of plants and direct contact via wounds, arising during the care and cultivation, increases the transmission of the pathogen (Dougherty et al. 1974; Kennedy et al. 1987; Griesbach and Olbricht 2002). The choice of chemicals effective against *Xhp* as sprays and disinfectants available on the market is limited at present. Copper preparations are recommended against bacterial blight on geraniums, but strong infections usually cannot be avoided, because the disease has epidemic character. Antibiotics such as streptomycin and erythromycin are not allowed to be used in plant protection in the majority of EU countries (Iacobellis et al. 2005). Therefore other options need to be considered. The mixture of plant extracts or etheric oils produced by plants together with chemical compounds such as copper increase their effectivity against the target pathogens (Zeller 2005). Plants reported here and their oils have a wide application range in medicine (Bruneton 1999). The chemistry of these oils including monoterpenes, sesquiterpenes and phenols is well documented in literature (Guenther 1972-98; Bruneton 1999). Some of the essential oils have very strong insecticidal (Isman 2000; Çalmaşur et al. 2005; Pavela 2005), antifungal (Wang et al. 2005) and antibacterial activity (Burt 2004; El-Kamali et al. 2005).

The aim of this study was to compare the effectivity of essential oils against *Xhp*, the best to be used in plant protection against bacterial blight on geraniums in future.

## Material and methods

In our experiments 33 essential oils obtained from various plants were tested for antimicrobial effectivity against *Xhp*. The list of essential oils used in the tests is shown in Table. The screening was performed in laboratory conditions on agar plates inoculated by *Xhp*. The reference strain *Xhp* CCM 612 was grown on nutrient meat-peptone medium and used as 2-day culture at a concentration corresponding to  $OD_{620} \approx 0.5$  in all tests. Each crude extract was dripped on the surface of agar plates in dosages of 1  $\mu$ l per 6 grounds. After treatment, the Petri dishes were incubated in a thermostat at 26 °C. The average of inhibitory zones was measured after 3 days and then the average of the obtained values was calculated. The effectivity of the essential compounds was directly proportional to the size of the inhibitory zone. Streptomycin and erythromycin in 0.02 – 0.2 % concentrations were used as controls (Kokošková 1992).

## Results and discussion

Out of 33 essential oils tested, 11 had very weak, 5 weak, 12 intermediate and 5 strong bactericidal activity against *Xhp*. Streptomycin and erythromycin at concentrations of 0.02 % were not sufficiently effective (zones 2.7-5.7 mm); therefore both antibiotics were used at the concentration of 0.2% (zones 5.8-7.2 mm). Plant extracts having lower inhibitory activity against *Xhp* than antibiotics (zones lower than 7.2 mm) were evaluated as extracts without effectivity, because only plant extracts better than antibiotics were considered as promising. Essential oils tested had more efficacy *in vitro* against *Xhp* than against *Erwinia chrysanthemi* and *Pseudomonas marginalis* pv. *marginalis*. Weak inhibitory activity against *Xhp* was found in essential oils from *Lavandula latifolia*, *Melaleuca quinquenervia*, *Mentha spicata*, *Tagetes bipinnata* and *Zingiber officinale* (zones 7-10 mm); medium inhibitory activity was found in essential oils from *Citrus aurantifolia*, *Eugenia caryophyllata*, *Lavandula angustifolia*, *Mellisa officinalis*, *Mentha arvensis*, *M. citrata*, *M. pulegium*, *Nepeta cataria*, *Origanum majorana*, *Rosmarinum officinalis*, *Thuja occidentalis* and *Thymus mastichina* (zones 11 - 20 mm); strong inhibitory effectivity was found in essential oils from *Artemisia absinthium*, *Ocimum basilicum* and *Salvia officinalis* (zones 21 - 30 mm); the strongest inhibitory activity had essential oils from *Origanum compactum* and *Thymus vulgaris* (zones 31 - 50 mm).

Among the essential oils examined those from *Origanum compactum* and *Thymus vulgaris* appeared to be the most efficient from both a bacteriostatic and bactericidal point of view, as reported also by other authors (Charai et al. 1996, Sivropoulou et al. 1996). Essential oils of *Origanum* sp. and *Thymus* sp. are rich in phenolic compounds, which are believed to be responsible for the marked antimicrobial activity (Zaika and Kissinger 1981). In fact, the phenolic compounds dissolve within the bacterial membrane and thus penetrate to the interior of the cells, where they interact with cellular metabolism (Juven et al. 1972).

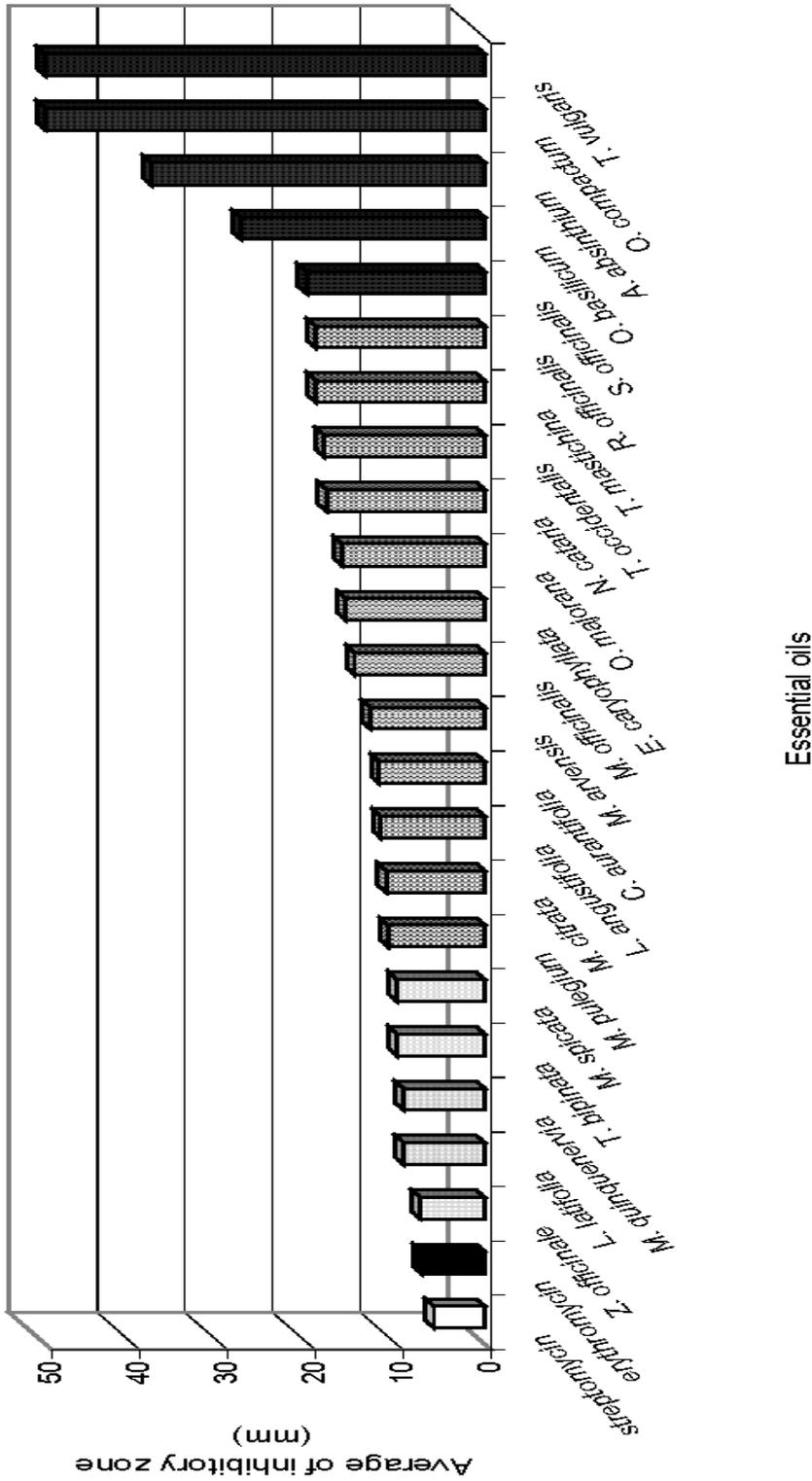
*A. absinthium*, *O. basilicum* and *S. officinalis* essential oils showed a weak bacteriostatic effect. It is likely that the unusual sensitivity of *Xhp* to the oils can be attributed to the complex composition. In particular, it is reasonable to hypothesise that there is a substance, or a pool of substances, able to attack species-specific membrane proteins.

The weak antimicrobial efficiency of *Lavandula latifolia*, *Melaleuca quinquenervia*, *Mentha spicata*, *Tagetes bipinnata* and *Zingiber officinale* essential oils tested in this study is in contrast with results obtained by other authors (Mazzanti et al. 1998). The presence of essential oils of mint caused a limited decrease in bacterial growth rates, as reported by other authors (Sivropoulou et al. 1995). However, comparison of the data obtained in this study with previously published results is not straightforward, considering that the composition of plant oils and extracts vary according to environmental conditions and plant species (Sivropoulou et al. 1995).

The work was supported by the Ministry of Agriculture of ČR, projects No. 320/5305 and MZE 0002700603.

**Table** Plant material used in the experiment

Oil	Plants	Family	Origin	Part
Amyris	<i>Amyris balsamifera</i>	Rutaceae	Haiti	Wood
Basil	<i>Ocimum basilicum</i>	Lamiaceae	Egypt	Herb
Bergamot	<i>Mentha citrata</i>	Lamiaceae	USA	Herb
Calamus	<i>Acorus calamus</i>	Araceae	Nepal	Root
Catnip	<i>Nepeta cataria</i>	Lamiaceae	Canada	Flower tops
Cedarleaf	<i>Thuja occidentalis</i>	Cupressaceae	Canada	Leaf/twig
Cedarwood	<i>Juniperus virginiana</i>	Cupressaceae	USA	Wood
Clary Sage	<i>Salvia sclarea</i>	Lamiaceae	USA	Flower/leaf
Clove bud	<i>Eugenia caryophyllata</i>	Myrtaceae	Madagascar	Bud
Cornmint	<i>Mentha arvensis</i>	Lamiaceae	India	Herb
Fir Needle	<i>Abies sibirica</i>	Pinaceae	Russia	Needle
Geranium	<i>Pelargonium graveolens</i>	Geraniaceae	Réunion	Leaf
Geranium	<i>Pelargonium roseum</i>	Geraniaceae	Madagascar	Leaf
Ginger	<i>Zingiber officinale</i>	Zingiberaceae	China	Root
Juniperberry	<i>Juniperus communis</i>	Cupressaceae	Croatia	Berry
Lavender a.	<i>Lavandula angustifolia</i>	Lamiaceae	France	Flower
Lavender l.	<i>Lavandula latifolia</i>	Lamiaceae	France	Flower
Lemon	<i>Citrus limonum</i>	Rutaceae	Italy	Peel
Lime	<i>Citrus aurantifolia</i>	Rutaceae	Mexico	Peel
Marjoram	<i>Thymus mastichina</i>	Lamiaceae	Spain	Flower/leaf
Marjoram, Sweet	<i>Origanum majorana</i>	Lamiaceae	Egypt	Flower/leaf
Melissa "rectified"	<i>Melissa officinalis</i>	Lamiaceae	Spain	Flower/leaf
Niaouli MQV	<i>Melaleuca quinquenervia</i>	Myrtaceae	Madagascar	Leaf
Oregano	<i>Origanum compactum</i>	Lamiaceae	Morocco	Herb
Patchouli	<i>Pogostemon cablin</i>	Lamiaceae	Indonesia	Leaf
Pennyroyal	<i>Mentha pulegium</i>	Lamiaceae	USA	Leaf
Rosemary	<i>Rosmarinus officinalis</i>	Lamiaceae	Morocco	Leaf
Sage	<i>Salvia officinalis</i>	Lamiaceae	Greece	Leaf
Spearmint	<i>Mentha spicata</i>	Lamiaceae	China	Flowg. herb
Spruce	<i>Tsuga canadensis</i>	Pinaceae	Canada	Needle
Tagetes	<i>Tagetes bipinnata</i>	Asteraceae	Madagascar	Flower
Thyme	<i>Thymus vulgaris</i>	Lamiaceae	Spain	Herb
Wormwood	<i>Artemisia absinthium</i>	Asteraceae	USA	Flowrg tops



**Figure** Effectivity of essential oils against *Xanthomonas hortorum* pv. *pelargonii* in vitro

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## Antibacterial activities of the essential oils from medicinal plants against the growth of *Clavibacter michiganensis* subsp. *michiganensis*

### Abstract

In the present study antibacterial activities of essential oils obtained from aerial parts of aromatic plants such as thyme (*Thymbra spicata* subsp. *spicata*), oregano (*Origanum syriacum* var. *bevanii*), mint (*Mentha spicata*), and lavender (*Lavandula stoechas* subsp. *stoechas*) were investigated against the seed-borne plant pathogenic bacterium, *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm* ICP7200), the causal agent of bacterial canker or wilt of tomato. By using the paper disc diffusion assay, all essential oils have shown antibacterial activity. Essential oils used in the paper disc diffusion assay varied in their antibacterial activity. Essential oil from thyme was the most effective in inhibiting the growth of *Cmm*, followed by those obtained from oregano and lavender. By using the micro agar broth dilution assay, the minimum bactericidal concentrations of the essential oil of thyme, oregano, ment and lavender were 10, 10, 25 and 50 µg/ml, respectively.

### Introduction

Plant diseases caused by bacteria are one of the major problems in crop cultivation in several agricultural commodities (1). At present, rapid and effective management of plant disease and microbial contamination in several countries is generally achieved by the use of synthetic pesticides and antibiotics. Chemical control of plant disease relies upon the use of antibiotics (such as streptomycin) in USA or copper compounds in the rest of the world; such control methods prevent bacterial multiplication but are not always adequate controls of seed-borne inoculum. Unfortunately, the frequent use of pesticides and antibiotics against plant and human pathogenic bacteria has led to the selection of resistant bacterial populations against antibiotics. The high cost of pesticides, development of pesticide/antibiotic resistant food-borne and plant pathogenic isolates, governmental restrictions on the use of antibiotics against plant pathogens in European countries, including Turkey, and the interest of environmental consideration raise the need to find alternative control methods. The antimicrobial properties of essential oils and their major constituents from a wide range of aromatic plant species have been assessed against the comprehensive range of microorganisms including bacteria, fungi and viruses (2,3).

Bacterial canker, caused by *Cmm*, is a recurrent and serious disease of field and greenhouse-grown tomatoes (*Lycopersion esculentum*) in several countries (4). The disease agent leads to vascular infections, wilting, chlorosis, and eventual death of the plant. Disease control is difficult because of the lack of commercially available resistant tomato cultivars.

Observations in the eastern Mediterranean region of Hatay province, Turkey, indicate the existence of wild types with a rich composition of indigenous aromatic and medicinal plant species. Therefore this research was conducted to evaluate the antibacterial potential of essential oils from oregano (*Origanum syriacum*), thyme (*Thymbra spicata*), mint (*Mentha spicata*) and lavender (*Lavandula stoechas* ssp. *stoechas*) (Table) growing in the region against bacterial canker of tomato agent (*Cmm*).

**Table** Inhibitory effect of plant essential oils and antibiotics on growth of *Clavibacter michiganensis subsp. michiganensis*. Diameter of inhibition zone including disc diameter of 6 mm. Numbers in parenthesis are standard errors of means. Means in the column followed by different letters are significantly different according to Duncan Multiple Range Test ( $P < 0.05$ )

Plants and agents	Inhibition zone (mm)	% increase of inhibition zone
<i>Thymbra spicata</i>	37.2 (2.62) F	83.6
<i>Origanum syriacum</i>	35.6 (1.78) F	82.8
<i>Mentha spicata</i>	31.3 (1.38) E	80.5
<i>Lavandula stoechas</i> subsp. <i>stoechas</i>	23.7 (1.27) D	74.2
Tetracycline (100 µg/ml)	18.7 (0.79) C	67.3
Ethanol (100%)	14.9 (0.43) B	59.0
Control (sterile medium)	6.1 (0.19) A	0

% increase was calculated by using formula;

% increase:  $100 - [(\text{inhibition zone in control plate}) / (\text{inhibition zone in treated plate}) * 100]$

## Material and methods

**Plant material and isolation of essential oils:** The plants used in this study were identified by Dr. I. Uremis. Voucher specimens have been deposited in the herbarium of the Plant Protection Department, MKU. Essential oils from air dried leaves of plants were extracted by steam distillation with Clevenger's apparatus for 2.5 h. After extraction, oil fractions were separated, dried over anhydrous sodium sulphate and stored in amber bottles at 4 °C until required.

**Test microorganisms and cultural methods:** The strain of *Cmm*, ICP7200, was preserved on modified nutrient yeast dextrose agar (NYA) at 4 °C. Inoculum suspensions were prepared from early log-phase cells, which were obtained by growing the bacteria in nutrient yeast extract broth in 25 ml sterile tubes and incubated at 27 °C on an orbital shaker at 200 rpm for 24 h. Bacteria were subsequently pelleted by centrifugation (twice, each at 3500 g for 5 min) and washed in sterile distilled water (SDW). The concentration was adjusted to  $10^8$  cfu ml<sup>-1</sup> by dilution to give an OD<sub>640</sub> of 0.12. These suspensions were used as required.

**Determination of antibacterial activity of the essential oils:** The antibacterial activity of the essential oils was determined by using the paper disc diffusion technique. Briefly, the test was performed in sterile Petri dishes (100 mm diameter) containing solid and sterile appropriate media. The surface of plates with appropriate media was inoculated with 200 µl of bacterial suspension prepared as described previously. Sterile filter paper (Whatman No.1) discs (6 mm in diameter) containing 10 µl of the tested essential oils were placed in the centre of the agar surface. Discs containing 10 µl sterile broth media were used as negative control. The reference antibiotics (tetracycline) amended discs, at 100 µg/ml concentrations, and 70% ethanol were used as positive control for comparison. The lid of each individual Petri dish was replaced immediately to prevent eventual evaporation. After allowing 1 h at room temperatures for the essential oils to diffuse across the surface, the plates were sealed with sterile Parafilm and incubated at 25 °C for 48 h. The antibacterial activity of oils and antibiotics was demonstrated by a clear zone of inhibition around the disc. The zone of inhibition was measured with the help of Vernier calipers.

The minimum inhibitory concentrations (MICs) of the essential oil against the test micro-organisms were determined by the broth micro dilution method. All tests were performed in LB broth supplemented with DMSO (Merck) (final concentration of 0.5%) to enhance the oil solubility. Bacteria were incubated overnight at 26 °C in LB broth. Test strains were suspended in LB to give a density of  $5 \times 10^5$  cfu ml<sup>-1</sup>, confirmed by viable counts. Dilutions of the essential oil ranging from 1 to 50 µg ml<sup>-1</sup> were prepared in 1 ml Eppendorf tubes. All determinations were performed in duplicate and two growth controls consisting of LB medium and LB with 0.5% (v/v) DMSO were included. All tubes were incubated at 26 °C for 24 h. Following incubation, 100 µl inoculum suspensions from each concentration of different oil were separately spread onto sterile NYA plates and incubated for further 48 h. The numbers of colonies on each Petri plate were counted. The MICs were determined as the lowest concentration of oil inhibiting the visible growth of bacteria on the agar plate.

Five replicate plates of each essential oil for each bacterium were used in all tests. The data were subjected to analysis of variance (ANOVA) by using SPSS statistic program (Version 11.05) and the significance between treatments was determined by means of Duncan's Multiple Range Test ( $P < 0.01$ ).

## Results and discussion

The antibacterial activity of the essential oils from each aromatic plant under study was estimated by using the paper disc diffusion technique and the response of *Cmm* to the essential oils is presented in Table 1. Differences between the plant essential oils were significant. Essential oils from *T. spicata* and *O. syriacum* had the highest inhibitory activity against the bacterium corresponding to 83.6% and 82.8 % increase in the zones of inhibition over the control (water). This was followed by essential oils obtained from mint and lavender (80.5 and 74.2% respectively). Efficacy of essential oils from thyme and oregano was comparable with the antibiotic tetracycline and ethanol even at the highest concentration (Table). The growth inhibition of test micro-organism was also evaluated by using the broth micro dilution method. The lowest MIC was determined against *Cmm* at 10  $\mu\text{g ml}^{-1}$  concentration of *Origanum* and thyme oil, followed by mint and lavender oils with an MIC of 25 and 50  $\mu\text{g ml}^{-1}$  respectively.

Previous studies had been conducted to assess the efficacy of essential oils from the medicinal plants, including those used in this study, against some phytopathogenic fungi and nematodes in Turkey (5,6,7) and in other countries (8,9,10,11). Antibacterial effects of essential oils have been examined mainly on human pathogens, spoilage microorganisms and dermatophytes. A relatively limited number of reports was found in the literatures on plant extracts or/and essential oils against phytopathogenic bacteria. To our knowledge there is no research on the evaluation of the efficacy of essential oils against *Psp*. Earlier studies have demonstrated the ability of different species of oregano and thyme oils to retard and inhibit the growth of various plant pathogenic bacteria such as *Agrobacterium tumefaciens*, *Cmm*, *Erwinia amylovora*, *E. caratovora*, *E. herbicola*, *Pseudomonas syringae*, *Pseudomonas viridiflava*, *Xanthomonas axonopodis* pv. *vesicatoria* (12,13,14,15,16,17,18). The results of this investigation showed that essential oils obtained from oregano, thyme, mint and lavender growing in the region have an antibacterial potential against the seed-borne tomato pathogen *Cmm*, as they showed similar antibacterial activity against halo blight of bean agent *Pseudomonas syringae* pv. *phaseolicola* (19). The mode of action of essential oils against bacteria is not known, however the involvement of essential oil components may disrupt the cell membrane of the bacterium and change its permeability, as reported by Sivropoulou et al. (20). Essential oils of plants belonging to the *Lamiaceae* family are rich in phenolic compounds, which are believed to be responsible for the marked antimicrobial activity. Essential oils of oregano, thyme and rosewood have been reported to induce rapid cell lysis of *Streptococcus pneumoniae* (21). Our results suggest that essential oils have the potential for use in bacterial control. Because of essential oils and/or their main components such as carvacrol, thymol and linalool have been reported to possess fungicidal and bactericidal activities (22,23), seed treatment with essential oils or their components could serve as a seed disinfectant. However, further experiments are needed to obtain information regarding the economic aspects and antibacterial activities of essential oils *in vivo* without phytotoxic effects on seed germination. Research on the chemical composition of the essential oils used and antibacterial activities against a variety of plant pathogenic bacteria and fungi are currently under investigation.

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## **Inhibition of soil borne *Xanthomonas campestris* pv. *malvacearum* in cotton by *Bacillus* spp.**

### **Abstract**

Oxolinic acid with known bactericidal efficacy against the genus *Xanthomonas* was used as a seed dresser to control bacterial blight of cotton caused by *Xanthomonas campestris* pv. *malvacearum* (*Xcm*). The bactericide inhibited the growth of *Xcm* on nutrient agar plates and induced resistance in seedling tissues up to 8 weeks after sowing. Control was improved when the seedlings, established from treated seeds, were sprayed with oxolinic acid suspension; they showed additive effects and provided much better disease control in cotton. The population of *Xcm* in the soil was reduced substantially when seeds were treated with oxolinic acid and/or *Bacillus* spp. Moreover, oxolinic acid was found to increase the population of an antagonistic strain of *Bacillus* in the rhizosphere of cotton. This strain suppressed the population of *Xcm* in the soil and efficiently controlled secondary infection throughout the cotton season. *Xcm* showed different levels of resistance to oxolinic acid.

### **Introduction**

Bacterial blight of cotton, caused by *Xcm*, is potentially one of the most damaging diseases in many cotton growing areas of the world (Innes 1983). In Sudan, bacterial blight infection is becoming more prevalent among the commercial varieties and across the irrigated areas. The disease is responsible for heavy shedding of the leaves and fruit abscission and is considered a major cause of lower yields observed in recent seasons. It can reduce seed cotton yield by 14-21% depending on the prevailing environmental conditions and the developmental stage at which infection takes place (Innes 1983; Ahmed et al. 1997). The control measures currently used include a combination of resistant cultivars, sowing date, legislative measures and chemical seed treatment (Ahmed et al. 1997).

Under the irrigated schemes, seed dressing with fungicides/bactericides, is being considered as a major component of the integrated disease management package for cotton and oxolinic acid was recently released for use to control seed borne *Xcm* (Ahmed et al. 1996). There is, however, an increasing preference for biological control methods due to undesirable side effects of chemicals that are harmful to the environment. Furthermore, secondary infection from soil borne inocula as well as from inocula on plant debris is difficult to control by chemical seed treatments. This work was therefore undertaken to determine the antagonistic activity of rhizosphere bacteria against the population of *Xcm* in the soil.

### **Materials and methods**

**Inoculum preparation:** The bacterium *Xcm* was cultured and maintained on nutrient agar (NA) medium. Bacterial suspensions were prepared from 48 h-old cultures, grown on Stolp medium, adjusted to an optical density of 0.06 at 660 nm and diluted to give approximately  $10^6$  cfu ml<sup>-1</sup>.

**In vitro tests:** The bactericide oxolinic acid, *Bacillus subtilis* and an antagonistic strain of *Bacillus* sp., isolated from soils treated with oxolinic acid in former tests, were evaluated for their *in vitro*-toxicity in inhibiting the growth of *Xcm* on NA plates. Different concentrations of the bactericide suspensions were spotted on solid nutrient agar medium in Petri dishes and streaked with 48 h bacterial cultures 2 days later. Inhibition was evaluated relative to the untreated control. Supernatants of *Bacillus* strains grown on NA medium (supplemented by 1% trypton, 0.5% yeast extract, 0.5% NaCl) at 30 °C for 48 h were added to fiber glass discs on NA Petri dishes. After 48 h at 30 °C, each treated Petri dish was streaked with a loop of *Xcm* culture.

**Enumeration and isolation of bacteria:** *Xcm* and *Bacillus* sp. were enumerated and isolated using nutrient agar plates from rhizosphere and non-rhizosphere soils of the cotton crop. Bacterial numbers in non-planted soil were determined by sampling from three replicates before sowing and then every 4 weeks. Soil samples were suspended in 10-fold sterile water dilution blanks. The soil suspensions were shaken vigorously for 10 min, serially diluted with sterile water and 0.1 ml of each dilution was plated in triplicate onto NA plates and incubated for 3-5 days at 25±2 °C.

**Field experiments:** The study was conducted at Gezira Research Station (GRS) experimental plot during 2000-2003 growing seasons. Artificial inoculation was applied in order to ensure a very high infection level. The seeds were shaken for 5 min in the bacterial suspension and the surplus fluid was decanted before the seeds were air dried at room temperature overnight. Inoculated seeds were treated with oxolinic acid, *B. subtilis*, the antagonistic strain of *Bacillus* sp. or a mixture of the two former. Untreated seeds and a standard seed treatment with metalaxyl/oxine copper mixture were used for comparison. Treated and untreated seeds were planted following heavy rains to ensure high relative humidity for at least one week after inoculation.

The experiments were established in a randomized complete block design with 4 replicates using a highly susceptible *Gossypium barbadense* commercial variety, Barakat 90. The standard cultural practices recommended for cotton production in Gezira were adopted. At four and eight weeks after sowing, disease incidence was recorded and half of the subplots received a single spray with oxolinic acid at the rate of 96 g a.i./ha. Four weeks later disease incidence was recorded and seed cotton yield was taken at harvest.

**Data analysis:** Data were statistically analyzed by ANOVA. Data in percentage was Arcsine transformed before analysis. Means were separated using Duncan Multiple Range test and differences between treatment means were considered significant when  $P < 0.05$

## Results

**In vitro test:** Oxolinic acid effectively inhibited the growth of *Xcm* at all concentrations tested. However, the higher concentrations (600-400 ppm) were more effective than the lower concentrations. In addition, the *Bacillus* strain isolated from soils that received oxolinic acid is as competent as the bactericide. The antagonistic capability was homogeneous among the strains isolated from the rhizosphere and non-rhizosphere soils (Table 1).

**Tab. 1** Effects of oxolinic acid and *Bacillus* sp. on growth of *Xcm*

Treatments	Inhibition rate				Positive (%)
	+++	++	+	-	
Oxolinic acid 20 WP (ppm) 600	25	5	0	0	100
400	21	6	3	0	100
200	17	8	3	2	93
100	9	13	5	3	90
50	0	10	12	8	70
<i>Bacillus subtilis</i>	8	11	6	5	83
Origin of <i>Bacillus</i> sp.					
Rhizosphere soil	26	4	0	0	100
Non Rhizosphere soil	23	6	1	0	100

Zone of inhibition: +++ = 9-12 mm; ++ 5-8 mm; + = 1-4 mm; - = no inhibition

**Recovery of bacteria from rhizosphere and non-rhizosphere soil:** Population of *Xcm* in the rhizosphere soil of the cotton crop was highly affected by seed treatment. The cfu g<sup>-1</sup> soil was substantially reduced by oxolinic acid and antagonistic *Bacillus* sp., but to a lesser degree by *B. subtilis* seed treatment compared to the untreated control. The efficacy of oxolinic acid and *Bacillus* sp. extended up to 20

weeks after planting (Figure 1). In the non-rhizosphere soil, the population of the bacterium increased with time. However, the antagonistic effect of *Bacillus* sp against *Xcm* was clearly indicated (Figure 2). The antagonistic *Bacillus* sp. was not detected in the rhizosphere soil of the untreated control and very few cfu were found in the rhizosphere of plants that received *B. subtilis* seed treatment. *Bacillus* sp. was consistently the numerically dominant bacterium in oxolinic acid treatments. The total number of *Bacillus* sp. cells increased rapidly in the first 4 weeks (Fig. 3) and the increase was more prominent in the oxolinic acid treatments. Oxolinic acid and *Bacillus* sp. increased the cfu g<sup>-1</sup> soil 12-20 weeks after planting by 75 and 68 %, respectively, compared to their particular populations at 4 weeks (Figure 3). In the non-rhizosphere soil, a similar trend of cfu g<sup>-1</sup> soil of total bacterial cells was observed. In this case the total number of *Bacillus* sp. cells was lower than in the rhizosphere soil (Fig. 4).

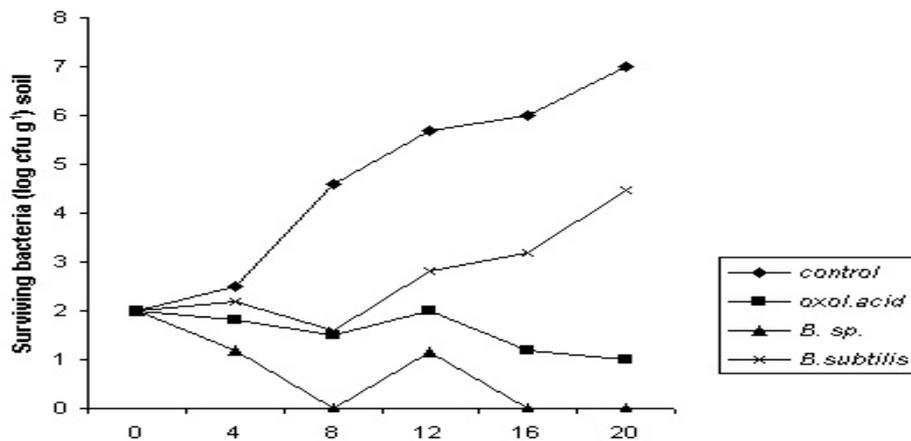


Fig. 1 Population of *X. c. pv. malvacearum* in the rhizosphere of cotton plants

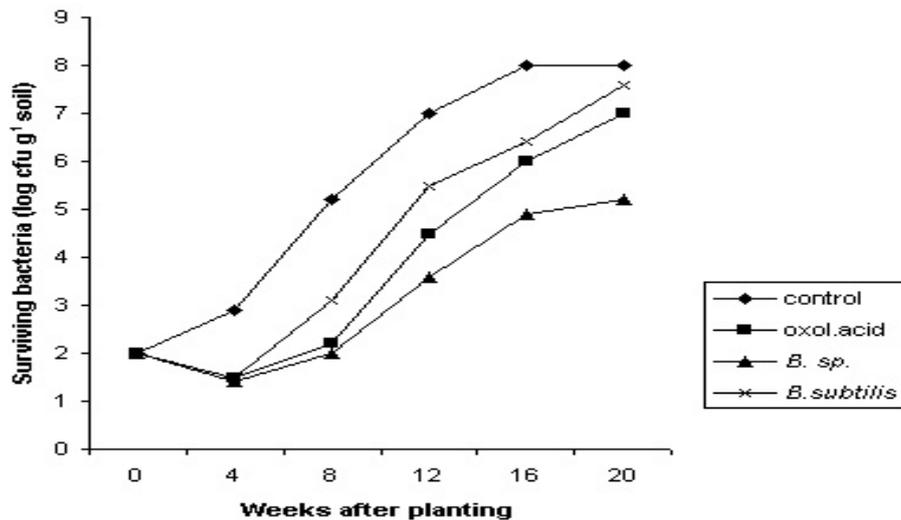


Fig. 2 Population of *X.c. pv. malvacearum* in the non-rhizosphere of cotton plants

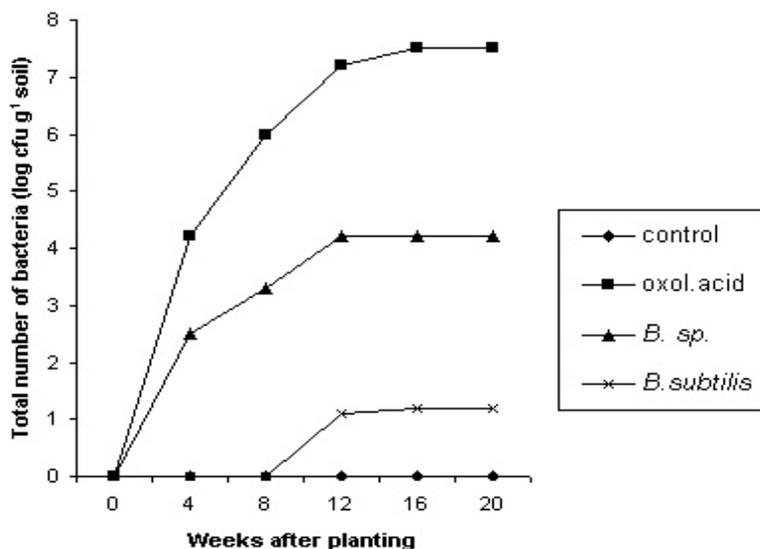


Fig. 3 Population of *Bacillus* sp. in the rhizosphere of cotton plants

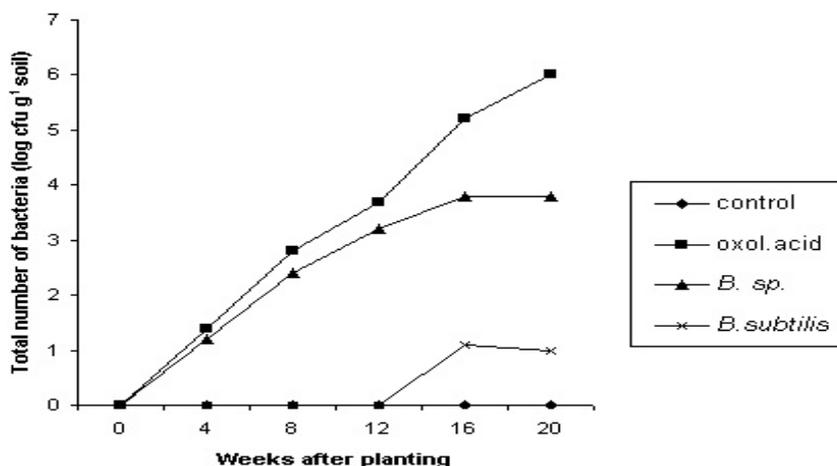


Fig. 4 Population of *Bacillus* sp. in the non-rhizosphere of cotton plants

Disease assessment: Seedling blight is largely controlled by bactericide/biocide seed treatment. In the season 2000/2001, seed treatment with oxolinic acid and *Bacillus* sp. reduced the percentage of infection of bacterial blight in the cotton variety Barakat 90 by 35 - 65% up to 12 weeks after planting (Tables 2, 3 and 4). Seed treatment with oxolinic acid and *B. subtilis* either singly or in mixture suppressed disease development up to 8 weeks after planting. *Bacillus* sp. was more effective as indicated by its consistently low values in the three seasons.

Control efficacy achieved by the foliar application of oxolinic acid was affected by the disease pressure encountered in the different seasons and the disease was suppressed by 14-38, 22-50 and 15-28 % in seasons 1, 2 and 3, respectively (Tables 2, 3 and 4). As expected, foliar application of the bactericide extended the period of low infection up to 12 weeks after planting. However, the concentration used was insufficient to suppress high levels of disease observed in the untreated control plots.

Effect on yield: Efficacy of the pesticides and biocides to control bacterial blight was reflected in good crop establishment and better crop yield. In the three seasons all treatments increased the yield of Barakat 90 significantly compared with the untreated control (Tables 2, 3 and 4). However, there was no significant difference between treatments and the supplementary spray of oxolinic acid; it significantly increased seed cotton yield over the unsprayed crop.

**Tab. 2** Effect of oxolinic acid and *Bacillus* on bacterial blight infection and seed cotton yield, variety Barakat 90, season 2000/2001; (% of infected plants. resp. yield in k/fed)

Treatments	Infection after		Infection after 12 weeks		Seed cotton yield (k/fed)	
	4 weeks	8 weeks	sprayed	unsprayed	sprayed	unsprayed
Oxolinic acid	0	16.5 b	18.3 c	54.8 c	11.1 b	9.6 b
<i>Bacillus subtilis</i>	0	11.0 bc	12.9 b	39.4 b	11.8 b	10.6 b
<i>Bacillus</i> spp.	0	7.0 c	7.0 a	21.3 a	12.1 b	11.8 bc
Oxolinic acid + <i>Bacillus subtilis</i>	0	8.6 c	9.1 ab	27.8 a	11.8 b	11.5 bc
Metalaxyl	0	20.4 bc	24.1 d	62.4 d	11.5 b	10.2 b
Untreated control	21.6	42.0 a	71.6 e	89.7 e	8.4 a	6.8 a
SE ±	-	1.9	1.25	2.4	0.72	0.47
CV %	-	16.8	21	26.5	24.8	25.3

**Tab. 3** Effect of oxolinic acid and *Bacillus* on bacterial blight infection and seed cotton yield, variety Barakat 90, season 2001/2002 (% of infected plants. resp. yield in k/fed)

Treatments	Infection after		Infection after 12 weeks		Seed cotton yield (k/fed)	
	4 weeks	8 weeks	sprayed	unsprayed	sprayed	unsprayed
Oxolinic acid	5 ab	10 a	19 a	69 b	15.6 b	13.5 b
<i>Bacillus subtilis</i>	14 bc	20 b	36 a	85 b	15.5 b	12.7 b
<i>Bacillus</i> spp.	0 a	16 a b	23 a	45 a	15.2 b	13.2 b
Oxolinic acid + <i>Bacillus subtilis</i>	9 b	26 bc	32 a	71 b	14.9 b	12.9 b
Metalaxyl/oxine copper	18 c	29 c	36 a	79 b	14.6 b	12.7 b
Untreated control	34 d	78 d	100 b	100 c	8.4 a	7.8 a
SE ±	1.8	2.7	5.6	6.7	1.8	1.62
CV %	24.3	33.4	27	22	18.4	19.6

**Tab. 4** Effect of oxolinic acid and *Bacillus* on bacterial blight infection and seed cotton yield, variety Barakat 90, season 2002/2003 (% of infected plants. resp. yield in k/fed)

Treatments	Infection after	Infection after	Infection after 12 weeks		Seed cotton yield (k/fed)	
	4 weeks	8 weeks	sprayed	unsprayed	sprayed	unsprayed
Oxolinic acid	6 a	28 ab	53.3 a	68 a	11.5 b	10.7 b
<i>Bacillus subtilis</i>	8 ab	25 a	46.7 a	74 ab	12.1 b	11.2 b
<i>Bacillus</i> spp.	3 a	21 a	46.3 a	65 a	11.9 b	11.3 b
Oxolinic acid + <i>Bacillus subtilis</i>	11 b	31 b	56.7	76 ab	11.2 b	10.5 b
Metalaxyl/ oxine copper	16 c	35 b	56.3 a	84 b	11.7 b	11.0 b
Untreated control	34 d	54 c	100 b	100 c	8.9 a	7.80 a
SE ±	1.6	1.4	7.3	3.6	0.46	0.65
CV %	36	23	23.7	28.3	26.5	30.70

## Discussion

Aerobic endospore forming bacteria of the genus *Bacillus* can be commonly found in soil and plant litter, where they are assumed to play an important role in the biological cycling of carbon and nitrogen (Ladd and Paul 1973). Vardavakis (1989) reported that *Bacillus* spp. were present as the most abundant genera

of heterotrophic bacteria. In the present study, *Bacillus* sp. was consistently the numerically dominant bacteria in oxolinic acid treatments. It always comprised a considerable fraction (over 50%) of the total bacterium detected on NA plates. *Bacillus* sp. are often reported to be present in low numbers in the rhizosphere compared with other bacteria, such as pseudomonads (Hasegawa 1985; Claus and Berkeley 1986; Young et al. 1995). In this study, the antagonistic strain of *Bacillus* sp. was abundantly isolated only from soils that had received oxolinic acid and in low numbers from soils treated with *B. subtilis*. These findings are consistent with those reported by Reddy and Rahe (1998) who reported that the numbers of introduced *B. megaterium* increased in the rhizosphere of soybean. The different seed treatments used, caused bacterial blight to appear less severe, with limited leaf lesions and abscission during the first 8 weeks from planting. This might support the low *Xanthomonas* population in soil during that particular time. Our findings clearly indicate the role of *Bacillus* in inhibiting growth of *Xcm* in the soil and thus reducing, in the long run, secondary infection of bacterial blight that might occur from soil inocula.

In these experiments, the pathogen challenge was continuous from lateral spread of the bacterium between plants in the same field or from autoinfection. Yet, seed dressing with biocides still proved effective over time although it was not sufficient enough to protect the plants up to the end of the season. A supplementary foliar spray of oxolinic acid was efficient in suppressing the disease up to 12 weeks after sowing which was reflected in higher yields. With such increase in yield, the use of bactericide and/or biocide would still be justifiable if bacterial blight was responsible for some yield losses. However, the use of oxolinic acid over the extremely large cotton production areas (1 million acre) will be restricted for its indispensable importance as a drug to some endemic human diseases in Sudan. The quinolone group to which this bactericide belongs is known to cause quick mutation in Gram-negative bacteria (Hikichi et al. 2001; Shtienberg et al. 2001). Cross resistance between quinolones and their sister fluoroquinolones was also reported specially under inadequate dosing in very high population (Hirai et al. 1986). *B. subtilis* was reported to control bacterial blight in cotton (Safiyazof et al. 1995).

In this study, *Bacillus* spp. gave good control of bacterial blight even after direct challenge with high soil inocula conducive to high disease incidence in the untreated control plots. This suggests that seed treatment with cultures of antagonistic *Bacillus* can be beneficial in the field. Further studies of their activity could lead to development of *Bacillus* products to control bacterial blight of cotton.

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## II. Biological control of bacterial diseases in field crops

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### Mechanisms of action for biocontrol of *Erwinia* soft-rot diseases

#### Abstract

*Erwinia carotovora* and *E. chrysanthemi* cause soft-rot and vascular diseases that exact significant yield loss in field crops such as potato and sugar beet. Antagonists (primarily saprophytic bacteria, apathogenic strains and phage) have been identified that suppress disease after endophytic, tuber, spermosphere and rhizospheres colonisation. A variety of mechanisms of action operate in antagonists, including lysis, production of antibacterial compounds (bacteriocins, antibiotics), competition (for nutrients and space) and blocking expression of pathogen virulence genes (quorum sensing quenching). Understanding these mechanisms opens the door to streamlining strain selection, optimising activity and co-opting these for novel applications such as transgenic crops.

#### Introduction

This short review considers *E. carotovora* subspecies (= *Pectobacterium*; Gardan et al. 2003) and *E. chrysanthemi* (= *Dickeya*; Samson et al. 2005) and focuses on potato and sugar beet, which are the most widely cultivated field crops afflicted by soft-rot diseases. The primary virulence mechanism of these pathogens is the excretion of pectolytic enzymes (e.g., cellulases, pectinases, proteases) that macerate host tissues causing the typical soft-rot symptoms (Perombelon and Kelman 1980; Barras et al. 1994; Laasik et al. 2005), but other factors (i.e., superoxide dismutases that shield against host defenses; Santos et al. 2001) are important and worthy of attention as potential biocontrol targets. Biocontrol has been studied for suppression of soft-rot diseases in all the main infection courts: endophytic, tuber (geocaulosphere), spermosphere and rhizosphere. The bacterial and bacteriophage antagonists that have shown efficacy operate via a diversity of mechanisms including antibiosis, lysis, competition and induced resistance. Plant growth promoting bacteria can indirectly suppress disease by enhancing host ability to resist and/or compensate for pathogen attack (Wang et al. 2006) but such examples are not considered in this review.

#### Competition

Competition for occupation of the infection court and/or limited nutrients is a foundation mechanism of biocontrol for most antagonists (Duffy 2001). Ability to effectively colonise the infection court be it natural openings such as tuber lenticels (Scott et al. 1996) or wounds (e.g., from harvesting; Kastelein et al. 1999) and during crop growth or postharvest (Colyer and Mount 1984) is a critical factor in achieving effective biocontrol. Aggressive competition for iron, an essential nutrient for all life that is critically limiting in most environments, may determine whether antagonists can establish in the infection court and exclude pathogenic *Erwinia* encroachment. Bacteria, antagonists and pathogens alike, produce chelators (e.g., fluorescent siderophores) for iron acquisition. Differential affinities of these metabolites for their primary target iron, and whether bacteria can utilise heterologous siderophores, determines the winner in these competitions (Peñalver et al. 1994; Loper and Henkels 1999). Competition events in agroecosystems are not isolated and potential antagonists that outcompete pathogens may not necessarily be dominant members of diverse microbial communities (Sessitsch et al. 2004).

## Phages

Bacteriophages are lytic viruses found ubiquitously in nature that have generally high specificity for bacterial genotypes killing from a few strains to most strains of a given pathogen species (Gill and Abedon 2003). Phages effective against *Erwinia* (Eayre et al. 1995; Tovkach 2002) can readily be isolated from standing water in agricultural fields by spreading onto 'lawns' of pathogen cultures where they manifest as clear plaques; and phages can be readily mass-produced for biocontrol application. Narrow host range of phages is attractive since non-target effects are minimised, but it can be a severe disadvantage since the strong selection pressure favours rapid development of resistant pathogen genotypes. This drawback can be overcome in part through application of a phage mixture (e.g., h-mutants; Flaherty et al. 2001) or genetic manipulation to stabilise host range (Nguyen et al. 2001). Managing native populations of phages in an integrated biocontrol approach deserves more attention (Ashelford et al. 2003). Phages, albeit distinct from pathogen phage, can also interfere with bacterial antagonists (Keel et al. 2002).

## Bacteriocins

Bacteriocins are antibiotic-like metabolites that are highly specific affecting only taxonomically related bacteria and acting similar to both antibiotics and lytic phage. It is thought bacteriocins have an evolutionary origin in phage. As with phage therapy, the high specificity of bacteriocins makes their deployment an attractive biocontrol option due to low non-target effects in the environment, but it can also present a high selection pressure for rapid resistance development in pathogen populations (Riley and Wertz 2002). Observations of *Erwinia* bacteriocins that enable the pathogen to compete in tuber wounds (Axelrood et al. 1988) have been exploited for biocontrol using near-isogenic, nonpathogenic strains (e.g., Out-mutants) with a competitive advantage (Costa and Loper 1994). Membrane bound ABC transporter proteins in *Erwinia* can expel antimicrobial metabolites serving as a pathogen self-defense mechanism (Llama-Palacios et al. 2002).

## Natural antibiotics

Plant-associated bacteria produce a diversity of antibiotic metabolites with broad-spectrum antimicrobial activity. Antibiotic biosynthesis is generally stimulated in iron-sufficient environments where siderophore production is repressed. Antibiotics enhance the ecological competence of the producing bacteria and in competitions with pathogens they can provide biocontrol (Raaijmakers et al. 2002). Antagonists that produce either phenazine or 2,4-diacetylphloroglucinol antibiotics effectively inhibit *E. carotovora* growth and reduce soft-rot disease severity on potato (Molina et al. 2004). *Erwinia carotovora* produces antibiotics, as well, (i.e., carbapenam; Holden et al. 1998) which may level the playing field in certain interactions. Antibiotic producing *Pseudomonas fluorescens* and *P. putida* strains applied to potato seed pieces effectively suppressed late decay caused by *E. carotovora* subsp. *atroseptica* in the field (Xu and Gross 1986). A threshold antagonist population of approximately  $10^6$  cfu/seed piece was needed for effective biocontrol (see Quorum Quenching below). Biocontrol treatments were ineffective in field trials against early decay caused by *E. carotovora* subsp. *carotovora*, presumably because antagonist population sizes were too low when this pathogen was predominant. This study neatly demonstrates the primary importance of competitiveness before other mechanisms can have a chance to operate. It also emphasises the need to clearly identify the target disease (Toth et al. 2001).

## Induced resistance

Non-specific systemic induced resistance (IR) to plant pathogens can be induced in plants by prior colonisation by non-damaging plant-associated bacteria as well as by wounding (e.g., challenge inoculation with incompatible or necrotising pathogens). Plant emitted volatile organic compounds (VOCs; e.g., methyl jasmonate) trigger IR in adjacent plants. Similarly, VOCs produced by *Bacillus subtilis* and *B. amyloliquefaciens* in the rhizosphere or even bacterial-free captured emissions trigger IR against *E. carotovora* subsp. *carotovora* (Ryu et al. 2004). Plants react to pathogens and other microorganisms by triggering an initial oxidative burst that confers transient resistance. *Bacillus mycoides* strain BacJ uniquely produces an as yet uncharacterised metabolite that triggers a second burst of active oxygen species conferring persistent IR in sugar beet to pathogenic strains of *E. carotovora*

subsp. *betavasculorum*, similar to that observed after challenge with incompatible apathogenic *E. carotovora* subsp. *betavasculorum* strains (Bargabus et al. 2003). Salicylate and other siderophores can trigger IR (Palva et al. 1994) and may be primary determinants in other antagonists (van Loon et al. 1998). The plant growth hormone ethylene plays a key role in IR (Geraats et al. 2003) and antagonists that target this pathway (Glick 2005) have yet to be explored for potential soft-rot biocontrol.

### Quorum-sensing quenching

Pectolytic phytopathogenic bacteria autoregulate expression of key virulence factors by means of *N*-acyl homoserine lactones (AHLs; von Bodmann et al. 2003; Whitehead et al. 2003; Smadja et al. 2004). Each cell excretes these signal molecules into the environment, and when cohabiting cells sense these a cascade of genes are switched on. Thus pathogens coordinate the expression of virulence factors for when enough cells are present to cause real damage, a process termed 'quorum sensing'. Premature pectinase gene expression in a small wound for example may trigger host defenses precluding the progression of infection. By waiting until enough bacteria accumulate in the infection court (i.e., a dense population) to overwhelm general host defenses, pathogens gain the upper hand. Soil inhabiting *Bacillus* strains were found that produce an acyl homoserine lactonase (Dong et al. 2002, 2004; Reimann et al. 2002) and are able to block virulence gene expression in *E. carotovora* and suppress potato soft-rot even without affecting pathogen growth (Molina et al. 2004). Heterologous expression of the responsible gene, *aiiA*, in transgenic bacteria (Molina et al. 2004) or plants (Dong et al. 2000, 2001) also resulted in effective biocontrol of soft-rot disease. Recently, other types of quorum quenching metabolites (i.e., acylases) have been identified in bacterial antagonists (Fray 2002; Kang et al. 2004; d'Angelo-Picard et al. 2005; Delalande et al. 2005; Uroz et al. 2003, 2005). *E. carotovora* possess a second distinct type of quorum sensing system centered around LuxS proteins (Xavier & Bassler 2005) and finding antagonists that block this signal molecule may open another biocontrol option. *Erwinia* recycles its own autoinducer molecules (Byers et al. 2002) and finding antagonists that may accelerate this turnover is as yet an unexplored option for biocontrol. Similarly, certain bacterial antagonists also utilise quorum sensing to autoregulate the expression of biocontrol genes such as those involved in antibiotic biosynthesis and can be adversely affected by quorum quenching (Pierson et al. 1998). Optimising quorum sensing in beneficial bacteria while at the same time avoiding signal interference from other bacteria, including pathogens, or autoinducer mimicry by plants (Teplitski et al. 2000; Lu et al. 2005) will also be critical to achieve effective biocontrol.

### Harnessing mechanistic insights to improve crop health management

Understanding the mechanisms behind effective biocontrol studies will be a key to future efforts aimed at ensuring transfer of knowledge to practitioners and realising commercial scale biocontrol of soft-rot diseases. The laborious and time-consuming strain selection process can be streamlined by pre-screening random isolates for expression of relevant genes such as those encoding antibacterial metabolites. The level of activity can be optimised with special formulations and/or alteration of the infection court (e.g., with mineral additives; Duffy and Défago 1999) in order to favour the expression of such biocontrol genes *in situ*. Along these lines, prescription biocontrol can be pursued matching specific antagonist isolates with crop cultivars and agro-environments (e.g., soil type) that favour expression of biocontrol genes (Ownley et al. 2003). Mixtures of antagonists (Kastelein et al. 1999) that favour rather than interfere with the activity of each component can be pursued. Novel applications such as transgenic crops with inducible expression of antagonist biocontrol genes may circumvent problems with antagonist establishment in the infection court. Native antagonist populations can be exploited and manipulated (Ashelford et al. 2003; Mazzola 2004) by following the population dynamics of genotypes with key biocontrol genes in order to effect sustainable control, thus precluding costs and potential risk factors involved in the introduction of biocontrol agents.

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## **Bacterial inoculants for suppression of rice bacterial blight in India: role of 2,4-diacetylphloroglucinol in disease suppression**

### **Abstract**

This study uses Amplified Ribosomal DNA Restriction Analysis (ARDRA) to highlight the prevalence of 2,4-diacetylphloroglucinol (DAPG)-producing strains of *Pseudomonas fluorescens* in crop rhizospheres of southern India and uses results from field test and *Phl* mutants to implicate DAPG production as an important mechanism in the biological suppression of rice bacterial blight in India.

### **Introduction**

Significant yield losses from diseases still occur in rice in spite of continuous improvements in rice breeding. Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* is one of the most devastating rice diseases of global occurrence and is particularly destructive causing annual crop losses that range from 10 to 50% in tropical Asia (Mew 1987). In India, planting of resistant rice cultivars has been the most successful disease management strategy. However, breakdown of varieties carrying a single R-gene is more frequent in the field because of the rapid evolution of sub-populations of the pathogen that overcome these resistances (Venkatesan and Gnanamanickam 1999; Brindha Priyadarisini et al. 2003). In recent years, we had constructed through molecular marker-assisted backcross breeding and transformation, transgenic elite indica rices (cv.CO39 and IR50) that carry a pyramid of genes for blast and BB resistance (*Pi-1+Pi-z+Xa21*) (Narayanan et al. 2002, 2004). These are yet to be approved for planting in rice fields.

In all these years there has been no major study on the biological suppression of BB in indica rices with bacterial agents. In our isolations made during 1997-2000, we encountered more and more of *Bacillus* spp in the rice rhizosphere of southern India. These strains inhibited *X. oryzae* pv. *oryzae* in laboratory assays and field experiments (Gnanamanickam 2004; Vasudevan, 2002; Vasudevan et al. 2002). More recently, using the PCR-based screening method (Raaijmakers et al. 1997), we identified fluorescent pseudomonads which produced 2,4-diacetylphloroglucinol (DAPG) in our tropical rice rhizosphere (Velusamy and Gnanamanickam 2003; Velusamy et al. 2004, 2005). In this paper, we provide a careful analysis on the occurrence of DAPG producing strains of bacteria and highlight the importance of DAPG production as a mechanism for the biological suppression of bacterial blight severities in rice.

### **Materials and methods**

Bacterial strains and analyses for DAPG production: Bacterial strains assembled from crop rhizosphere of four southern states of India were available for this study. Batch 1 consisted of 637 strains of which 27 strains were PCR-positive (by the method of Raaijmakers et al. 1997) for DAPG production and also inhibited the growth of *X. oryzae* pv. *oryzae*. Batch 2 consisted of 724 strains of which 67 strains inhibited the growth of *X. oryzae* pv. *oryzae* and were also positive for DAPG production when analyzed with the new PCR primers (B2BF and BPR4) developed by Gardener et al. (2001). These positive strains that shared a 629 bp DNA fragment were subjected to amplified ribosomal DNA restriction analysis (ARDRA). Full length 16s rRNA gene sequence information was obtained for the strains by PCR amplification with the conserved eubacterial primer 8F and 1492R using PCR conditions described by Weisburg et al. (1991). Reaction mixtures were incubated with *Hae* III and *MSP* I (at 37°C) and with *Taq* I (at 60°C). Digested products were subjected to electrophoresis on 2% agarose gel in 0.5% TBE for 2-4 h at 75V. Banding patterns were visualized by ethidium bromide staining and by viewing under a uv transilluminator.

A strain of *Pseudomonas fluorescens* CHAO (gift from Dr. G. Defago, ETH-Zentrum, Zurich), known to produce DAPG, was used as a positive control in all PCR based strain selections.

Suppression of bacterial blight by DAPG-producing strains in net-house and field experiments: Selected DAPG-producing bacterial strains from batch 1 were evaluated for their ability to suppress the bacterial blight (BB) disease in a net-house experiment during July-December, 2002 and in a field experiment carried out during July-December, 2003. These experiments were conducted at the Regional Agriculture Research Station (RARS), Pattambi, Kerala. Rice seeds of a bacterial blight-susceptible cultivar (cv. IR24) were seed-coated by bacterization (Gnanamanickam and Mew 1992) separately with cell suspensions of DAPG-producer strains at  $10^8$  cfu/ml in 0.1% carboxymethylcellulose (cmc). Seeds coated with plain 0.1% cmc solution served as the untreated check. At the time of transplanting, the seedlings were given a root-dip in respective bacterial cell suspensions (containing  $10^8$  cfu/mL) (or in sterile distilled water in the case of untreated control). When the seedlings were 35 and 45 days old, they received two additional foliar spray applications with respective bacteria at  $10^8$  cfu/mL of spray fluid suspension (prepared in 0.1% carboxymethylcellulose (cmc)). In these experiments each treated plot was covered with a plastic sheet on all four sides before spraying, in order to avoid cross-contamination among different bacterial strains used as treatments. Plants raised from bacteria-treated and untreated seeds were clip-inoculated with  $10^6$  cfu/ml of *Xoo* (Kauffmann et al. 1973) when they were 45 d old. Length of bacterial blight lesions that developed were measured 14 d after the clip-inoculation and reduction in disease severity was derived from reductions in lesion length. The reduction in disease incidence in the treated plants was calculated and the data was analyzed for statistical significance using the least significant difference (LSD) test (Gomez and Gomez 1976).

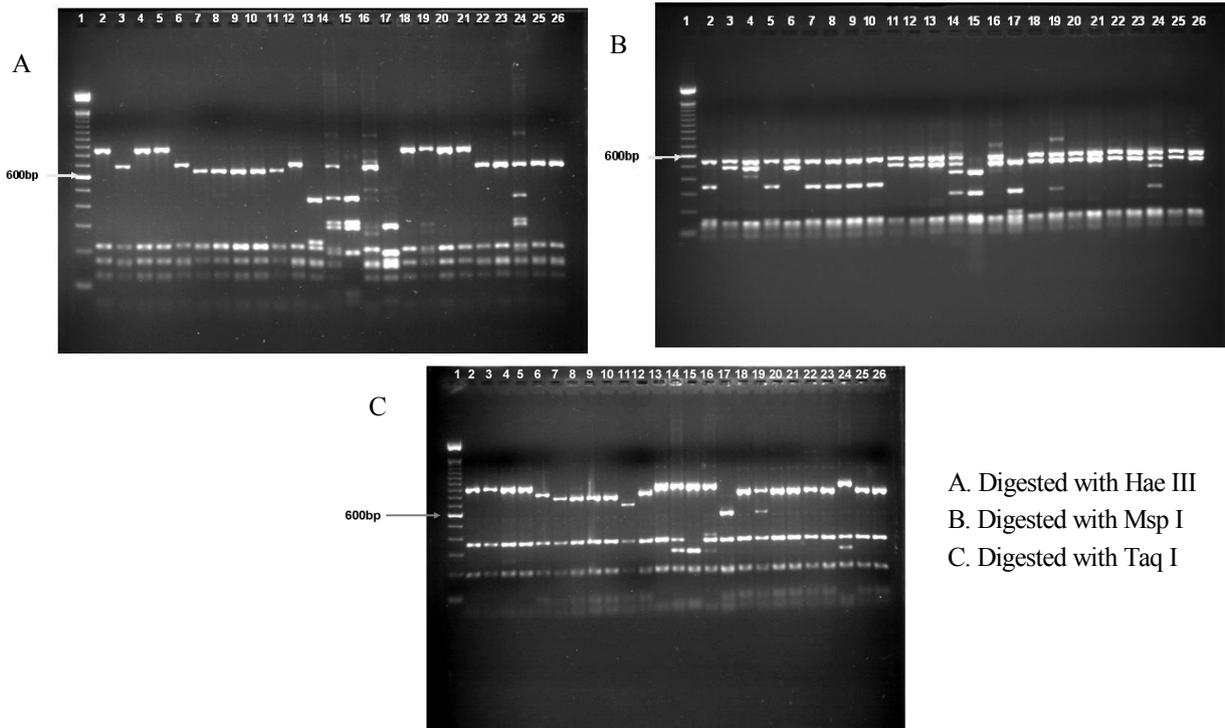
Generation of PhI<sup>-</sup> mutants of DAPG-producing *P. fluorescens* strain PTB9: To make a critical assessment of the role of DAPG in bacterial blight suppression, PhI<sup>-</sup> mutants were generated by transposon mutagenesis. Transposons (in donor strain S17-1( $\lambda$ pir)/pUT•Km::phl TR#1) were mobilized into DAPG-producing *P. fluorescens* strain PTB9 by the method of Bangera and Thomashow (1999). A total of 1,125 transconjugants were screened *in vitro* by dual plate assay against the rice bacterial blight pathogen, *X. oryzae* pv. *oryzae* to identify mutants defective in antibiotic activity. Five of the fifty strains that lacked the ability to inhibit *X. oryzae* pv. *oryzae* in the laboratory were selected and checked with PCR to confirm the insertion of the transposon.

Greenhouse test to evaluate PhI<sup>-</sup> mutants and the wild type *P. fluorescens* PTB9 for rice bacterial blight suppression in cv. IR24 rice: During July-November 2004, a greenhouse experiment was carried out in our University greenhouse at Chennai, to evaluate the biocontrol efficacy of the DAPG-producing wild type strain *P. fluorescens* PTB9 and its mutants that were defective in their antibiotic activity, PTB9a, PTB9b, PTB9c, PTB9d and PTB9e. Small batches of 5 g seeds of IR24 rice were seed-coated by bacterization as described in the previous section. Both bacteria-treated and untreated leaves were inoculated with the pathogen. Reductions in BB lesion lengths were measured.

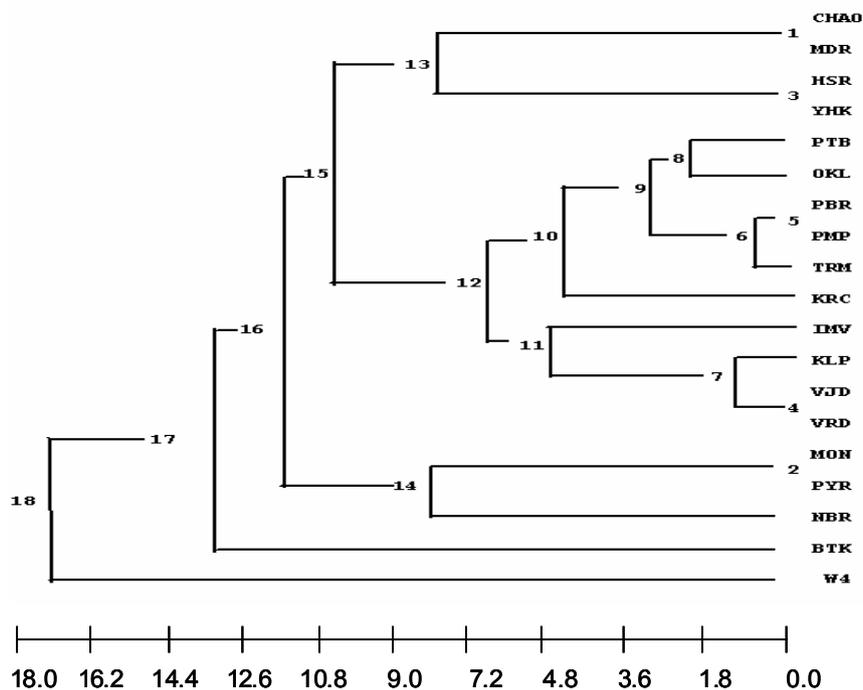
## Results

Detection of DAPG production by PCR-based screening methods: When the PCR method that used forward (PhI2a) and reverse (PhI2b) primers, 27 strains (in batch 1) showed a characteristic 745-bp DNA fragment amplification. PCR products of a DAPG-producing reference strain, *Pseudomonas fluorescens* CHAO also had this characteristic 745-bp fragment while in the rest of the non-producers, no PCR products were amplified. In batch 2, strains that were positive for DAPG production had a common DNA fragment of the size of 629 bp. On the basis of their biochemical profile, the DAPG-producing strains were identified as strains of *P. fluorescens* (data not shown).

ARDRA analysis: Three of the 25 DAPG-producing strains analyzed revealed the same RFLP pattern as that of the *P. fluorescens* CHAO, the reference strain (Figure 1a). The remaining strains formed two major groups that were different as observed in the phylogenetic analysis (Figure 1b).



**Fig. 1a** Amplified ribosomal DNA restriction analysis (ARDRA) for different strains of *Pseudomonas fluorescens*



**Fig. 1b** Phylogenetic analysis of 16 rDNA banding patterns for DAPG – producing *P. fluorescens* strains

Evaluation of DAPG-producers for biological suppression of rice BB in net-house and field plots: In the net-house experiments, all 27 strains (batch 1) of *P. fluorescens* suppressed BB and the disease reductions ranged from 8.2 to 58.8%. Nine strains (five rice strains and 4 non-rice strains) reduced BB incidence by more than 50% and among them the most efficient strains (rice strain PTB9) reduced BB by 58.8%.

When these 27 DAPG-producing *P. fluorescens* strains (batch 1) were evaluated for BB suppression in a field experiment planted with cultivar IR24 rice, the mean leaf BB lesion length in bacteria treated plants ranged from 7.8 to 21.9 cm while in the untreated plants, it was 22.0 cm (Table 1). The untreated plants showed severe BB disease symptoms with long and spreading blight lesions, while the plants treated with some of the DAPG producer strains were relatively healthy and had shorter lesions of less than 3 cm. Of the 27 strains, 7 strains, IMV 14, PTB 9, MDR 7, KAD 7, VEL 17, VGP 13 and PDY 7 showed more than fifty percent (56.74%, 64.46%, 54.43%, 53.9%, 50.8%, 51.2% and 51.9%, respectively) BB suppression compared with untreated plants. Maximum BB suppression (64.46%) was afforded by strain PTB 9, a rice-associated strain.

**Tab. 1** Evaluation of 2,4-diacetylphloroglucinol (DAPG)-producing *Pseudomonas fluorescens* strains for biological suppression of BB in rice cv. IR24. Net-house and field experiments, RARS, Pattambi, Kerala, India

DAPG-producing <i>P. fluorescens</i> strain	Diam. of inhibition of <i>Xoo</i> in dual plate assay (cm)	Net-house experiment		Field experiment	
		Mean BB lesion length (cm) <sup>†</sup>	Percent BB suppression	Mean BB lesion length (cm) <sup>†</sup>	Percent BB suppression
KAD7	3.6	9.04**	56.87	10.15**	53.93
IMV14	4.1	8.66**	56.68	9.53**	56.74
IMV2	2.8	13.25**	36.78	14.72**	33.18
BGR19	3.0	15.20**	27.48	12.62**	42.71
PTB9	4.4	8.64**	58.78	7.83**	64.46
MON1	4.2	16.83**	19.70	13.85**	37.13
TVM8	1.2	17.54*	16.32	16.85**	23.51
VEL17	3.5	16.07**	23.33	11.07**	50.75
VEL10	2.8	17.39*	17.03	18.17*	17.52
GDY4	2.1	14.52**	30.73	12.90**	41.44
GDY7	2.3	10.15**	51.57	12.05**	45.30
TRP5	1.7	13.90**	33.68	14.42**	34.54
TRP18	2.3	16.87**	53.15	11.38**	48.34
MDR9	2.1	14.53**	30.68	16.90**	23.29
MDR7	4.1	8.99**	57.11	10.04**	54.43
STR7	1.8	9.82**	19.51	14.09**	36.04
VGP13	3.5	9.35**	55.39	11.19**	51.21
MDR16	1.4	13.08**	37.60	11.87**	46.12
PDY7	3.8	8.63**	58.83	9.50**	51.88
VLB7	1.7	13.91**	33.64	20.03 <sup>ns</sup>	9.08
KVR5	2.5	9.24**	55.92	13.42**	39.08
TNI13	3.6	15.50**	26.05	15.82**	23.19
KOV8	3.0	12.85**	38.69	19.89 <sup>ns</sup>	9.71
RJP31	2.4	18.80 <sup>ns</sup>	10.31	21.92 <sup>ns</sup>	0.50
KOV3	1.8	16.52**	21.18	20.13 <sup>ns</sup>	8.62
PDU1	2.9	19.25 <sup>ns</sup>	8.16	19.43 <sup>ns</sup>	11.80
PDU9	2.7	17.41*	16.94	21.58 <sup>ns</sup>	2.04
Untreated Check	0.0	20.96	0.00	22.03	0.00
LSD <sub>0.05</sub>		3.0		3.4	
LSD <sub>0.01</sub>		4.0		4.5	

<sup>†</sup>Each figure is a mean of 40 observations; \*\*Reduction in lesion length significant at 1% level; \*significant at 5% level and <sup>ns</sup>= not significant.

Loss of Phl production results in loss of biocontrol efficiency: In PCR analysis, five Phl<sup>-</sup> mutants (PTB9a, 9b, 9c, 9d, 9e) of *P. fluorescens* PTB9 generated through transpositional mutagenesis (*Tn5-Km*), defective in DAPG production, showed transposon insertion. Their heat-lyzed bacterial DNA did not show the amplification of the characteristic 745 bp fragment whereas the DNA of the wild type strain PTB9 had the fragment.

In the greenhouse assay, rice plants raised from seeds treated with PhI<sup>-</sup> mutants, PTB9 a to e had bacterial blight lesions of 15 to 25 cm length while the plants raised from seeds that were treated with the wild type strain *P. fluorescens* PTB9 had shorter lesions of 3-5 cm (Table 2). The wild type strain afforded a bacterial blight reduction of 59.52% while the mutants (PTB9 a,b,c,d,e) defective in DAPG production afforded 19.65, 17.11, 23.77, 20.81 and 18.11 percent bacterial blight reductions, respectively.

**Tab. 2** Evaluation of *Pseudomonas fluorescens* PTB9 and its PhI<sup>-</sup> mutants for suppression of bacterial blight (BB) in IR24 rice. Greenhouse experiment, Chennai, southern India, July-November, 2004

Bacterial strain	Mean BB lesion length (cm) <sup>1</sup>	Difference in lesion length from control (cm) <sup>2</sup>	Percent BB suppression
Wild type	7.66**	11.27**	59.52
<i>Pseudomonas fluorescens</i> PTB9			
PhI <sup>-</sup> mutants			
PTB9a	15.21 <sup>ns</sup>	3.72 <sup>ns</sup>	19.65
PTB9b	15.69 <sup>ns</sup>	3.24 <sup>ns</sup>	17.11
PTB9c	14.43 <sup>ns</sup>	4.50 <sup>ns</sup>	23.77
PTB9d	14.99 <sup>ns</sup>	3.94 <sup>ns</sup>	20.81
PTB9e	15.50 <sup>ns</sup>	3.43 <sup>ns</sup>	18.11
Check	18.93	0.00	0.00
LSD 5%	5.8	5.8	5.8
1%	6.6	6.6	6.6

\*\*Reduction in lesion length significant at 1% by LSD method of analysis; <sup>ns</sup> = not significant. <sup>1</sup>Mean of three replications; <sup>2</sup>Mean of normalized lesion lengths (mean lesion length in untreated control/check subtracted from mean lesion length in bacteria-treated plants)

## Discussion

This is perhaps the first systematic research effort in India to investigate the possible use of *Pseudomonas* strains and to highlight a role for 2,4-diacetylphloroglucinol (DAPG) in the suppression of bacterial blight. Therefore, this is an important land mark study on the biological control of bacterial blight both in India and the whole tropics. There has been no previous report from India on the production of DAPG by plant-associated bacteria or on its suppression of rice bacterial blight.

DAPG production has been a very well known mechanism in the biological control of some of the major fungal pathogens of the temperate regions and had been assigned much importance as the factor that has contributed to the “take-all” decline in wheat (Raaijmakers et al. 1997, 1999; Raaijmakers and Weller 1998). Further, its antibacterial activity against soft-rot *Erwinia* was also previously known. Yet, in the present study production of DAPG has been implicated as an antibacterial compound involved in the suppression of one of the most important and devastating bacterial crop diseases of the tropics, the bacterial blight of rice. Results presented in Table 2 show bacterial blight suppression by DAPG-producing strains and among these, a superior rice-associated strain of *P. fluorescens* PTB 9 was most impressive in its performance. It suppressed BB by 58.8% in the net-house and by 64.5% consistently in greenhouse, net-house and field experiments carried out in 2 locations over a period of 2 years (2002-04). The generation and use of PhI<sup>-</sup> mutants further clarifies the role of DAPG in bacterial blight suppression (Table 2). The PhI<sup>-</sup> mutants have shown substantial loss of their ability to protect the rice plants against *X. oryzae* pv. *oryzae*.

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## **Status of research on biological control of tomato and groundnut bacterial wilt in Vietnam**

### **Abstract**

Bacterial wilt (BW) caused by *Ralstonia solanacearum* is one of the most destructive diseases on many crops in Vietnam. The disease regularly causes significant damage to tomato and groundnut. Until recent years, some efforts have been initiated on biological control research of bacterial wilt on tomato and groundnut. Two strains of *Pseudomonas fluorescens* (B16 and VK58) and one strain of *Bacillus subtilis* (B.16) were identified as promising for biological control of bacterial wilt. They were tested and showed high effectiveness in controlling bacterial wilt on tomato in both greenhouse and field conditions in Northern Vietnam. In a field trial conducted on tomato with B16, VK 58 and B.16 in Hanoi, B.16 showed high effectiveness for BW control. In the field trial conducted on tomato and groundnut with B16, VK58 and B.16 in Hanoi, Bac Giang and Bac Ninh provinces disease incidence was reduced by biocontrol treatment, the best with B.16. In these field trials the tomato yield increased by 3.3 – 4.1 tons/ha and groundnut yield also increased by 0.7 – 0.94 tons/ha upon bio-control treatments. Antagonistic B.16 has a positive effect on growth, yield of groundnut and can replace 20% mineral NPK fertilizer without significant changes in crop yield. This research is now continued extensively in Vietnam. Initial results have indicated that research on biological control of bacterial wilt should be encouraged in the future.

### **Introduction**

Bacterial wilt caused by *R. solanacearum* is one of the most destructive bacterial diseases on many economically important crops in Vietnam including groundnut, potato, tomato, sesame, tobacco, and eggplant. Vietnam climatic conditions are favorable for the development of crop diseases and pests. Bacterial wilt causes significant damage to tomato and groundnut under favorable weather conditions. In the past, research on bacterial wilt in Vietnam mainly concentrated on host-plant resistance. Many agricultural chemicals have been used to prevent the disease, but their residues in the soil cause environmental problems. Only in very recent years, some research efforts have been initiated on biological control of bacterial wilt of tomato and groundnut. One of the most important criteria for choosing antagonistic microbial strains is the persistence of the organisms in the root systems of host plants.

### **Material and methods**

Two strains of *P. fluorescens* (B16 from National Seoul University, Korea, VK58 from Vietnam National Science & Technology Center) were cultured on Luria-Bertani (LB) medium and one strain of *B. subtilis* (B.16 from Vietnam Agricultural Science Institute-VASI) was cultured on King's medium B (KB) for 1-2 days at 28 – 30 °C. They were identified to be promising for biological control of bacterial wilt.

Tomato and groundnut seeds were obtained from the Plant Genetic Resource Center (VASI) and Legume Research and Development Center (VASI). Seeds were dipped in suspensions of B16, B.16 and VK58 with 10<sup>8</sup> cfu/ml (expt. in the greenhouse) and with 10<sup>9</sup>cfu/ml (expt. in the fields) for 1 h. Then the seeds were sown in the greenhouse or in the fields.

Trials for effectiveness of bacterial wilt control by B16, B.16, and VK58 on tomato and groundnut were conducted under greenhouse and field conditions. In the greenhouse the experiment was designed with 3 replications and 50 plants/treatment and, in the field with 50 m<sup>2</sup>/treatment and 3 replications. B.16 was identified by microscopy, DNA and RAPD.

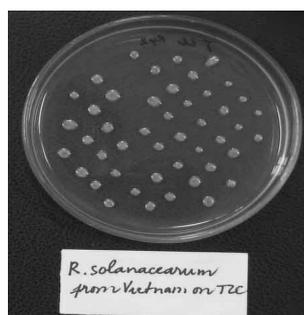
## Results and discussion

Status of bacterial wilt of tomato and groundnut in Vietnam: In Northern Vietnam, groundnut is grown mostly in Nghe An, Thanh Hoa, Bac Giang, Ha Tay, Hai Phong and other provinces. It is cultivated mainly under rainfed conditions in hilly upland areas and along river banks in dry land cropping systems. Tomato is grown in Hanoi, Ha Tay, Bac Ninh, Hai Phong and other provinces in Northern Vietnam mainly along river banks in delta region and lowland cropping systems. Bacterial wilt occurs in all areas growing groundnut and tomato. The disease incidence tends to increase fast and varies greatly from place to place, depending on cropping patterns and soil conditions (Table 1). The disease incidence on tomato is higher than on groundnut at any location where bacterial wilt was observed.

**Tab. 1** Sown area, yield groundnut and tomato, and disease incidence of BW in Northern Vietnam

Crops	Sown area (ha)	Yield (tones/ha)	Wilt incidence (%)
<u>Groundnut (2000)</u>			
North	108.3	1.49	5-47
North Mountain and midland	71.6	1.86	8-51
Red River delta	97.7	2.00	2-41
North Central coast	84.0	1.48	3-43
Vinh Phu	60.8	1.90	8-29
Bac Ninh	8.3	2.30	5-20
Hanoi	10.4	1.89	5-28
Hai Phong	20.3	2.22	10-27
Ha Tay	30.4	2.18	13-32
<u>Tomato (2000)</u>			
Hanoi	60.2	40.4	20-46
Bac Ninh	70.0	42.5	15-30
Vinh Phu	56.0	39.1	20-52
Hai Phong	70.0	41.0	22-35
Ha Tay	79.0	42.8	21-38

Characteristics of *R. solanacearum* on TZC medium and hypersensitive reaction caused by the pathogen: Isolates of *R. solanacearum* were collected from 14 provinces in Vietnam. The suspensions were spread on TZC medium (containing 1g casein hydrolysate, 10 g peptone, 5 g glucose, 15 g agar and 50 mg of triphenyl tetrazolium chloride per liter) and incubated at 28 °C. The colonies of *R. solanacearum* were fluidal, smooth, and white or creamy with a pink color center (Figure 1). A positive hypersensitive reaction was given by a rapid collapse in 24 - 48 h followed by dry, light-brown necrosis of the tobacco leaf tissue (Figure 2).



**Fig. 1** *R. solanacearum* on TZC medium



**Fig. 2** HR of *R. solanacearum* on tobacco

Antagonistic effect of useful bacterial strains (B16, B.16, and VK58) on media: *P. fluorescens* B16, VK58 and *B. subtilis* B.16 are effective against plant root pathogens, such as *R. solanacearum*. B16, VK58 and B.16 were cultured at 28–30 °C with *R. solanacearum* on TZC media. After 3 - 5 days, B.16, B16 and VK58 formed non-bacterial rings, indicating their antagonistic activity against *R. solanacearum*. The results are illustrated in Figure 3 of VK58 and B.16, B16 and VK58 (Figure 4) and B.16 (Figure 5).

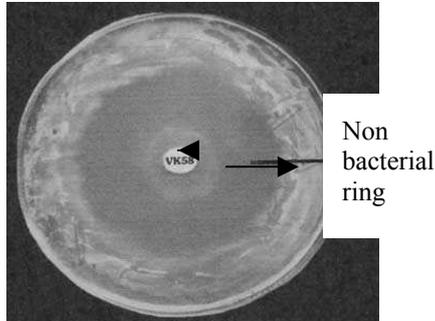


Fig. 3 VK58 on TZC/KB with BW

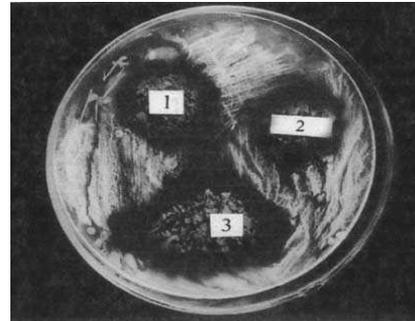


Fig. 4 1-B.16; 2- VK58; 3-B16 on TZC/KB with BW

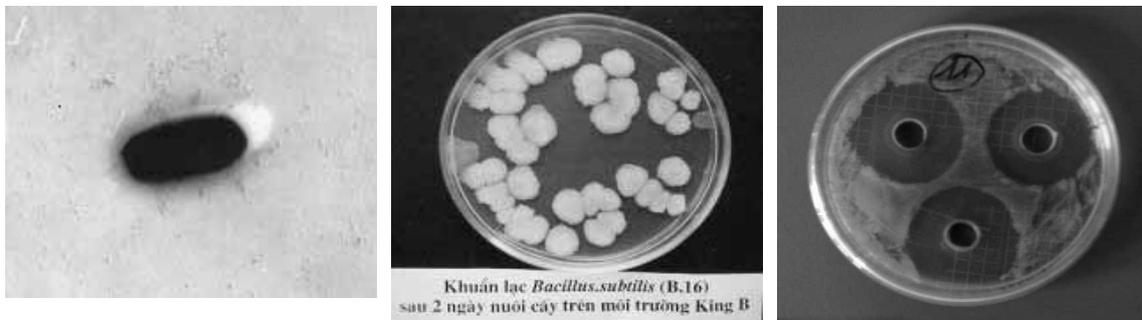


Fig. 5. B.16 cell, microscopically viewed, B.16 on KB, and TZC medium with BW. On King's B medium, B.16 cells have a circular margin, corrugation, creamy color, ellipse, gram-positive, rod-shape and are slowly moving. A characteristic cell is determined by its morphology as *Bacillus subtilis* species

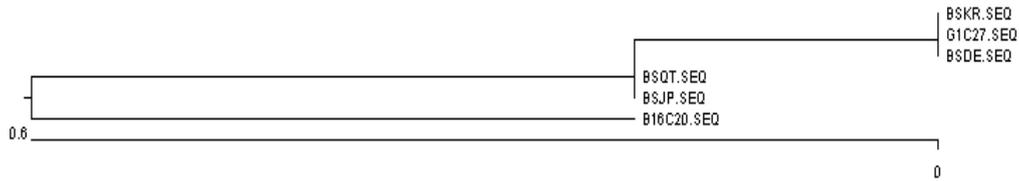
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SQ SEQUENCE 482 BP; 124 A; 104 C; 163 G; 91 T;

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AACTACGTGC CAGCAGCCGC GGTAATACGT AGGTGGCAAG CGTTGTCCGG AATTATTGGG
CGTAAAGGGC TCGCAGGCGG TTTCTTAAGT CTGATGTGAA AGCCCCGGC TCAACCGGGG
AGGGTCATTG GAAACTGGGG AACTTGAGTG CAGAAGAGGA GAGTGAATTCCACGTGTAG
CGGTGAAGTG CGTAGAGATG TGGAGGAACA CAGTGGCGA AGGCGACTCT CTGGTCTGTA
ACTGACGCTG AGGAGCGAAA GCATGGGGAG CGAACAGGAT TAGATACCCT GGTAGTCCAC
GC //
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Fig. 6 DNA sequencing of 16S rRNA gene of B.16

Using Kit Api 50CHB/E, Api20E and sequencing of DNA fragment of the 16S rRNA gene the strain B.16 was determined as *B. subtilis*. B.16 was registered in the manageable international gene bank in 2000, the name is AJ973635.



**Fig. 7** Dendrogram of B.16 compared with others *B. subtilis* species. BSKR- from Korea; BSDE- Germany; BSJP- Japan; BSQT- International; B.16C20 - from Vietnam

Figure 7 shows that B.16 is located in the cluster of *B. subtilis* race with other *B. subtilis* species but in a different subcluster which means different origins. The results show that B.16 is determined as *B. subtilis* species.

Trial for effectiveness in bacterial wilt control by B16, B.16, and VK58 on tomato and groundnut under greenhouse conditions

Tomato and groundnut seeds were dipped in the suspension of B16, VK58 ( $10^8$ cfu/ml) for 25-30 minutes and sown in trays at 25-28 °C. The 15 to 18-day-old seedling roots were inoculated with *R. solanacearum* ( $3 \times 10^7$ cfu/ml) with 5 ml bacterial suspension/plant. The degree of plant wilting and death was recorded until 45 days after inoculation. The results were presented in Figures 8, 9 and Table 2., showing that B16, VK58 and B.16 significantly reduced the bacterial wilt incidence on tomato and groundnut.



**Fig. 8** BW bio-control effect by VK58 and B16 on tomato

CT1: control, inoculation BW; CT2: inoculation BW + VK58; CT3: inoculation BW + B16



B.16 Control (no biocontrol); B16

**Fig. 9** Effect on bacterial wilt on groundnut by B16 and B.16

CT1: inoculation BW + B.16; CT2-control- inoculation BW; CT3- inoculation BW + B16

Figures 8 and 9 show that in the control treatment (infected with *R. solanacearum*, BW) all plants were infected, wilted and died (tomato). The plants grew slowly and were stunted (groundnut); but in treatments with B.16, B16 and VK58 the percentage of surviving plants was higher.

**Tab. 2** Effect of seed treatment with antagonistic bacteria on tomato and groundnut wilt in the greenhouse

Treatment	Disease incidence (%)	Reduced incidence
<u>Tomato</u>		
Control (no seed treatment)	90	HS*
Seed treatment with <i>P. fluorescens</i> -B16	40	R
Seed treatment with <i>B. subtilis</i> -B.16	38	R
<u>Groundnut</u>		
Control (no seed treatment)	86	HS*
Seed treatment with <i>P. fluorescens</i> -B16	56	R
Seed treatment with <i>B. subtilis</i> -B.16	52	R

\*HS: highly susceptible; R: resistant; *R. solanacearum* with 10<sup>7</sup>cfu/ml and B16, B.16 with 10<sup>8</sup>cfu/ml

Table 2 shows that trial with B16 and B.16 on tomato had a disease incidence of 40% and 38% (control was 90%), and on groundnut 56% and 52 %, respectively (control was 86%). Trials with B16 and B.16 on tomato appeared more effective than those on groundnut.

Field trial for effectiveness of VK58, B16, and B.16 in bacterial wilt control: The experiments were carried out on tomato and groundnut at the Dong Anh district, Hanoi over two seasons (summer/autumn and winter/spring). The results are shown in Table 3.

In a field trial conducted in Hanoi, in comparison with the control, the disease incidence was reduced by B16, B.16, and VK58 in tomato, and in groundnut in summer/autumn and in winter/spring 2004-2005, the tomato yield increased by 3.3 – 4.1 tons/ha and groundnut yield also increased by 0.7 - 0.94 tons/ha.

The second experiment was carried out at the Tu Son district, Bac Ninh province, and Viet Yen district, Bac Giang province, and Dong Anh district, Hanoi in winter/spring of 2004-2005 on tomato and groundnut. The results are shown in Table 4. Table 4 shows that antagonistic B16, VK58 and B.16 in trials on tomato disease incidence was reduced at VK58, B.16 and B16 treatment in three provinces. The yield in all trials with treatment with antagonistic bacterial strains was higher than control yields (no treatment). The treatment by B.16 gave the highest tomato yield.

**Tab. 3** Effect of antagonistic bacteria on tomato and groundnut wilt in the field

Treatment	summer/autumn 04		winter/spring 04-05	
	Disease Incidence (%)	Yield (tons/ha)	Disease Incidence (%)	Yield (tons/ha)
<u>Tomato</u> (Polish variety)				
Control (no seed treatment)	85	41,30 <sup>a</sup>	80	41,50 <sup>a</sup>
Seed treatment with B16	50	44,70 <sup>c</sup>	56	45,80 <sup>c</sup>
Seed treatment with VK58	48	44,02 <sup>b</sup>	52	45,25 <sup>b</sup>
Seed treatment with B.16	46	45,08 <sup>d</sup>	47	46,40 <sup>d</sup>
<u>Groundnut</u> (Sen Nghe An)				
Control (no seed treatment)	89	2,00 <sup>a</sup>	85	2,30 <sup>a</sup>
Seed treatment with B16	67	2,71 <sup>bc</sup>	66	2,80 <sup>c</sup>
Seed treatment with VK58	64	2,63 <sup>b</sup>	64	2,62 <sup>b</sup>
Seed treatment with B.16	61	2,75 <sup>c</sup>	62	2,96 <sup>d</sup>

*R. solanacearum* with 10<sup>8</sup>cfu/ml and B16, B.16 and VK58 with 10<sup>9</sup>cfu/ml

**Tab. 4** Effect of seed treatment with antagonistic bacteria on tomato wilt in three provinces (winter/spring 2004/05)

Treatment	Bac Giang		Bac Ninh		Hanoi	
	DI* (%)	Yield (tons/ha)	DI (%)	Yield (tons/ha)	DI (%)	Yield (tons/ha)
Control (no seed treatment)	86	41,8 <sup>a</sup>	89	41,1 <sup>a</sup>	87	41,3 <sup>a</sup>
Seed treatment with B16	52	46,1 <sup>c</sup>	55	45,8 <sup>b</sup>	53	45,6 <sup>c</sup>
Seed treatment with V58	48	45,7 <sup>b</sup>	51	45,4 <sup>c</sup>	49	45,0 <sup>b</sup>
Seed treatment with B.16	45	46,5 <sup>d</sup>	48	46,2 <sup>d</sup>	47	46,3 <sup>d</sup>
±SE		±2,9		±2,6		±2,3

DI\*: disease incidence; *R. solanacearum* with 10<sup>8</sup>cfu/ml and B16, B.16 and VK58 with 10<sup>9</sup> cfu/ml

Relationship between antagonistic B.16 and chemical fertiliser on groundnut in the green house: Experiments with three treatments: (control -no B.16, B.16 + 100% NPK; B.16 + 80% NPK) on groundnut. The results are shown in Table 5. The antagonistic B.16 has a positive effect on growth, yield of groundnut and could replace 20% mineral NPK fertiliser without significant change in crop yield.

**Tab. 5** Relationship between antagonistic B.16 and chemical fertiliser

Treatment	spring		winter	
	Wilt incidence (%)	Pod yield (tons/ha)	Wilt incidence (%)	Pod yield (tons/ha)
Control (no B.16)	78%	2.52 <sup>a</sup>	70%	2.38 <sup>a</sup>
B.16+ 100%NPK	46%	3.02 <sup>b</sup>	42%	2.80 <sup>b</sup>
B.16 + 80% NPK	44%	2.61 <sup>a</sup>	38%	2.43 <sup>a</sup>
CV%		1.3		2.9
LSD5%		0.88		1.45

*R. solanacearum* with 10<sup>8</sup>cfu/ml and B.16 with 10<sup>9</sup> cfu/ml

## Conclusion and suggestions

Two strains of *P. fluorescens* (**B16 and VK58**) and one strain of *B. subtilis* (**B.16**) are promising for biological control of bacterial wilt in the greenhouse and in the field. In the field trial conducted on tomato and groundnut with B16, B.16 and VK58 in Hanoi, Bac Giang and Bac Ninh provinces the tomato yield increased from 3.3 to 4.1 tons/ha and the groundnut yield from 0.7 to 0.94 tons/ha. **B.16** has a higher effect in disease control, a positive effect on growth and yield of groundnut and can replace 20% NPK fertiliser without significant changes in crop yield. These initial results indicate that research on biological control of bacterial wilt should be intensified in the future.

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## Characterization of the interaction of antagonistic bacteria and of silicon (SiO<sub>2</sub>) with tomato infected with *Ralstonia solanacearum*

### Abstract

Out of 44 bacterial rhizosphere strains screened by *in vitro* assays for their antagonistic potential against *Ralstonia solanacearum* and for their rhizosphere competence in tomato, the five strains *Bacillus pumilus* A8, *B. atropheus* A9, *B. subtilis* CH6, *Pseudomonas fluorescens* CMR03 and *P. fluorescens* CRS02 were selected. Enzymatic fingerprinting revealed differences between *Pseudomonas* and *Bacillus* strains. In *ad plantam* experiments, bacterial wilt severity and wilt incidence were reduced by up to 41% and 68%, respectively, while fresh weight increased by over 170% compared to infected control plants. In split-root experiments of the moderately resistant genotype King Kong2 treated with the antagonist *B. pumilus* A8, pathogen populations in stems were reduced by more than two orders of magnitude from 10<sup>5</sup> to 10<sup>2</sup> cfu/g fresh weight, indicating antagonist-triggered induced resistance. Immuno-fluorescence microscopy and immuno-tissue prints revealed a decrease in the number of fluorescent vessels after *B. atropheus* A9 treatment of the infected, highly susceptible genotype NHG3 indicating reduced production of phenolic compounds. The fluorescence in vessels of the A9-treated, susceptible genotype NHG167 seemed to be caused by an increase of arabinogalactan-protein epitopes in midstems, which were also visible in tissue prints. Additionally, the tolerance to high bacterial numbers was increased in genotype NHG167 after treatment with *B. atropheus* A9.

Silicon (SiO<sub>2</sub>) treatment reduced wilt incidence expressed as area under disease progress curve (AUDPC) by up to 26.8% in the susceptible genotype L390 and by up to 56.1% in the moderately resistant genotype King Kong2, whereas in the latter genotype also bacterial numbers were significantly reduced in midstems. Since silicon was increased in roots, but not stems, these observations indicate an induced resistance, which was accompanied by reduced degradation of homogalacturonan and an increased staining for arabinan and galactan side chains of rhamnogalacturonan I in cell walls.

### Introduction

Bacterial wilt caused by *Ralstonia solanacearum* (Yabuuchi et al. 1995) is a devastating disease attacking over 450 crop species in the tropics and subtropics, among them commercially important crops such as peanut, banana, tobacco, ginger and geranium (Grimault et al. 1994; Swanson et al. 2005). But, it is also a menace for potato and ornamental production in non-tropical countries. Control of bacterial wilt is difficult, since no possibility for chemical control exists and host plant resistance is not stable due to high genotype x strain x environment interactions. Since the pathogen additionally is highly variable with an extraordinary ability to survive in diverse environments and has an extremely wide host range, only an integrated approach seems promising. Therefore, biological control and soil amendment with silicon were tested for their effect on bacterial wilt in tomato.

Compared to the various attempts of biological control of fungal diseases by antagonists, less studies on biocontrol of bacterial diseases in general, and only few on bacterial wilt specifically, were conducted. Antagonistic micro-organisms and soil amendments with nutritional elements were shown to have disease suppressing effects in various pathosystems. Thus, a beneficial effect of silicon by increasing resistance has been reported in many crops when challenged with fungal pathogens, such as in cucumber, oat, rice, wheat, barley and sugarcane, which are silicon accumulator plants (Kunoh and Ishizaki 1975; Chérif et al. 1992; Samuels et al. 1994; Savant et al. 1999; Rodrigues et al. 2003). But so far, the effect of silicon in tomato, a silicon non-accumulator plant, and on a bacterial pathogen was only recently shown in a hydroponic culture system (Dannon and Wydra 2004). The modes of action of silicon in disease control as well as of bacterial antagonists against *R. solanacearum* have not been clarified to date. Therefore, the aim of the present studies was to identify effective antagonists against *R. solanacearum*

and to test the effect of silicon in suppression of a bacterial pathogen in the silicon non-accumulator plant tomato, as well as to elucidate the modes of action of these treatments.

## Materials and methods

Tomato genotypes, bacterial strains, silicon (SiO<sub>2</sub>) sources and methods used are described in detail in Dannon and Wydra (2004), Diogo (2005), Semrau (2005) and Beri (2005).

## Results and Discussion

### Biological control by antagonistic bacteria

**Characterization of antagonists *in vitro*:** Forty-four strains of rhizosphere bacteria isolated from tomato fields in Thailand were screened *in vitro* in agar plate tests for antagonism against *R. solanacearum*, indicated by inhibition zones produced by the production of antimicrobial compounds, and in tube tests for rhizosphere competence (data not shown). According to their efficacy to suppress the pathogen and their growth in the tomato rhizosphere, the strains *Bacillus pumilus* A8, *B. atropheus* A9, *B. subtilis* CH6, *Pseudomonas fluorescens* CMR03 and *P. fluorescens* CRS02 were selected. Characterization of enzyme production of the five strains revealed a moderate activity of esterase and esterase lipase in all the strains (Table 1). The pseudomonads CH6, CMR03 and CRS02 were characterized by a strong reaction for alkaline phosphatase and a moderate activity of acid phosphatase and naphthol-AS-BI-phosphohydrolase. These enzymes showed only weak activity in the *Bacillus* strains, except for CH6 which also showed a colour reaction of maximum intensity for alkaline phosphatase. For *Bacillus* strain A8 a maximum reaction of lipase was observed.

**Tab. 1** Enzymatic fingerprint of antagonist strains *B. pumilus* A8, *B. atropheus* A9, *B. subtilis* CH6, *P. fluorescens* CMR03 and *P. fluorescens* CRS02.

Enzyme	Reaction				
	A8	A9	CH6	CMR03	CRS02
Control	- <sup>a</sup>	-	-	-	-
Alkaline phosphatase	+ -	(+)	+++	+++	+++
Esterase	+	+	+	+	+
Esterase Lipase	++	++	+	++	++
Lipase	+++	+ -	-	+ -	+
Leucine arylamidase	-	+ -	-	+	++
Valine arylamidase	-	+ -	-	-	-
Cystine arylamidase	+ -	-	-	+ -	-
Trypsin	-	(+)	-	-	-
α-chymotrypsin	-	-	-	+ -	-
Acid phosphatase	+ -	(+)	+ -	+	+
Naphthol-AS-BI-phosphohydrolase	(+)	+ -	+ -	+	+
α-galactosidase	-	-	-	-	-
β-galactosidase	+ -	-	-	-	-
β-glucuronidase	-	-	-	-	-
α-glucosidase	-	+ -	(+)	-	-
β-glucosidase	+	(+)	+ -	-	-
N-acetyl-β-glucosaminidase	-	-	-	-	-
α-mannosidase	-	-	-	-	-
α-fucosidase	-	-	-	-	-

<sup>a</sup> “-” corresponds to a negative reaction and “+ + +” to a reaction of maximum intensity. “+ -”, “(+)”, “+” and “+ +” were intermediate reactions in the order of increasing intensity. Only “+”, “+ +” and “+ + +” were considered as positive reactions; tests were conducted with api<sup>®</sup>ZYM (bioMérieux<sup>®</sup>SA, Marcy-l’Etoile, France)

**Effect of antagonists *in planta*:** Comparing the bacterial antagonist treatments of the five selected strains, disease severity expressed as area under the disease severity progress curve (AUD<sub>s</sub>PC) was significantly reduced in the highly susceptible genotype NHG3 by all strains except CMR03: CH6, CRS02 > A8 > A9 (data not shown). In the susceptible genotype NHG167 only strains A8 and A9 led to a significantly

reduced disease severity in infected plants compared to non-treated plants, whereas in the moderately resistant genotype King Kong2 the AUD<sub>s</sub>PC was similar in the pathogen-inoculated control and the antagonist treatments. Comparing treatments by evaluation of wilt incidence, the AUD<sub>i</sub>PC was significantly lower in all plants treated with antagonistic bacteria compared to non-treated plants in genotype NHG3: A8 > CH6 > A9 > CRS02 > CMR03. In both genotypes King Kong2 and NHG167 treatments with the bacterial antagonists showed no effect except treatment with strain CH6 which evoked a significantly higher level of AUD<sub>i</sub>PC in genotype NHG167 compared to the *R. solanacearum* control of the same genotype.

Antagonist treatments led to a fresh weight increase from 223 to 297 % of infected plants of genotype NHG3 compared to non-treated plants, with three out of five treatments being significant (Table 2). The application of the potential plant growth promoting rhizosphere bacteria (PGPR) to non-infected plants did generally not display a growth promoting effect compared to the control plants without pathogen and antagonist treatment.

**Tab. 2** Fresh weight [in g and %] of aerial parts of eight-week-old tomato plants of the moderately resistant genotype King Kong2, the susceptible NHG167 and the highly susceptible NHG3 four weeks after inoculation with *R. solanacearum* strain To-udk2, treated with *Bacillus sp.* and *Pseudomonas sp.* antagonist strains

	King Kong2		NHG167		NHG3	
	Fresh weight [g] <sup>a</sup>	%	Fresh weight [g]	%	Fresh weight [g]	%
Rs + A8b	37.0 ± 4.4 ac	117	34.6 ± 3.7 ab	116	25.8 ± 3.8 a	297
Rs + A9	35.0 ± 4.4 a	110	33.7 ± 3.5 ab	113	19.4 ± 3.1 ab	223
Rs + CH6	36.2 ± 4.0 a	114	26.1 ± 4.5 ab	87	24.4 ± 4.3 a	274
Rs + CMR03	37.4 ± 4.4 a	118	34.9 ± 4.0 ab	117	25.0 ± 4.6 a	287
Rs + CRS02	40.9 ± 4.1 a	129	34.1 ± 5.1 ab	114	21.6 ± 4.0 ab	248
A8	45.5 ± 2.7 a	95	47.9 ± 3.7 a	110	30.1 ± 4.8 a	102
A9	48.8 ± 3.6 a	101	51.0 ± 5.2 a	117	37.3 ± 4.9 a	127
CH6	42.4 ± 3.0 a	88	38.7 ± 6.1 ab	89	18.1 ± 4.3 ab	62
CMR03	44.2 ± 2.4 a	92	46.9 ± 4.0 ab	108	25.6 ± 4.9 a	87
CRS02	47.6 ± 3.5 a	99	48.1 ± 3.9 a	111	24.5 ± 5.0 a	83
Rs	31.7 ± 4.6 a	100	29.9 ± 5.0 b	100	8.7 ± 3.1 b	100
H <sub>2</sub> O	48.1 ± 3.3 a	100	43.5 ± 3.8 ab	100	29.4 ± 4.7 a	100

<sup>a</sup> means of four repeated trials ±SE; in case of Rs means of three repeated trials ±SE; <sup>b</sup> Rs = *R. solanacearum*, A8 = *B. pumilus*, A9 = *B. atropheus*, CH6 = *B. subtilis*, CMR03 = *P. fluorescens*, CRS02 = *P. fluorescens*, H<sub>2</sub>O = tap water; <sup>c</sup> means followed by the same letters are not significantly different at p = 0.05

The application of the five potential antagonistic bacterial strains significantly reduced the numbers of *R. solanacearum* cells in midstems of tomato genotype NHG3 from  $1.45 \times 10^7$  cfu/g (control without antagonist) to minimal  $2.5 \times 10^3$  cfu/g (CMR03-treated) (data not shown).

Comparing symptom development, plant growth and pathogen quantification, best results were achieved with antagonistic strain *B. pumilus* strain A8, which significantly decreased AUD<sub>s</sub>PC and AUD<sub>i</sub>PC, and increased the fresh weight of infected plants of genotype NHG3. In split-root experiments infected plants of tomato genotype King Kong2, treated with antagonistic strain *B. pumilus* A8, the reduced pathogen numbers in midstems by two orders of magnitude, indicate the effect of a resistance induction through the antagonist treatment at the roots (data not shown).

Immunohistological characterization of resistance reaction: After inoculation of genotype NHG3 with *R. solanacearum*, an increased fluorescence due to reaction with antibody LM2, specific for arabinogalactan proteins (AGPs), in vessels of stem sections and an increased staining in stem tissue prints indicated the production of AGPs, belonging to the hydroxyproline-rich glycoproteins (HRGPs), which are known to be involved in resistance (photos not shown). However, in the antagonist

*B. atropheus* A9 treatment, the number of fluorescent vessels in this genotype was significantly reduced compared to the *R. solanacearum* control. This decrease, which is not reflected in the tissue prints, should be due to a decrease in autofluorescent phenolic substances, which were, to a lesser extent, produced in A9-treated, infected plants.

In the infected susceptible genotype NHG167, fluorescence microscopy revealed weaker staining of probably phenolic substances, and more intense fluorescence of vessel cell walls caused by an increase of AGP epitopes in midstems, when treated with silicon. The increase in AGPs was confirmed by tissue prints (photos not shown). This observation indicates that antagonist strain A9 has the potential to trigger increased synthesis of AGPs. Moreover, since disease development in NHG167 was reduced after *B. atropheus* treatment despite a higher number of pathogens in midstems compared to the *R. solanacearum* control, AGPs seem to be a tolerance factor. Comparing disease development, pathogen numbers in midstems and AGP-localisation, the application of *B. atropheus* strain A9 to infected plants of NHG3 significantly reduced the AUD<sub>s</sub>PC and AUD<sub>i</sub>PC, indicated by a slower development of disease severity and wilt incidence, lowered the bacterial numbers of *R. solanacearum* in midstems and led to a decreased number of fluorescent vessels and fluorescent xylem tissue areas probably due to less phenolic production in midstem sections.

#### Effect of silicon amendment

**Symptom development:** Disease severity and wilt incidence expressed as area under disease progress curves (AUDPC) of silicon-treated plants were significantly lower than of non-treated plants of the susceptible tomato genotype L390 with 16.1 and 26.8%, respectively, and genotype King Kong2 with 41.3 and 56.1%, respectively, in comparison to non-treated plants grown in hydroponic culture (Table 3).

**Tab. 3** Area under disease progress curves (AUDPC) for disease severity and wilt incidence in treatments with silicon (*Rs* + Si) and treatments without silicon (*Rs* -Si) in tomato genotypes L390 (susceptible) and King Kong2 (moderately resistant)

Treatments	Disease severity		Wilt incidence	
	AUDPC L390 <sup>1</sup>	King Kong2	L390	King Kong2
<i>Rs</i> + Si <sup>2</sup>	33.87 ± 1.69 aA <sup>3</sup>	16.97 ± 2.45 aB	704.16 ± 46.40 aA	135.42 ± 36.32 aB
<i>Rs</i> - Si	40.40 ± 1.71 bA	28.91 ± 3.16 bB	962.50 ± 62.50 bA	308.25 ± 14.57 bB

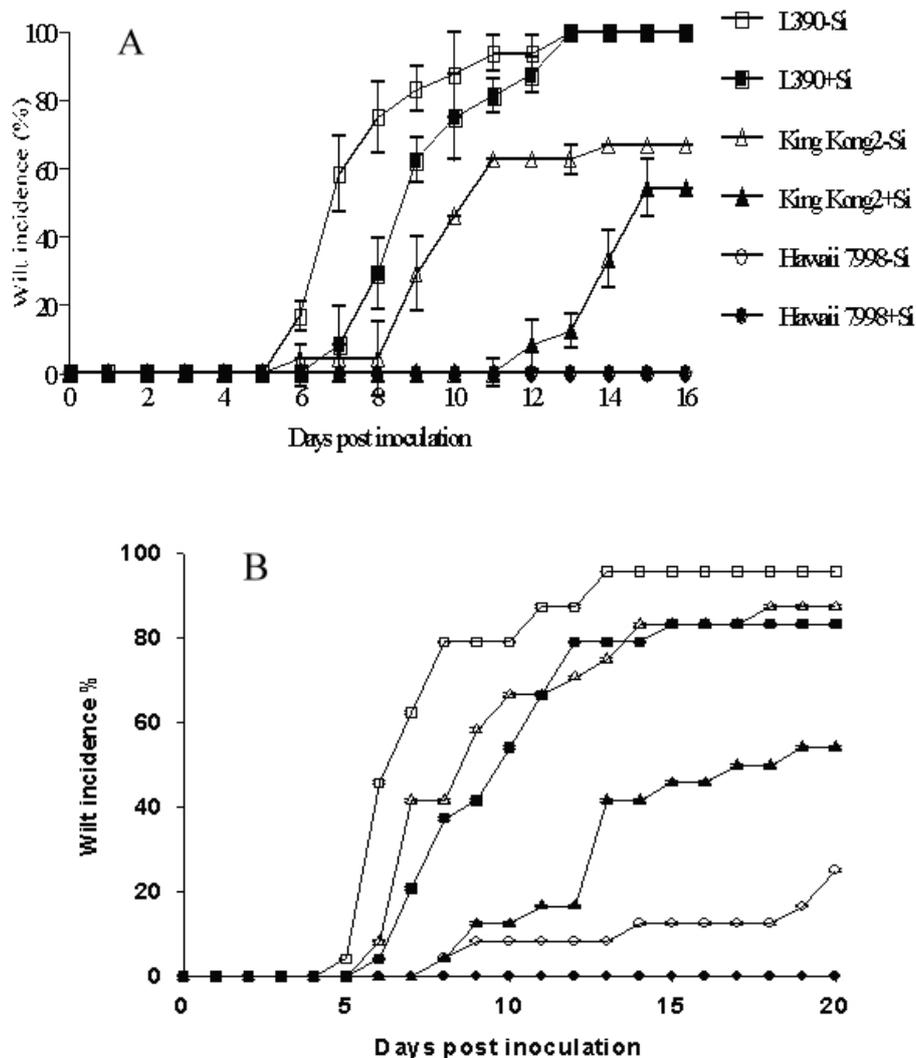
<sup>1</sup>Means of three repeated trials ± SE; <sup>2</sup>*Rs* +Si = silicon-treated plants inoculated with *R. solanacearum*, *Rs* -Si = non-silicon-treated plants inoculated with *R. solanacearum*. Disease severity was defined as the average of disease classes of all plants of a treatment (-Si or +Si) at an evaluation date, while wilt incidence was the proportion of dead plants (disease class 5) out of the total number of plants in the treatment; <sup>3</sup>Means followed by the same letter are not significantly different with Tukey test at 5%. Small letters vertically refer to comparisons between treatments with the same genotype and capital letters horizontally to comparisons between genotypes with the same treatment

In genotype King Kong2, disease severity increased more slowly in silicon-treated than in non-treated plants (data not shown), and wilt incidence development was retarded by 6 days (Figure 1A). At the end of the trials, 46% of the plants of King Kong2 had survived in silicon treatments and 33% in treatments without silicon. A more expressed effect of silicon was observed when plants were grown in peat moss substrate (Figure 1B), where final wilt incidence was reduced by 38.1% to 49.4% in the silicon-treated plants of King Kong2 compared to 87.5% plant death in the non-amended plants. No dead plants occurred in the silicon-treated Hawaii7998 compared to 33.3% plant death in the treatment non-amended with silicon.

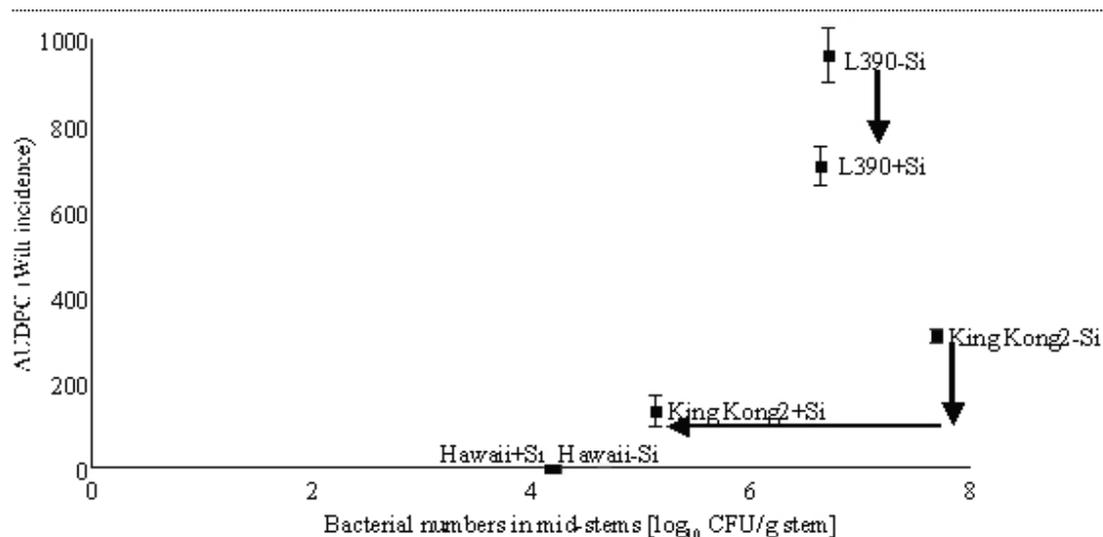
Effect of silicon on symptom development and bacterial number: Plotting wilt incidence against bacterial numbers at 5 dpi, the effect of silicon application on the reduction of bacterial numbers in genotype King Kong2 is demonstrated, whereas a disease reducing effect occurred in both genotypes L390 and King Kong2 (Figure 2). A reduced symptom development in genotype L390 at similar bacterial population numbers in silicon-treated plants indicates an increased tolerance induced by silicon amendment.

Relation between silicon content and bacterial number: Silicon treatment increased the silicon concentration in roots of tomato plants by about 80%, while no significant difference was observed comparing stems of +Si plants to -Si plants (data not shown). Negative correlations were found between the silicon content in roots and bacterial numbers in stems of genotypes King Kong2 and Hawaii 7998, and between silicon in roots and bacterial numbers of roots and leaves of genotype Hawaii 7998, suggesting an induced resistance (Dannon and Wydra 2004).

Modes of action of silicon-induced resistance: Immunohistochemical analysis of stem sections of genotype King Kong2 revealed a degradation of methylesters of homogalacturonan of cell walls and increases of autofluorescent phenolics and degraded cells after infection with *R. solanacearum* (photos not shown). In stem sections of plants treated with silicon diminished the degradation of homogalacturonan, decreased autofluorescence of phenolics and increased staining for arabinan and galactan side chains of rhamnogalacturonan I, a major constituent of the pectic substances of cell walls, was observed.



**Fig. 1** Bacterial wilt symptom development expressed as wilt incidence in silicon-treated (+Si) and non-treated (-Si) plants of tomato genotypes L390 (susceptible), King Kong2 (moderately resistant) and Hawaii 7998 (resistant), inoculated with *R. solanacearum* grown in hydroponic culture (A) and in peat moss substrate (B)



**Fig. 2** Distance between tomato genotypes L390, King Kong2 and Hawaii 7998 in regard to the AUDPC based on wilt incidence and bacterial numbers in the midstems at 5 days after infection with *R. solanacearum* strain To-udk2 in silicon-treated and silicon non-treated plants. Data are from means of the AUDPC based on wilt incidence and of bacterial numbers in the midstems at 5 dpi. Wilt incidence = percentage of dead plants (class 5) in a treatment at an evaluation date

Analysing stem extracts of genotype King Kong2 for the effect of polygalacturonase-inhibiting proteins which were described for the first time to be effective against a bacterial pathogen (Wydra et al. 2005) revealed an increase in PGIPs' effect in silicon-treated, infected plants compared to non-silicon-treated plants (data not shown).

## Conclusions

Biological control and silicon amendment both revealed a disease-reducing effect through induced resistance and induced tolerance, depending on the tomato genotype. The combination of both treatments is under investigation. Among the modes of action, resistance mechanisms on cell wall level seem to be involved, with modifications in the structure of homogalacturonans and side chains of rhamnogalacturonans I, lower production of phenolic substances and increases in AGPs in silicon- and antagonist-treated, infected plants compared to non-treated plants. An increase in PGIPs after silicon treatment may be involved in increased resistance of genotype King Kong2 to bacterial wilt.

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## **Biological control of potato bacterial wilt caused by *Ralstonia solanacearum* in Ethiopia: Determination of biovars of *Ralstonia solanacearum***

### **Abstract**

*Ralstonia solanacearum* is a very destructive pathogen that causes wilt in potato and many other solanacean crops in Ethiopia. In order to select effective antagonistic biocontrol agents for *R. solanacearum* strains, it is necessary to characterize the population of pathogenic strains. Therefore, 62 strains collected from wilted potato, tomato and pepper plants and potato tubers from the major potato producing regions of Ethiopia were characterized culturally and classified physiologically, based on their capacity to oxidize 3 disaccharides (lactose, maltose and cellobiose) and 3 hexose alcohols (mannitol, sorbitol and dulcitol). The results of this study indicated that all virulent strains from Ethiopia produce fluidal and irregular colonies with red centre and whitish periphery on triphenyl tetrazolium chloride (TZC) medium and irregular, fluidal, and creamy white colonies on casamino acids-pepton-glucose (CPG) medium. Physiologically, 19 strains were grouped to biovar I and 43 strains to biovar II. Previous studies from Ethiopia reported the availability of only biovar II of *R. solanacearum*. Thus for biovar I this is the first report concerning the Ethiopian *R. solanacearum* population.

### **Introduction**

*R. solanacearum* E.F. Smith (Yabucchi et al. 1995), the causal agent of bacterial wilt, produces a severe and devastating disease affecting many crops in tropical and temperate regions (Hayward 1991). In Ethiopia it is a very destructive pathogen that causes wilt on potato and many other solanacean crops with incidence on potato as high as 63% in major potato growing areas (Bekele 1996).

To date, no effective control methods exist for potato bacterial wilt disease. Plant breeding, field sanitation, crop rotation and use of bactericides had only limited success (Ciampi-Panno et al. 1989). An increasing number of reports have indicated that biological control of potato bacterial wilt could be achieved using antagonistic micro-organisms. In order to select effective antagonistic biocontrol agents for the *R. solanacearum* strains, it is necessary first to characterize the variability of the strains. Therefore, the objective of this study was to culturally and physiologically characterize Ethiopian *R. solanacearum* populations.

### **Materials and methods**

**Bacterial strains and growth conditions:** 62 strains collected from potato producing regions of Ethiopia from infected potato, tomato, and pepper plants and potato tubers were cultured on tetrazolium chloride (TZC) medium (Kelman 1954) and on casamino acids-pepton-glucose (CPG) medium. Cultures were maintained in sterile water at room temperature and revived by plating a loopful on TZC agar medium and CPG agar (0.1% peptone, 0.01% casamino acids (Difco), 0.05% glucose, and 1.5% (wt/volume) agar at 30°C.

**Cultural and physiological tests:** Isolates were culturally characterized by growing them on TZC and CPG medium at 30°C and recording the colony characters. The oxidation of sugars and sugar alcohols was tested on the basal medium according to Hayward (1964). Lactose, maltose, and cellobiose solutions were filter sterilized, while mannitol, sorbitol and dulcitol were autoclaved for 20 min as 10% (w/v) solutions. Five ml of each sugar and sugar alcohol solution were added to 45 ml of Hayward's medium and 10 ml of the resulting amended medium was dispensed into test tubes (Hayward 1964).

A suspension of each strain was prepared by inoculating 300 µl of sterile water with a loopful of cells (Williamson et al. 2002) from each strain grown on CPG for 48 h at 30°C. The test tubes with Hayward's

medium were inoculated with 30 µl of the suspension, incubated at 30°C and checked for acid production (yellow colour) (Hayward 1964) at various intervals for up to 5 weeks.

## Results and discussion

All virulent strains of *R. solanacearum* from Ethiopia produce fluidal and irregular colonies with a red centre and whitish periphery on triphenyl tetrazolium chloride (TZC) medium after 48 h of incubation. However, when the strains lost their virulence upon storage, the colony becomes smaller and round with deep colour. On CPG medium Ethiopian isolates produce larger and whitish fluidal colonies which turn brown after 48 h of incubation. The colony appearance on TZC is typical to *R. solanacearum* (Kelman 1954), in agreement with a report from Ethiopia by Yaynu (1989) 17 years ago.

Marked differences were observed in the ability of strains from Ethiopia to oxidise disaccharides and sugar alcohols. Based on Hayward's classification scheme (Hayward 1964), 43 of 62 strains were classified as biovar II and 19 as biovar I (Table). Biovar II strains produced acid from lactose, maltose and cellobiose but failed to oxidise mannitol, sorbitol and dulcitol, while biovar I strains oxidized none of the disaccharides and sugar alcohols even after 5 weeks of incubation.

**Table** Oxidation of carbohydrates by strains of *Ralstonia solanacearum* from Ethiopia

Number of strains	Carbohydrate <sup>a</sup>						Biovar
	Lactose	Maltose	Cellobiose	Dulcitol	Mannitol	Sorbitol	
19	-	-	-	-	-	-	I
43	+	+	+	-	-	-	II

<sup>a</sup>+ = reaction; - = negative reaction

Previous studies from Ethiopia reported the availability of only biovar II of *R. solanacearum*. Thus biovar I is observation the first time in the Ethiopian *R. solanacearum* population. Since this biotype is present in most parts of the world (He et al. 1983) this result is not surprising because Ethiopia has been introducing several thousands of potato genotypes (Berga et al. 1994) from the International Potato Center (CIP) and other parts of the world to develop high yields and adaptable cultivars with resistance to major stresses. Hence biovar I strains might have been introduced to Ethiopia from other parts of the world with latently infected tubers.

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## Resistance to *Erwinia carotovora* introduced to *Solanum tuberosum* from wild species

### Abstract

Resistance of potato tubers to soft rot, caused by the bacterium *Erwinia carotovora* subsp. *atroseptica* (Eca), was introduced to tetraploid potatoes from diploid hybrids of *Solanum tuberosum*, *S. chacoense*, *S. yungasense* and *S. phureja*.

### Introduction

Potato blackleg and soft rot are caused by bacteria: *E. carotovora* subsp. *atroseptica*, van Hall, 1902 (Dye 1969), *E. carotovora* subsp. *Carotovora*, Jones, 1901 (Bergey et al. 1923) and *E. chrysanthemi* (Burkholder et al. 1953); synonyms are respectively: *Pectobacterium carotovorum* subsp. *atrosepticum*, *P. carotovorum* subsp. *carotovorum* and *P. chrysanthemi* (Hauben et al. 1998). Distribution of different *Erwinia* species depends on the temperature, but in general in temperate climate *Erwinia carotovora* is common and the global losses due to *Erwinia* spp. were estimated to 50 to 100 million USD (Pérombelon and Kelman 1980). Chemical control is not applied in practice and control of this disease is mainly based on prevention: planting of healthy seeds and avoiding the risks of damage and wetting of tubers during harvest as well as preventing anaerobic conditions in storage and transport (Pérombelon 2000). As another possibility to control soft rot of potatoes by a biological method we tested the level of resistance to *Erwinia* species in a tetraploid potato cultivar, because its resistance is relatively low (Krauze et al. 1982) and breeding of resistant cultivars would be a good solution to minimise the losses caused by these bacteria.

### Results and Discussion

Resistance of potato tubers to soft rot, caused by the bacterium *Erwinia carotovora* subsp. *atroseptica* (Eca), was introduced to tetraploid potatoes from diploid hybrids of *Solanum tuberosum*, *S. chacoense*, *S. yungasense* and *S. phureja* (Lebecka et al. 2004).

Six clones were selected (out of 1353 individuals) in the course of breeding programs, based on their good agronomical characteristics (yield, phenotypic appearance, starch content, chipping quality) and presence of additional resistance to *Synchytrium endobioticum*, PV.Y, PLRV, PV.M and resistance to *Phytophthora infestans*. The high level of resistance to Eca found in these clones was confirmed in two consecutive years of evaluation using the point inoculation method described by Lebecka et al. (2004). The five-year-mean values of rotten tissue diameter ranged from 5.8 to 6.8 mm in resistant clones (Table), while the average value for susceptible cultivar Irys was 16.2 mm. Screening for resistance to Ecc indicated a high resistance of four clones (mean diameter of rotten tissue from 5.3 to 7.9 mm) and medium resistance of two other clones (9.9 and 10.5 mm) as compared with 14.0, 17.4 and 16.9 mm measured for the susceptible cv. Irys and the susceptible tetraploid parental clones PS 646 and PW 378, respectively.

**Table** Soft rot symptoms of six potato clones, their parents and standards, expressed as diameter of rotten tissue of inoculated tubers with *Erwinia carotovora* subsp. *atroseptica* and *Erwinia carotovora* subsp. *carotovora*

Clone/cultivar	Pedigree or type of tested material	Diameter of rotten tissue (mm) after inoculation with	
		<i>Eca</i> (five-year-mean ± SD)	<i>Ecc</i> (one-year-mean)
E97-908	PS646 x DG88-9	5.8 ± 0.3	7.9
E97-678	PW378 x DG88-9	5.9 ± 0.9	5.7
E97-1954	M62564 x DG88-9	6.4 ± 1.7	6.5
E97-572	PW378 x HT/HZ84PH151	6.7 ± 1.7	5.3
E97-2075	M62564 x DG 94-112	6.8 ± 0.7	10.6
E97-1112	Cv. Glada x HT/HZ84PH151	6.8 ± 1.4	9.9
DG 88-9	Resistant parent	6.1 ± 0.9	6.7
HT/HZ84PH151	Resistant parent	6.4 ± 0.5	8.4
DG 94-112	Resistant parent	8.3 ± 1.7	10.5
Cv. Glada	Resistant parent	8.5 ± 2.2	10.9
M62654	Medium-resistant parent	9.2 ± 1.5	12.8
PS646	Susceptible parent	11.5 ± 3.5	17.4
PW378	Susceptible parent	11.7 ± 3.8	16.9
USA 249	Resistant standard	8.6 ± 2.3	16.3
Cv. Irys	Susceptible standard	16.2 ± 4.0	14.0

The source of resistance to potato soft rot demonstrated its value in a breeding program and some of these clones will be used as parental lines in breeding new cultivars.

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### III. Biological control of bacterial diseases in horticulture

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#### Strategies for biological control of soilborne pathogenic bacteria and practical efficacy of different methods

##### Abstract

Two bacterial models with different biocontrol strategies are presented. For *Ralstonia solanacearum*, probably the most dangerous soilborne pathogenic bacterium, biocontrol against race 1 and race 3 strains has been studied but no universal and successful treatment is available yet. The second one is the biocontrol of the pathogen *Agrobacterium tumefaciens*, the best example of how an efficient strain can provide optimal results in most cases, working quite well in different hosts, countries and soil types. The use of *A. radiobacter* strain K84 and its derivative strain K1026 demonstrates that the strain selected as biocontrol agent is really effective in a commercial scale and that there are different mechanisms involved in controlling the pathogen.

##### Introduction

Soilborne plant pathogenic bacteria are responsible for several economically important diseases, such as bacterial wilt caused by *R. solanacearum* and crown gall caused by *A. tumefaciens*. Both pathogens were described at the beginning of the last century, but they are still causing high economic losses world-wide every year and they have a difficult chemical control due to the lack of efficient bactericides commercially available.

Biological control of plant pathogenic bacteria is relatively new when compared to chemical control. However, biocontrol is more likely to be successful in systems with some degree of environmental control and in some aspects the rhizosphere is more stable than other plant environments. Consequently, the possibilities of success in the rhizosphere are, at least in theory, higher than for other parts of the plant. *A. radiobacter* strain K84 was the first registered biocontrol agent against a soilborne bacterial pathogen, in Australia in 1973 and later in the USA in 1979. In spite of the time lapsed, only 13 bacteria are currently registered in the USA, four against bacterial pathogens and ten against fungi. None is registered in the EU where there is one under evaluation.

In this paper, two bacterial models with different biocontrol strategies are presented. The first one refers to the pathogen *R. solanacearum*, probably the most dangerous soilborne pathogenic bacterium, for which biocontrol against race 1 and race 3 strains has been studied by many authors obtaining quite different results; for this reason no universal and successful treatment is available yet (Trigalet et al. 1994; López and Biosca 2005). The second one is the biocontrol of the pathogen *A. tumefaciens*, the best example of how an efficient strain can provide optimal results in most cases, working quite well in different hosts, countries and soil types. The use of *A. radiobacter* strain K84 and lately its derivative strain K1026 demonstrates that, when the strain selected as the biocontrol agent, it is really effective in a commercial scale and that there are different mechanisms involved in controlling the pathogen; the method can remain as effective as at the beginning for more than 30 years until now.

Biocontrol of *Ralstonia solanacearum*: Strains of *R. solanacearum* race 1 and 3 are responsible for bacterial wilt in a large number of host plants from more than 50 families, including solanaceae such as potato and tomato. The disease is widespread around the world. The severe losses caused by *R. solanacearum* in most tropical and subtropical countries prompted to study the possibilities of biocontrol already in the middle of the last century. Averre and Kelman in 1964 demonstrated that the severity of bacterial wilt was influenced by the ratio of virulent to avirulent cells of the pathogen. Since

then, several approaches to bacterial wilt biocontrol have been developed based on the use of different strains and strategies. However, the lack of profound knowledge of the mechanisms of each biocontrol system and/or on the pathogen survival in different soils and its interaction with the root system is probably the cause of inconsistency of the results. Some non-exhaustive examples of biocontrol of race 1 and 3 strains of this pathogen are shown in the Table, but they are not currently utilised in practice according to the available information.

There is a large spectrum of strategies from the selection of natural antagonists or competitors to the use of GEM's, phages, or integrated control. Biocontrol strains efficient in laboratory assays often fail under natural conditions, probably due to poor competitive ability and/or poor edaphic adaptation (López and Biosca 2005). There are only few examples of efficient biocontrol in the field, such as those reported by Ciampi-Panno et al. (1989) on potato in Chile, by Jinnah et al. (2002) in Bangla

**Table** Some strategies used for biocontrol of *R. solanacearum* race 1 and 3

<b>• Wild strains or spontaneous mutants</b>	
<i>Avirulent strains or mutants of R. solanacearum</i>	Averre and Kelman (1964); Trigalet and Trigalet-Demery (1990)
<i>Bacillus sp.</i>	Anuratha and Gnanamanickam (1990)
<i>Pseudomonas fluorescens</i>	Jinnah et al. (2002)
<i>P. cepacia</i>	Aoki et al. (1991)
<i>P. glumae</i>	Wakimoto (1987)
Plant Growth Promoting Rhizobacteria (PGPR).	Guo et al. (2004)
<b>• Genetically engineered microorganisms</b>	
Tn 5 non-pathogenic mutants	Trigalet and Trigalet-Demery (1990)
Omega hrp-non pathogenic mutants	Frey et al. (1994); Smith et al. (1998)
<b>• Phage therapy</b>	
Phages isolated from soil and water	Jones et al. (2005)
<b>• Integrated control</b>	
Combination of several methods and biocontrol.	Anith et al. (2004); Momol et al. (2005)

Desh, and by Guo et al. (2004) in China on tomato. Recently, more environmentally friendly approaches intend to modify the natural soil communities trying to favour other microorganisms that can compete or kill the pathogen (van Elsas et al. 2005). The experience of several authors suggests that the integrated control combining several methods can provide higher efficacy. In this context, the use of PGPR, complemented with an inducer of resistance as Actigard and different types of soil amendments has provided good results (Anith et al. 2004).

In conclusion, many different types and strains of biocontrol agents are available against *R. solanacearum* and likely a combination of several biocontrol agents, multiple subsequent applications and the inclusion of the biocontrol in an integrated control scheme could give a better control than individual strains.

Biocontrol of *A. tumefaciens*: *Agrobacterium* spp. includes three main bacterial species (corresponding to the biovars 1, 2 and 3) that cause crown gall disease in about 643 plant species. Tumors can appear on roots, crown and less frequently in aerial parts. The disease is world-wide distributed and in the EU *Agrobacterium* sp. is considered as a “quality pathogen”. It causes important economic losses mainly in fruit trees, grapevines and ornamentals. The molecular bases of the mechanism of crown gall induction is quite well known and the complete genome of the *A. tumefaciens* strain C58 has been sequenced (Goodner et al. 2001; Wood et al. 2001).

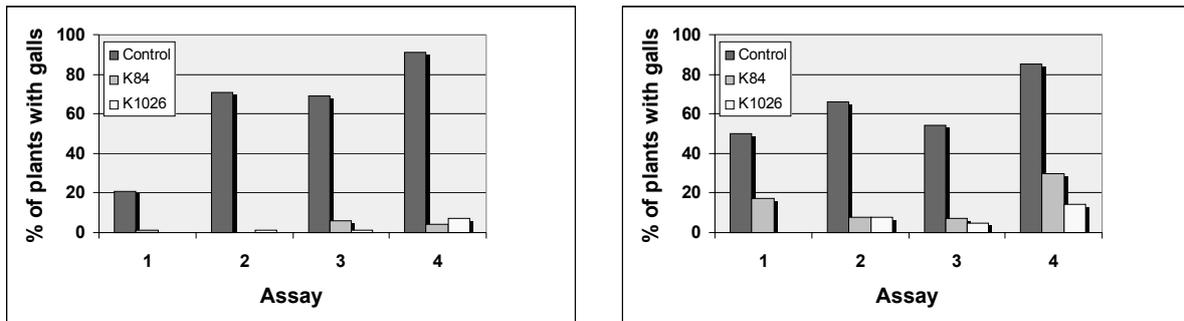
The history of crown gall biocontrol developed from the field observation that the proportion of pathogenic and non-pathogenic bacteria on the roots correlated with the incidence of galled plants (New

and Kerr, 1972). When the non-pathogenic strain K84 was co-inoculated with pathogenic strains on the roots, it inhibited the appearance of tumors and was also efficient in seed inoculations (Kerr, 1972).

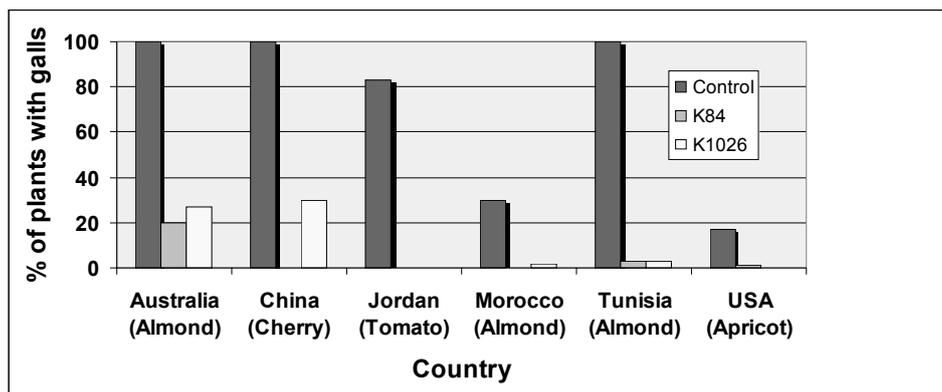
Strain K84 has been utilised at least in 12 countries for more than 30 years in more than 20 cultivated plants. It is commercialised in different formulations and registered in Australia, New Zealand, Canada and USA. More than 1 million plants are treated every year in USA. Strain K84 is not recommended for crown gall control on apple and grapevine. It does not control latent infections and it is not efficient against some pathogenic strains resistant to agrocin 84. A risk of transfer of pTi plasmid to strain K84 was inferred from the results of a biocontrol assay. Later, the transfer of pAgK84 plasmid from strain K84 to pathogenic strains has been demonstrated *in vitro* and *in vivo* in different countries creating new pathogenic strains producing tumours and agrocin 84, then resistant to agrocin 84 and not controlled by K84 (Penyalver et al. 2000). To avoid the transfer of pAgK84 from K84 to pathogenic strains, a safer strain was constructed by making a deletion in pAgK84 in the Tra region that codified for the transfer of the plasmid. The new developed strain was named K1026 and has not foreign DNA (Jones et al. 1988). Figure 1 shows the efficacy of strains K84 and K1026 in controlling pathogenic strains either sensitive or resistant to agrocin 84 in Spain, and the Figure 2 shows their efficacy on different crops in several other countries.

**Strains sensitive to agrocin 84**

**Strains resistant to agrocin 84**



**Fig. 1** Efficacy of strains K84 and K1026 in Spain



**Fig. 2** Efficacy of strains K84 and K1026 on different crops in several other countries

The study of the mechanisms of biocontrol by strains K84 and K1026 during more than 30 years revealed that there is a complex interaction between the biocontrol agent, the pathogen and the plant. Both organisms are shown to be good root colonizers. The main mechanism evolved in biocontrol by strain K84 is the production of the highly specific antibiotic, agrocin 84, a potent inhibitor of protein synthesis. Pathogenic strains are sensitive or resistant to agrocin 84. Its production and the immunity of strain K84 is encoded in the pAgK84 plasmid. The production of agrocin 84 by K84 has been demonstrated *in vivo* in bacteria colonizing roots. Pathogenic strains sensitive *in vitro* to agrocin 84 are usually controlled *in vivo*, and resistant strains can sometimes be controlled, too (Penyalver et al. 2000).

Production of agrocin 84 is the main factor involved in the control, but both biocontrol agents produced agrocin 434, other antibiotic substances are active only against biovar 2 strains. Both biocontrol agents also produced a hydroxamate siderophore named ALS84 that showed antagonistic activity *in vitro* against pathogenic strains only under iron-limited conditions. In the biocontrol of pathogenic strains resistant to agrocin 84, the biocontrol agents seem not to block the attachment of the pathogen to the root surfaces and the subsequent root colonization. Investigations on some other possible mechanisms involved in the biocontrol such as the physical blockage or competition for wounds, the formation of biofilms on wounds or roots, the induction of systemic resistance or the interference of the plant cell transformation by strains K84 and K1026 are under way. The final annotation of the incoming complete genome of strain K84 will provide new insights into the biocontrol mechanisms.

Strain K1026 was registered in Australia at the end of 1988 and became the first, live, GEM to be registered for commercial release to the public. It has been utilised for standard practice in more than 350 Australian nurseries. About 1.5 million fruit and walnut trees and 3 million ornamentals are treated per year in this country. In the USA it was registered since 2000, and 3 million fruit trees and 3 million walnut trees, berries and ornamentals are treated per year. It has been recently registered in Turkey, and soon will be in Tunisia and Algeria. The application in the EU presented by FuturEco S.L., Spain, is now in final stages.

Strain K1026 is not considered as a GEM in several countries. It is not toxic for animals, humans or plants. It does not grow at 37 °C and the produced antibiotic agrocin 84 only affects *Agrobacterium* strains. The method can be integrated in standard cultural practices and it is compatible with other treatments. Therefore, as no toxicological problems have been reported when using K84 for more than 30 years in several countries, no risk is expected when using the strain K1026.

## Conclusions

The knowledge of the bacteria-plant interaction is necessary to design efficient biocontrol methods for soilborne pathogens. The lasting success of a biocontrol agent seems to be correlated with multiple mechanisms of action.

It is necessary to follow the hidden life of the biocontrol agent in soil and rhizosphere and its interaction with the pathogen, even in successful systems.

There is a need of evaluation of genetic stability of the biocontrol agents as well as of GEM's in soil and plants. There is also a need of risk assessment for biocontrol agents as well as for GEM's (toxicity studies, soil persistence, impact on microbial community, interactions with crops and weeds). In practice, it is required, in some models, to integrate several biocontrol agents and treatments for an economically acceptable level of control.

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### **Screening biocontrol agents for control of seed-borne bacterial pathogens of carrots and brassicas**

As part of an EC co-funded project to identify and develop seed treatments for organic vegetable production (STOVE), a number of potential biocontrol agents (BCAs) were examined for their efficacy in controlling *Xanthomonas hortorum* pv. *carotae* and *X. campestris* pv. *campestris*, the causal agents of bacterial blight of carrot and black rot of brassicas, respectively. Seed-borne bacterial pathogens present particular experimental difficulties due the relatively low (but epidemiologically significant) levels of infestation found in naturally infested seed lots. Potential BCAs were initially screened in vitro for inhibition/antagonism against the target pathogens. The best potential BCAs from the first screening were then applied to naturally infested seed and their effects on pathogen transmission (from seed to seedling) were assessed in glasshouse experiments. Finally the most effective was screened in a field trial (carrot) or further glasshouse transmission experiments (brassicas). The results obtained to date had been presented.

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## Biological control of black rot of vegetable brassicas, caused by *Xanthomonas campestris* pv. *campestris*, with endophytic *Bacillus* strains

### Abstract

*Bacillus* strains have been successfully used in the control of black rot of vegetable brassicas, caused by *Xanthomonas campestris* pv. *campestris*, in Tanzania and Zimbabwe. Of several methods of application tested, dipping roots in a suspension of *Bacillus* prior to transplanting was the best. One of the promising *Bacillus* strains (strain BB) showed endophytic ability and could be re-isolated from plants growing under greenhouse and field conditions. Preliminary results suggested that induced resistance was one of the mechanisms involved in the control of black rot by the tested strain BB.

### Introduction

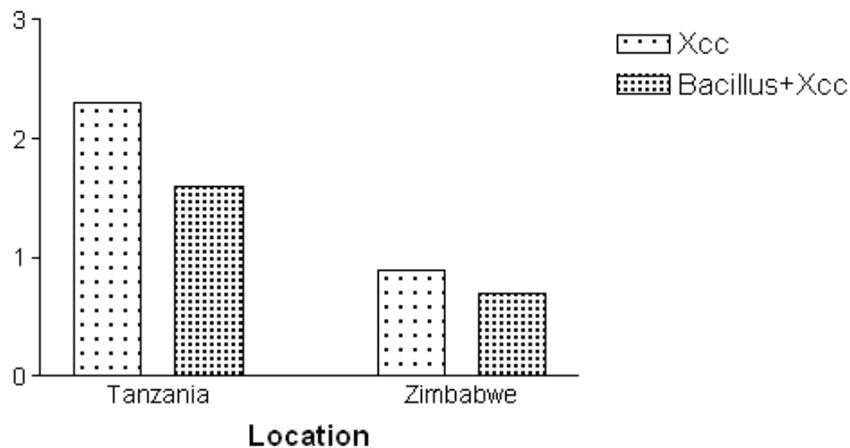
Brassica species (e.g. cabbage, broccoli, cauliflower, spinach, Brussels sprouts and kale) are health-giving, nutritious and among the most widely consumed vegetables in the world. However, black rot, caused by the bacterial pathogen, *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson (Xcc), continue to be the major disease constraint to sustained production of brassicas and other crucifers and in many parts of the world (Onsando 1992; CAB 2003; Massomo et al. 2005). Characteristic symptoms of the disease are V-shaped chlorotic to necrotic lesions at the borders of the leaves and blackening of the vascular tissues (CAB 2003). Diseased leaves and heads have a poor market value, are unsuitable for storage as they quickly rot after harvest (Massomo 2002). Satisfactory control of black rot can only be obtained with an integrated control strategy where some of the most recommended measures are: use of pathogen-free seeds, crop rotation and resistant varieties (CAB, 2003). However, varieties popular with the consumers often lack satisfactory resistance and effective chemicals are also not available for controlling the disease.

In recent years, the use of antagonistic bacteria, able to internally colonise healthy plant tissues, have been shown to have a good potential to act as biological control agents (Pleban et al. 1995; Chen et al. 1995). Internal colonisation of plant tissues by endophytic bacteria [Bacterial endophytes are defined as internal plant colonists which do not cause any visible damage to their hosts and which can be re-isolated from surface disinfected plant material or internal plant extracts (Hallmann et al. 1997)] seems to be a natural phenomenon and according to Kloepper et al. (1999), such bacteria might improve plant health. One of the advantages of using endophytes in biocontrol is that once inside the host, they are better protected against environmental stresses and microbial competition compared to other plant colonisers (Schulz and Boyle 2005).

Biological control of bacterial pathogens using antagonistic, endophytic bacteria: Endophytic bacteria that are antagonistic to bacterial and fungal pathogens have been identified, and biological control, using endophytic bacteria against bacterial pathogens, has been reported in various agricultural crops including vegetables (Kawalek and Schaad 1988; Assis et al. 1997; 1998), cereals (Poon et al. 1977), tubers (Sessitsch et al. 2004) and fruit trees (Cao et al. 2005). In vegetables, a few studies have been conducted on brassicas. For instance, an avirulent strain of *X. c.* pv. *campestris* was used in biological control of black rot in cabbage (Kawalek and Schaad 1988). Also Assis et al. (1997, 1998) showed that black rot of cabbage and kale could be controlled under field conditions with endophytic strains of *Kluyvera ascorbata* and *Bacillus* spp.

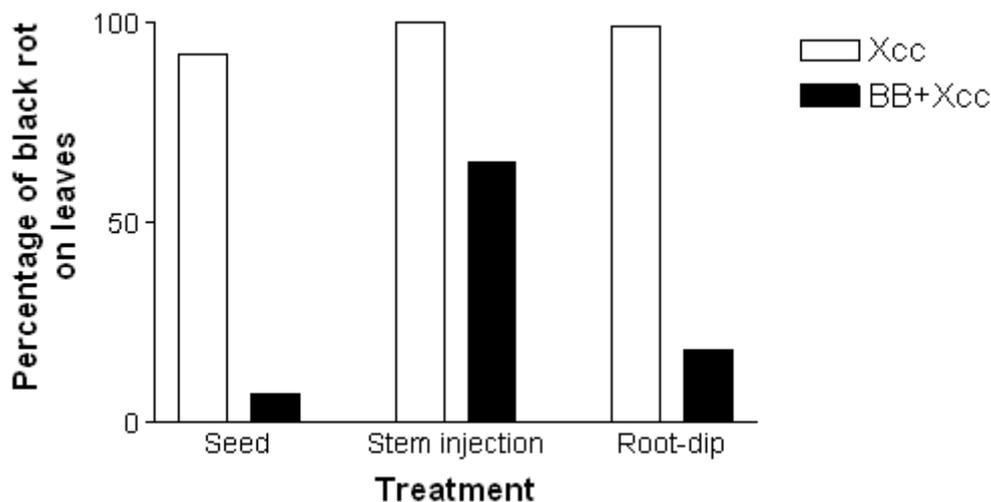
**Biological control of black rot of brassicas with *Bacillus* strains in Tanzania and Zimbabwe:** In Tanzania and Zimbabwe, brassicas are among the most consumed and cultivated vegetables, with cabbage (*Brassica oleracea* var. *capitata*) as the main cultivated *Brassica* crop (Faostat 2004; Massomo et al. 2005). Strategies to control black rot are, however, difficult to implement because most of the cabbage varieties used are open pollinated and susceptible to black rot; many resource poor farmers do not have access to healthy seeds; and due to land shortage and an increase in the frequency of cabbage cropping, farmers do not take advantage of the beneficial effect of crop rotation (Massomo et al. 2005).

To reduce losses caused by black rot in vegetable brassicas, the Plant Pathology Section at the Royal Veterinary and Agricultural University has developed a control strategy where *Bacillus* spp. is used as biological control agents. The work started in 1992, when 393 *Bacillus* strains were isolated from *Brassica* seeds and other plant materials (Mguni 1996). Five of these strains showed promising potential against black rot on cabbage under growth chamber and field conditions (Mguni 1996). From 1998-1999, a number of field experiments were conducted in Zimbabwe to evaluate the biological control efficacy of one selected *Bacillus* strain (BB) with endophytic ability against the black rot pathogen in different brassica crops (cabbage, cauliflower, rape and broccoli) (Wulff et al. 2002). Except for the rainy season, promising control was obtained with strain BB in the four brassica crops (Wulff et al. 2002). Additional experiments conducted in Tanzania also showed that symptoms and losses caused by black rot on cabbage could be reduced in different growing seasons when the antagonist (strain BB) and other *Bacillus* strains were applied especially via the roots (Massomo et al. 2004). Figure 1 shows reduction of internal black rot symptoms under field conditions by *Bacillus* strains in Tanzania and Zimbabwe.



**Fig. 1** Control of black rot of cabbage under field conditions in Tanzania and Zimbabwe with *Bacillus* species. Internal black rot index (Wulff et al. 2002): 0 = No discoloration, no symptoms on the heart leaves (healthy plants); 1 = vein discoloration extends < ½ of the stem, no symptoms on the heart leaves; 2 = vein discoloration extends > ½ of the stem, no symptoms on the heart leaves; 3 = vein discoloration of stem and 1-3 of the heart leaves and 4 = vein discoloration of stem and on more than 3 heart leaves

**Inoculation methods:** Bacterial endophytes can be introduced into plants in different ways. In our system, we have tried seed inoculation, foliar treatment, stem injection and root-dipping prior to planting alone or in various combinations (Mguni 1996; Massomo et al. 2004). While under greenhouse conditions (Figure 2), all the inoculation methods provided effective control of Xcc (Mguni 1996), under field conditions, neither foliar nor seed treatment gave significant control (Massomo et al. 2004). However, root inoculation, alone or in combination with other treatments (seed and foliage application), was shown to be, under field conditions in Tanzania, the most effective way to apply the endophyte to control Xcc in cabbage (Massomo et al. 2004).

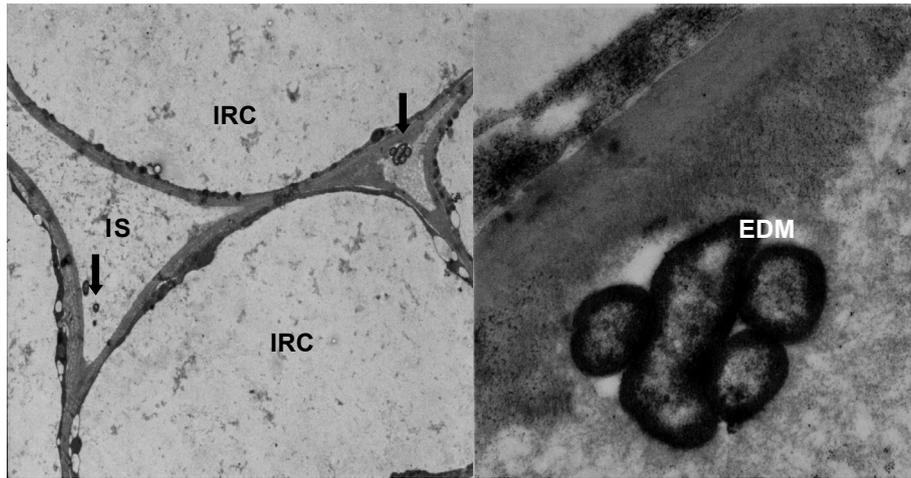


**Fig. 2** Efficacy of three inoculation methods (seed inoculation, stem injection and root-dip treatment) in the control of black rot of cabbage under greenhouse conditions with *Bacillus subtilis*, strain BB

Location and population dynamics of the endophytic strain BB in the plant: In treated plants, population levels of strain BB were generally low with the highest levels in the upper root and base of the stem, and considerably lower levels in the aerial plant parts (Wulff et al. 2003). A similar trend has also been shown by Pleban et al. (1995) using a different endophytic strain of *Bacillus*. Using transmission electron microscopy (TEM), BB was found to colonise predominantly the intercellular spaces of roots and stem tissues of cabbage seedlings (Wulff et al. 2003).

The population level of the BB strain was found to be higher under greenhouse conditions (seed inoculation) than in plants grown under field conditions (root-dip method) (Wulff et al. 2003). The levels of BB in the plant tissues in the greenhouse varied from Log 4.6 CFU/g fresh weight (in root tissues) to Log 1.9 CFU/g fresh weight (in leaf tissues) (Wulff et al. 2003). However, 35 days after inoculation, BB was undetectable in the greenhouse-grown cabbage seedlings. Although under field conditions, the population of BB within cabbage roots was low and fluctuated between Log 1 and Log 2 CFU per g fresh root, the strain could be detected throughout the twelve week growing season (Wulff et al. 2002).

Mechanisms of disease control: In our system (BB x Xcc), it seems probably that induced resistance (IR) is one of the mechanisms operating to control Xcc as i) the strain BB effectively controlled black rot (Mguni 1996; Wulff et al. 2002; Massomo et al. 2004), but was not found colonizing the same tissues of the host plant as the pathogen (Wulff et al. 2003); ii) the presence of BB in the intercellular spaces was often followed by accumulation of an electron dense material (EDM) (Figures. 3a, b), which probably originated from the host plant. Accumulation of EDM was reported by Benhamou et al. (1996) in peas and considered to be phenol compounds involved in plant defense reactions; iii) when *Arabidopsis* plants were root treated with living or dead cells of BB, the colonisation of *Arabidopsis* leaves by Xcc was reduced in BB treated *Arabidopsis* plants (Massomo 2002).



**Fig. 3a, b** Transmission electronic microscopy combined with immuno-gold labelling of root tissues of cabbage seed inoculated with *Bacillus subtilis*, strain BB. **3a-b**: Bacterial cells (arrows) are located in the intercellular spaces of the inner root cortex. **3b**: Magnification of boxed area of figure **3a** showing electron dense material involving the bacterial cells in the intercellular space of the inner root cortex. Arrows: bacterial cells; IS: intercellular space; IRC: inner root cortex; EDM: electron dense material. Magnifications: x 2492 (3a) and x 29400 (3b)

### Conclusions and future prospects

*Bacillus* strain BB was successfully used as a biological control agent against black rot of brassicas in field trials carried out during different growing seasons in Tanzania and Zimbabwe. Xcc colonises systemically the vascular system of host and since strain BB has been demonstrated to be endophytic in vegetable brassicas, the present work demonstrates the potential of employing endophytes to control of vascular pathogens. Induced resistance seems to be one of the mechanisms involved in the control of black rot by strain BB. However, further studies need to be done to confirm this hypothesis.

The promising results obtained with strain BB deserve additional work to improve its efficacy as a biological control agent, i.e. through the development of appropriate formulations, through combined applications with other biocontrol agent(s) and with other component(s) of an integrated black rot management strategy. Further studies need to be conducted to determine the toxicological risks for humans and for the environment of using BB, as well as other *Bacillus* strains, as biocontrol agents.

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## Biological control of bacterial pathogens on tomato plants

### Abstract

Natural extracts were shown to be useful for biocontrol of *Cmm*, *Pst* and *Xv* tomato as bacterial pathogens. No negative effect of these natural substances was observed on tomato plants. The use of these natural substances appears to be particularly interesting for protecting tomato plants in the greenhouse. Moreover, the natural substances seem to be also useful in the field prior to bacterial inoculation. The antibacterial activity of these natural substances was effective at least for 10 days, indicating that they may provide a substitute for copper treatments that are usually applied in organic agriculture

### Introduction

The biological control of parasites in organic agriculture is based on the use of natural antagonists and substances present in nature. On tomato plants, bacterial pathogens are a serious problem almost wherever this horticultural crop is cultivated. Amongst them, *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), causal agent of tomato bacterial canker, *Pseudomonas syringae* pv. *tomato* (*Pst*), causal agent of tomato bacterial speck and, *Xanthomonas vesicatoria* (*Xv*), causal agent of tomato bacterial spot, are the most dangerous. To control these phytopathogenic bacteria in organic agriculture, preventive strategies can be developed; treatment with cupric and some natural substances, and good agronomical practices (healthy seeds, fertilization, irrigation, etc.) are suggested (Balestra 2003; Varvaro et al. 2001).

Due to the recent EU restriction on copper use in organic agriculture (Reg. EU n° 473/2002) and the increased transportation of vegetal material among the EU and non EU countries, the effectiveness of natural bactericidal/bacteriostatic compounds attain relevant importance in controlling tomato bacterial pathogens especially in organic agriculture.

The aim of this research was to verify (*in vitro* and *in vivo*) the possibility of a biocontrol by different natural substances to control the most dangerous bacterial pathogens (*Cmm*, *Pst*, *Xv*) of tomato plants.

### Materials and methods

As potential natural substances effective against tomato bacterial pathogens, vegetal extracts from species of *Liliaceae* and *Moraceae* plants were used. *Liliaceae* plants were chosen for their antimicrobial properties, well known in human health for several beneficial properties; the efficiency is related to sulphur substances and among these, allicin has the most evident antimicrobial activity (Cavallito and Bailey 1944; Holzgartner et al. 1992). *Moraceae* plants were chosen because several of their extracts showed antibacterial activity (Abegaz et al. 2000; Wongkham et al. 2001).

As tomato bacterial pathogens, strains characterized by a higher level of virulence of *Cmm* (n°21), *Pst* (n°14), *Xv* (n°697), from the collection of the Department of Plant Protection, University of Tuscia, Viterbo, were used.

*In vitro* spot tests and well tests were carried out (Balestra et al. 1998). Bacterial strains were used at 10<sup>6</sup> and 10<sup>8</sup> cfu/ml concentration; natural extracts of *Liliaceae* plants were used at a concentration of 1%, those of *Moraceae* plants at 30%. Tests were performed on NA and NSA medium. After distribution of each bacterial suspension (100 µl per Petri dish), vegetal extracts (4 drops, 30 µl each) were placed and Petri dishes were incubated at 25 ± 2°C for 48-72 h; developing inhibition halos were observed and measured daily with a stereomicroscope (Balestra et al. 1998).

*In vivo* tests were carried out in the greenhouse on tomato plants of cv. S. Marzano, 1 month old. Temperature (T) was maintained at 25 ± 2 °C in the light and at 15 ± 2°C in the dark for 12 h, respectively; relative humidity (RH) was maintained between 70-80% during the whole experiments. The extract of *Liliaceae* plants was used at a concentration of 1%; those of *Moraceae* plants at 10%. Bacterial strains (*Cmm* 21, *Pst* 14, *Xv* 697) were used at 10<sup>5</sup> and 10<sup>8</sup> cfu/ml concentration.

Each natural substance was uniformly sprayed on the surface of tomato plants; 24 h later, plants were abundantly irrigated and covered for 2 h with plastic bags to favour stomatal opening. Then the bacterial pathogens were sprayed on the tomato plants and the plants were covered with plastic bags for another 2 h. Plants were monitored daily for 15 days. After the appearance of typical symptoms the number of necroses per cm<sup>2</sup> of tomato leaf was counted per tomato plant inoculated with *Pst* and *Xv*; the diseased leaf area (cm<sup>2</sup>) was measured for the *Cmm* inoculations. Tomato plants infected with the same *Cmm*, *Pst* and *Xv* bacterial concentrations, but not previously treated by natural substances, were maintained separately at the same climatic conditions, as control plants. Each host-pathogen/natural substance combination was repeated three times. All data obtained were subjected to statistical (ANOVA) analysis.

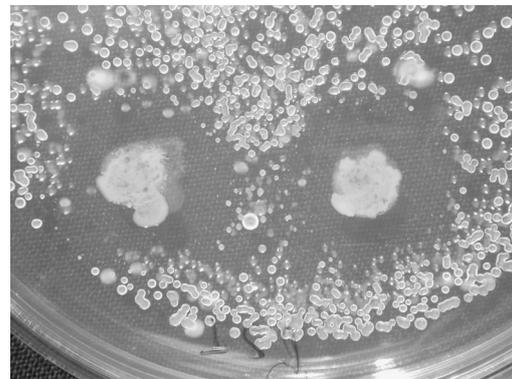
**Results**

*In vitro* (spot and well) tests, both vegetal extracts showed antibacterial activity against the different tomato bacterial strains used. *Lilaceae* extract (1%) showed antibacterial activity against *Cmm* and *Xv* pathogens when the bacterial concentration was 10<sup>6</sup> cfu/ml but not at 10<sup>8</sup> cfu/ml. Independent of the bacterial concentration (10<sup>6</sup> and 10<sup>8</sup> cfu/ml), a weak bactericidal activity of *Lilaceae* extract was recorded towards the *Pst* 14 bacterial strain (Figure 1, Table 1).

*Moraceae* extract (30%) revealed remarkable antibacterial activity against the bacterial pathogens tested (*Cmm*, *Pst*, *Xv*) at both concentrations (10<sup>6</sup> and 10<sup>8</sup> cfu/ml) used (Figure 2, Table 1).



**Fig. 1** Growth inhibition (halos) of *Cmm* 21 (10<sup>6</sup> cfu/ml) by using *Liliaceae* extract at 1%, in spot tests (2x)



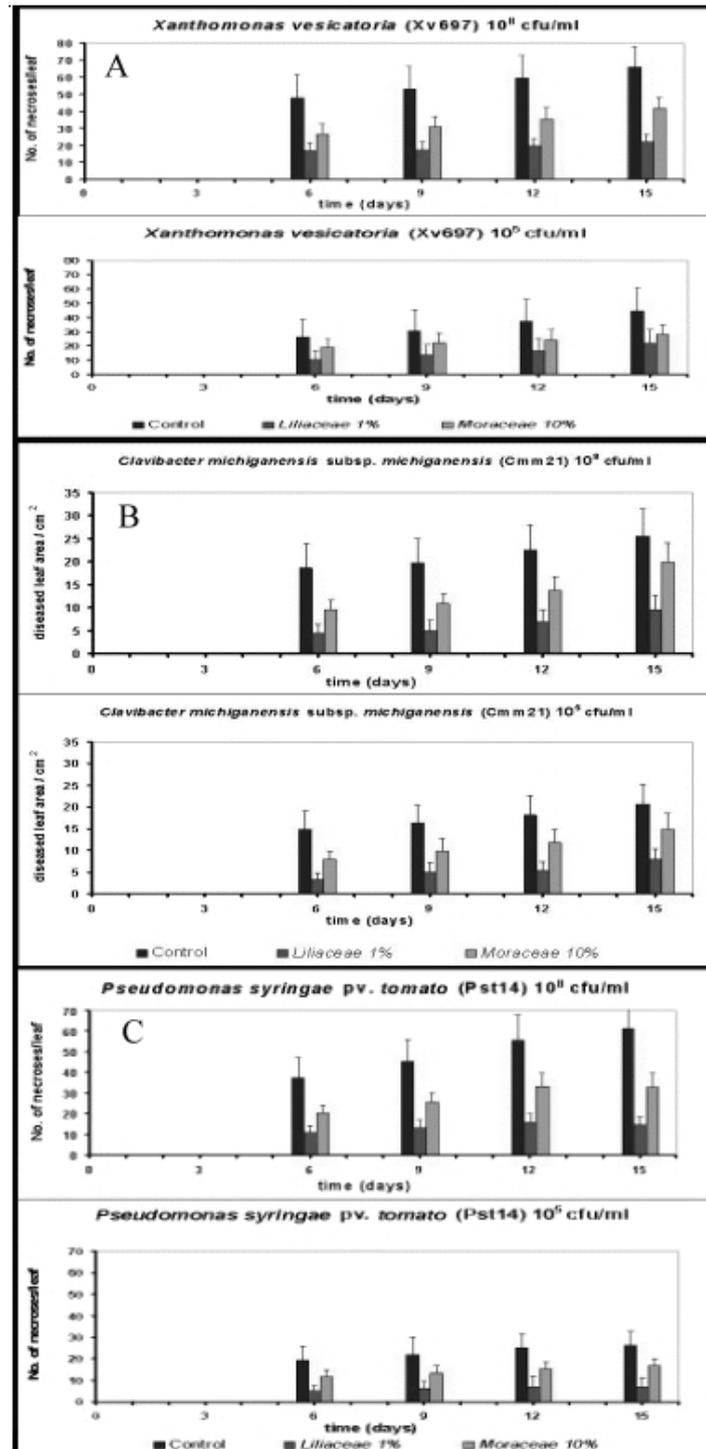
**Fig. 2** Growth inhibition (halos) of *Pst* 14 (10<sup>8</sup> cfu/ml) by using *Moraceae* extract at 30%, in spot tests (4x)

**Tab. 1** *In vitro* biocontrol of tomato bacterial pathogens (*Pst*, *Xv*, *Cmm*) by using *Liliaceae* (1%) and *Moraceae* (30%) extracts, in spot tests

	Bacterial pathogens					
	<i>Pst</i> 14 10 <sup>8</sup> cfu/ml	<i>Pst</i> 14 10 <sup>6</sup> cfu/ml	<i>Xv</i> 697 10 <sup>8</sup> cfu/ml	<i>Xv</i> 697 10 <sup>6</sup> cfu/ml	<i>Cmm</i> 21 10 <sup>8</sup> cfu/ml	<i>Cmm</i> 21 10 <sup>6</sup> cfu/ml
<i>Liliaceae</i> 1%	0.04±0.03	0.03±0.02	-	0.16±0.13	-	0.23±0.11
<i>Moraceae</i> 30%	0.15±0.13	0.21±0.18	0.13±0.7	0.19±0.17	0.16±0.12	0.20±0.15

**Legend:** *Pst* 14: *Pseudomonas syringae* pv. *tomato*; *Xcv* 697: *Xantomonas vesicatoria*; *Cmm* 21: *C. m.* subsp. *michiganensis*. The values shown are the mean (± SD).

In *in-vivo* tests with both natural substances confirmed their antimicrobial activity against *Cmm*, *Pst*, and *Xv* as bacterial pathogens. With respect to strain *Cmm21*, disease was reduced particularly by using *Liliaceae* against higher bacterial concentrations ( $10^8$  cfu/ml) but by both natural extracts (*Liliaceae* and *Moraceae*) at a lower concentration ( $10^5$  cfu/ml) (Figure 3). On *Pst14* strain both natural substances were particularly effective at  $10^5$  cfu/ml bacterial concentration (Figure 3). Also with respect to *Xv697* strains, both *Liliaceae* and *Moraceae* extracts were able to reduce the necroses/cm<sup>2</sup> with particular significance when the bacterial inoculum was of  $10^5$  cfu/ml (Figure 3).



**Fig. 3** *In vivo* biocontrol of *Cmm21* (A), *Pst14* (B) and *Xv697* (C) bacterial pathogens, by using *Liliaceae* (1%) and *Moraceae* (10%) extracts on tomato plants

## Discussion

We were able to show that natural extracts could be useful for biocontrol of *Cmm*, *Pst* and *Xv* tomato as bacterial pathogens. No negative effect of these natural substances was observed on tomato plants. The use of these natural substances appears to be particularly interesting for protecting tomato plants in the greenhouse. Moreover, the natural substances seem to be also useful in the field prior to bacterial inoculation. The antibacterial activity of these natural substances was effective at least for 10 days, indicating that they may provide a substitute for copper treatments that are usually applied in organic agriculture.

Further studies are necessary to evaluate field-application of these natural substances and the possibility of applying them together with copper compounds, as a means of reducing the use of cupric ions in controlling tomato bacterial pathogens.

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## **Biocontrol of bacterial canker disease of tomato by *Pseudomonas fluorescens***

### **Abstract**

Use of plant growth promoting rhizobacteria in managing bacterial canker disease of tomato was studied in the present work. Tomato seeds were treated with five strains of *Pseudomonas fluorescens* and subjected to seed germination and seedling vigour. Among the *P. fluorescens* strains tested only three strains (*Pf3*, *Pf7a* and *Pf11*) which showed enhancement in the seed quality parameters like seed germination, seedling vigour, were further subjected to estimation of one of the defense related enzyme phenylalanine ammonia lyase with total phenol content. The same three strains were recorded for maximum disease protection under greenhouse conditions. The level of PAL and total phenol contents increased significantly upon the *P. fluorescens* treatment. The rate of reduction in the bacterial canker disease incidence was directly proportional to level of PAL and total phenol content. The possible use of these *P. fluorescens* strains in effective management of bacterial canker of tomato are discussed in the present work.

**Key words:** Tomato, bacterial canker, *Pseudomonas fluorescens*, phenylalanine ammonia lyase (PAL)

### **Introduction**

Tomato (*Lycopersicon esculentum* Mill.) is an important vegetable crop throughout the world. India is the second largest producer of vegetables in the world next to China. The production of tomato seeds free from the pathogen is of great economic importance to both growers and the seed industry. An important bacterial disease of tomato is bacterial canker, caused by *Clavibacter michiganensis* ssp. *michiganensis*, a very destructive harmful disease of the solanaceous family, that results in complete loss of the crop (Boudyach et al. 2001). *P. fluorescens* Trevisan Migula is one of the most important antagonists of *Fusarium* in cucumber (Liu et al. 1995), and *Xanthomonas oryzae* pv. *oryzae* in rice (Vidhyasekaran et al. 2001). Unsuccessful attempts to manage this disease by chemotherapeutics prompted us to develop alternative strategies. Therefore, the present studies were aimed to study the possible use of *P. fluorescens* in managing the bacterial canker disease of tomato.

### **Materials and methods**

Seeds: Tomato seeds of variety PKM-1, commonly known as *Madanapalli* tomato, were used for all experiments.

Effect of *P. fluorescens* on seed germination and seedling vigour of tomato under laboratory conditions: Five *P. fluorescens* strains, *Pf1*, *Pf3*, *Pf7a*, *Pf11* and *Pf15*, were obtained from the culture collection of the Department of Applied Botany, Seed Pathology and Biotechnology, University of Mysore, Mysore, India and sub-cultured on nutrient media for 48 h by incubating at 26±2°C. The 48 h old cultures of *P. fluorescens* strains were centrifuged at 10000 rpm for 5 min. The washed bacterial pellet was suspended and the OD was adjusted to 0.45 (A<sub>610</sub> nm) to obtain 1x10<sup>8</sup> cfu/ml. Seeds were gently shaken in the bacterial suspension on an orbital shaker. Germination tests were carried out by the paper towel method and seedling vigour was calculated according to Abdul Baki and Anderson (1973). The experiment was repeated three times. Strains which gave high germination and vigour were selected for further experiments.

Determination of phenylalanine ammonia lyase activity and total phenol content: Eight-day-old seedlings from *P. fluorescens* treated and untreated seeds were root-dip inoculated with the bacterial pathogens and after 21 h the seedlings were subjected to enzyme extraction. PAL activity was assayed according to Lisker et al. (1983). The enzyme activity was spectrophotometrically determined by the production of *t*-cinnamic acid from L-phenylalanine. Protein content of the extracts was determined according to

Bradford (1976) using BSA as standard. The enzyme activity was expressed as  $\mu\text{mol}$  of *t*-cinnamic acid  $\text{mg}^{-1}$  protein  $\text{h}^{-1}$ . Phenol content was estimated according to Malick and Singh (1980). As standard, gallic acid was used. Phenol content of the extract was expressed as  $\text{mg}$  phenol/ $\text{g}$  material. The experiment was conducted in four replicates and repeated thrice.

Effect of *P. fluorescens* strains on disease incidence: Tomato seeds were treated with pure cultures of *Pf3*, *Pf7a* and *Pf11*, were blot dried, sown in pots (10 seeds/pot in 20 replicates) and pots were maintained in greenhouse conditions. Broth cultures of *C. michiganensis* ssp. *michiganensis* (48 h old) ( $1 \times 10^8$  cfu/ml) were inoculated to the first pair of true leaves (eight-day-old) of the tomato seedlings, and repeated for two days. The plants were monitored several weeks after the challenge inoculation for visible symptoms and canker incidence was recorded. Experiments were conducted in 20 replicates of 10 plants each and repeated thrice. Average disease incidence was calculated and tabulated. Data were transformed to arcsine, analysis of variance (ANOVA) was carried out for the transformed values, and means were compared for significance using Duncan's Multiple Range Test (DMRT;  $p=0.05$ ).

## Results

Effect of *P. fluorescens* strains on seed germination and seedling vigour of tomato seeds under laboratory conditions: An improvement in seed germination and seedling vigour was observed following *P. fluorescens* seed treatment. This improvement is strain specific and not uniform with all *P. fluorescens* strains. Three among five *P. fluorescens* strains, as fresh suspensions, significantly ( $p=0.05$ ) enhanced seed germination under laboratory conditions compared with the control. Mean shoot length of seedlings increased significantly, when the seeds were treated with *P. fluorescens* strains *Pf3*, *Pf7a* and *Pf11* (Table 1).

**Tab. 1** Effect of seed treatment with *P. fluorescens* strains on seed germination and seedling vigour of tomato under laboratory conditions

Treatment	Germination (%)	MRL (cm)	MSL (cm)	VI
Control	77±1.08 <sup>c</sup>	6.1±0.21 <sup>a</sup>	2.7±0.07 <sup>b</sup>	678 <sup>c</sup>
<i>Pf1</i>	77±1.08 <sup>c</sup>	6.2±0.04 <sup>a</sup>	2.7±0.07 <sup>b</sup>	685 <sup>c</sup>
<i>Pf15</i>	80±1.22 <sup>bc</sup>	6.2±0.10 <sup>a</sup>	3.1±0.14 <sup>a</sup>	744 <sup>ab</sup>
<i>Pf3</i>	80±1.08 <sup>bc</sup>	6.6±0.04 <sup>a</sup>	3.1±0.04 <sup>a</sup>	776 <sup>a</sup>
<i>Pf11</i>	83±0.75 <sup>a</sup>	6.2±0.17 <sup>a</sup>	3.1±0.04 <sup>a</sup>	771 <sup>a</sup>
<i>Pf7a</i>	83±1.68 <sup>a</sup>	6.3±0.23 <sup>a</sup>	3.2±0.12 <sup>a</sup>	789 <sup>a</sup>

Values are means  $\pm$  SE of four replicates of 100 seeds each and were repeated thrice; MRL – mean root length; MSL – mean shoot length; VI – Vigour index; The values in the column followed by same letter(s) are not significantly different according to ANOVA (DMRT;  $p = 0.05$ )

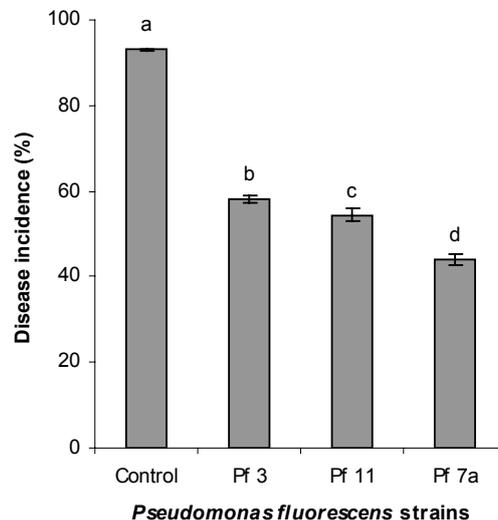
Effect of seed treatment with *P. fluorescens* strains on PAL and total phenol content: Seedlings raised from *Pf11* strain treated seeds and pathogen inoculated showed higher PAL activity (approximately 4 times) as compared to the control. PAL activity increased with *P. fluorescens* seed treatment over the control. Among the three treatments *Pf7a* showed a significant increase in PAL activity over the control (Table 2). Total phenol content in the *P. fluorescens* treated seedling was higher than the control (Table 2).

**Tab. 2** Effect of seed treatment with *P. fluorescens* strains on the PAL activity and total phenol content

Treatment	PAL activity ( $\mu\text{M tCA}/\text{mg protein/h}$ )	Total phenol (mg/g) tissue
Control	1464.36±0.30 <sup>b</sup>	0.0541±0.00083 <sup>d</sup>
<i>Pf3</i>	2467.33±0.19 <sup>b</sup>	0.0958±0.00083 <sup>b</sup>
<i>Pf11</i>	2226.93±0.10 <sup>b</sup>	0.0808±0.0008 <sup>c</sup>
<i>Pf7a</i>	5352.03±0.62 <sup>a</sup>	0.1883±0.00016 <sup>a</sup>

Values are means  $\pm$  SE of three independent experiments with four replications each. The values in the column followed by same letter(s) are not significantly different according to ANOVA (DMRT;  $p = 0.05$ ).

Effect of seed treatment with *P. fluorescens* strains on the bacterial canker incidence under greenhouse condition: Seed treatment with *P. fluorescens* strains effectively reduced the canker incidence. Disease incidence in the control was 93 % whereas it was significantly ( $p=0.05$ ) reduced upon *P. fluorescens* treatment. The disease incidence in the Pf7a treated was 44% while in the Pf3 and Pf11 treated the disease incidence was 58 % and 54 %, respectively (Figure).



**Figure** Effect of seed treatment with *P. fluorescens* strains on the bacterial canker incidence under greenhouse conditions; Values are means  $\pm$  SE from three repeated experiments with twenty replicates of 10 seeds/pot each; the values in the column followed by same letter(s) are not significantly different according to ANOVA (DMRT;  $p = 0.05$ )

## Discussion

Seed treatment with plant growth promoting rhizobacterial strains for the management of bacterial canker disease in tomato was reported in the present study. On treatment with *P. fluorescens* strains, a gradual increase in the germination of tomato seeds and vigour of seedlings was observed. Similar results were reported by Murphy et al. (2000) and Raj et al. (2003). The results of the present studies revealed a decrease in the bacterial canker disease and an increase in the level of defense related enzyme PAL. Similar studies were reported by Padmaja et al. (2004) for cotton and *Xanthomonas axonopodis* pv. *malvacearum* interaction. The level of PAL is known to play a crucial role for the degree of host resistance (Umesha 2005). It is obvious from the present study that among the *P. fluorescens* strains tested, Pf3, Pf7a and Pf11 showed a significant reduction in canker incidence and increase in PAL and phenol level.

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## **Biological control potentials of fluorescent pseudomonad strains against bacterial blotch disease (*Pseudomonas tolaasii*) of cultivated mushroom**

### **Abstract**

Mushroom growing is endangered by some serious diseases in Turkey because of insufficient growing techniques. Bacterial Blotch disease caused by *Pseudomonas tolaasii* is the most serious problem of cultivated mushroom (*Agaricus bisporus*). The objective of this study is to identify effective antagonistic fluorescent pseudomonads (FP) for biological control of this disease. In this study, 38 fluorescent pseudomonad (FP) strains were tested for biological control of Bacterial Blotch disease of cultivated mushroom caused by *P. tolaasii*. FP strains such as 12, 13, 37, 50, 51, 74, M4/2, M5/3, and 39A, which were sprayed on freshly harvested detached mushroom caps, decreased the disease severity of Bacterial Blotch by 70-100%. The selected candidate FP strains (M4/2, M5/3, and 39A) effectively inhibited the disease development on detached mushroom caps were tested for biological control of *P. tolaasii* under mushroom growing house conditions. The suspensions of FP strains ( $10^{11}$  cfu/ml) were applied at three different growing stages of mushroom: five days after casing soil, at the stage of button, and before the harvest. A suspension of  $10^8$  cfu/ml of the pathogen was sprayed one day after the first application of the antagonists. A randomized complete block design was applied with five replications. Disease severity was estimated by a 0-3 scale. FP strains M-4/2 and M-5/3 decreased the severity of disease caused by *P. tolaasii*, by 88.07% and 85.33 %, respectively, when FP strains were applied at the stage of button.

### **Introduction**

Bacterial blotch disease, which is one of the most common diseases in all mushroom growing countries, causes qualitative and quantitative losses in mushroom crops. During the survey made in mushroom growing houses in 1993, the disease was observed in 20% of the houses in Turkey (Bora et al. 1993, 1994). From the survey in 1997-1999, *P. tolaasii* was observed in 35% of the total surveyed growing houses (Fidan et al. 1999).

Hypochlorite is commonly recommended for controlling the disease. Although the pulverisation with chlorine compounds suppresses the disease to some degree, it causes a yield decrease (Olivier and Guillaumes 1981) and browning on the caps (Wong and Preece 1985) and it is found to be risky with respect to human health.

In recent years, biological control studies on the disease have been intensified (Munjal et al. 1989; Khanna et al. 1990). In this context, several biopreparations, such as Victus or Concord, and including *P. fluorescens* as active organism had been registered (Fermor et al. 1991).

The objective of this study is to identify antagonistic fluorescent pseudomonads (FP) that are effective for the biological control of brown blotch disease of cultivated mushroom, and to determine the application frequency of FP strains necessary for effective biocontrol against *P. tolaasii*.

### **Material and methods**

**Bacterial pathogen:** The isolate of *P. tolaasii* was obtained and identified from infected mushroom caps in a commercial farm.

**Bacterial biocontrol agents:** 38 fluorescent pseudomonad (FP) strains, isolated from healthy mushroom caps, were tested for biological control of Bacterial Blotch disease of cultivated mushroom caused by *P. tolaasii*.

Growth material and conditions: Commercially prepared pasteurised compost provided by a private company in İzmir and *A. bisporus* str 512 were used in the study. In vivo trials were performed in the mushroom growing room of the Department of Plant Pathology of Ege University. Hard-plastic trays were 25 x 40 x 16 cm in size and filled with compost (5kg/tray). The trays were cased by sterile peat 14 days after the spawn development period.

### ***In vivo* tests**

Efficacy of FP strains against *P. tolaasii* on detached mushroom caps: The suspensions of FP strains ( $10^{11}$  cfu/ml) were sprayed on freshly harvested detached mushroom caps. A suspension of *P. tolaasii* ( $10^9$  cfu/ml) was sprayed on the caps one hour later. Detached caps were kept at high RH (90%) and 24 °C for 2 days. Disease severity was evaluated by a 0-3 scale: (0): healthy caps, (1): small spots up to 3, (2): numerous small spots, (3): extended blackening and rotting (Bora and Özaktan, 2000).

Efficacy of FP strains against *P. tolaasii* in mushroom growing room: FP strains decreased the disease severity of Bacterial Blotch by more than 70% on detached mushroom caps, tested for biological control of *P. tolaasii* under mushroom growing house conditions. The suspensions of FP strains ( $10^{11}$  cfu/ml) were applied at three different growing stages of mushrooms as 100 ml/tray:

- First application: 5 days after casing; 100 ml/tray
- Second application: at pinhead stage; 100 ml/tray
- Third application: a few days before harvest; 100 ml/tray

A suspension of  $10^9$  cfu/ml of the pathogen was sprayed one day after the first application of the antagonists. The pathogenic inoculum was sprayed with 60 ml per tray. The experiment was conducted with 5 replications and as a positive control only pathogen suspensions were sprayed. At harvest, the average of disease severity was calculated by using a 0-3 disease scale (Bora and Özaktan 2000).

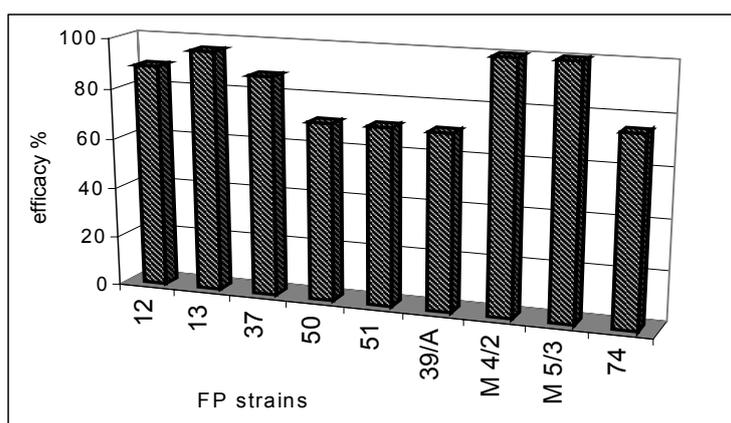
### ***In vitro* tests**

The mode of action of FP strains, which inhibited the disease severity of *P. tolaasii* on mushroom caps by 70 to 100%, was investigated under *in vitro* conditions. The strains of FP were placed at four different points at equal distance from the margin of the Petri dish (9 cm Ø), containing King's Medium-B, incubated for 48 h, to allow development of bacterial colonies. A bacterial suspension of *P. tolaasii* ( $10^9$  cfu/ml) was sprayed onto the bacterial colonies. After incubation at 24 °C for 3 days, the inhibition zones developed between the colonies of FPs and *P. tolaasii* were evaluated using a 0-5 scale (Geels and Schippers 1983). The antagonistic FP strains were also plated at four different points of Petri dishes containing 80 µM FeCl<sub>3</sub>/L added to King's Medium-B. After 48 hours, the dishes were sprayed with the bacterial suspension of *P. tolaasii* ( $10^9$  cfu/ml). If no inhibition zone developed between the colonies of FP and *P. tolaasii* within 5 days, it was considered that the antagonism may be due to the production of siderophores. In vitro tests were conducted with 3 replications.

### **Results and discussion**

In this study, 38 fluorescent pseudomonad (FP) strains were tested for biological control of Bacterial Blotch disease of cultivated mushroom caused by *P. tolaasii*. FP strains such as 12, 13, 37, 50, 51, 74, M4/2, M5/3, and 39A, which were sprayed on freshly harvested mushroom caps, decreased the disease severity of Bacterial Blotch by 70-100% (Figure).

The selected candidate FP strains (M4/2, M5/3, and 39A), which effectively inhibited the disease development on detached mushroom caps, were tested for biological control of *P. tolaasii* under mushroom growing house conditions. Efficacy of antagonistic FP strains on the development of bacterial blotch disease, applied to the mushrooms at 3 different growing stages, is shown on Table. All antagonistic FP isolates, applied at the different development stages of mushroom, inhibited bacterial blotch disease infections on the caps at rates of 69.63%-87.56%.



**Figure** *In vivo* efficacy of FP strains against the pathogen (*P. tolaasii*) on detached mushroom caps

**Table** Effect of antagonistic FP strains against *P. tolaasii*, applied at three different stages of mushroom development

Antagonists	Applica- tion period*	Disease severity in replications (%)					Mean Disease Severity (%)	Efficacy (%)
		1	2	3	4	5		
M-4/2	U1	2.90	2.90	4.40	2.90	1.40	2.90 bc**	87.56
	U2	4.10	2.00	6.10	1.70	0.00	2.78 bc	88.07
	U3	9.60	3.40	5.90	5.20	1.30	5.08 bc	78.21
M-5/3	U1	3.70	4.90	7.40	12.10	5.70	6.76 bc	71.01
	U2	5.10	2.80	0.00	9.20	0.00	3.42 c	85.33
	U3	8.20	5.70	0.00	4.10	1.40	3.88 bc	83.36
39/a	U1	12.60	0.00	3.70	14.20	1.20	6.34 bc	72.81
	U2	4.60	5.80	16.00	5.70	0.00	6.42 bc	72.46
	U3	8.10	13.70	4.20	5.50	3.90	7.08 b	69.63
Pathogen alone (+)		17.00	26.00	41.70	19.70	12.20	23.32 a	---
Without pathogen (-)		0.00	0.00	0.00	0.00	0.00	0.00 d	---

\*T1: First antagonist application, 5 days after casing; T2: Second application of antagonists at the stage of pinhead development; T3: Third application of the antagonists at the beginning of harvest; \*\*Means followed by the same letter are not significantly different ( $P > 0.05$ ) according to Fisher's protected least significant difference (LSD= 6.945) test

The values shown in Table 1 reveal that strain M-4/2 showed the most successful antagonistic effect among the 3 FP isolates, but also that of others as M-5/3 and 39/a. With respect to the effect of application frequency, in general, the first and second applications of M-4/2 and M-5/3 were more inhibitory of the bacterial blotch disease than the others (Bora and Özaktan 2000). All three antagonistic bacteria were equally effective whether applied two or three times.

*P. tolaasii* is one of the pathogens which is difficult to control and sometimes cause blotch epidemics in mushroom farms. Due to the ineffectiveness of chemical control agents and breeding resistant varieties (Geels et al. 1991; Royse and Wuest 1980), biological control studies become more important. Since these antagonistic bacteria belong to the same taxonomical group as the pathogen, it is predicted that the fluorescent pseudomonads will have a superior effect in biological control as compared to other bacterial spp. (Baker and Scher 1987). This also explains why FP strains are inhibitory for *P. tolaasii* (Miller and Spear, 1995). Moreover, these antagonistic bacteria require the same ecological conditions as the pathogen. If the adequate amount of these antagonistic bacteria is present in casing soil, they are able to compete successfully for the space and nutrition (Compeau et al. 1988).

Since the FP strains 39/a and M-5/3 were not inhibitory *in vivo* against *P. tolaasii*, it has been concluded that the biological control mechanism of these two antagonists is likely to results from competition. M-4/2 produces siderophores, thus  $Fe^{+3}$  competition is one possibility.

A biological control of 50% in disease severity represents an important increase in yield (Fermor 1986). As a general result of the present study, FP strains, tested in different developmental stages of mushrooms, prevented the disease by 69.63%-88.07%.

For effective biological control of *P. tolaasii*, two applications of FP suspension, the first immediately after casing and the second at the pinhead development stage, will provide adequate control and additional applications are not required. Further work is now needed in the development of this research to evaluate the commercial use of FP strains in farms.

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## Control of Asiatic citrus canker and citrus bacterial spot with bacteriophages in Florida

### Introduction

Asiatic citrus canker, caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*), is a serious threat to Florida's \$8.5 billion citrus industry. The pathogen has been introduced to Florida at least three times since the beginning of the 1980's, and the disease is currently under eradication. However, recent hurricane events hindered the eradication efforts and distributed the pathogen over wide areas. One of several approaches tested for controlling the disease is the use of bacteriophages. After the establishment of a phage collection, disease control trials were carried out in the greenhouse. Since the current regulations do not allow field research with citrus canker in Florida, field trials were conducted with citrus bacterial spot disease (CBS), incited by *Xanthomonas axonopodis* pv. *citrumelo* (*Xacm*).

### Results

Establishment of a bacteriophage collection: A collection of 81 phages active against *Xac* was made and consisted of (i) 13 *Xac* phages received from other collections (USDA Fruit Lab, Beltsville, MD and OmniLytics, Inc., Salt Lake City, UT);, (ii) four phages of other xanthomonads found to lyse *Xac*, and (iii) 67 phages isolated from diseased tissue collected in Florida and Argentina. The phages constituted 13 groups based on host range.

Greenhouse disease control experiments: The effect of a single application of a mixture of four phages on citrus canker disease development was evaluated. Young Duncan grapefruit plants were pruned and the uniformly emerging new flush was inoculated with a  $5 \times 10^6$  cfu/ml suspension of either Xac65 (a phage-sensitive Miami strain of *Xac*), or its phage-resistant mutant. The plants were either sprayed with the phage mixture at  $2 \times 10^8$  plaque forming units (PFU) /ml (treated plants) or with sterilized tap water (control plants) 18 hours before inoculation. Disease severity was assessed 2-3 weeks after inoculation using the Horsfall-Barratt (HB) scale. The HB values were converted to percent defoliation and statistically analyzed using ANOVA and Duncan's multiple range tests.

The disease severity caused by the phage-sensitive strain (Xac65) was significantly reduced in all three experiments (data not shown). Interestingly, the severity of disease caused by the phage-resistant mutant was also significantly reduced in one of three experiments.

Control of citrus bacterial spot in field trials: Due to the restrictions associated with working on citrus canker, field trials cannot be carried out in Florida. Therefore, field trials were conducted using CBS as a model system. The causal agent, *Xacm*, is closely related to *Xac*; both cause leaf and stem lesions, affect only young plant tissues and their phage sensitivity profiles overlap.

Trials were set up to evaluate the efficacy of bacteriophages for suppressing naturally occurring CBS in a commercial citrus nursery. Three test sites were set up in the nursery with three phage-treated and three control plots in each. The phage-treated plots were sprayed twice-weekly at dawn with a mixture of three phages at  $2 \times 10^8$  PFU/ml supplemented with 0.75% (w/v) non-fat dry milk powder using a non-CO<sub>2</sub>, backpack sprayer. Disease severity was assessed biweekly.

In sites 1 and 2 disease progress, as measured by AUDPC, was significantly reduced on phage-treated plants (data not shown). In site 3 the AUDPC reduction was not statistically significant, although in three of the six assessments disease severity was significantly lower in the phage treated plots. In all sites a

significant reduction in disease severity occurred on the new flushes of growth after the plants were pruned.

### **Conclusions**

A collection of 81 bacteriophages was established (i) from known *Xac* phages, (ii) by screening *Xanthomonas* phages on *Xac* and (iii) by isolating phages from diseased plants.

In greenhouse experiments a single application of a phage mixture significantly reduced disease severity caused by a phage-sensitive *Xac* strain.

In field trials twice-weekly bacteriophage applications suppressed naturally occurring citrus bacterial spot development in two of three sites. Marked disease suppression was observed on the emerging new flushes of growth indicating that the presence of phages hindered the establishment of new infections.

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### **Possibility for biological control of pectinolytic erwinias in hyacinth using antagonistic bacterial isolates**

The pectinolytic *Erwinia* species, *Erwinia chrysanthemi* (*Ech*) and *Erwinia carotovora* (*Ec*), are plant pathogenic bacteria responsible for soft rot disease among many important crops including flower bulbs such as hyacinth, freesia and *Zantedeschia*. Spread and development of the disease can only be controlled by application of an integrated strategy that includes testing the propagation material, hygienic measures and use of chemical, physical and biological control agents. Within this study, the possibilities to control pectinolytic erwinias with antagonistic microorganisms have been explored.

The bacteria were isolated from hyacinth bulbs and antagonists were selected *in vitro* on the basis of the ability to inhibit growth of *Ech* and *Ec* strains on LB agar medium. Also the isolates capable of degrading of acyl-homoserine lactones (AHLs), the signalling molecules involved in quorum sensing of *Ech* or *Ec*, were chosen to analyse the possibility for interference with the production of pectinolytic enzymes by degradation of these molecules. Selected isolates were identified by 16S rDNA sequencing, characterised by the BIOLOG system and their ability to degrade different AHLs. These isolates were classified into following genera: *Serratia*, *Pantoea*, *Pseudomonas*, *Erwinia* and *Bacillus*. The results of *in vitro* analysis provide a promising base for further studies. The best antagonists will be tested for reduction of soft rot in leaf- and bulb bioassay.

## IV. Implementation of biological strategies in integrated disease management systems

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### Integrated management of tomato bacterial spot in Florida

#### Abstract

In order to develop more sustainable and integrated strategies for reducing tomato bacterial spot severity, we investigated various combinations of biological control agents, including bacterial antagonists, plant growth promoting rhizobacteria (PGPR), host-specific bacteriophages, and systemic acquired resistance (SAR) inducers in greenhouse and field experiments. Copper hydroxide was applied as a standard bactericide treatment, while untreated plants served as the untreated control. Results from the greenhouse experiments showed insignificant effects of antagonistic and PGPR strains on disease severity. Therefore, we further investigated combinations of SAR compounds (harpin protein, acibenzolar-*S*-methyl), and formulated bacteriophages for controlling tomato bacterial spot in north and central Florida fields. In three consecutive seasons, the combination of formulated phages and acibenzolar-*S*-methyl (ASM) provided reduction in disease pressure and resulted in more efficient foliar disease control than the other treatments. Although there was no significant increase in yield by this combination, there appeared to be a trend of higher yield harvested from plots treated with phage, either alone or in combination with SAR compounds, than in plots receiving no phage. When results of phage- and corresponding non-phage treatments were grouped and compared by using SAS CONTRAST analysis, phage-treated plants produced significantly more marketable fruits than plants not receiving phage. Based on these experiments, the combination of ASM and bacteriophage may be an effective new tool for tomato growers to manage bacterial spot.

#### Introduction

Bacterial spot, caused by *Xanthomonas euvesicatoria*, *X. perforans*, and *X. vesicatoria*, is one of the most devastating tomato (*Lycopersicon esculentum* Mill.) diseases in tropical and subtropical regions where environmental conditions favor infection and disease development (Bouzar et al. 1999; Scott and Jones 1984). In such conditions defoliation and fruit spotting occurs, causing significant reduction in fruit yield and quality of fresh-market and processing tomatoes (Pohronezny and Volin 1983; Pernezny et al. 1996). In Florida, current tomato bacterial spot management strategies have failed to provide satisfactory control due to the endemic nature of the pathogen (Jones et al. 1986) and the occurrence of streptomycin-resistant (Stall and Thayer 1962) and copper-tolerant strains (Marco and Stall 1983). In addition, there are no commercially available tomato cultivars resistant to the predominant pathogenic races (*X. euvesicatoria* race T1 and *X. perforans* race T3) (Jones et al. 1995; Jones et al. 1998). Recently, activation of natural plant defense mechanisms by systemic acquired resistance (SAR) inducers (Ji et al. 1996; Qui et al. 1997; Louws et al. 2001; Romero et al. 2001), application of antagonistic bacteria (Wilson et al. 2002) and bacteriophages (Flaherty et al. 2000, Balogh et al. 2003) were reported as effective alternatives in control of bacterial diseases.

In order to integrate some of those methods to optimize the effects of bacteriophages, SAR compounds, PGPRs and copper-mancozeb in control of tomato bacterial spot, we studied various combinations under greenhouse and field conditions.

## Material and methods

**Greenhouse experiments:** Two PGPR strains, *B. pumilus* B122 and *P. fluorescens* B130, applied alone or in combination with foliar applications of either harpin protein (Messenger, Eden Bioscience Corp., Bothell, WA), acibenzolar-*S*-methyl (Actigard 50WG, Syngenta Crop Protection Inc., Greensborough, NC), unformulated bacteriophage (phage) mixture (Agriphage, OmniLytics Inc., Salt Lake City, UT), or a suspension of one of the antagonists (*P. syringae* Cit7 or *P. putida* B56) were tested for efficacy in control of tomato bacterial spot disease in the greenhouse (Table 1). The PGPR (B122 or B130) suspension, or a tap water control was drenched in a potting mix (50 ml/pot), while foliar treatments were applied with a hand-held sprayer until run-off at concentrations indicated in Table 1. Untreated plants (UTC) and plants treated with copper hydroxide (Kocide 2000, Griffin Corp., Valdosta, GA) were used as control treatments. The treatments were applied to tomato plants of the cultivar Florida 47 before they reached the four-leaf stage. The plants were inoculated with a copper-sensitive strain of *X. perforans* race T3 (91-118). Disease intensity was measured by counting the number of lesions per plant, one and two weeks after inoculation. The data were transformed using log transformation. Statistical analysis was performed with the SAS software version 9.0 (SAS Institute, Inc. Cary, NC).

**Tab. 1** Application timing, concentration of treatments on tomato bacterial spot, greenhouse experiments.  
<sup>z</sup>Timing of treatment application before inoculation; d, days; h, hours. <sup>y</sup>Concentration of active ingredient

Treatments	Application timing <sup>z</sup>		Concentration
	1 <sup>st</sup>	2 <sup>nd</sup>	
<u>Drench treatments</u>			
Water	-	-	-
<i>Bacillus pumilus</i> B122	14 d	7 d	10 <sup>8</sup> cfu/ml
<i>Pseudomonas fluorescens</i> B130	14 d	7 d	10 <sup>8</sup> cfu/ml
<u>Foliar treatments</u>			
Harpin (Messenger)	7 d	1 d	0.0018 % <sup>y</sup>
Acibenzolar- <i>S</i> - Methyl (Actigard)	9 d	4 d	0.003 % <sup>y</sup>
<i>P. syringae</i> Cit7	7 d	2 h	10 <sup>8</sup> cfu/ml
<i>P. putida</i> B56	7 d	2 h	10 <sup>8</sup> cfu/ml
Bacteriophage (Agriphage)	-	2 h	1 % v/v
Copper hydroxide (Kocide 2000)	-	1 d	0.135 % <sup>y</sup>
Untreated control	-	-	-

**Field experiments:** Based on results from greenhouse experiments, the following compounds were selected for control of tomato bacterial spot in the field: harpin protein, acibenzolar-*S*-methyl (ASM), and bacteriophages (phage) (Table 2). Harpin and ASM, which are SAR inducers, were applied every 14 days either alone or in combination with other treatments. ASM was limited to six applications per season. When included in the same treatment, harpin and ASM were alternated and applied every other week. The phage mixture contained six different phages specific to *X. perforans* race T3 strain 91-118 and was formulated in order to prolong phage longevity (Balogh et al. 2003). The commercial phage preparation containing ca. 10<sup>10</sup> plaque forming units (PFU)/ml was used at a 1% concentration. A standard bactericide treatment consisting of copper hydroxide + mancozeb (Cu-Mz) was applied once prior to inoculation and then every 7 days while untreated plants served as an untreated control (UTC). Experiments were conducted in north and central Florida fields during fall 2001, spring and fall 2002. Foliar disease severity was assessed by estimating the percent leaf area affected (necrotic tissue) by bacterial spot using the Horsfall-Barratt (HB) rating scale (Horsfall and Barratt 1945) starting one month after inoculation and every second week thereafter. Fruits were harvested twice, graded into medium,

large and extra-large size fruits (Florida Tomato Committee standards, 2002) and the weight for each class and the total marketable yield were calculated. The data were analyzed statistically using SAS software and differences between means were determined by Duncan's multiple range test ( $P \leq 0.05$ ).

## Results and discussion

In the greenhouse experiments, the PGPR and antagonistic strains, as well as harpin, when applied alone or in combination, had no significant effect on bacterial spot intensity. However, when harpin was applied in combination with phage, the number of spots was significantly reduced (Obradovic et al. 2005). ASM, phage or copper hydroxide treatments alone significantly reduced the number of spots per plant compared to the untreated control. A single application of unformulated phages was inconsistent in control of bacterial spot. A possible explanation for inconsistency could be the limited survival of unformulated phage in natural conditions. Balogh et al. (2003) indicated the importance of phage formulation in order to obtain higher efficacy. Although ASM completely prevented the occurrence of typical symptoms of the disease, necrotic spots similar to a hypersensitive reaction (HR) were observed on plants treated with ASM alone (Obradovic et al. 2005). Electrolyte leakage and population dynamics experiments confirmed that ASM-treated plants responded to inoculation by eliciting an HR. Application of phages in combination with this compound suppressed a visible HR and provided excellent disease control. Although we were unable to quantify populations of the bacterium on the leaf surface, indirectly we determined that host specific bacteriophages reduced populations of a tomato race 3 strain of the pathogen on the leaf surface of ASM-treated plants to levels that did not induce a visible HR. Therefore, integrated use of ASM and phages could be a base for an alternative management strategy against bacterial spot on tomatoes (Obradovic et al. 2005).

**Tab. 2** Treatments for control of tomato bacterial spot in the field

Treatments	Concentration	Timing of application <sup>x</sup>	Field experiments				
			Fall 2001		Spring 2002		Fall 2002
			AUDPC <sup>y</sup>	Yield (t/ha)	AUDPC	Yield (t/ha)	AUDPC
UTC <sup>r</sup>	none	none	164.5 ab <sup>y</sup>	60.8 a	161.0 a	29.8 abc	91.38 a
Cu-Mz <sup>q</sup> (C)	0.19 % + 0.18 %	1	177.6 a	61.3 a	124.3 bc	34.6 abc	66.63 b
Harpin <sup>t</sup> (H)	0.0018 %	2	159.3 bc	66.2 a	152.3 a	27.3 c	85.38 a
Harpin, ASM (HA)	w	w	147.0 bcd	61.4 a	133.0 b	30.4 abc	nt
Harpin, ASM, Cu-Mz	w	w	143.5 cd	61.1 a	nt	nt	nt
ASM <sup>u</sup> (A)	0.003 %	3	133.9 de	65.5 a	131.3 b	27.0 c	57.88 b
Phage <sup>v</sup> (P)	1 %	4	137.4 d	69.4 a	108.5 cd	34.3 abc	53.50 bc
Phage, Cu-Mz (PC)	w	w	nt	nt	98.0 d	36.1 ab	38.63 cd
Phage, harpin (PH)	w	w	152.3 bcd	71.8 a	105.0 d	37.9 a	54.63 bc
Phage, harpin, ASM (PHA)	w	w	112.9 f	66.1 a	99.8 d	31.9 abc	nt
Phage, ASM(PA)	w	w	117.3 ef	67.9 a	101.5 d	30.1 abc	34.75 d

<sup>r</sup>Untreated control. <sup>q</sup>Copper-Mancozeb (Kocide 2000 + Manzate 75DF, Griffin Corporation, Valdosta, GA).

<sup>t</sup>Harpin protein (Messenger, Eden Bioscience). <sup>u</sup>Acibenzolar-S-methyl (Actigard 50 WG, Syngenta Inc.).

<sup>v</sup>Agriphage (AgriPhi Inc. Logan, UT). <sup>w</sup>Applied respectively in concentration and timing indicated for particular treatment.

<sup>x</sup>Application timing: 1 – once prior inoculation, after that weekly; 2 - two applications before inoculation, then in two-week intervals; 3 - two applications before inoculation, then in two-week intervals, limited to 6 applications; 4 – once prior inoculation, then twice a week at dusk. <sup>y</sup>Area under disease progress curves.

<sup>z</sup>Means followed by the same letter within a column are not significantly different according to Duncan's multiple range test,  $P=0.05$  level. nt – not tested

Based on the results from the greenhouse experiments, in which we compared bacterial antagonists, PGPRs, SAR inducers and phages (Obradovic et al. 2005), we focused on application of SAR inducers and host specific bacteriophages for disease control experiments in the field (Table 2). In the field experiments, conducted during three consecutive seasons, combination of ASM and phage or ASM, phage and harpin, significantly reduced foliar symptoms of the disease compared to the other treatments (Obradovic et al. 2004). Although no significant difference in yield was observed between treatments (Table 2), there appeared to be a trend of higher yield harvested from plots treated with the phage, either alone or in combination than in plots receiving no phage applications. Therefore, we analyzed the data by using the SAS CONTRAST statement, grouping the phage treatments (phage, phage-harpin, phage-harpin-ASM and phage-ASM) and comparing them with either Cu-Mz treatments (Cu-Mz and harpin-ASM-Cu-Mz) or corresponding non-phage treatments (UTC, harpin, harpin-ASM and ASM). As a result we observed that phage applications significantly improved yield (Obradovic et al. 2004). Application of formulated phages contributed to greater stability of phage particles on leaf surfaces (Balogh et al. 2003) and enhanced efficacy of this treatment. Being naturally occurring, environmentally friendly, and highly specific to the pathogen, phages represent a very promising alternative to streptomycin or copper-based bactericides. Based on these experiments, the combination of ASM and phage may be an effective new tool for tomato growers to manage bacterial spot. The fact that ASM can trigger a natural defense response against other tomato diseases (Benhamou and Belanger 1998; Momol et al. 2003) increases the potential benefit of this compound in integrated disease management.

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## Management of bacterial spot on tomatoes with bacteriophages

### Abstract

Bacterial spot of tomato, caused by several *Xanthomonas* spp., is a serious disease in tropical and subtropical climates as well as in certain temperate climates. For many years, only tank mixes of copper bactericides and mancozeb were used for controlling bacterial spot. Recently, bacteriophages have been used quite extensively in Florida to control this disease. The strategy used with bacteriophages for bacterial spot control differed from those used previously. The bacteriophages were applied as a mixture of several different phages so that resistant bacterial strains would not build up. We also determined that the timing of bacteriophage application was a critical factor. Applications made in the mid-morning resulted in poor control, whereas applications made prior to sunrise or close to sunset resulted in a significant reduction in disease and increase in yield compared to the standard copper-mancozeb treatment. Further improvement of bacteriophage efficacy resulted from modifications in the formulation and timing of application. We have determined that phage populations plummet during sunlight hours in non-formulated phage suspensions, but are affected to a much lesser extent when powdered milk is added or if the phage suspension is applied in the evening.

### Introduction

Bacterial spot of tomato, caused by *Xanthomonas euvesicatoria*, *X. perforans*, and *X. vesicatoria*, is a serious disease in tropical and subtropical climates as well as in certain temperate climates. Bacterial spot causes frequent and severe yield and quality losses in fresh-market and processing tomato production due to defoliation and spotting of fruit (Pohronezny and Volin, 1983). In Florida, the disease is endemic (Jones et al. 1986) and is caused primarily by *X. euvesicatoria* race T1 and *X. perforans* race T3 (Jones et al. 1995, 1998). Growers apply copper plus mancozeb at least twice weekly in an attempt to control this disease. However, the disease is not controlled when environmental conditions are optimal for disease development (Jones et al. 1991; Marco and Stall 1983). Furthermore, control is hampered in part by the presence of copper-tolerant strains and the endemic nature of the pathogen in some locations (Ritchie and Dittapongpitch 1991; Stall et al. 1986). Antibiotics such as streptomycin have been ineffective for many years as a result of the development of resistant strains (Thayer and Stall 1961).

Management of this disease may be achieved by integration of cultural practices, chemical control and resistance breeding. Presently, there are no commercial tomato cultivars that are resistant to both races of the bacterium. Additionally, appearance of antibiotic and copper-resistant bacterial strains in the field hindered the efficacy of chemical control (Marco and Stall 1983; Thayer and Stall 1961).

Due to the adaptability and population diversity of the pathogen it frequently overcomes disease control strategies (Bouzar et al. 1999; Jones et al. 1995). As a result of poor efficacy in disease control, disease management strategies have focused on identifying alternative methods for tomato bacterial spot management. New approaches have placed greater reliance on biological technologies that could be effectively used in integrated disease management programs. Use of bacterial biocontrol agents (Byrne et al. 1996; Ji et al. 1996, 2006; Jindal and Thind 1993, 1994; Liu 1998; Wilson et al. 1997) and compounds that induce systemic acquired resistance (SAR) in the plant (Louws et al. 2001; Qui et al. 1997) have been reported as effective alternative tools for disease control.

Bacteriophages (phages) have long been proposed as plant disease control agents (Moore 1926) and have been used in several plant-bacterium pathosystems (Coons and Kotila 1925; Kotila and Coons 1925; Saccardi et al. 1993; Tanaka et al. 1990; Thomas 1935; Zaccardelli et al. 1992). As early as 1926 (Moore

1926), phages were used to control Stewart's disease in corn (Thomas 1935). Civerolo and Kiel (1969) reduced bacterial spot disease of peach seedlings caused by *Xanthomonas pruni* using phage applications. However, phages were abandoned due to the emergence of bacterial mutants resistant to the phages.

## Results and discussion

Jackson (1989) hypothesized that using mixtures of bacteriophages would overcome problems with phages experienced in the past. In collaborative efforts, we tested mixtures of bacteriophages for control of the bacterial spot disease on tomato on overhead-irrigated tomato transplants and observed that irrigation water containing bacteriophages specific to the bacterial spot pathogen reduced disease incidence compared to a copper bactericide (Somodi et al. 1997).

Following the success in the greenhouse with tomato transplants, the next step was to conduct field tests to determine the efficacy of bacteriophage applications. An initial field experiment was conducted in which bacteriophages were applied in the mid-morning. We were unsuccessful in controlling bacterial spot in that particular study. We speculated that the bacteriophages were ineffective because the bacteriophages were quickly degraded as a result of UV. Many field studies have demonstrated that microbial pesticides, more specifically virus species are inactivated by exposure to high temperature, alkaline or acidic pH, sunlight, and rain/dew (Ignoffo and Garcia 1992; McGuire et al. 2001). Ultraviolet-A and ultraviolet-B spectra (280-400 nm) of sunlight have been reported to be the most destructive environmental factor in bacteriophage degradation (Ignoffo and Garcia 1994). Additionally, most bacteriophages are sensitive to desiccation.

In field trials in 1997 and 1998, we attempted to minimize problems resulting from UV or desiccation. This was accomplished by making twice weekly spray applications of a mixture of four phages specific to *Xanthomonas euvesicatoria* T1, and *X. perforans* T3 in the early-morning (prior to sunrise) to minimize the effects of UV. In the field study, disease severity of bacterial spot was reduced on plants by an average of 17% compared to control plots receiving no treatment, whereas a copper-mancozeb application, the standard chemical treatment, caused only 11% reduction on treated plants (Flaherty et al. 2000). Additionally, phage-treated plants were found to be significantly more vigorous than copper-treated and non-treated ones and they yielded significantly more extra large fruits as well.

We have attempted to improve bacteriophage application by identifying compounds that could be added to the bacteriophage suspension to extend phage longevity on the leaf surface. Balogh (2002) tested many chemicals in greenhouse and field experiments which were categorized as UV inhibitors, reactive oxygen inhibitors and various compounds which affect virus survival and adherence to leaf surfaces. He identified several formulations that enhanced bacteriophage longevity on leaf surfaces in the greenhouse (Balogh et al. 2003). Two of the formulations, 0.5% Cascrete (a casein product) with 0.5% sucrose, and 0.25% pregelatinized corn flour (PCF) with 0.5% sucrose, significantly extended the phage activity on the leaf surface compared to the non-formulated treatment. In field tests, the formulated phage significantly reduced bacterial spot disease severity compared to the control and the non-formulated phage (Balogh et al. 2003). All formulated bacteriophage treatments were significantly better than the standard copper-mancozeb treatment. In several greenhouse tests a formulation consisting of skim milk and sucrose was tested and gave equally good or better results for controlling bacterial spot, while Cascrete performed best in one field experiment. Of the three formulations tested, skim milk was easiest to prepare and apply (Balogh et al. 2003) and resulted in greater efficacy against the bacterial spot pathogen (Obradovic et al. 2004).

We also determined that the timing of phage application affects efficacy (Balogh et al. 2003). Applications made close to sunset resulted in significantly less disease on plants compared to morning phage applications.

Despite the improved efficacy in disease control, none of the treatments achieved a significant increase in yield. This could have been the result of close proximity of the plots and the highly contagious nature of phage infection: phages were detected in many non-treated plots in the middle of the season in the field experiments. In order to alleviate interference between plots, field plots were separated by greater

distances (Obradovic et al. 2004). In those experiments the formulated phage mixtures effectively controlled bacterial spot and resulted in significant increase in yield.

In summary, our results indicated that the efficacy of phage treatment could be increased significantly compared to standard copper bactericide-EBDC combinations by use of protective formulations and by proper timing of applications. Nevertheless, further research is needed to optimize formulations, application frequency, phage dose and the constitution of phage mixtures so that phage treatment could stand as a valid alternative to chemical bactericides for treatment of bacterial plant diseases in the future.

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### **Integrated management of bacterial wilt on field-grown tomatoes**

New tactics are developed for the management of bacterial wilt on tomatoes caused by *Ralstonia solanacearum* (Rs). Thymol used for soil fumigation consistently provided significant reductions in bacterial wilt incidence and resulted in significantly higher tomato yield in repeated field trials. To develop practical application methods, thymol was applied through drip irrigation lines and it reduced significantly the disease incidence, indicating application of thymol through drip irrigation is a feasible method for bacterial wilt control. Application of Acibenzolar-S-methyl (ASM) significantly reduced bacterial wilt incidence on moderately resistant tomato cultivars, such as Neptune, BHN 466 and FL 7514. While ASM was not effective on susceptible cultivars when high concentrations of Rs were used, ASM in conjunction with pre-plant soil fumigation by thymol significantly improved disease reduction on a susceptible cultivar Solar Fire compared with either thymol or ASM applied alone. Other biorational products reduced disease incidence significantly in greenhouse experiments when used alone or in combinations with other compounds. Sorghum-sudan and rye as cover crops reduced Rs population in artificially infested fields to undetectable levels in a two-year study. These studies indicate that thymol and ASM have the feasibility to be used as a fumigant and a plant activator, respectively, in an integrated management program of bacterial wilt of tomatoes.

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## **Effects of different drying techniques on the inhibitory effect of *Pantoea agglomerans* strain EH-24 bioformulation on fire blight**

### **Abstract**

The strain of *P. agglomerans* Eh-24 was isolated from blossoms collected from healthy pear orchards in the Aegean Region and has been extensively studied as a potential biological control agent of disease in the last decade in Turkey. In orchard trials conducted in 1999 and 2000, talc-based formulations of the strain Eh-24 reduced the percentage of blighted blossoms on pear orchards by 63.16% and 76.25%, approximately (Özaktan et al. 2004). However the commercial utilization of this antagonistic bacterium as biological control agent (BCA) requires the development of a practical bioformulation of the produced biomass. The drying step in development of bioformulation of BCA is one of the most critical problems. Recently various drying techniques have also been tested for obtaining wettable dry powders for easy storage, transport and application.

In this study, the effectiveness of drying techniques on the inhibitory effect of *Pantoea agglomerans* Eh-24 bioformulation was determined by using laminar air flow, spray dryer and fluid bed dryer as drying methods. Before bioformulation, *Pantoea agglomerans* strain Eh-24 was grown in a stirred tank bioreactor using sucrose and heat disintegrated baker's yeast (HDBY) as medium. A stirring rate 500 rpm was used. The temperature and pH during fermentation were kept at 24 °C and 6.8, respectively. Each batch was centrifuged at 6000 rpm for 20 minutes and biomass was used for talc based formulation.

### **Introduction**

Some antagonistic and other beneficial organisms have been very effective in the laboratory but fail at some stage in the field, even after development of a product for marketing. Common causes of this failure are poor stability of the product during storage prior to application, too little active material actually reaching the field target, and rapid degradation of the active material. Formulation plays a vital role in helping to solve these problems and in making an organism effective (Burgess 1998).

So far we have focused on the production of wettable dry powder bioformulation and application of *Pantoea agglomerans* strain Eh-24 because of a project which has been undertaken by the State Planning Organization of Turkey, which is entitled "Investigations on the Scale-Up Production of Some Antagonistic Bacterial Bioformulations and Their *In-Vivo* Performance in the Agricultural Biocontrol".

Wettable dry powders are normally spray dried or lyophilized biomasses with practically no free moisture. Lyophilization is seldom used for large scale productions because of the cost, and its use is limited to research and development (Burgess 1998). Spray drying is a standard process and readily adaptable to formulate fungal spores and other organisms amenable to drying at ambient or higher temperatures; however, the literature search revealed that it is somehow rarely used for drying of Gram-negative bacteria.

In this paper, data on optimization of the spray drying process will be presented. Furthermore, this technique will be compared with drying in a fluid bed dryer and in laminar air flow.

### **Materials and methods**

Organisms: Strain Eh-24 of *P. agglomerans* used for this study had originally been isolated from healthy pear orchard in the Aegean Region and is effective in biological control of *Erwinia amylovora* in pear orchards (Özaktan et al. 1999). The organism was stored at +4 °C in NGBA medium containing 8 g nutrient broth, 20 g glycerol and 18 g bacteriological agar in 1 liter. Before preparation of inocula, the

organism was transferred to SNA medium (sucrose nutrient broth) containing 50 g sucrose, 8 g nutrient broth and 16 g bacteriological agar in 1 liter, from NGBA and incubated at 24 °C overnight.

Inoculum production and growth conditions: Inoculation growth was carried out in conical flasks of 250 ml, using 100 ml of autoclaved SNB medium (50 g sucrose, 8 g nutrient broth in 1 liter). The flasks were inoculated with a loop of cultures of strain Eh-24 and incubated at 24 °C with an agitation rate of 140 rpm.

Production in 3 liter bioreactor: The inoculation samples were taken after 16 hours of incubation and viability counts were performed. A 3 liter stirrer tank bioreactor containing 2.5 liter of medium (5% of sucrose and 3% of heat disintegrated baker's yeast-HDBY) was inoculated with 62.5 ml of inoculum, i.e. 2.5% inoculation volume. After fermentation for 18 h in the bioreactor at 500 rpm and 24 °C, viability counts were performed and determined to be  $1 \times 10^9$  cfu/ml. The bacterial suspension was centrifuged for 20 minutes at 6000 rpm.

Development of bioformulation of *P. agglomerans* strain Eh-24: The bacterial pellet was re-suspended in 0.1 M MgSO<sub>4</sub> in a 1:1 (w/v) ratio and then combined with 15% (v/v) glycerol, which was included to stabilize the bacterial cells. Thereafter, the suspension was mixed with an equal volume of autoclaved 1.5% Na-alginate. The bacteria–Na alginate mixture was combined with sterilized talc at the ratio 1:4 (v/v). A wetting agent (Ca–lignosulphate) was added (7% w/w) to the mixture (Özaktan et al. 2004).

### **Drying techniques**

Drying in laminar air-flow cabinet: The bacterial bioformulation, whose procedure was described above was spread thinly over a glass sheet and air dried in a laminar air-flow cabinet at 24 °C for 1 h to form a slightly moist powder (22 % moisture content). After drying, the bacterial formulation was blended in a Waring Blender to create a powder (Özaktan et al. 1996).

Fluid bed drying: The experimental system used in the fluid bed drying process has six stainless steel cylindrical, vertical beds carrying drying cups having a grid support at the bottom to hold granular matter. The drying cups were located directly above the cylindrical beds. The air is introduced below the grid through a suction blower. The flow rate of the air in each bed was measured using a mobile orifice. Air flow rate could be adjusted by means of dampers. Air was drawn into the fluidization chamber through an electrical heater to set the desired air temperature. Air velocity was 2 m/sec and the air temperature was 40 °C.

Spray-drying: The bacterial suspension produced in the bioreactor was formulated with maltodextrin (MDX 29) and subsequently homogenized using a Silverson L4R mixer at low speed. In order to obtain a stable mixture, the water soluble part of dry weight of the formulation was set at 20%. A laboratory spray dryer (Lab plant SD04, Lab Plant Ltd., England) was used for this drying method. The following conditions were used: inlet temperature,  $120 \pm 2$  °C; outlet temperature,  $90 \pm 2$  °C by adjusting the peristaltic pump to 5%. During the spray drying process the size of spray nozzle was 0.5 mm.

Immature pear fruit test (IPFT): Immature pear fruits were harvested in mid-July 2005, 6-8 weeks after bloom and kept in cold storage for use until October. The bioformulations which were obtained by three different drying techniques were tested for their ability to suppress *E. amylovora* by depositing log phase suspension in wells cut in the cheeks of disinfected pear halves. Pears were surface disinfected with 70% ethanol, sliced and cored using a sterile cork borer number 1. Fruits were inoculated with 60 µl of suspension of bacterial bioformulation. After 2-3 h, the fruits were inoculated with 40 µl of suspension of *E. amylovora*. Treated pears were incubated at 28 °C in plastic boxes lined with moistened paper towels. The first signs of infection, ooze drops in the well or on the cut ends, were usually evident in unprotected control pears two days after inoculation. The pears were observed daily for about four more days (Beer et al. 1984; Vanneste et al. 1996; Özaktan et al. 1996).

### **Results**

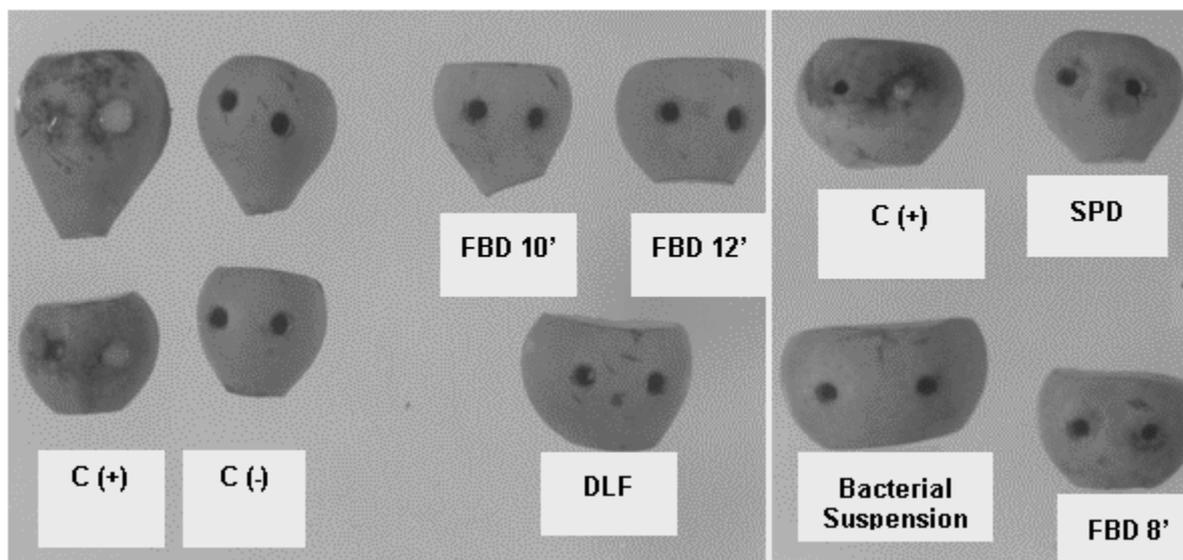
To prove whether spray drying (SPD) can be substituted for other drying techniques, it was compared with fluid-bed drying (FBD) and drying with air flow (DLF). In order to clarify the effect of drying techniques on viability and antagonistic effect of bioformulation, the immature pear test was performed.

Drying in fluid bed dryer for 10 min revealed better viability compared to the other process in FBD. The viability of antagonistic bacteria in bioformulation obtained by the spray drying technique was found to be less than those by other by drying techniques, because of high temperature (see Table). Drying in laminar flow was found as effective as drying in FBD for 10 min in terms of viability, but the process took much more time with respect to the drying period.

**Tab. 1** Effect of drying techniques on the viability of *Pantoea agglomerans* Eh-24

<b>Bioformulation steps</b>	<b>cfu/ml</b>	
Bacterial suspension with some preservative additives	8x10 <sup>8</sup>	
Steps of bioformulation	cfu/g	Humidity (%)
Bioformulation before drying	6 x10 <sup>8</sup>	33.4
FBD in 8 min	2 x10 <sup>7</sup>	23.4
FBD in 10 min	2 x10 <sup>8</sup>	20.1
FBD in 12 min	6 x10 <sup>6</sup>	15
DLF in 1 h	8 x10 <sup>7</sup>	22
SPD in 5 sec	1x10 <sup>6</sup>	12

The bioformulations which were obtained by three different drying techniques differed in their ability to suppress the development of fire blight (*Ea*) in the immature pear fruit assay (Figure). The talc-based bioformulation of *P. agglomerans*, which was dried in FBD for 10 min, almost completely suppressed the development of *Ea* in the immature pear fruit test (IPFT). The bioformulation dried by spray drier had no effect on suppression of *Ea* in IPFT compared with the negative control.



**Figure** Effect of drying techniques on the antagonistic effect of *Pantoea agglomerans* Eh-24, immature pear fruit test

**Discussion**

The most common drying processes used with living organisms are freeze and spray drying. While the former is expensive and mostly limited to research use, the latter is often used, especially for spore forming organisms (Burges 1998). Stephan and Zimmerman (1998) demonstrated that submerged spores of various entomopathogenic fungi can be spray dried without loss of viability and that temperature is a key factor in the spray drying.

Since it is not common to use spray drying for Gram-negative bacteria, this study employed this drying technique without much success. As shown in the results of immature pear fruit tests, the bioformulation obtained with the spray drying technique resulted in the lowest antagonistic effect compared with others due to too much loss of viability. Stephan and Zimmerman (2001) worked with submerged spores and demonstrated that the inlet temperature is not essentially influencing the viability of dried submerged spores. When the outlet temperature was adjusted to 45 °C the viability was higher than 80% even with an inlet temperature of 135 °C. In the same manner, Kassa et al. (2004) studied submerged spores of the same strain and their results agreed with those of Stephan and Zimmerman (1998). In order to obtain high viability, the outlet temperature should be reduced to 40 °C to enable a high antagonistic effect.

Stephan and Zimmerman (1998) also indicated that spraydrying of spore forming bacteria without the use of protectants results in complete loss of spore viability. Likewise, we have observed that spray drying of our strain without using maltodextrin as a protectant resulted in loss of all cell viability. Hence in our further experiments the viability of the bioformulation was improved with maltodextrin.

Besides drying with spray drying, the fluid bed drying (FBD) technique and drying in laminar flow was studied. As shown in the results, drying in FBD for 10 min gave better biocontrol activity and viability compared with the other process in FBD. On the other hand, drying in the laminar flow was as effective as drying in FBD for 10 min, but it takes much more time for drying.

Although the spray drying technique is one of the most important industrial drying techniques, it is crucial to find appropriate protectants and temperature regimes in order to allow for drying without loss of viability when spray drying microorganisms (Stephan and Zimmerman 1998). The results presented in this study point to the potential for future improvement of the spray drying technique when contrasted with the fluid bed drying technique. Such improvements on the spray drying technique will be conducted by the use of different protectants, i.e., bentonite, skimmed milk powder, and starches such as hydroxyethyl-starch, soluble starch and yeast extract. Moreover, it should be mentioned that the outlet temperature of the spray dryer should be around 40 °C.

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## **A novel natural anti-microbial product for use as an agricultural bactericide and fungicide**

### **Abstract**

A new natural fungicide/bactericide has been developed on the basis of the so-called LP-system, an anti-microbial system active in bovine milk. The activity of the LP-system (lactoperoxidase system) is the enzymatic formation of reactive oxygen molecules which react with proteins in micro-organisms, such as bacteria and fungi. Those micro-organisms are then killed. The currently developed formulation is applied as a curative contact fungicide and initially targeted to control powdery mildew in greenhouse vegetables and ornamentals. Results are presented from various crops. The product can also be used to control bacterial diseases. Initial test results on fire blight in apple are given.

The registration in the EU of a product based on naturally occurring substances is discussed as well as possibilities to control other fungal and bacterial plant diseases.

### **Introduction**

A novel natural bactericide/fungicide has been developed on the basis of the lactoperoxidase system, an anti-microbial system active in milk. The enzyme lactoperoxidase and other heme-containing peroxidases fulfill an important role in the non-immune defense of higher organisms against micro-organisms (Tenovuo 1985; Reiter 1985). Together with hydrogen peroxide and halides ( $Cl^-$ ,  $Br^-$ ,  $I^-$ ) or the pseudohalide thiocyanate ( $SCN^-$ ), they form potent natural anti-microbial systems. The halides and thiocyanate can be oxidised by the peroxidases to reactive oxidising compounds (e.g.  $IO^-$ ,  $SCNO^-$ ), also called reactive oxygen species (ROS), which attack sulfhydryl groups in essential proteins in micro-organisms. The oxidised molecules are active against and may kill viruses, gram-positive and gram-negative bacteria, yeasts, fungi and probably also mycoplasmas and possibly other parasites.

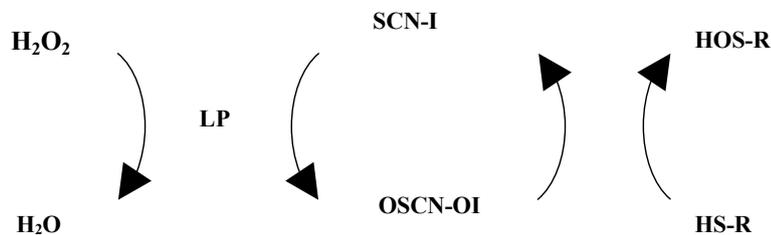
Peroxidase systems are present in plants, animals and man and are an important part of the non-immune defence against micro-organisms, mainly against bacteria. They can be found in body-secretions such as saliva, tear fluid, bronchial, nasal and intestinal secretions and also in milk. Bovine milk contains high concentrations of a heme peroxidase, the so-called lactoperoxidase. From this the term lactoperoxidase system or LP-system is derived. In milk the LP-system functions as a natural preservative agent and inhibits bacterial growth or kills them. The LP-system is used as a preservative in a number of food and non-food applications in dairy products, functional foods, personal care products, and feed and veterinary specialties.

### **The LP-system for disease control**

An agricultural disease control product has been developed by Koppert Biological Systems B.V. and DMV-International B.V. Koppert is a leading developer and producer of beneficial arthropods, such as bumblebees, and bio-pesticides. DMV-International, a subsidiary of Campina, a large Dutch dairy company, is focused on the development and production of milk ingredients.

The companies collaborated in the development of this new agricultural fungicide and bactericide based on the LP-system. Lactoperoxidase can be extracted from milk or whey in a highly purified form and can be used as a basic component in applications of the LP-system. The enzyme by itself has no activity, but needs a hydrogen peroxide source and a (pseudo) halide to be able to form the reactive molecules. In milk mainly thiocyanate ( $SCN^-$ ) is transformed into hypothiocyanite ( $SCNO^-$ ), a short-lived oxidation product that is active against bacteria.

Our research focused mainly on developing a formulation with activity against fungal diseases, particularly powdery mildews. There were some indications that the addition of another halide, iodide, would enhance the activity of the LP-system against fungal organisms (Pruitt and Reiter 1985) and we found that at a certain ratio of thiocyanate and iodide the LP-system was able to kill fungal material, such as mycelium and spores. This has led to the formulation of a new product for control of fungal diseases, called Enzicur. This formulation also has an activity against bacteria.



### Mode of action

The mode of action is based on the oxidation of iodide and thiocyanate into the short-lived reactive oxygen species (ROS) hypothiocyanite ( $SCNO^-$ ) and hypoiodite ( $IO^-$ ). These react with sulfhydryl groups in proteins and the nucleotides NADH and NADPH. The oxidation of these compounds results in major changes in these molecules and loss of functions. The cytoplasmic membrane, sugar, and amino acid transport systems, and glycolytic enzymes may be damaged. The result of such damage may be cell death or inhibition of growth, respiration, active transport, or other vital metabolic functions.

In bacteria, the outer membrane, the cell wall, and the cytoplasmic membrane are changed and possibly damaged by the reactive products. Transport systems of amino acids and other compounds are also inhibited. Several glycolytic enzymes are inhibited by the products of the LP-system. As a result of the above-mentioned reactions cells are inhibited in growth and killed. These processes depend on the concentrations of the LP-system reaction products and duration of the exposure. Most of the research done on the activity of the LP-system has been performed on bacteria and only some on fungi and yeasts. In the case of powdery mildew fungi it is not known how the system works in detail. We have seen that spores are killed (no germination anymore) and sometimes collapse, probably due to leakage. Also mycelium is killed and collapses for the same reason.

ROS may have an effect of a few minutes up to some hours depending on the exposure, dosage, and target organism. Direct contact between the ROS solution and the target sites is very important. The product shows a direct effect by contact and has no persistent or preventative effect, nor any systemic effect. When the water evaporates, the activity ceases.

### Control of fungal plant diseases

The formulated product Enzicur has been tested primarily against powdery mildews in various crops. Approximately 600 species of powdery mildew exist that infest as many plant species of which a number are important agricultural crops. Resistance to chemical fungicides is a big problem and new solutions have to be developed to be able to obtain sufficient control. Powdery mildews form haustoria that are inside the plant tissue, the other structures of the fungus develop on the leaf surface. Therefore, contact between the ROS and the fungal material can be achieved. Fungal growth inside plant tissue or vessels is difficult to contact with the ROS. Why these small molecules are not systemically transported is not known. Apparently they cannot pass through the plant epidermis or waxy surface.

In order to improve the contact between the watery spray solution containing the ROS and the lipophilic mycelium we have developed an adjuvant based on vegetable oil. This adjuvant, which also contains an emulsifier and a spreading agent, improves the contact and thereby the efficiency of the product.

Enzicur can be used as a curative control product and gives direct control of the disease, whereby the mycelium and spores are killed and even disappear after 1-2 days, probably due to leakage of the cells.

Control is as effective as with chemical fungicides. Examples of trials in cucumber and tomato are given in Figures 1 and 2. Enzicur was compared with bitertanol (Baycor Flow) as a reference product. The applications were done on a naturally occurring powdery mildew infestation when leaves were covered 10-20% with the disease. Assessments were made 2-3 and 7-8 days after spraying, assessing the leaf surface covered with powdery mildew.

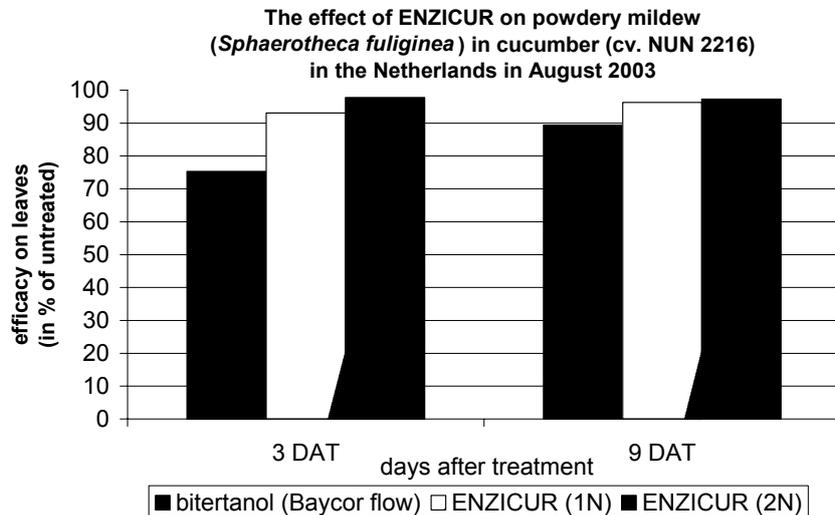


Figure 1

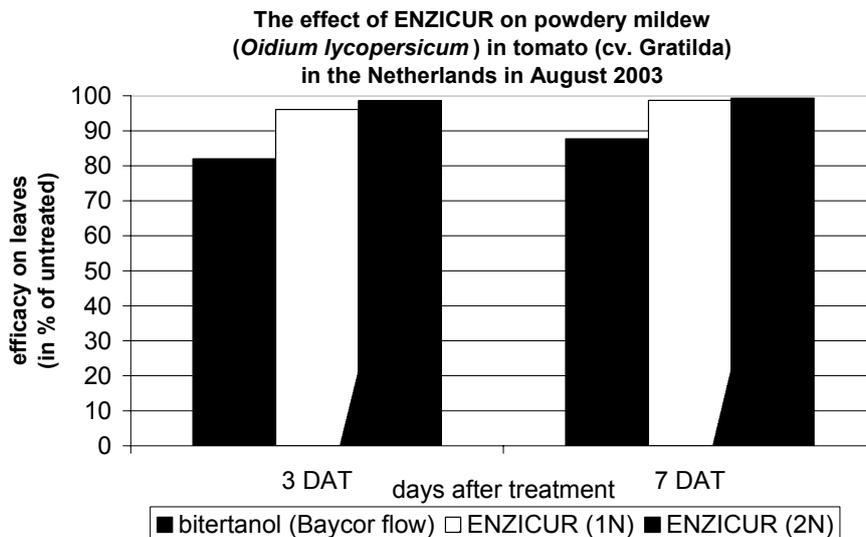


Figure 2

In cucumber *Sphaerotheca fuliginea* was controlled up to 80% after 2 days and to 95% after 8 days. The same was seen in tomato against *Oidium lycopersicum*. The reference product acts slower. It works by inhibition of fungal growth, but reaches similar control levels and gives a longer period of control. Enzicur has no persistent effect and must be repeated when the diseases appear again.

In sweet pepper, strawberry, rose and oak similar results have been achieved, so Enzicur is able to control *Sphaerotheca* spp., *Erysiphe* spp., *Leveillula* spp. and *Microsphaera* spp. Besides powdery mildew we have seen that this formulated LP-system can also kill *Botrytis*, *Fusarium* and *Verticillium* species and probably many more fungi.

## Control of bacterial plant diseases

The formulation has been tested *in vitro* on a number of bacteria that cause plant diseases and we have seen high mortality in *Erwinia*, *Clavibacter*, *Xanthomonas* and *Pseudomonas* species (no data given). The difficulty in controlling bacterial diseases is the prerequisite of direct contact between the spray solution and the bacteria. When bacteria can be reached on the surface of the plant, good results can be expected. Fire blight in apple is an example where bacteria can be reached on the petals before they germinate and enter the plant tissue.

In collaboration with Agroscope FAW Wädenswil and Andermatt Biocontrol AG (both Switzerland) the effects of Enzicur on *Erwinia amylovora* were tested in 2004 and 2005.

*In vitro* the solution kills the bacteria within one minute of exposure, even at lower dosages than the recommended field rate for control of fungi. Agar tests showed complete inhibition of bacterial growth. Tests on flowers, however, did not result in adequate control. In a field trial in Germany (Dr. C. Scheer, KOB) results were about 30% of the control, far lower than the reference product streptomycin and not sufficient. Enzicur is able to kill the *E. amylovora* bacteria, but in order to have good field results the formulation should be improved for this particular use, together with an efficient application strategy.

## Discussion and conclusions

A specific formulation and application has been developed with the LP-system for use as an agricultural fungicide/bactericide. Successful control of powdery mildews can be achieved with this formulation. Active substances and product registration are pending in the EU and individual member states. The formulation and its use are patented in the EU and several other countries.

Two products have been developed: a fungicide for foliar applications in greenhouse vegetables and ornamentals and a fungicide for dipping treatments of flower bulbs such as tulip, gladiolus, lily etc., for control of storage diseases such as *Fusarium oxysporum* and *Penicillium* spp. The product also has bactericidal effects and applications in the field have to be further studied.

The LP-system is a natural system and all the ingredients of the two products are natural chemicals and food approved. The activity is enzyme-based whereby the reactive components are formed in the spray solution. This differs from most chemical pesticides where the active ingredient is in the product. Since all formulation components occur in nature our products have a very safe profile towards animals, plants, and the environment. They fit well in IPM systems since they are safe for beneficial arthropods and for pollinators, like honeybees and bumblebees.

From the mode of action it can be concluded that LPS has a multiple-site activity. Many proteins and enzymes are inactivated in a micro-organism and, as a result, the organism dies. Apart from the oxidation of proteins, other important metabolic processes are disturbed or inhibited. Resistance is very unlikely to develop due to multiple targeted sites. Moreover, since this is a system present in nature in many organisms as an anti-microbial system, development of resistance is deemed almost impossible. Only a few bacteria are resistant to peroxidase systems, such as oral streptococci and lactic bacteria, micro-organisms that evolved in conjunction with the peroxidase systems.

This new product offers a chance for control of bacterial diseases where the bacteria can be reached and killed before they enter the plant tissue. Fire blight in apple seems to be a case in which this could be achieved with an improved application strategy and possibly a new formulation.

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## ***Bacillus subtilis* strain QST 713, bacterial disease control in fruit, vegetable and ornamental production**

### **Abstract**

*Bacillus subtilis* QST 713, a naturally occurring bacterial strain, discovered and commercialized by AgraQuest, Inc., USA, has been shown to possess significant efficacy against a broad spectrum of economically important diseases in fruit, vegetable and ornamental production. In addition to activity against fungal pathogens, *B. subtilis* QST 713 also provides control of key bacterial pathogens such as *Erwinia amylovora* (fire blight of pome fruit), *Xanthomonas campestris* (bacterial spot of tomato and pepper) and *Pseudomonas* and *Xanthomonas* spp. (bacterial spots of ornamentals). *B. subtilis* QST 713 works through novel, multiple modes of action that involve the biological action of *B. subtilis* competing for nutrients on the host surface in addition to the antimicrobial activity of lipopeptide metabolites produced by *B. subtilis* causing permeability changes of the cytoplasmic membrane and subsequent disintegration of the pathogen cells. As determined by US-Environmental Protection Agency and international regulatory authorities, *B. subtilis* QST 713 is exempt from the requirement of a tolerance because there are no synthetic chemical residues, and it is safe to workers and the environment. As a result, treated fruit and vegetables can be exported throughout the world without restrictions. *B. subtilis* QST 713 is also safe to non target; beneficial organisms have been shown to be an effective tool for disease control in organic crop production and in integrated disease control programs contributing to resistance management and reduction in the use of synthetic fungicides.

### **Description and registration status**

*Bacillus subtilis* is a rod-shaped, gram positive, aerobic, motile bacterium which is ubiquitous in nature. The bacterium can also produce an endospore. *B. subtilis* is commonly found in various ecological niches including soil, water and air. The US-Environmental Protection Agency and international regulatory authorities have classified *B. subtilis* QST 713 as a microbial fungicide. The commercial formulated product and QST 713 technical product contain living *B. subtilis* strain QST 713 as the active ingredient. *B. subtilis* QST 713 was approved in the United States as a foliar fungicide in 2000 and is currently registered in 16 countries under the trade name, Serenade® (Table 1).

**Tab. 1** *Bacillus subtilis* QST 713 Global registration status. Bold type indicates regulatory approval for commercial use

1999	2000	2001	2002	2003	2004	2005	2006>
Chile	USA	Mexico	Costa Rica	Japan	Guatemala	France	Africa
		New Zealand		Israel	Honduras	Italy	Germany
		Puerto Rico		Philippines	Switzerland	Korea	Canada
					Turkey	Ecuador	Spain
					Argentina	Columbia	Greece
							Belgium
							Australia
							UK

### Crop uses and disease spectrum

*Bacillus subtilis* QST 713 efficacy has been demonstrated on over 30 crops in 20 countries against a broad spectrum of fungal and bacterial pathogens. Currently, the major commercial uses are in the crops listed in Table 2. *B. subtilis* QST 713 can be applied alone, in tank mixtures or in rotation programs with other fungicides. Therefore, it is ideally suited for “high” value, fruit and vegetable production particularly for export markets with requirements for organic or reduced chemical-input certification.

**Tab. 2** *Bacillus subtilis* QST 713: global commercial use

Crop	Disease	Pathogen
Tomato/Pepper	Bacterial Leaf Spot	<i>Xanthomonas campestris</i>
	Powdery Mildew	<i>Leveillula taurica</i>
	Early Blight	<i>Alternaria solani</i>
Grapes	Gray Mold	<i>Botrytis cinerea</i>
	Powdery Mildew	<i>Uncinula necator</i>
	Sour Rot	Multiple pathogens
Cucurbits	Powdery mildew	<i>Erysiphe/Sphaerotheca</i> spp.
	Gummy Stem Blight	<i>Didymella bryoniae</i>
Banana	Black Sigatoka	<i>Mycosphaerella fijiensis</i>
Mango	Anthracnose	<i>Colletotrichum gloeosporioides</i>
Lettuce	Leaf Drop	<i>Sclerotinia</i> spp.
Apples/Pears	Fire Blight	<i>Erwinia amylovora</i>
Beans	White Mold	<i>Sclerotinia sclerotiorum</i>
Ornamentals	Bacterial spots	<i>Pseudomonas/Xanthomonas</i> spp.

### Modes of action

*Bacillus subtilis* QST 713 works through novel modes of action that are manifested by the bacterium colonizing the leaf surface and competing with the pathogen for nutrients and space and physically preventing attachment and penetration of the pathogen. In addition, *B. subtilis* QST 713 produces three groups of metabolites known as lipopeptides, (iturins, agrastatins/plipastatins, and surfactins) that act in a synergistic manner to destroy pathogen germ tubes and pathogen membranes. The iturins and plipastatins have been reported to have fungicidal activity. *B. subtilis* QST 713 is the first strain reported to produce iturins, plipastatins and surfactins and two new compounds, the agrastatins. Studies at AgraQuest on the effects of the individual groups of lipopeptides on pathogen spore germination compared to mixtures of the groups provided a better understanding of the role of these metabolites. Morphological differences were observed in spores treated with different lipopeptide groups. The iturin group resulted in inhibition of spore germination and was dependent upon the concentration of iturins present. The iturins were most effective on *Botrytis cinerea* spores with an EC<sub>50</sub> as low as 15 ppm (50% inhibition of spore germination). The EC<sub>50</sub> of *Monilinia fructicola* spores occurred at 30 ppm and for *Alternaria brassicicola* the level required was 25 ppm. Exposure of the spores to the iturin/plipastatin group resulted in an abnormal appearance in which the spore had a large bubble-like growth replacing the normal appressorium. This effect was most notable with *A. brassicicola* spores in which the EC<sub>50</sub> was 5 ppm. Investigation of the effects of combining the groups of lipopeptides gave further explanation for the efficacy observed with *B. subtilis* QST 713. Addition of concentrations as low as 1 ppm agrastatin/plipastatin to 10 ppm iturin provided a significant reduction in spore germination; reduced to approximately 5% spore germination for *M. fructicola*. The surfactin group was found to have no effect on spores at the highest rate tested (250 ppm). Addition of 25 ppm surfactin to 20 ppm iturin reduced spore germination from 85% to less than 5%. Surfactin at 25 ppm added to agrastatin/plipastatin reduced germination from 100% to 10%. Given the novel, multiple, modes of action, *B. subtilis* QST 713 is utilized as a resistance management tool in rotation programs with chemical fungicides, such as the strobilurin and triazole groups, which are highly susceptible to resistance development due to a specific metabolic site, modes of action.

## Formulation

*Bacillus subtilis* QST 713 is formulated as a wettable granule and aqueous suspension product containing from 1 to  $7 \times 10^9$  cfu/gram depending upon the formulation. It can be applied in conventional application equipment, requires no special storage conditions and has a shelf life of more than 2 years. *B. subtilis* QST 713 formulations have been shown to be compatible in mixtures with commonly used synthetic fungicides (e.g., sulfur, copper hydroxide, mancozeb, chlorothalonil, azoxystrobin, myclobutanil) and are approved for use in organic agriculture under the guidelines established by the Organic Materials Review Institute (OMRI-USA), Institute for Marketecology (IMO-Switzerland) and BCS Öko-Garantie (BCS-Germany).

## Use in integrated disease management programs

Because of the excellent environmental profile, broad disease control spectrum and safety to non-target, beneficial organisms (Table 3), *Bacillus subtilis* QST 713 is ideally suited for use in integrated pest management (IPM) programs that utilize many approaches such as cultural practices, classical biological control and other fungicides.

**Tab. 3** Summary of *Bacillus subtilis* strain QST 713 ecological toxicity studies

Non Target Test Organism	Toxicity [mg/kg]	Toxicity Rating <sup>1</sup>
Avian oral (quail)	LD50 > 5000	5
Freshwater fish (trout)	LC50 = 162	3
Honey bee larvae	LC50 >10,000	5
<i>Daphnia</i>	EC50 = 108	3
<i>Hymenoptera</i> parasitic wasp	LC50 > 30,000	5
Lady beetle	LC50 > 60,000	5
Lacewing larvae	LC50 > 60,000	5

<sup>1</sup>Rating according to Hodge and Sterner scale of 1 to 6 where 1 is "extremely toxic", 6 is "relatively harmless"

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## Biological control agents as tools against some emerging bacterial plant diseases in Italy: a concrete perspective ?

### Abstract

This work was carried out to confirm and/or evaluate the efficacy of three *Pseudomonas* spp. strains against fire blight (*Erwinia amylovora*), southern bacterial wilt of tomato (*Ralstonia solanacearum*), bacterial spot/canker of stone fruits (*Xanthomonas arboricola* pv. *pruni*) and grapevine crown gall (*Agrobacterium vitis*). In the laboratory, there was a trend towards a higher colonisation of apple and pear flowers when Na-alginate (0.5%) was added to suspensions of freeze-dried cells of wild-types and rifampicin-resistant mutants of IPV.-BO G19 (*P. putida*) and IPV.-BO 4027C (a non-fluorescent *Pseudomonas* sp.) strains; *E. amylovora* populations on apple flowers were significantly reduced by strain IPV.-BO 4027C. In a field experiment, performed on actively growing shoots of pear scions ‘Abbé Fétel’ during Spring-Summer 2005, different treatments were also tested, including copper compounds the resistance inducers prohexadione-Ca (Regalis) and acibenzolar-S-methyl (Bion), and Serenade and BS-F4 (both based on *Bacillus subtilis*) etc. The strain IPV.-BO G19 plus Na-alginate was the most effective in reducing shoot blight incidence by 65% six weeks after wound inoculation with *E. amylovora*. A virulent *R. solanacearum* bv. 2, race 3 strain was inhibited by both these antagonists *in vitro*. When the pathogen was inoculated 48 h after their application (approx. 10<sup>8</sup> cfu/ml) to the root apparatus of tomato plants grown in a climatic chamber, bacterial wilt progression rate was clearly reduced; in particular, the strain IPV.-BO G19 was able to delay symptom expression and to reduce disease incidence by 100% and 37%, 9 and 21 days after inoculation, respectively.

Under glasshouse conditions, BS-F4, Bion and IPV.-BO G19 reduced relative incidence of grapevine crown gall by 58%, 54% and 48%, respectively. The IPV.-BO G19 strain was also effective against *X. arboricola* pv. *pruni*: when sprayed on plum fruitlets it reduced disease incidence by 90% eight days after experimental inoculation with the pathogen.

### Introduction

In this last decade, several bacterial diseases are considered as “emerging” in Italy (Iacobellis *et al.*, 2004). The so-called emerging diseases can be referred to as “new diseases”, as well as “old diseases”: among their causal agents, some are subject of phytosanitary legislation in the EU (EU Directive 2002/89, November 28<sup>th</sup>, 2002) and others (e.g. *Agrobacterium vitis*) are not listed as quarantine pests, but are commonly regarded as harmful, widespread pathogens (“quality organisms”) that can reduce the value of propagation materials. In international trade, they might be included among the so-called “regulated non-quarantine pests” (International Standard of Phytosanitary Measures, ISPM 16-FAO). The great battlefield created by these emerging problems does require great efforts directed to the search of modern integrated, eco-friendly and sustainable control programmes. Besides breeding resistant genotypes (Lespinasse and Aldwinckle 2000) or the innovative induction of defence responses by treating plants with suitable triggering molecules (Durrant and Dong 2004), another promising approach is the use of beneficial biological agents, isolated from the natural environment and commonly defined as antagonists, whose activity is based on different mechanisms of action (Cook 2000).

The aim of our studies was to confirm and/or evaluate the effectiveness of different bacterial strains used as biological control candidates against fire blight (*Erwinia amylovora*, *Ea*), southern bacterial wilt (*Ralstonia solanacearum*, *Rs*), grapevine crown gall (*A. vitis*, *Av*) and bacterial spot/canker of stone fruits (*Xanthomonas arboricola* pv. *pruni*, *Xap*).

## Materials and methods

**In vitro tests:** The fluorescent strain IPV.-BO G19 (*Pseudomonas putida*) and the non-fluorescent strain IPV.-BO 4027C (*Pseudomonas* sp.) were tested in solid media (MM-agar and KB-agar) for their ability to inhibit the growth of *Ea*, *Rs*, *Xap*, *Av*, *Pseudomonas syringae* pv. *syringae* (*Pss*) and *Erwinia carotovora* subsp. *carotovora* (*Ecc*).

## Experiments with *E. amylovora*

**Colonization of apple and pear flowers:** The experiments were carried out on detached flowers of apple ‘Rome Beauty’ and pear ‘Abbé Fétel’ kept in Eppendorf tubes containing sterile distilled water. Aqueous suspensions (approx.  $10^{5-6}$  cfu/ml) of rifampicin resistant mutants (Rif<sup>r</sup>) of the strains IPV.-BO G19, IPV.-BO 4027C and the fluorescent strain IPV.-BO 3371 (*Pseudomonas mendocina*) were sprayed on freshly opened flowers; Na<sup>+</sup>-alginate (sodium salt of alginic acid; 0.5%) was added to the bacterial suspensions. After incubation in a humid chamber, five flowers per time point were washed in 10 mM MgSO<sub>4</sub>. Bacterial populations in each flower were determined by plating tenfold dilution in 10 mM MgSO<sub>4</sub> on KB-agar plates amended with 20 µg/ml of rifampicin (recovery medium).

The population level of the *Ea* strain OMP-BO 1077.7/94 Rif<sup>r</sup>, sprayed on freshly opened ‘Abbé Fétel’ flowers as an aqueous suspension (approx.  $10^6$  cfu/ml) 24 h after application of the two freeze-dried strains IPV.-BO 4027C and IPV.-BO 3371 (approx.  $10^6$  cfu/ml) plus Na<sup>+</sup>-alginate (0.5%) and water (control), was monitored for 96 h by colony counting on recovery medium plates.

**Colonization of pear leaves:** Pear scions ‘Abbé Fétel’ (1 scion x 2 replicates per treatment) were sprayed with an aqueous suspension (approx.  $10^6$  cfu/ml) of the freeze-dried mutants IPV.-BO G19 Rif<sup>r</sup> and IPV.-BO 4027C Rif<sup>r</sup> amended with Na<sup>+</sup>-alginate (0.5%). At 1, 24, 48, 96 h and 7 days after application, 20 leaves/treatment were detached, washed in 10 mM MgSO<sub>4</sub> for 45 min at 200 rpm and centrifuged (15 min, 10,000 g). Bacterial populations were determined by plating on recovery medium tenfold dilution of the pellets resuspended in 1 ml of 10 mM MgSO<sub>4</sub>.

**CAT, SOD and POD activity:** Pear scions ‘Abbé Fétel’ (2 scions x 3 replicates per treatment) were sprayed with an aqueous suspension of strains IPV.-BO G19 and IPV.-BO 4027C, the biofungicide BS-F4 (based on *Bacillus subtilis*, *Bs*, Agribiotec srl, Italy) and water (control). After 1, 24, 48, 72 h and 6 days, approx. 200 mg of leaves were randomly collected, immediately frozen in liquid nitrogen and stored at -80°C. Frozen tissues were finely ground in liquid nitrogen and used for the extraction of the enzymes, whose activity was determined according to the protocol of Sofo *et al.* (2004): catalase (CAT) and superoxide dismutase (SOD) activity was related to the fresh weight of leaf tissue; guaiacol peroxidase (POD) activity was related to total soluble proteins from leaf samples.

## Experiments with *R. solanacearum*

**Efficacy on tomato plants:** Tomato plants ‘Moneymaker’ (4 plants x 3 replicates per treatment) were individually grown in pots. The root apparatus of each plant (third leaf stage) was strongly cut and soaked for 10 min in aqueous suspensions (approx.  $10^8$  cfu/ml) of the strains IPV.-BO G19 and IPV.-BO 4027C grown on KB-plates for 24 h; deionized water was used as a control. After 48 h in a climatic chamber ( $26 \pm 1^\circ\text{C}$ ), each pot was drenched with 60 ml of the bacterial suspension (approx.  $10^8$  cfu/ml) of *Rs* bv. 2, race 3 strain IPV.-BO 5836, grown on SP-agar for 48 h. Bacterial wilt progression was monitored for three weeks; then bulk samples of disks from the basal part (8-10 cm) of symptomless stems were analysed to detect the endophytic presence of the pathogen. Extracts from stem disks were used for direct isolation on TZ-agar and for PCR assays using the primers of Seal *et al.* (1993).

**Experiments with *A. vitis*: Efficacy on vines:** Grapevines ‘Ancellotta/420A’ (5 vines x 5 replicates per treatment) in pots were wounded with an electric drill; 3 holes (4 mm in diameter, 8–10 cm from each other) drilled on the rootstock to the depth of the pith (Burr *et al.*, 1995) were charged with 60 µl suspensions (approx.  $10^8$  cfu/ml) of the strains IPV.-BO G19, IPV.-BO 4027C the *Pseudomonas fluorescens* (*Pf*) strains CR330d and Pf 1–3 (from N. Lemanova, “SPM Group” SA, Moldavia), and the biofungicides BS-F4 and Serenade (*Bs* QST 713, Agraquest, USA). After about 30 min, a suspension (approx.  $10^8$  cfu/ml) of the vitopine *Av* strain IPV.-BO 5159 grown on YMA for 48 h at 27°C was

applied in the holes and the inoculation sites were wrapped with Parafilm. During two weeks before pathogen inoculation the growth retardant Regalis (prohexadione-Ca, 10% WG, BASF, Germany) and Bion (acybenzolar-S-methyl 50 WG, Syngenta, Switzerland) were sprayed at 7 day intervals (1 x 75 ppm, 1 x 50 ppm and 2 x 100 ppm, respectively). The seed grapefruit extract DF100 (Smaksteknikk AS, Norway) (200 ppm), copper oxychloride (2 g Cu<sup>++</sup>/L, negative control) and water (positive control) were applied before inoculation with *Av*, as well as the antagonist suspensions.

Experiments with *X. arboricola* pv. *pruni*: Efficacy on plum fruitlets: Detached plum fruitlets 'Angelino' (5 fruits x 2 replicates per treatment) were sprayed with a suspension of strain IPV.-BO G19 (approx. 10<sup>8</sup> cfu/ml) and, within 1 h, they were inoculated with a suspension (approx. 10<sup>8</sup> cfu/ml) of *Xap* strain IPV.-BO 2959, grown on GYCA for 24 h at 27°C. Water and streptomycin (100 ppm) were used as positive and negative controls, respectively. The fruitlets were incubated in a humid chamber at 26°C for two weeks to determine disease incidence and severity (% of water-soaked surface).

Field experiments with *E. amylovora*: Efficacy on pear scions: Pear scions 'Abbé Fétel' (5 one-year old scions x 4 replicates per treatment) were sprayed with suspensions (approx. 10<sup>7</sup> cfu/ml) of the freeze-dried strains IPV.-BO G19 and IPV.-BO 4027C plus 0.5% Na<sup>+</sup>-alginate, and with the biofungicides BS-F4 and Serenade (approx. 10<sup>9</sup> cfu/ml). After 24 h, the three youngest apical leaves of 6 actively growing shoots per scion were wounded (3 wounds per leaf) with a special hand device and sprayed with the *Ea* strain OMP-BO 1077.7/94 (approx. 10<sup>7</sup> cfu/ml). Application of BS-F4 and Serenade was repeated 24 h after inoculation. During three weeks before inoculation, Regalis was applied at 8–10 day intervals (3 x 100 ppm); during two weeks before inoculation, Bion was applied at 8 day interval (2 x 100 ppm); several copper compounds (Genrame, Kelal Cubig, Naturam 5, Ossiclor 50, see Table) were applied 24 h before inoculation; BS-F4 and Serenade were applied a few hours after application of Naturam 5. DF100 was sprayed 24 h before (200 ppm) and after (100 ppm) pathogen inoculation. Water and streptomycin (100 ppm) were used as positive and negative controls, respectively.

**Table** Copper compounds tested

Commercial name		a. i.	Cu %	Dosage (Cu g/l)
Ossiclor 50	WP	Copper oxychloride	50	0.5
Naturam 5	CS	Copper peptidate	5	0.120
Kelal Cubig	CS	Cu <sup>++</sup> EDTA	10	0.120
Genrame	CS	Copper chelate	8	0.120

## Results and discussion

When tested in solid medium, the fluorescent strain IPV.-BO G19 and the non fluorescent strain IPV.-BO 4027C, were able to inhibit the growth of *Ea*, *Rs*, *Xap*, *Av*, *Pss* and *Ecc*. The antimicrobial activity of these two antagonists was reported by Galasso et al. (2002) and Biondi et al. (2004): our results give further evidence on the broad spectrum of effectiveness of these bacteria.

The two lyophilized Rif<sup>r</sup> mutants IPV.-BO G19 and IPV.-BO 4027C supplemented with 0.5% of Na-alginate were able to colonize pear and apple flowers reaching a population of approx. 10<sup>8</sup> cfu/ml (Figure 1); they were also able to reduce the *Ea* population level on pear flowers in the laboratory (from 10<sup>6-7</sup> to approx. 10<sup>5</sup> after 4 days). Both mutants, with and without 0.5% Na-alginate, were able to survive on pear leaves with a population of approximately 10<sup>5</sup> cfu/ml and 10<sup>4</sup> cfu/ml, respectively, 13 days after application (Figure 2). The functional uses of Na-alginate (stabilizer, thickener, gelling agent, emulsifier, etc.) may have enhanced the survival ability of the antagonists, and in particular, on leaves.

An increase of CAT, SOD and POD activity was not detected after application of the biofungicide BS-F4 and of strains IPV.-BO G19 and IPV.-BO 4027C, even if there was a significant trend towards an increase of the CAT level after application of the latter. This slight physiological change could be considered as an acquired resistance reaction which does appear after application of resistance inducers (Hammerschmidt and Becker 1997), but, such a preliminary finding needs to be substantiated by further experiments.

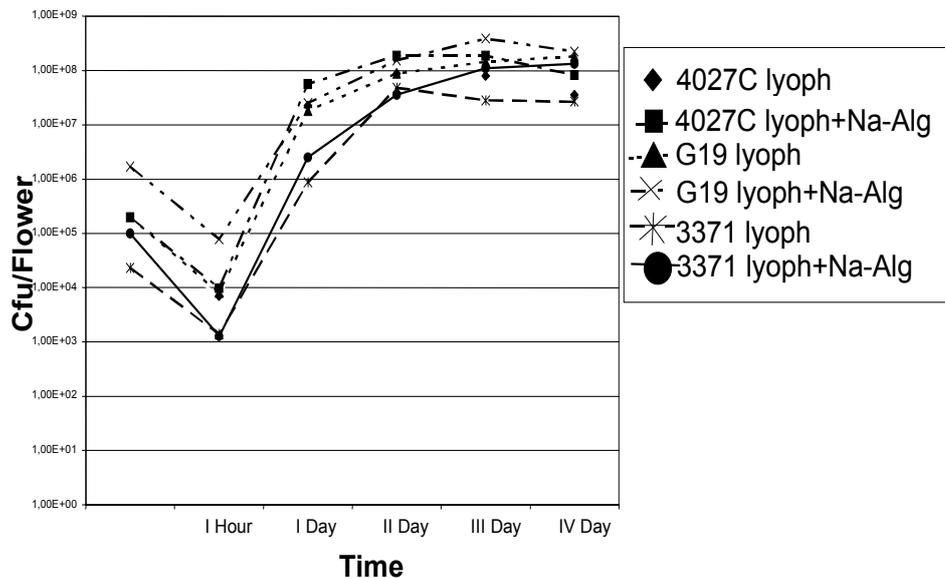
In the field, application of IPV.-BO G19 plus Na<sup>+</sup>-alginate, Naturam 5/Serenade, Serenade and Regalis, significantly reduced fire blight incidence on pear shoots by 65%, 59%, 56% and 54%, respectively; in the same experiment, streptomycin reduced disease incidence by 91% (Figure 6). In particular, the decrease of disease severity level (data not shown) gives further evidence of the ability of Regalis in triggering a plant defence response (Bazzi et al. 2003).

Under controlled conditions, IPV.-BO G19 was able to delay symptom expression in tomato plants experimentally inoculated with *Rs* and to reduce disease incidence by 100% and 37%, 9 and 21 days after inoculation, respectively (Figure 3, 4). On the other hand, the pathogen was isolated from all the samples of symptomless stems but it was not detected by PCR performed on stem extracts from plants treated with IPV.-BO G19. This means that the pathogen population level was likely under the sensitivity threshold of the detection method (approx. 10<sup>3</sup> cfu/ml).

Under glasshouse conditions, BS-F4, Bion and IPV.-BO G19 reduced relative incidence of grapevine crown gall by 58%, 54% and 48%, respectively (Figure 5). These preliminary results do encourage further investigation on biological control and induction of plant defence responses against the disease. The anti-tumour efficacy of these agents is being tested on vines under vineyard conditions.

On plum fruitlets, IPV.-BO G19 was able to reduce disease incidence by 90% and 20%, 8 and 13 days after inoculation with *Xap*. In particular, after 13 days, disease severity was approx. 2% (12% on positive control) (data not shown). Further experiments are being planned under field conditions on peach.

In conclusion, the fluorescent pseudomonad IPV.-BO G19 confirms its efficacy against fire blight, reveals its versatility as a potential biocontrol candidate against different phytopathogenic bacterial species and gives new interesting clues for further research.



**Fig. 1** Average of the mutant strains IPV.-BO G19 Rif, IPV.-BO 4027C Rif and IPV.-BO 3371 Rif populations on apple flowers. The bacterial suspensions from lyophilized bacteria were used with and without Na<sup>+</sup>-alginate

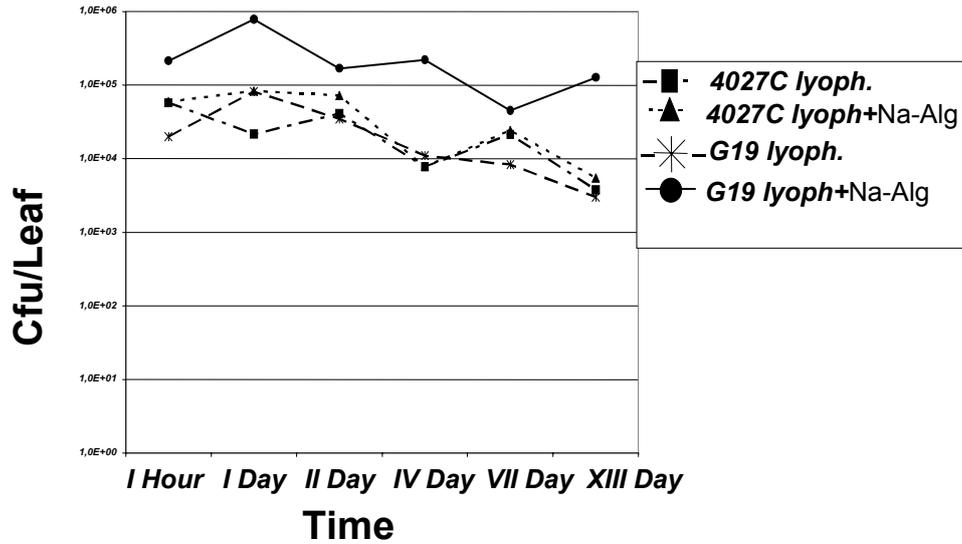


Fig. 2 Average of the mutant strains IPV.-BO G19 Rif<sup>r</sup> and IPV.-BO 4027C Rif<sup>r</sup> populations on pear leaves. The bacterial suspensions from lyophilized bacteria were used with and without Na<sup>-</sup>-alginate

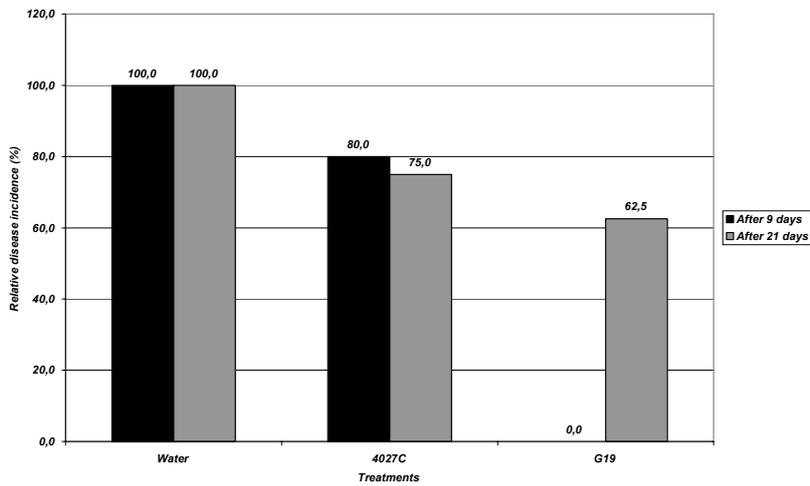


Fig. 3 Southern bacterial wilt of tomato: Relative disease incidence 9 and 21 days after inoculation with *R. solanacearum* (Duncan's test, p 0.05)

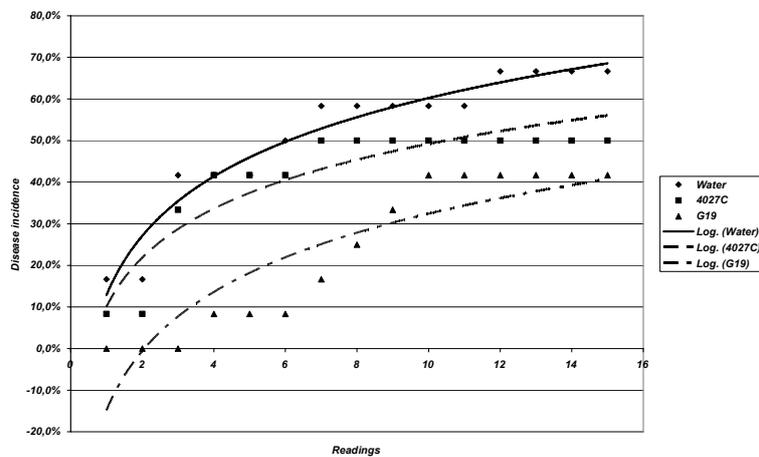


Fig. 4 Southern bacterial wilt of tomato: Disease development 21 days after pathogen inoculation

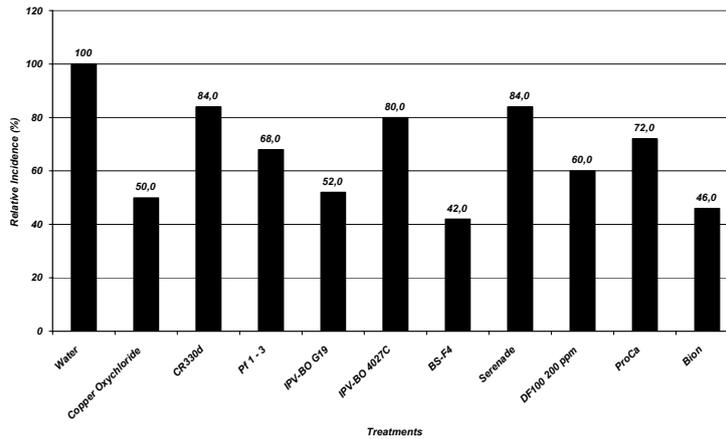


Fig. 5 Relative crown gall incidence 12 weeks shoots after *A. vitis* inoculation (LSD test, p 0.05)

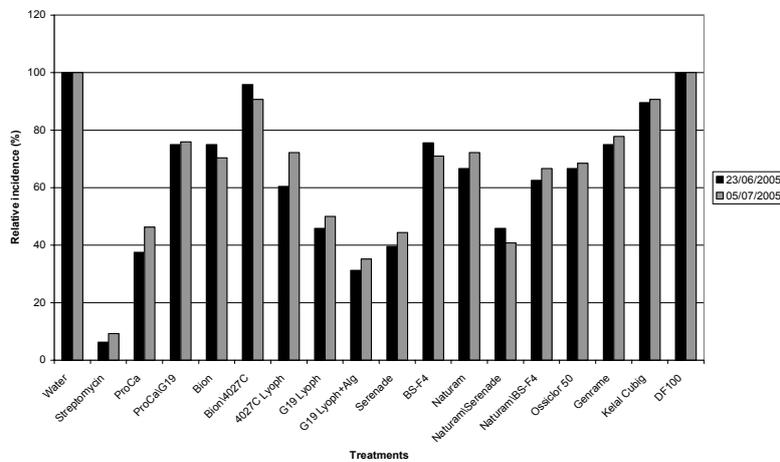


Fig. 6 Relative fire blight incidence on pear 27 and 39 days after inoculation with *E. amylovora* (Duncan's test, p 0.05)

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## **Biocontrol of phytopathogenic bacteria by using organic compost**

### **Abstract**

The biocontrol activity of the bacterial strains isolated from organic compost has been revealed in this study. Gram-negative strain IF25 showed a wide control activity of different bacterial plant pathogens. It was characterised by the BIOLOG Identification System. The results suggest that IF25 may be referred to as *Burkholderia pyrrocinia* species that is included in the *Burkholderia cepacia* complex.

### **Introduction**

Phytopathogenic bacteria are a serious threat in organic agriculture; to control them copper compounds, natural antagonists and treatment with various substances, combined with appropriate cultural practices, are suggested (Balestra 2003, 2004; Muganu et al. 2005; Varvaro et al. 2001). In organic agriculture the sources of soluble nutrients in soils are decomposition of plant residues, manures, compost and other organic amendments. Such compost amended soils have been noticed to be suppressive against plant diseases in a diversity of cropping systems (Postma et al. 2003). The possibility to increase the disease suppressive properties of compost by its inoculation with bacteria antagonistic to dangerous bacterial pathogens might be considered as a method to increase the effectiveness of composting in organic use. Here we report the results obtained with *in vitro* studies on bacterial strains isolated from vineyard soil amended with compost with respect to their biocontrol activity on different bacterial pathogens.

### **Materials and methods**

Bacterial strains, characterized by potential biocontrol activity from soil amended with organic compost, were isolated in the summer of 2004, 4 months after compost distribution.

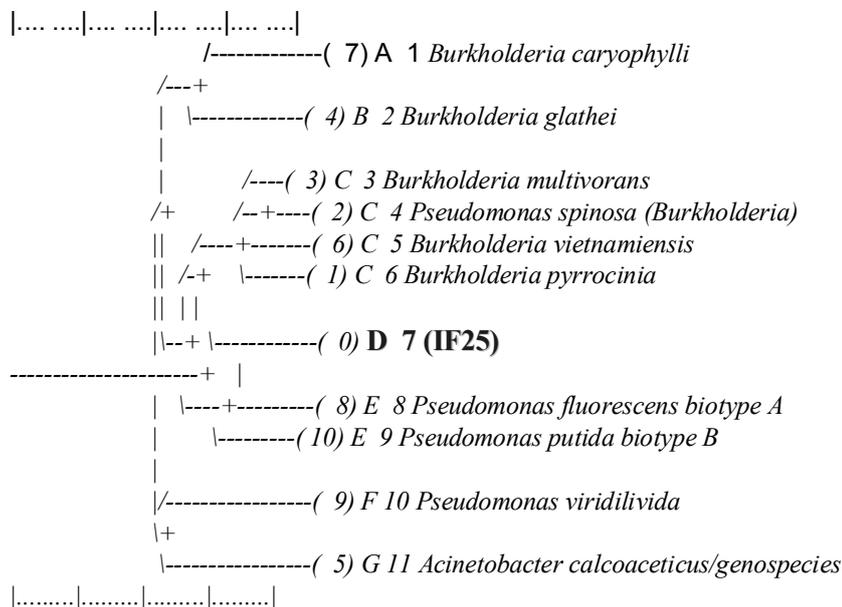
Bacterial strains with potential biocontrol activity were isolated from soil and cultivated on different agar plate media, supplemented (NSA) or not (NA) by 5% sucrose, or on selective media (PCAT) (Richardson et al. 2002). In particular, PCAT medium has been developed for the isolation of bacteria of the *Burkholderia cepacia* complex (Bcc) from environmental samples (Vermis et al. 2003). The potential biocontrol agents (BCA) were then purified on NSA and maintained at 5 °C on NA slant medium, supplemented by glycerol 2% (NGA). Bacterial strains, isolated from vineyard soil, amended by compost, were characterised on the basis of their morphological, physiological and biochemical traits (Viillard et al. 1998). All tests were performed at 28 °C. Moreover, the BIOLOG (GN-GP) system was used to characterise potential BCA by their oxidation test with respect to 95 different carbon sources. All tests (inhibition and identification) were repeated three times and submitted to statistical analysis.

Bacterial isolates were assayed by using different *in vitro* tests (spot and well) (Balestra et al. 1998) for several bacterial pathogens (*Agrobacterium tumefaciens*, *Brenneriae* spp., *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas avellanae*, *P. viridiflava*, *P. s. pv. actinidiae*, *P. s. pv. syringae*, *P. s. pv. savastanoi*, *P. s. pv. tomato*, and *Xanthomonas vesicatoria*). Bacterial strains used as BCA were applied at 10<sup>8</sup> cfu/ml, the phytopathogenic bacteria at 10<sup>6</sup> cfu/ml. On NSA medium, after distribution of the phytopathogenic bacterial suspension (100 µl per Petri dish), each BCA (4 drops, 30 µl each) was placed opportunely (Lavermicocca et al. 1999) and Petri dishes were incubated at 25 ± 2°C for 48-72 h; developing inhibition halos were observed and measured daily with a stereomicroscope.

**Results**

After preliminary investigations (morphological, physiological, cultural) the attention was focused on the bacterial strains obtained from PCAT medium. On PCAT selective medium,  $\log 3.30 \pm 0.1$  per cfu g<sup>-1</sup> (soil d.w.) were counted. Bacterial isolates appeared as white or beige opaque shining colonies, with an intact margin, and about 10% were considered for selection. Occasionally some strains showed minimal growth upon primary and secondary plating. Such growth was scored as negative and strains were rejected.

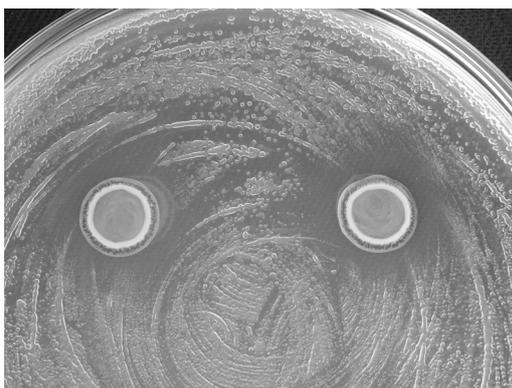
Two presumed *Burkholderia* strains, IF21 and IF25, were Gram-negative, non-spore-forming, rod-shaped motile bacteria. Another strain, IF22, was a Gram-positive, non-spore-forming, rod-shaped bacterium. The phenotypic characterization of the isolated strain was performed by the BIOLOG system. Strain IF21 was identified as *Pseudomonas* spp., whereas strain F22 was not identified. The metabolic profile of strain IF25 more closely resembled the strain (Biolog database) of Bcc genomovar IX (*Burkholderia pyrrocinia*) (SIMILARITY 0.48 and DISTANCE 8.34) (Figure 1).



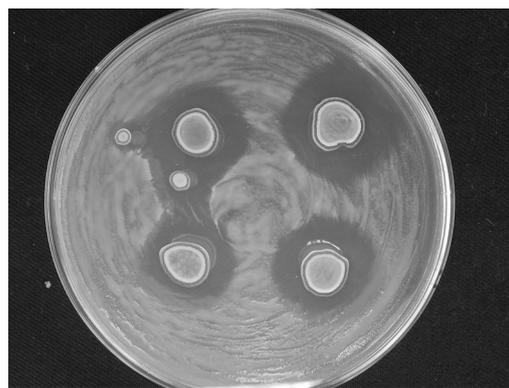
**Fig. 1** UPGMA tree was constructed on the basis of Microlog database and 10% Microlog distance

Like all the *Burkholderia* species (Viillard et al. 1998; Vermis et al. 2003) strain IF25 did not use erythritol, maltose or D-turanose. IF25 assimilated the following substrates as carbon sources: glycerol, L- arabinose, galactose, D-glucose, D- fructose, D-mannose, inositol, mannitol, sorbitol, D-arabitol, and gluconate. Moreover, strain IF25 was oxidase-negative, and able to grow at 41 °C and on a variety of media without producing diffusible pigments. In addition, the IF25 strain, like all *Burkholderia pyrrocinia*, was able to assimilate D-cellobiose, D-fucose, L-threonin and D-trehalose.

By spot and well tests great biocontrol activity of IF25 strain was shown (Figures 2-3); no biocontrol activity was recorded with IF21 and IF22 bacterial strains (Table 1). The effectiveness of IF25 appeared as inhibition halo ranging between 0.1-1.95 cm. It was able to inhibit the growth of all phytopathogenic bacterial strains tested except one of *P. avellanae* and of *B. nigrifluens* (Table 1).



**Fig. 2** Inhibition halos obtained in spot tests with IF25 strain ( $1 \times 10^8$  cfu/ml) on *Pseudomonas syringae* pv. *syringae* ( $1 \times 10^6$  cfu/ml) bacterial growth (4x)



**Fig. 3** Inhibition halo's obtained in spot tests by using IF25 strain ( $1 \times 10^8$  cfu/ml) on *Xanthomonas vesicatoria* ( $1 \times 10^6$  cfu/ml) bacterial growth (2x)

**Table** *In vitro* results obtained by spot tests. Data of 3 replicates,  $P=0.05$ .

		Pst	Cmm	Xcv	Psa	Pss	Pv.	Pa144	Pa 3872	Bn	Bq	Br	P. sav
BCA	IF21	-	-	-	-	-	-	-	-	-	-	-	-
	IF22	-	-	-	-	-	-	-	-	-	-	-	-
	IF25	1,34 $\pm 0,13$	1,95 $\pm 0,41$	1,64 $\pm 0,21$	0,04 $\pm 0,02$	1,48 $\pm 0,19$	0,04 $\pm 0,02$	0,04 $\pm 0,02$	-	-	0,02 $\pm 0,01$	0,51 $\pm 0,08$	1,40 $\pm 0,16$

BCA (Biocontrol agents isolated form organic compost).

Bacterial pathogens: *Pseudomonas syringae* pv. *tomato* 14 (Pst); *Clavibacter michiganensis* subsp. *michiganensis* 21 (Cmm); *Xanthomonas vesicatoria* (Xcv); *Pseudomonas syringae* pv. *actinidiae* (Psa); *Pseudomonas syringae* pv. *syringae* (Pss); *Pseudomonas viridiflava* (Pv.); *Pseudomonas avellanae* 144 (Pa144); *Pseudomonas avellana*, 3872 (Pa3872); *Brenneria nigrifluens* (Bn); *Brenneria quercina* (Bq); *Brenneria rubrifaciens* (Br); *Pseudomonas savastanoi* (P. sav).

## Discussion

The increasing use of organic compost shows its great potential to solve different problems reusing organic debris and improving the fertility and the biological activity of soils (Pinamonti 1998). Moreover, also by the here-obtained results, it may have potential in biocontrol of phytopathogenic bacteria reducing the risk of diseases caused by dangerous micro-organisms especially in organic agriculture.

The biocontrol activity of the bacterial strains isolated from organic compost has been revealed in this study. Gram-negative strain IF25 showed a wide control activity of different bacterial plant pathogens. It was characterised by the BIOLOG Identification System. The results suggest that IF25 may be referred to as *Burkholderia pyrrocinia* species that is included in the *Burkholderia cepacia* complex (Bcc). Bcc currently comprises at least nine species that are an object of growing interest because of their capacity to act both as plant growth promoting, biocontrol and bioremediation agents (O'Sullivan and Mahenthiralingam 2005).

The IF25 strain was particularly promising for field application considering its biocontrol activity against dangerous phytopathogenic bacteria, which cause bacterisoses at the rhizosphere and phyllosphere level of different herbaceous and arboreous host plants.

Taking into account that many *Burkholderia* species have very similar 16S rDNA sequences (Viallard et al. 1998) and the here reported results of IF25 strain as BCA, future aims will be the characterisation of the sequence of the 16S rRNA gene of IF25 isolates and testing it in *in vivo* to confirm its biocontrol activity against different and dangerous bacterial pathogens.

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## **Efficacy of different control strategies for preventing crown gall in the nurseries**

Evaluation of the efficacy of different control strategies has been the main topic of our research on crown gall during the last years. The relevant results of our work are briefly illustrated below. The screening for resistant genotypes showed that Mr.S. 2/5 was the least sensitive to crown gall among the most common peach rootstocks. This rootstock is a *Prunus cerasifera* x *Prunus spinosa* hybrid that has a low compatibility with some peach varieties and for this reason it is not widely used. Solarization was performed both in naturally infected and in artificially inoculated soils during a four year experiment performed in different geographic areas. Results showed that this method strongly reduces but does not eliminate the agrobacteria in the treated soil and that this effect is more evident in sandy than in clay soil. Moreover, agrobacteria survive on the roots of weeds growing around the borders of solarized plots where they may represent a dangerous source of soil re-contamination. A great number of non-pathogenic agrobacteria was isolated from peach tumors and was evaluated for antagonistic activity. Many were able to inhibit tumorigenic strains in vitro but not in vivo. The majority of the strains produced siderophores but no one produced agrocin-like compounds, thus showing that the ability to compete for iron is a mechanism that alone is not sufficient to prevent plant infection.

The efficacy of the biocontrol strain K84 in preventing peach crown gall was monitored for three years in several nurseries located in southern Italy. In one single nursery K84 insensitive tumorigenic strains caused a disease outbreak. Molecular analyses showed that these strains originated by plasmid transfer (pAgK84) from K84 strain to autochthonous tumorigenic agrobacteria. Except for this single case, the use of the biocontrol strategy always was highly effective in peach nurseries where it still represents the most reliable way to prevent crown gall. Selection of dangerous transconjugant agrobacteria can be avoided by the use of a K84 derivative strain (named K1026) in which the region coding for pAgK84 transfer has been deleted. It is considered as a genetically modified microorganism and EU legislation does not allow its use in agriculture.

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## **Biophototonic technology for inactivation of the tomato pathogen *Clavibacter michiganensis* subsp. *michiganensis*: Preliminary results**

### **Abstract**

The data support the assumption that *Cmm* might be inactivated by the photosensitization method which is completely safe, reproducible, non-mutagenic, and environmentally and human friendly. Actually, the combination of two non-toxic constituents, organic dye and visible light, might effectively contribute to inactivation of Gram-positive *Cmm* bacteria and be used as a new approach for the pathogen control in seeds

### **Introduction**

Bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is an important disease in tomato crops. *Cmm* is a seed-borne plant pathogenic bacterium which causes tomato wilt (Thyr 1969; Shoemaker and Echandi 1976). The disease is responsible for major economic losses in commercial tomato production worldwide. Disease incidence in Lithuania approached 10% and is the most frequent disease of greenhouse grown tomatoes (Vasinauskienė 2002). Seeds and plants must be free of the pathogen and, in practice the pathogen is controlled by tomato seed treatment with hot water (Blood 1933; Shoemaker and Echandi 1976), hydrochloric acid (Thyr et al. 1973; Dhanvantary 1989), or sodium hypochlorite (Shoemaker and Echandi 1976). The methods used for inactivation of these pathogens are ecologically inert but not always effective.

Photosensitization is based on the concept that photoactivable compounds, or photosensitizers (PS), can be preferentially localized in living organisms. Subsequently activated by light of the appropriate wavelength it might induce singlet oxygen and free radicals that are toxic to cells of the target microorganisms (Dougherty et al. 1998). So far the research indicates that bacteria, viruses, fungi and yeast can be killed by photosensitization (Lukšienė et al. 2004; Lukšienė 2005).

Taking the above into account, the present study was focused on the possibility to inactivate *Cmm* bacterial cells using biophototonic technology – photosensitization.

### **Materials and methods**

**Bacterial strain:** The strain *Cm8* used in this study was obtained from the Federal Centre for Breeding Research on Cultivated Plants, Institute of Resistance Research and Pathogen Diagnostics, Aschersleben, Germany.

**Bacterial growth and photosensitization procedure:** The *Cmm* culture was grown on potato dextrose agar medium for four days and transferred into nutrient broth medium with the addition of 1% glucose to a final volume of 25 ml at the initial optical density of 0.03 at 670 nm. Growth of bacteria continued at room temperature with aeration. The bacterial suspension (1.5 ml) was placed in a 16 mm well of 24 well tissue culture plates. PS was added to the culture medium at the beginning of the exponential phase, incubated for 20 min in a thermostat and was afterwards illuminated.

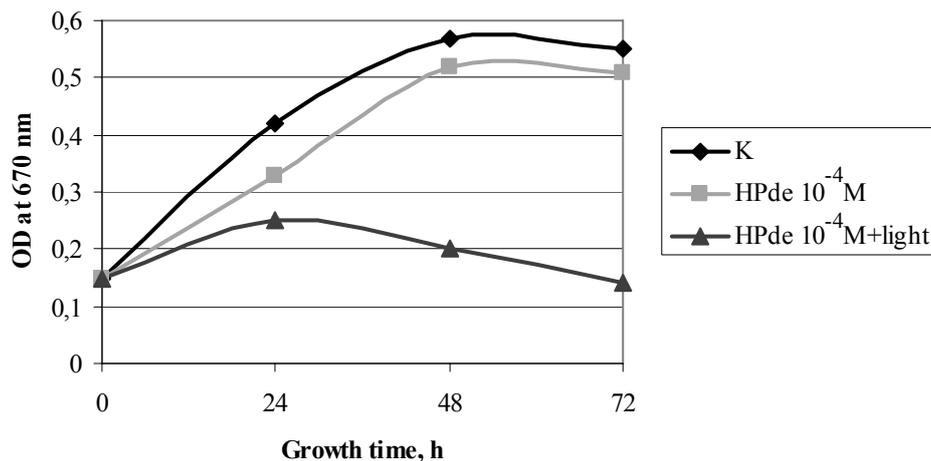
Application of the photosensitizer was performed in the dark. Bacterial growth was determined by the increase in optical density at 670 nm, as a function of time. Growth of the bacterial culture under the same conditions and light exposure but without any photosensitizer served as control.

**Illumination:** The light source used for illumination of bacteria consisted of a tungsten lamp (500 W), an optical system for light focusing, an optical filter for UV and infrared light elimination ( $370\text{nm} < \lambda < 680$

nm). Hematoporphyrin dimethyl ether (HPde) was used as a photosensitizer at a concentration of  $10^{-4}$ M. Optical density at 670 nm was chosen as parameter to evaluate growth of bacteria. Control and treated samples were started to be measured after 24 h of illumination.

## Results and discussion

The results of this study revealed that photosensitized HPde has photokilling activity on the growth of *Cmm* bacteria. The photosensitization was found when the *Cmm* culture was treated with HPde and visible light. *Cmm* showed sensitivity to HPde treatment alone (dark toxicity) and this increased after following illumination with visible light (Figure).



**Figure** Effect of photosensitisation on the growth of *Clavibacter michiganensis* subsp. *Michiganensis*

Numerous investigators demonstrated possible practical usefulness of photosensitization in a wide field of different sciences: virology, microbiology, immunology and dermatology (Lukšienė 2003). Photosensitization is successfully applied for the detection and treatment of tumours in clinical research and positive results were obtained for eradication of Gram-positive *Staphylococcus aureus*, *Propionibacterium acnes* bacteria (Orenstein et al. 1997; Ashkenazi et al. 2002). The method offers great potential use for inhibiting development of fungi and might be used for sterilization and decontamination of various surfaces (Lukšienė et al. 2004). According to the data of other authors, porphyrin-type photosensitizers are active killing agents used in light against most Gram-positive bacteria (Nitzan et al. 1983, 1987; Malik et al. 1990). Published results confirm our preliminary investigations and prompt further studies in the field of phytobacteriology.

## Conclusions

The presented data support the idea that *Cmm* might be inactivated by the photosensitization method which is completely safe, reproducible, non-mutagenic, and environmentally and human friendly. Actually, the combination of two non-toxic constituents, organic dye and visible light, might effectively contribute to inactivation of Gram-positive *Cmm* bacteria and be used as a new approach for the pathogen control in seeds.

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## **Biological control of bacterial plant diseases in Venezuela**

The use of agrochemicals in a significant way affected the sustainability of agriculture due to the environmental contamination by the indiscriminate use of these products, which have reduced the biodiversity of the ecosystem and have brought about problems to public health. Biological control based on microbial antagonism, induced resistance in plants, and the use of plant extracts with antagonistic properties is one of the most promising strategies within integrated disease control that influence the production of our plantations. The objectives of the present study were:

- To identify alternative biological control of phytopathological bacteria in Venezuela
- To evaluate the bactericidal effect of extracts of plants on pathogenic bacteria
- To evaluate a biopreparation of *Trichoderma harzianum*

Martínez and Colmenárez (3), at *in vitro* level, evaluated the effect of plant extracts with bactericidal, anti-inflammatory, healing, astringent and disinfectant properties on the development of isolated colonies of *Xanthomonas axonopodis* pv. *allii* on onion cultures. The extracts were prepared from *Caesalpinia coriaria* (fruit), *Allium sativum*, (bulb), *Bixa orellana* (fruit), *Pterocarpus officinalis* (sap) and *Zingiber officinale* (rhizome). Between 90 and 100% inhibition of bacterial growth was found in the first four treatments and 55% in the last.

In another experiment extracts of 12 plant families were evaluated for their bactericidal effect on pathogenic bacteria in *Mangifera indica*, *Helianthus annuus*, *Carica papaya* and *Musa* sp. The tests were performed with sterilized filter paper discs and three drying periods (moist, dried for 24 and for 48 h); six discs impregnated with a different plant extract were placed on Petri dishes where pure bacteria cultures were growing. The best bactericidal effects *in vitro* for the control of *Erwinia* were obtained with *Eryngium foetidum*, *Ruellia tuberosa* and *Momordica charantia*. The extract of *Melicocca bijuga* had bactericidal effect on *Pseudomonas* that attacks banana trees. None of the extracts had an effect on *Pseudomonas syringae* pv. *helianthi* (2).

A biopreparation of *T. harzianum* directed to leaves, shoots, and on the suckers of banana trees decreased the inoculum of *Pectobacterium carotovorum* (syn. *Erwinia carotovora* ssp. *carotovora*) diminished the incidence of disease up to 80%, and increased the yield by 40% (1).

One alternative for biological control with phytopathological bacteria in Venezuela is the application of *T. harzianum*. This is necessary for the existence of diverse commercial levels of the product. Also the use of natural extracts would be an excellent medium-term alternative, since in our country the production of these products has not been patented.

On the other hand, *P. agglomerans* has been identified in different plants and *P. fluorescens* in natural substrates for seedlings. It has to be analyzed whether *P. agglomerans* is acting as a phytopathogenic and proliferating organism in cereals, ornamentals, vegetables, *Musa* spp. and *Aloe vera* in Venezuela, demonstrating the evolution of the bacterium from an epiphytic to a pathogenic state in the mentioned crops. Thus for biological control of bacteria in plants the pathogenic strains of *P. agglomerans* have to be studied and compared with the other strains of which no pathogenic effects in other parts of the world are reported, since factors like variability and environmental conditions can influence the virulence.

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## **V. Molecular approaches in biocontrol of bacterial diseases**

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### **Molecular biology of plant-associated bacteria involved in biocontrol**

In this introductory lecture, our recent progress on the molecular biology of biocontrol mechanisms of plant-associated is presented. Genetic tools for fluorescent pseudomonads, *Erwinia* and *Agrobacterium* species, as well as Gram-positive biocontrol organisms have been developed and are now available in many laboratories. More importantly, the complete and, in part, fully annotated genome sequences of many relevant biocontrol organisms have been published and are easily accessible via the internet. A systems approach to combine these sequence information with transcriptome analyses of various biocontrol systems is the desirable major point for the current and future research. Recent advances in the molecular biology of biocontrol bacteria are discussed. In addition, an example for a resistance mechanism of a plant-pathogen, *Erwinia amylovora*, towards a biocontrol organism, *Pantoea agglomerans*, is introduced and general consequences are presented.

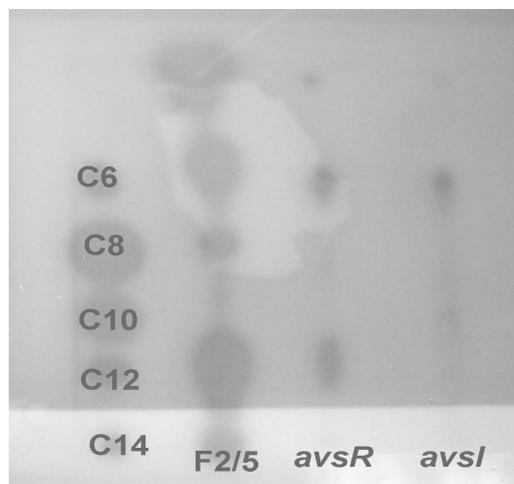
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### Identification of genetic mechanisms that affect *Agrobacterium vitis*-plant interactions including biological control

Crown gall disease of grapevines occurs in major viticulture regions worldwide causing reduced vine growth and possible death. In addition to causing crown gall, *Agrobacterium vitis* causes a grape-specific necrosis and a hypersensitive response (HR) on tobacco (Burr and Otten 2000). One non-tumorigenic strain, F2/5, is able to prevent crown gall infection when applied to wounds on grape prior to application of tumorigenic strains (Staphorst et al. 1985). Single gene mutations in F2/5 result in altered necrosis, HR and biological control phenotypes suggesting that their underlying mechanisms are related (Herlache et al. 2001). We have identified three *luxR*-like genes, *aviR*, *avhR* and *avsR* that affect the expression of HR and necrosis and one that affects biological control. Therefore we have strong evidence that these grape/*A. vitis* interactions are regulated by a complex quorum-sensing system (Zheng et al. 2003; Hao et al. 2005; Hao and Burr 2006). At least six *N*-acyl-homoserine lactone autoinducers were found to be produced by F2/5 including those with long acyl side chains that are characteristic for all *A. vitis* strains examined thus far (Li et al. 2006).

In addition to *luxR*-type genes a *luxI* (AHL synthase gene) homolog, *avsI*, was discovered that is responsible for the production of long-chain AHLs. Disruption of *avsI* resulted in loss of long-chain AHLs, a greatly reduced grape necrosis phenotype and loss of HR. The *avsI* mutant was complemented with cloned *avsI*. In addition grape necrosis and the tobacco HR were complemented in the *avsI* mutant by the addition of AHLs extracted from the wildtype strain (Hao and Burr 2006).



**Figure** Identification of AHLs produced by F2/5 and corresponding *avsR* and *avsI* mutants using thin-layer chromatography separation overlaid with the *Agrobacterium tumefaciens* sensor strain NTL4 (Cha et al. 1998)

Other genes found to be associated with the HR, necrosis and, biological control phenotypes include additional regulatory genes, a gene cluster that is associated with production of long-chain polyunsaturated fatty acids, and a gene that is homologous to those encoding a ClpA (ATP dependent Clp) protease. Additional ORFs associated with these responses on grape and tobacco, have been identified. A significant outcome from this research will be the determination of how F2/5 controls grape crown gall and how the control can be implemented in commercial agriculture. The research will also broaden our basic knowledge of how bacteria interact with plants. The completed sequencing of several Rhizobiaceae species including *A. tumefaciens* C58 and *A. vitis* S4, will provide valuable clues into the evolutionary development of these bacteria and their responses on plants.

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## Genes associated with and mechanism of biological control of grapevine crown gall by *Agrobacterium vitis* strain F2/5

*A. vitis* causes crown gall disease in grapevine, which can be severe in grapevine growing regions worldwide. *Vitis vinifera* L. cultivars are highly susceptible to freeze injury, providing wounds important for infection (Burr and Otten 1999; Otten and Burr 2006). Phytohormone concentration within the plant appears to play a role in tumor development, as does natural wound healing. Via an unknown mechanism, the nontumorigenic strain, F2/5, prevents crown gall in grapevine tissue (Creasap et al. 2005). M852, a Tn5 mutant of F2/5, lacks the biological control ability, does not induce an HR on tobacco, and is reduced in its ability to cause grapevine necrosis (Herlache et al. 2001). The disrupted gene has been identified as homologous to an ATP-dependent Clp protease, *clpA*, with similarities to *A. tumefaciens* (86%), and *Sinorhizobium meliloti* Rm1021 (83%) *clpA* genes. With primers designed from the genome sequence of *A. vitis* strain S4, the ORF of *clpA* was amplified from F2/5 DNA, sequenced, and confirmed similar to *clpA* in S4 (97%). Screening of this site-directed mutant has shown that this gene is involved in biological control, and this *clpA* homolog will be cloned into an expression vector and used to complement M852 and the site-directed mutant  $\mu$ *clpA*. Additionally, to determine whether *clpA* is solely responsible for the lack of biological control, the genes immediately upstream and downstream have been sequenced and identified as a *clpS* homolog and ORFD, respectively. Site-directed mutants of these genes have been tested for biological control, HR, and necrosis.

Additional work in *Ricinus* determined that F2/5 prevented tumor development by tumorigenic strain K306. This indicates that the biocontrol activity is not grapevine specific. *Ricinus* stems were inoculated with either the tumorigenic K306 *A. vitis* strain carrying p35S*gusint* or K306(p35S*gusint*) and F2/5. Isolation of mRNA from inoculated *Ricinus* stems indicated that F2/5 prevents expression of the *gus* mRNA (Zäuner et al. 2006).

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## Sensitivity to biological control of *Agrobacterium vitis* is related to its Ti plasmid

### Introduction

Crown gall caused by *Agrobacterium vitis* is a common disease of grapevine (*Vitis vinifera*) worldwide. Most *V. vinifera* cultivars are highly susceptible to and may be killed by crown gall. The disease has been and continues to be an economic problem for grape growers. Typically, infections are initiated at wound sites on trunks that are caused by alternating freezing temperatures. Wounds release chemical signals that are perceived by the bacterium and result in the induction of virulence (*vir*) genes encoded by the Ti plasmid. In addition, some chromosomal genes are also involved in the infection process (Gelvin 2000).

The disease on grapevines usually develops at grafts or on the trunks and to a lesser extent on canes. Galls are generally not observed on grape roots. Severely infected plants show reduced vigor and productivity, with substantial loss of trunks and sometimes of entire vines. The bacterium survives systemically in vines and can be disseminated in latently infected propagation materials. Today the most effective way to prevent the disease is the use of clean-indexed nursery stock. Shoot-tip cultures may be used for the establishment of bacterium-free mother plants. Because shoot-tips are generally free from the bacterium it is possible to produce *A. vitis*-free vines starting from actively growing shoot tips (Burr et al. 1998). Healthy plants however can be contaminated in nurseries and vineyards. Unfortunately, chemical controls are not effective for grape crown gall (Burr et al. 1998).

Although many bacterial isolates can inhibit the growth of *A. vitis* strains, so far biological control in the field has not been effective. In the middle of the eighties, several non-pathogenic *A. vitis* strains were isolated in South Africa (Staphorst et al. 1985). They produced bacteriocin-like substances and seemed to be antagonistic to *A. vitis* strains. With one of those strains, F2/5, when applied to wounded grape tissue prior to the pathogen *A. vitis*, tumor formation was prevented (Burr and Reid 1993). The mechanism by which F2/5 prevents gall formation is not fully understood. The mechanism of control is not associated with bacteriocin production or with competition for attachment sites (Burr et al. 1997). In this paper, we describe how the Ti plasmid of the target *A. vitis* and its chromosomal background contribute to the success of biological control of grape crown gall.

### Materials and methods

**Bacterial strains and media:** Bacterial strains used in this study are listed in Table 1. All strains were cultivated on YEB medium containing (per 1000 ml) yeast extract 1 g, peptone 5 g, beef extract 3 g, sucrose 1 g, and magnesium sulfate 0.5 g. Hybrid strains were constructed either with transformation with pTi DNA or by *in planta* mating as indicated in Table 1. A856(p35Sgusint) was made by an *in vitro* crossing between A856 and S17-1 (p35Sgusint) and selection for 50 mg/l kanamycin and 10 mg/l K-tellurite resistance.

**Plant inoculations:** The cultivar Chasselas was chosen for these experiments because of its sensitivity to the pathogen. Shoots of young grapevine plants (20-25 cm in height) were inoculated in spring or early summer by wounding the stems with a sterile lance and placing a 10 µl drop of suspension of strain F2/5 containing about 10<sup>8</sup> colony forming units (cfu)/ml. One day later wounds were inoculated with 10 µl of suspension of pathogen strains containing 10<sup>8</sup> cfu/ml. Sterile distilled water was applied as negative control. Tumor formation was scored by examination of the inoculated sites 3 to 4 weeks later. After 8 weeks the tumors were cut off and weighed. All experiments were repeated at least three times.

**In vitro inoculation of grape tissue:** Stem tissue from the third internode below the apex of a rapidly growing shoot was surface sterilized with 10% Chlorox for 15 min, rinsed with sterile distilled water, cut

into 1 cm segments and inserted vertically in a jar containing half-strength MS medium solidified with 0.6% agar. The cut surfaces were inoculated by placing a 4 µl droplet of 10<sup>9</sup> cells ml<sup>-1</sup> of bacterial suspension. Control sections were inoculated with sterile distilled water. Strain F2/5 was inoculated 1 day before the pathogen strain was applied. Samples were removed 4 days after inoculation and prepared for GUS staining.

**GUS assay:** *In vitro* grape internodes were inoculated with A856 and A856(p35Sgusint), alone and after F2/5 as described above. To measure GUS activity, sections of the inoculated ends of internodes were cut by hand with a sharp knife and stained with a solution of 1 mg/ml of 5-bromo-4-chloro-3-indolyl-D-glucuronic acid (X-gluc) as described (Süle et al. 1994). Sections were incubated at 37 °C for 3 days and microscopically examined for the presence of blue cells.

**Tab. 1** Bacterial strains

Designation	Characteristics*	Origin or reference
<i>A. tumefaciens</i> C58	Wild-type from cherry	Nester, E. (Seattle, USA)
<i>A. tumefaciens</i> A136	pTi plasmid less C58	Knauf et al. 1983
<i>A. tumefaciens</i> UBAPF2	pTi-, and pAt-less C58	Hynes et al. 1985
<i>A. vitis</i> Ag162	Wild-type from grapevine	Knauf et al. 1983
<i>A. vitis</i> AB3	Wild-type from grapevine	Szegedi et al. 1988
<i>A. vitis</i> AB4	Wild-type from grapevine	Szegedi et al. 1988
<i>A. tumefaciens</i> A856	pTiAg162 (octopine Ti plasmid) in A136 obtained by transformation	Knauf et al. 1983
<i>A. tumefaciens</i> UBAPF2(pTiAB3)	pTiAB3 (octopine Ti plasmid) in UBAPF2 obtained after <i>in planta</i> mating between AB3 and UBAPF2	Otten et al. 1995
<i>A. tumefaciens</i> UBAPF2(pTiAB4)	pTiAB4 (nopaline Ti plasmid) in UBAPF2 obtained after <i>in planta</i> mating between AB4 and UBAP2	Otten, De Ruffray 1994
<i>E. coli</i> S17-1(p35Sgusint)	Plant transformation vector with an intron-containing <i>gus</i> marker gene, Sm <sup>r</sup> Km <sup>r</sup>	Vancanneyt et al. 1990
<i>A. tumefaciens</i> A856p(TiAg162,p35Sgusint)	Km <sup>r</sup>	This study

## Results

**Gall inhibition:** Treatment of wounds on grape shoots with F2/5 prior to wild-type *A. vitis* strains and *A. tumefaciens* strains with *A. vitis* pTi inhibited growth of galls. Only UBAPF2 (pTiAB4) induced a few very small swellings when wounds were pretreated with F2/5 (Table 1). Isolation of the pathogenic bacteria from these degenerated tumors was unsuccessful. Control plants pre-treated with water developed large tumors. The weight of tumors largely depended on the strains used. Ag162 and A856 formed much smaller tumors than AB3 and AB4. Generally *A. tumefaciens* strains with *A. vitis* Ti plasmids produced smaller galls than their respective wild-type *A. vitis* strains.

**F2/5 prevents expression of GUS activity in infected plant cells:** To detect if treatment with F2/5 inhibits the expression of T-DNA-encoded genes, we infected *in vitro* shoot segments with *A. vitis* Ag162 and *A. tumefaciens* A856 both containing p35Sgusint. The *gusAint* gene in p35Sgus-intron plasmid construction allowed us to assay expression of GUS activity within the transformed plant cells without expression of GUS activity in the bacteria. Segments cut from infected shoot ends were stained with X-Gluc four days after inoculation. Infection of these plants without F2/5 resulted in most but not all plants in the production of GUS activity, as indicated by sectors of blue-stained tissue. However, when plants were infected first with F2/5 and then with Ag162(p35Sgusint) or A856(p35Sgusint), we never detected GUS activity indicating that the T-DNA was not expressed. Similar results were obtained by Burr et al. (1997). The inhibition of the expression of GUS activity in grape cells infected with Ag162(p35Sgusint) and A856(p35Sgusint) after F2/5 indicates that F2/5 inhibits the genetic transformation of grape cells. The fact that not all control plants showed blue stained cells is probably related to the weak virulence of Ag162 and A856 on Chasselas as indicated in tumor weights (Table 2). Earlier experiments showed that

this strain is a limited host-range strain with a deletion on the pTi (Knauf et al. 1983). New experiments using the highly virulent strains AB4(p35Sgusint) and UBAPF(pTiAB4, p35Sgusint) are in progress.

**Tab. 2** Effect of F2/5 on tumor development of different wild type *A. vitis* strains and strains of *A. tumefaciens* with *A. vitis* pTi on grape shoots

Bacterial strains	Pre-treatment with F2/5.	Pre-treatment with water.
	Mean fresh wt. (mg) galls/plant*	Mean fresh wt. (mg) galls/plant*
C58 <i>A. tumefaciens</i> wild type	120*	112
A136, UBAPF2 ( <i>A. tumefaciens</i> plasmidless)	0	0
Ag162 (wild type <i>A. vitis</i> )	0	75
A856 ( <i>A. tumefaciens</i> with pTi of Ag162)	0	60
AB4 (wild type <i>A. vitis</i> )	0	1826
UBAPF(pTiAB4) ( <i>A. tumefaciens</i> with pTi of AB4)	3.5	370
AB3 (wild type <i>A. vitis</i> )	0	567
UBAPF(pTiAB3) ( <i>A. tumefaciens</i> with pTi of AB3)	0	347

\*Weight of tumors after two months

## Conclusions

In this study, hybrid strains prepared from *A. tumefaciens* chromosomal background and *A. vitis* Ti plasmids were controlled by F2/5 on grape shoots. The hybrid strains, producing normal galls on grape shoots, were not sensitive to agrocin, and transferred their T-DNA to grapevine as verified by the GUS assay. However, when they were mixed with F2/5, gall formation and T-DNA transfer to grape tissues were inhibited. In the control experiment the mother strain of *A. tumefaciens* C58 with the chromosomal background of the hybrid strains was not inhibited in gall formation. As expected the wild-type *A. vitis* strains with the pTis of the hybrid strains were perfectly inhibited by F2/5. We conclude from this experiment that the biological control on grape shoots by F2/5 depends on pTi of *A. vitis*, and that the genetic bases for the sensitivity to biological control may be located on the Ti plasmid of *A. vitis*.

Since the chromosome influences the weight of tumors (Table 2), it could also influence the sensitivity of the strains to biological control. It seems possible that the chromosomal background of the grapevine-specific *A. vitis* strains may have features particularly adapted to grapevines and it would be interesting to see if biological control can be effective against strains having Ti plasmids of *A. tumefaciens* in *A. vitis* chromosomal background. However, so far, all attempts to transform *A. vitis* strains with Ti plasmid DNA from *A. tumefaciens* have been unsuccessful.

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## Interactions between *Agrobacterium radiobacter* and dicot and monocot plants

### Introduction

Bacteria of the genus *Agrobacterium* are known to infect di- and monocotyledonous plants and to induce tumors on these plants. *Agrobacterium tumefaciens* transfers a part of its Ti plasmid DNA (T-DNA) into plant cells, thus initiating proliferation of host cells. Before the T-DNA is transferred, bacteria must attach to the plant cell surface. All known genes participating in the attachment process (*cel*, *att*, *chvA*, *chvB*, *exoC*) are localized on the chromosome (1). *A. tumefaciens* mutants, defective in attachment, are avirulent, suggesting that agrobacterial attachment to the plant cell surface plays an important role in the plant-microbe interaction. It is known that *A. radiobacter* is a natural antagonist of *A. tumefaciens*. *A. radiobacter* strain 5D-1 was isolated from wheat roots after surface sterilization in Russia (Saratov region) in 1986 (2) and re-isolated from wheat roots in 1994 as *A. radiobacter* 5D-2 (3). Both strains showed similar culture characteristics and patterns of *EcoRI*, *HindIII*-DNA restriction fragments and gave a positive reaction with 5D-1 strain antibodies. Thus *A. radiobacter* possibly belongs to rhizosphere-associative bacteria.

### Results and discussion

Inoculation of wheat seeds, tomato stem, and sunflower plantlets with *A. radiobacter* 5D-1: Bacterial inoculation is an old, traditional and ecologically safe method of increasing plant yield and plant protection against pathogens (3). Inoculation of sunflower plantlets with *A. radiobacter* 5D-1 suspension increased the sunflower height (104-113% of the control), grain yield (124-191% of the control), and dry weight (120-213% of the control). The effect of wheat seedling inoculation on dry weight in the field experiments was lower (104-112% of control). In field experiments, a noticeable positive effect was recorded after triple inoculation of the wheat seedlings at the different stages of growth.

Testing *A. radiobacter* 5D-1 for indolyl-3-acetic acid (IAA) production showed that in the presence of tryptophan (up to 200 mg/L) the concentration of IAA ranged from 9 to 24 mg/L medium. We selected a set of IAA-negative mutants for *A. radiobacter* 5D-1. The observed sufficiently high IAA-production of *A. radiobacter* 5D-1 may provide one reason for the positive effect this strain has on plants. *A. radiobacter* 5D-1 failed to induce tumors on dicotyledonous plants (*Kalanchoë*, tomato, sunflower), but pre-treatment of the stems of tomato seedlings with *A. radiobacter* 5D-1 protected them from *A. tumefaciens*-mediated tumor induction. *A. radiobacter* 5D-1 was shown to attach to wheat roots to a greater extent (more than 3 fold), than associative rhizobacteria (*Azospirillum brasiliense* Sp245) and the pathogenic agrobacteria *A. tumefaciens* LMG187 (more than 13 fold) (Table). The observed sufficiently high plant surface colonization by *A. radiobacter* 5D-1 may provide a second explanation for the positive effect this strain has on plants. We do not exclude other types of the positive effects, such as bacteriocin production.

**Table** Attachment of *A. tumefaciens* LMG 187 (M) and *A. radiobacter* 5D-1 (M) and attachment-minus mutants (m) to wheat root surface evaluated by radioisotope methods

Strains	attached cells (10 <sup>6</sup> cell/g root)	
	M	m
<i>A. radiobacter</i> 5D-1	2	67
<i>A. tumefaciens</i> LMG 187	5	2

Adsorption and attachment of agrobacteria to the plant cell surface: Bacterial adsorption and attachment to the plant cell surface is the first real contact in the interaction and the real background for competition on plant cell surface. It seems that agrobacteria and rhizobia attachment-minus mutants (m) lack the capacity to induce tumor and nodules on dicotyledonous plants (1,3). It is considered that both non-pathogenic and pathogenic agrobacteria cannot adsorb and transform monocot plant cells, since receptors for binding *Agrobacterium* to the monocot cell surface are absent (4).

Using the radioisotope method, we found that *A. radiobacter* 5D-1 attached to rice roots (cv. Nipponbare) 6-12-fold more actively than did pathogenic strains (*A. rhizogenes* and *A. tumefaciens*). The percentage of the attached *A. radiobacter* 5D-1 cells to rice roots in comparison with not attached cells was dependent on the rice cultivars (1.6 to 4.9%) and much less dependent on the wheat cultivars (31 to 39%). We tested the ability of *A. rhizogenes* LMG150 to attach to the roots of various rice cultivars. The cultivars Lemont, IR54, and Taipei 309 had a higher number of attached cells to the rice root surface, as distinct from Nipponbare. We obtained a set of Tn5 mutants of *A. radiobacter* 5D-1 that was defective in attachment to the rice root surface.

The attachment of *A. tumefaciens* and *A. radiobacter* to wheat roots proceeded most actively during the initial 120 min, beginning from the first minute after inoculation. By means of scanning electron microscopy (SEM) we revealed that 15-30 min after inoculation some of the adsorbed cells produced connecting threads for the attachment to the plant cell surface (Figure). It seems that these fibril-like structures (FLS) can be produced by bacterial cells without plants, in free-living conditions on a glucose-containing medium. We showed that non-pathogenic bacteria can produce a lot of FLS which connect bacterial cells with a nylon membrane and with each other in model systems, where bacterial cells are separated from a glucose solution by a nylon membrane.

This type of attachment belongs to the theoretical mono-layer type of adsorption. But using SEM we showed that the attachment process proceeded differently (Figure). The surface of wheat roots is not covered by one layer of bacteria, but the bacteria were located in two and more layers at the attachment place. It looked like the "mosaic type" of attachment. The age of bacterial cell populations was also important for the number of surface attached cells. The *A. radiobacter* 5D-1 cells growing up to 20-22 h (stationary phase) have higher attachment ability (AA) content. We could block the attachment process of agrobacterial cells to wheat roots with different substances. For example, treating the *A. radiobacter* 5D-1 with the respiration inhibitor carbonyl cyanide m-chlorophenylhydrazine dramatically decreased the number of attached cells.



**Figure** Attachment of *A. radiobacter* 5D-1 cells to wheat root surface

### **Bacterial surface polysaccharides are involved in the attachment process of *A. radiobacter* 5D-1 to the plant cell surface**

Succinoglucan (EPS I): Succinoglucan (exopolysaccharide I, EPS I) is a higher molecular weight polymer composed of polymerized octasaccharide subunits. *A. tumefaciens*, *A. rhizogenes*, *A. radiobacter*, and *R. meliloti* can produce succinoglucan. Which nature have the *A. radiobacter* FLS? We have strong genetic evidence for an involvement of *A. radiobacter* 5D-1-calcofluor-binding polysaccharides in the attachment process to wheat root hair tips. By using Tn5 mutagenesis we obtained a number of *A. radiobacter* 5D-1 mutants, which did not produce calcofluor-binding polysaccharides. The *cfw*-binding minus mutants (227) were shown to have reduced adsorption ability as examined with a radioisotope method, and Tn5 mutants 5005 with overproduction of *cfw*-binding polysaccharides have more adsorption affinity to wheat roots. Thus we showed that surface *cfw*-binding polysaccharides of *A. radiobacter* 5D-1 are involved in the adsorption process to wheat roots. But it is not clear which subunits are involved in fibril-like structures expressed during the attachment of *A. radiobacter* 5D-1 to the plant cell surface.

Cellulose fibril structures: Pathogenic agrobacteria were reported to produce cellulose fibrils composed of cyclic  $\beta$ -glucan when attaching to the plant cell surface. Fibrils connecting *A. radiobacter* 5D-1 cells with the plant cell surface may be either thick and short (length comparable with bacterial cell length) or long and thin (Fig. 1). Non-pathogenic *A. radiobacter* 5D-1 can not regenerate them when treated with the respiratory inhibitors and enzymes. Cellulose-minus mutants of *A. radiobacter* 5D-1 had weaker attachment ability to wheat roots compared with the wild strain. Treatment of the wild strain 5D-1 with cellulase decreased the attachment ability by 1.5 fold. By transmission electron microscopy we showed that FLS on *A. radiobacter* 5D-1 surface could be labeled with cellulase-colloidal gold complex (data not shown). Now, more evidence is available concerning the cellulose nature of *A. radiobacter* FLS.

### **Bacterial surface proteins involved in the attachment process of *A. radiobacter* 5D-1 to monocot plant cell surfaces**

It is known that some bacterial surface proteins like agglutinins, lectins, rickadhesin or polypeptides are involved in the adsorption process of agrobacteria to plant cells. Treating the cells with trypsin, glutaraldehyde, or EDTA (chelating agent for divalent cations) that affect the *A. radiobacter* 5D-1 protein structures, decreased the adsorption to a great extent. The agrobacterial surface molecules (rickadhesin, *vir*-depending and *tra*-depending pilus, flagella) were evaluated as attachment-mediating molecules by transmission electron microscopy (TEM) and radioisotope methods. *A. radiobacter* 5 D-1 cells showed practically no adsorption to wheat roots when grown on a calcium-free medium. Addition of 1.4 mM  $\text{CaCl}_2$  to the growth medium increased the number of *A. radiobacter* 5D-1 cells attached to wheat roots by 30% in comparison with the control (without Ca) and 7 and 14 mM  $\text{CaCl}_2$ . However, we did not record any stimulating effect of Ca on pathogenic agrobacterial attachment to wheat roots. Possibly,  $\text{CaCl}_2$ -dependent agrobacterial proteins mediating the attachment process to legume root hairs are not involved in the attachment to wheat roots.

*vir*- and *tra*- depending pilus: A number of studies proposed that one or several genes, located in the *virB* and *tra* regions of the agrobacterial Ti plasmid, are responsible of the assembly of an extracellular channel linking the bacterium with the plant cells (1). We assumed that some agrobacterial VirB and Tra surface-located proteins possibly play a role in the attachment processes. We found that *A. tumefaciens* *virB2* and *tra* (R10) defective mutants did not express pilus precursor proteins, cannot form *vir*- and *tra*-depending pili, but display similar attachment to wheat root hairs in comparison with the wild type strain. It seems that *vir*-depending and *tra*-depending pili are not involved in the agrobacterial attachment process to monocot plant cell surfaces.

Flagella: *Agrobacterium* possesses several surface flagella. It is assumed that flagella do not contribute directly to the establishment of infection, because non-flagellate *A. tumefaciens* mutants retain their virulence and ability to attach to *Zinnia* mesophyll cells (5). That agrobacteria require flagella to attach to the plant surface has not been established, though non-flagellate mutants of nonpathogenic strains attach to the surface of wheat roots at a lower level than the wild type (3).

Using Tn5 mutagenesis, we isolated *A. radiobacter* 5D-1 chemotactic, flagella-negative and non-motile mutants. The attachment ability of the flagella-negative mutant 362 was significantly lower, though the chemotaxis mutant 195 had the same attachment ability as the parent strain. We observed that the flagella-minus mutant 362 elaborated FLS during its contact with the plant surface like the wild strain 5D-1. The attachment ability of these mutants decreased dramatically. Thus, we can assume that flagella of *A. radiobacter* are involved in the attachment process to the monocot cell surface.

### **Monitoring *A. radiobacter* 5D-1 in the wheat rhizosphere**

When plants are inoculated with specific bacterial strains, it is essential that a reliable method is used for identification and monitoring the applied bacteria under soil and rhizosphere conditions.

We worked out and applied in field conditions a monitoring method for plant-associated bacteria, based on the combination of genetic and immunological methods. This procedure is based on revealing the genetically marked strains (spontaneous double antibiotic resistance) which are then identified by means of antibodies specific to the carbohydrate antigens of the cell surface. Monitoring the cell number of strain 5D-1 in the rhizosphere of wheat was done under field conditions. We found that after inoculation

a low number of the applied *A. radiobacter* cells was observed (10000 per g soil). Possibly, the low number of the applied bacteria was the main reason for the neutral effect of a single *A. radiobacter* inoculation. We have obtained a positive result only in the case of triple inoculation, depending mainly on the concentration of bacteria in the inoculum.

We showed that the attachment behavior of *A. radiobacter* 5D-1 and its attachment-minus and over-attachment mutants defined in laboratory conditions and colonization capacity under field conditions are directly correlated.

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## Genomic analyses of niche specific traits in plant-associated *Pseudomonas*

### Abstract

Development and application of biocontrol technologies can be enhanced by a detailed understanding of the biology and ecology of pathogens and biocontrol agents. Bacterial plant pathogens such as *Pseudomonas syringae* establish intimate relationships with host cells that allow them to colonise the apoplast of susceptible host plants. In contrast, non-pathogenic *Pseudomonas* such as *Pseudomonas fluorescens* and *Pseudomonas putida* are unable to multiply to high levels in the plant apoplast, but are highly effective colonists of other plant-associated niches such as root and leaf surfaces. Non-pathogenic *Pseudomonas* can have significant effects on plant growth and plant disease resistance through their interactions with plant cells and plant-associated organisms. We have analysed genome sequence data from multiple strains of animal pathogenic, plant pathogenic and non-pathogenic *Pseudomonas*, along with data from more than 200 other complete bacterial genome sequences to identify genes and traits that may be involved in adaptation to life on plants. These analyses have taken four main forms: (1) Identification of over- and under-represented protein domains; (2) Identification of unique domains and domain architectures; (3) Identification of candidate secreted proteins; and (4) Identification of conserved promoter elements and regulators.

### Introduction

Bacteria in the genus *Pseudomonas* are potential agents for rhizoremediation, biocontrol, industrial biotechnology and plant growth promotion. However, practical applications of these strains have been hindered by the fact that many strains fail to establish natural populations following field release, or fail to consistently express desirable traits in a natural context (Bloemberg and Lugtenberg 2001; Walsh et al. 2001; Haas and Keel 2003; Kuiper et al. 2004; Haas and Defago 2005). One explanation for the low success rate of *Pseudomonas* in biocontrol applications is that desirable traits are poorly or variably expressed in response to variation in environmental conditions, which can affect both trait-specific regulators and global regulatory systems. A second is that strains are unable to colonise local niches and compete with endogenous microbial populations or with target pathogens. Researchers have attempted to overcome these limitations by using high inoculum densities, localised application, by engineering gene expression and by adding organic amendments. However, these approaches have generally been developed on an empirical basis for individual strains and traits. The availability of genome sequence data and high-throughput functional genomic and metabolomic technology offers new opportunities to gain deeper insight into niche specificity in *Pseudomonas* and to optimise *Pseudomonas* performance based on knowledge of *Pseudomonas* ecology, physiology and gene expression.

Five hypotheses about plant-colonisation genes in *Pseudomonas*: The need for improved understanding of plant colonisation and biocontrol trait expression by non-pathogenic *Pseudomonas* is mirrored by a need for greater understanding of niche specificity and plant colonisation by plant pathogenic bacteria. The genus *Pseudomonas* provides an ideal context in which to investigate and compare niche specific adaptations in pathogenic and non-pathogenic bacteria. This genus contains both pathogenic and plant growth-promoting bacteria and genome sequence data is available for at least 10 strains, including three plant pathogens (*Pseudomonas syringae* pv. *tomato* DC3000, *Pseudomonas syringae* pv. *syringae* B728a and *Pseudomonas savastanoi* (*syringae*) pv. *phaseolicola* 1448a) and four non-pathogenic, plant-colonising strains (*Pseudomonas fluorescens* SBW25, *Pseudomonas fluorescens* Pf-5, *Pseudomonas fluorescens* Pf0-1 and *Pseudomonas putida* KT2440). Online resources describing *Pseudomonas* genomes are available at

- [www.pseudomonas.com](http://www.pseudomonas.com), [pseudomonas-syringae.org](http://pseudomonas-syringae.org), [pseudo.bham.ac.uk](http://pseudo.bham.ac.uk), [www.sanger.ac.uk/Projects/P\\_fluorescens/](http://www.sanger.ac.uk/Projects/P_fluorescens/) and [http://www.genoscope.cns.fr/externe/English/Projets/Projet\\_FY/organisme\\_FY.html](http://www.genoscope.cns.fr/externe/English/Projets/Projet_FY/organisme_FY.html).
- We have conducted comparative analyses of *Pseudomonas* genomes based on five hypotheses about plant colonisation genes:
- Protein domains associated with plant colonisation traits are **over-represented** in *Pseudomonas* genomes compared to genomes of bacteria that do not colonise plants.
- Protein domains associated with functions that are of little or no importance for plant colonisation, but which are important in other ecological niches are **under-represented** in the genomes of plant-associated *Pseudomonas*.
- *Pseudomonas* have evolved and acquired novel plant colonisation genes that can be recognised in the form of **unique protein domains** and **unique protein domain architectures** that are not conserved in other bacterial genomes
- **Secreted proteins** and **membrane proteins** are likely to have key roles in *Pseudomonas*-plant interactions, interactions with other plant-associated microorganisms, stress tolerance and nutrient acquisition.
- Expression of many plant colonisation traits is regulated and coordinated by key **global regulators** such as RpoN, GacS and ECF sigma factors.
- All of these hypotheses can be investigated using currently available bioinformatic tools, generating predictions that can be validated using experimental techniques.

Comparative analyses of *Pseudomonas* genomes – general principles: Two considerations underpin all comparative genomic analyses – the phylogenetic relationships of the strains being studied, and the ecological niches occupied by these strains. Quantitative predictions such as domain number and regulator number must be weighed against genome size and complexity. Current phylogenetic analyses indicate that *Pseudomonas* strains belong to the superkingdom bacteria, phylum Proteobacteria, class  $\gamma$ -Proteobacteria, order Pseudomonadales, family Pseudomonadaceae and genus *Pseudomonas*. As complete genome sequence data is now available for a large number of organisms (more than 240 completed bacterial genomes, 300 completed genomes (Bernal et al. 2001), we can compare the properties of individual or groups of *Pseudomonas* genomes to higher taxonomic groupings, or to specific taxonomic groupings. We have chosen to base many of our analyses on protein domain predictions derived from completed genomes, in particular data corresponding to the Pfam protein domain database (Bateman et al. 2002, [www.sanger.ac.uk/Pfam](http://www.sanger.ac.uk/Pfam)), as this dataset provides both evolutionary and functional information about the structure and function of bacterial proteins and genomes.

We can phrase comparative genome queries in a variety of ways depending on the hypothesis being tested. For example to analyse whether *P. syringae* pv. *syringae* B728a possesses unique features not present in other organisms we might ask: “What protein domains or domain architectures are present in *P. syringae* pv. *syringae* B728a and not in other completed genome sequences?” Alternatively, we might want to consider *Pseudomonas* as a taxonomic unit and ask “What protein domains are over-represented in *Pseudomonas* genomes compared to other completed bacterial genomes?” Finally, if we are exploring specific phenomena, such as lateral gene transfer between bacteria and eukaryotes, we might ask, “What domains and domain architectures are found in *Pseudomonas* and Eukaryote or Archaea genomes, but not in other completed bacterial genomes?” An answer to this question is shown in Table 1.

**Table** Pfam domain architectures found in *Pseudomonas* and Eukaryote or Archaea genomes, but not other completely sequenced bacterial genomes. Analyses were performed in September 2005 using Pfam 18.0.

Architecture	Species distribution	Examples
AMP-binding – PP-binding - Condensation - AMP-binding - PP-binding - Condensation - AMP-binding - PP-binding	<i>Claviceps purpurea</i> ; <i>Streptomyces hygroscopicus</i> ; <i>P. syringae</i> 1448A; <i>P. syringae</i> B728a	gi 71733810 ref YP_273984.1 ; Q4ZVI3; Mannopectimycin peptide synthetase MppA. (Q643C7); D-lysergyl-peptide-synthetase. (O94205);
ART	<i>Gallus gallus</i> ; <i>Homo sapiens</i> ; <i>Macaca fascicularis</i> ; <i>Mus musculus</i> ; <i>Oryctolagus cuniculus</i> ; <i>Pan troglodytes</i> ; <i>Pongo pygmaeus</i> ; <i>Pseudomonas syringae</i> ; <i>Rattus norvegicus</i> ;	Q87WF8; Q88BP8; Q87WF6; Type III effector HopPtoS3. (Q87WF6); Type III effector HopO1-1. (Q88BP8); ADP-ribosyltransferase 1. (Q6NTD2*); GPI-linked NAD(P)(+)--arginine ADP-ribosyltransferase 1 precursor(EC 2.4.2.31) (Mono(ADP-ribosyl)transferase). (P52961*); GPI-linked NAD(P)(+)--arginine ADP-ribosyltransferase 1 precursor(EC 2.4.2.31) (Mono(ADP-ribosyl)transferase). (Q03515*);
Aegerolysin	<i>Agrocybe aegerita</i> ; <i>Aspergillus fumigatus</i> ; <i>Aspergillus nidulans</i> FGSC A4; <i>Clostridium bifermentans</i> ; <i>Neurospora crassa</i> ; <i>Pleurotus ostreatus</i> ; <i>Pseudomonas aeruginosa</i> ;	Q9I710; Aegerolysin Aa-Pri1 precursor. (O42717); PriA (Pleurotolysin A). (Q8X1M9); Hypothetical protein. (Q9I710); Related to Hemolysin. (Q8WZT0*); Hemolysin-like protein. (O32337);
Carotene_hydrox	<i>Agrobacterium aurantiacum</i> ; <i>Alcaligenes sp</i> ; <i>Arabidopsis thaliana</i> ; <i>Brassica campestris</i> ; <i>Capsicum annuum</i> ; <i>Citrus unshiu</i> ; <i>Crocus sativus</i> ; <i>Erwinia herbicola</i> ; <i>Flavobacterium sp.</i> ATCC 21588; <i>Glycine max</i> ; <i>Lycopersicon esculentum</i> ; <i>Narcissus pseudonarcissus</i> ; <i>Oryza sativa</i> ; <i>Pantoea agglomerans</i> pv. <i>milletiae</i> ; <i>Pantoea ananas</i> ; <i>Pantoea stewartii</i> ; <i>Paracoccus marcusii</i> ; <i>Picrophilus torridus</i> ; <i>Pseudomonas putida</i> ; <i>Sulfolobus solfataricus</i> ; <i>Tagetes erecta</i> ; <i>Vitis vinifera</i> ; <i>marine bacterium</i> P99-3;	Q88HV7; B-carotene hydroxylase. (P94792*); Beta-carotene hydroxylase. (Q01332*); Beta carotene hydroxylase. (Q6KYT6); Carotene hydroxylase. (Q9RLH6); protein. (Q8VUJ3*);
Copper-bind - Copper-bind	<i>Haloarcula marismortui</i> ; <i>Halobacterium sp</i> ; <i>Pseudomonas aeruginosa</i> ;	Q9I036; Halocyanin-like. (Q9HPH3); Halocyanin-like. (Q5V2Z9); Halocyanin-like. (Q5V4M1); Hypothetical protein. (Q9I036*);
DAP_epimerase	<i>Methanopyrus kandleri</i> ; <i>P. fluorescens</i> Pf5; <i>P. fluorescens</i> SBW25;	PFLU2104; Q4K9F2; Diaminopimelate epimerase (EC 5.1.1.7) (DAP epimerase). (Q8TY71);
LRR_1 - Pkinase_Tyr	<i>Arabidopsis thaliana</i> ; <i>Dictyostelium discoideum</i> ; <i>Homo sapiens</i> ; <i>Lymnaea stagnalis</i> ; <i>Neurospora crassa</i> ; <i>Oryza sativa</i> ; <i>Pseudomonas aeruginosa</i> ; <i>Pseudomonas syringae</i> ; <i>Rattus norvegicus</i> ; <i>Solanum demissum</i> ;	Q9I0Y0; Q880J6; Putative receptor-like protein kinase. (O22938*); Putative LRR receptor-like kinase 2. (Q6ZIW9); Receptor protein kinase-like. (Q56YS9); Putative receptor protein kinase. (Q75KZ6); Similar to <i>Dictyostelium discoideum</i> (Slime mold). Pats1 (Hypotheticalprotein roco2). (Q8SSS9);
VOMI	<i>Caenorhabditis briggsae</i> ; <i>Caenorhabditis</i>	gi 71737040 ref YP_275499.1 ;

Architecture	Species distribution	Examples
	<i>elegans</i> ; <i>Cyprinus carpio</i> ; <i>Gallus gallus</i> ; <i>Homo sapiens</i> ; <i>Mus musculus</i> ; <i>P. syringae</i> 1448A;	Hypothetical protein Y39F10C.1. (Q9TYM7*); Hypothetical protein CBG14364. (Q619B8); Hypothetical protein C08G5.3. (Q9TZL4*); Hypothetical protein T02H6.4. (Q9N5E6*); Hypothetical protein Y57G11A.2. (O45861);

We can also approach these analyses from an ecological standpoint, framing our queries in terms of niche specificity. For example we might ask “What protein domains and domain architectures are found in plant-associated Proteobacteria, but not in other bacterial genomes?” The corresponding list of “plant-associated Proteobacteria” would include *Pseudomonas*, *Agrobacterium*, *Rhizobium*, *Xanthomonas*, *Ralstonia*, *Erwinia*, and *Xylella*. Alternatively, we could focus only on a subset of Proteobacteria that are commonly found in the rhizosphere, this would include *Pseudomonas putida*, *aeruginosa* and *fluorescens*, along with *Erwinia carotovora*, *Agrobacterium*, *Ralstonia*, *Acinetobacter* and *Burkholderia* but exclude *P. syringae* and *Xylella spp.* The list of possible permutations is very large, and the form of the query determines the size and composition of the final dataset. Our analyses to date have focused on traits associated with plant colonisation and with strain, species and genus-specific traits in *Pseudomonas* (Studholme et al. 2005).

**Over-represented domains:** The aim of over-represented domain analyses is to identify protein domains that are present in exceptionally large numbers in a genome or subset of genomes. Expansion of a domain family may give a corresponding increase in regulatory or nutritional flexibility, enhanced stress responses or enable bacteria to evade host recognition by varying surface proteins. We might therefore expect a significant proportion of over-represented domains to correspond to niche-specific traits. Other domains commonly observed in over-represented domain analyses include phage, transposon and IS element-associated domains.

We identified over-represented domains in *Pseudomonas* by comparing domain numbers across *Pseudomonas*, Pseudomonadales,  $\gamma$ -Proteobacteria, Proteobacteria, bacteria and all organisms. Over-represented domains in *Pseudomonas* genomes include domains involved in nutrient uptake and nutrient utilization, chemotaxis, and transcriptional regulation. Individual species show expansions in particular domains, for example, membrane transport domains over-represented in *P. syringae* and other *Pseudomonas* genomes relative to other bacteria include **BPD\_transp\_1 (PF00528)** and **Sugar\_tr (PF00083)**. **ABC\_tran (PF00005)** is over-represented in *P. syringae* and *P. putida*, and **BPD\_transp\_2 (PF02653)** is over-represented in *P. syringae*, but not in other *Pseudomonas* species (Studholme et al. 2005). *P. fluorescens* genomes contain a large and diverse assortment of biosynthetic and biodegradative domains, which may reflect their ability to make a wide variety of antimicrobial compounds and to degrade complex organic molecules in the environment (Studholme and Preston, unpublished).

**Under-represented domains:** While over-represented domains correspond to a probable expansion in functional properties, under-represented domains are likely to represent loss of function. For example, the amino acid permease domain **AA\_permease (PF00324)** is over-represented in both *P. aeruginosa* PAO1 and *P. putida* KT2440, with 21 examples in each genome, but is under-represented in *Pst*DC3000, which contains only 4, which are similar to transporters for proline, GABA, ethanolamine and aromatic amino acids, omitting transporters with potential roles in the transport of D-serine/D-alanine/D-glycine; arginine/ornithine/putrescine; cadaverine; lysine; histidine; threonine; choline; glutamate and cysteine. The number of **AA\_permease** domains in *Pst* is similar to the number present in the fastidious vascular pathogen *Xylella fastidiosa* which contains only 3. Some studies have suggested that the composition of xylem sap closely resembles the composition of the leaf apoplast. Hypotheses that could account for the low number of **AA\_permease** domains and the inability of *P. syringae* to use certain amino acids as C sources include: (1) Metabolically preferred N sources are available in the niches colonised by *P. syringae*; (2) Certain N sources are not available to *P. syringae*; (3) negative selection imposed by bacteria-plant interactions favours loss of **AA\_permease** domains or (4) Domain numbers have been reduced as *P. syringae* has evolved through a series of evolutionary bottlenecks.

Unique domains and domain architectures: In addition to counting domain numbers, we can analyse the presence and organisation of domains in *Pseudomonas* genomes, investigating whether unique domains or domain architectures are present (Studholme et al. 2005). Unique architectures can arise by domain duplication, recombination or deletion. Many of the unique architectures in *Pseudomonas* genomes are predicted to be secreted adhesins and toxins or non-ribosomal polypeptide and polyketide synthases. Genes encoding these functions are large, complex and frequently contain domain repeats giving them a large potential for evolution by recombination and duplication. Further analyses of these synthases may lead to the identification of new bioactive compounds, including antibiotics, surfactants and siderophores, all of which have been shown to have key roles in biocontrol (Haas and Defago 2005). *Pseudomonas*-specific domains identified using domain analyses include well-characterised domains, such as the domain corresponding to the *P. syringae* type III-secreted protein HrpZ, as well as a number of conserved proteins of unknown function such as DUF1534, DUF1652 and DUF1654.

Domain architecture analyses may also provide insight into the evolution of novel host colonisation factors and evidence of horizontal gene transfer. Table 1 shows domain architectures that are present in *Pseudomonas*, Eukaryotes and Archaea, but which are not conserved in other complete bacterial genomes. This list includes the ART domain, which corresponds to the *P. syringae* type III secreted effectors HopPtoS3 and HopO1-1 that are injected into eukaryote cells where they mimic and modulate cellular functions in order to promote endophytic colonisation (Guo et al. 2005). Some architectures in Table 1, such as **DAP\_epimerase (PF01678)** and **AMP-binding - PP-binding - Condensation - AMP-binding - PP-binding - Condensation - AMP-binding - PP-binding** may represent convergence of paraphyletic genes on a common architecture by recombination or deletion, others, such as the **Vomi (PF03762)**, **Aegerolysin (PF06355)**, **Carotene\_hydrox (PF03897)** and **Copper\_bind (PF00127)** domains may be examples of domains that have a common ancestry, and which have been transferred between kingdoms.

Secreted and membrane proteins: We can predict the cellular location of many bacterial proteins by scanning the proteome for conserved secretion signals (**Tat**, **Sec**) or for domains found in secreted proteins. Candidate autotransported proteins can be identified by searching for Pfam domains - **Autotransporter (PF03797)**, **VacA (PF02691)** and **ShlB (PF03865)**. Other secretion pathways, such as the type I, type III and type IV pathways have less well-defined signals, but candidates can be identified by proximity, regulation and by functional analyses. We have used a wide range of bioinformatic tools to profile the secreted proteins of *Pseudomonas* genomes (Preston et al. 2005). Information on cellular location can be used to identify candidate extracellular and membrane proteins that are involved in interactions with the external environment and with plants and plant-colonising microorganisms, and can be integrated with other domain analyses to identify novel and niche-specific proteins for future analysis.

Global regulatory networks: A final bioinformatic approach for analysing gene function is to identify how genes are regulated. Identification of genes co-regulated by the alternate sigma factor HrpL has proved an efficient means of identifying virulence genes and secreted proteins in *Pseudomonas syringae* genomes (Fouts et al. 2001; Chang et al. 2005). The majority of these genes have a conserved cis-acting motif known as a “Hrp box” in their promoters, which can be identified from genome sequence data using Hidden Markov Models (HMMs). We are using HMMER software such as Promscan to identify regulatory elements in the promoters of *Pseudomonas* genes (Studholme and Dixon 2003, 2004; Preston et al. 2005; <http://www.promscan.uklinux.net/>). We can use promoter prediction data to build hypotheses as to which environmental and physiological factors affect gene expression *Pseudomonas*; to interpret phenotypes associated with regulatory mutants; to connect genes of unknown function with genes of known function in a common regulon, and thereby formulate hypotheses about function; and to identify candidate secreted proteins as discussed above.

## Conclusions

Bioinformatic predictions and comparative genomic analyses can be used to identify over- and under-represented domains, unique domain architectures, secreted proteins and co-regulated genes from bacterial genome sequence data. These analyses provoke new hypotheses regarding the evolution and function of bacterial genes, and identify new targets for directed mutagenesis and biochemical investigations. Bioinformatic approaches can be combined with high-throughput metabolomic, phenoarray and functional genomic technology to profile both bacteria and their plant habitats, and to

generate hypotheses about the role of niche-specificity and natural selection in generating *Pseudomonas* genome features.

Genomic and 'omic analyses can benefit applied research into bacterial biocontrol in a wide variety of ways. These include improved techniques for pathogen detection and multi-locus sequence-typing, risk analyses of candidate biocontrol agents, identification of novel elicitors of induced resistance, and identification of novel bioproducts for pathogen control. In addition, 'omic technology can be used to investigate the mode of action of biotic and abiotic anti-bacterial treatments and to develop assays to screen for novel anti-microbial compounds and biocontrol agents. A final application will be to revisit the question of why some bacterial biocontrol agents are more effective than others in the field or in artificial formulations, and to use this information to devise and optimise strategies for effective, safe and durable disease control.

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## Monitoring the biocontrol agent of fire blight *Pseudomonas fluorescens* EPS62e, by means of Real-Time PCR

### Abstract

Real-Time PCR has been developed in order to detect and quantify the biological control agent of fire blight *Pseudomonas fluorescens* EPS62e after field release. RAPD and U-PCR were used to find natural genomic markers within the EPS62e genome which differentiate the biocontrol strain against other *P. fluorescens* strains. Two differential amplified fragments were characterized as SCAR markers, and no similarities against known sequences from the GenBank database were found. Two SCAR primer pairs were designed, the SCAR 450 primer pair which amplified a 177 bp fragment and the SCAR 900 primer pair which amplified a 392 bp fragment. Both primers were selected for their specificity against EPS62e, as they amplified neither the 162 strains of *P. fluorescens* tested, nor the 71 strains of other closely related species analysed. Then, a Real-Time PCR was designed within each SCAR sequence developed, minimising the length of the amplification product and designing a TaqMan<sup>®</sup> probe for each fragment. The specificity of both designs was verified. Finally, the new molecular monitoring method was validated against a classical microbiological monitoring method based on dilution plating on selective media and colony forming units counting. The experiment was carried out on blossoms of Golden apple trees in the field and on detached branches in the greenhouse. Blossoms were sprayed with a EPS62eNal mutant suspension and were periodically sampled. Each sample was doubly evaluated by means of Real-Time PCR and dilution plating methods. There were no significant differences between both techniques regarding to the EPS62e population level estimated. Moreover, the biocontrol agent colonised and survived well on apple flowers in field conditions, reaching population values between 10<sup>7</sup> to 10<sup>8</sup> cfu/blossom and remaining stable at this level in immature fruit 55 days after inoculation.

### Introduction

Fire blight is a serious bacterial disease caused by *Erwinia amylovora* that affects a wide range of hosts belonging to the rosaceous family. Traditionally, the disease has been controlled by chemical bactericides such as copper derivative compounds or certain antibiotics. Nevertheless, consumer concerns about pesticide residues in food have favoured the development of alternative methods like biological control. *Pseudomonas fluorescens* EPS62e was selected in a screening procedure for its high efficacy in controlling *E. amylovora* infections on immature pear fruit, flowers, and whole plants (3,13). The registration for future commercialisation of a new biological control agent involves the development of monitoring methods able to assure its specific detection and quantification after field release (12). Monitoring techniques will also provide with information about the impact of the formulation, application techniques and environmental conditions on the ecological behaviour of the biological control agent. Culture-based methods such as cfu counting or most probable number estimations have been commonly used to monitor microorganisms. Nevertheless, they present a lack of specificity when working with biological control agents since many of them belong to species that are common inhabitants of plants (8). Therefore, spontaneous mutants resistant to certain antibiotics have been used to achieve discrimination at the strain level (2,7).

Molecular monitoring methods based on the detection of nucleic acid targets have largely enhanced the discrimination level compared to culture-based methods. The development of PCR-markers can be achieved by using techniques able to detect polymorphisms such as random amplified polymorphic DNA (RAPD). This technique provides a pattern of amplified products that can differentiate the target strain from others. However, the use of RAPD requires the isolation of the target strain, and several studies have improved the detection by the development of sequence specific amplified regions (SCAR) (4,6). Even though a SCAR PCR-marker of the biological control agent can assure its specific detection, it did

not allow its quantification. Therefore, other molecular methods providing quantification have been developed, such as quantitative competitive PCR (QC-PCR) (14,15) and more recently Real-time PCR (1,10). Real-time PCR has the advantage of quantifying the amplified products at the beginning of the amplification reaction, where small differences in the starting quantity of different samples can be detected.

The aim of this work was to develop a molecular monitoring method for the biological control agent *P. fluorescens* EPS62e based on the detection of SCAR specific molecular markers, the development of a Real-time PCR within the SCAR marker and its validation against a culture-based method during apple blossom colonisation under greenhouse and field conditions.

## Materials and methods

**Bacterial strains, growth media and DNA extraction:** Strains used in this work are listed in table 1. For routine use, bacteria were cultured in Luria-Bertani agar (LB) at 25 °C for 24 h. Media was supplemented with 50 mg L<sup>-1</sup> of nalidixic acid when working with the spontaneous mutant *P. fluorescens* EPS62e Nal and with 50 mg L<sup>-1</sup> of econazole to prevent fungal growth from plant extract samples. DNA was extracted by isopropanol precipitation as described by Llop et al. (9)

Development of SCAR markers:

RAPD and unspecific PCR (U-PCR) were performed to detect molecular polymorphisms that differentiated *P. fluorescens* EPS62e from 8 other *P. fluorescens* strains. U-PCR differed from RAPD because it used unspecific primer pairs with a longer but not randomised sequence. Several RAPD and U-PCR primers were tested using the thermal cycle conditions described by Moënne-Loccoz et al. (11) with minor modifications (13). Two differential amplified fragments of approximately 900 and 1000 bp were selected from the RAPD and U-PCR patterns, excised from agarose gel, cloned and sequenced for SCAR primer designs. The sequences obtained were compared to the known sequences from GenBank database to find possible similarities. Then, a primer pair was designed for each sequence, SCAR 450F (5'-GGCGCGCAACTGCTTT), SCAR 450R (5'-CGGTTAGATCCGACAAGATTAGAG), SCAR 900F (5'-CTCGCGTTGAGAGCAGAGAAC) and SCAR 900R (5'-TGGGACTATCGCTCACCATTG). The specificity of SCAR 450 and SCAR 900 was tested with 162 strains of *P. fluorescens*, 75 strains of closely related species (Table 1) and 61 field samples from pear plant extracts collected in a commercial orchard at Zaragoza (Spain).

**Development of Real-time PCR:** Two primer sets and TaqMan® probes were designed into each SCAR fragment. A SCAR primer was maintained and a new one was designed to minimise the amplified fragment length and optimise Real-time PCR. The reaction was carried in a final volume of 20 µL containing 1× PCR TaqMan Buffer A (PE Applied Biosystems), 6 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 0.3 µM of each primer, 0.2 µM probe, 1 U AmpliTaq Gold DNA Polymerase (PE Applied Biosystems) and 1 µL of the extracted DNA. The thermocycle conditions consisted of an initial denaturation step at 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Specificity of Real-time PCR was tested against 67 *P. fluorescens* strains and 30 strains of closely related species (Table 1). Finally, three standard curves were developed based on EPS62e cells diluted in plant extract (type A), EPS62e purified DNA diluted in plant extract (type B) and EPS62e purified DNA used directly in PCR (type C). Their accuracy in quantification was then compared.

**Tab. 1** Bacterial strains

Species	Code	Origin <sup>a</sup>
<i>Erwinia amylovora</i>	662, 665	UPN
<i>E. amylovora</i>	EPS101, EPS102	UdG
<i>Pantoea agglomerans</i>	15 strains isolated from rosaceous plants	UdG
<i>P. agglomerans</i>	850	CECT
<i>Pseudomonas corrugata</i>	124T	CECT
<i>Pseudomonas fluorescens</i>	EPS62e, EPS62e Nal	UdG
<i>P. fluorescens</i>	157 strains isolated from rosaceous plants	UdG
<i>P. fluorescens</i>	Q2-87	USDA
<i>P. fluorescens</i>	CHAO	IPS

Species	Code	Origin <sup>a</sup>
<i>P. fluorescens</i>	JBR1-70	WAU
<i>Pseudomonas putida</i>	324T, 385, 845, 4064, 4518, 4584, 4633	CECT
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	45 strains isolated from rosaceous plants	UdG
<i>P. syringae</i> pv. <i>tomato</i>	DC3000	(5)
<i>P. syringae</i> pv. <i>phaseolicola</i>	3635-95	UPN
<i>Ralstonia solanacearum</i>	125	CECT
<i>Shewanella baltica</i>	323T	CECT

<sup>a</sup>UPN, Universidad Pública de Navarra (Spain); UdG, Universitat de Girona (Spain); CECT, Colección Española de Cultivos Tipo (Spain); USDA, United States Department of Agriculture-Agricultural Research Service (USA); IPS, Institute of Plant Sciences (Switzerland); WAU, Wageningen Agricultural University (The Netherlands)

**Validation of Real-time PCR for monitoring *P. fluorescens* EPS62e on apple blossoms:** Monitoring assays were performed on blossoms of Golden Delicious apple cultivar at Maine-et-Loire (France) under greenhouse and field conditions. Three independent trials were carried out under greenhouse conditions, where branches containing dormant flower buds were collected and allowed to bloom in 1% sucrose at 22°C and natural light. Immediately after blossom, flowers were sprayed with a EPS62e NaCl suspension at 10<sup>8</sup> cfu mL<sup>-1</sup> until run-off point. A field trial was performed by spraying a set of trees during bloom with a EPS62e NaCl suspension at 10<sup>8</sup> cfu mL<sup>-1</sup>. Periodically, flowers were collected and homogenised in an extraction buffer (0.14 M NaCl, 0.26 M NaH<sub>2</sub>PO<sub>4</sub>·2 H<sub>2</sub>O, 0.75 mM Na<sub>2</sub>HPO<sub>4</sub>·12 H<sub>2</sub>O, 2% PV.P-10, 1% mannitol, 10 mM ascorbic acid, 10 mM L-glutathione reduced) for 60 s. All samples were doubly analysed by Real-time PCR and the dilution plating method in selective media.

## Results

**Development of SCAR markers:** RAPD and U-PCR gave rise to two fragments which clearly differentiated EPS62e from the other *P. fluorescens* strains analysed. The sequences of the two fragments did not show significant similarities to known sequences from the GenBank database. The SCAR 450 and SCAR 900 primer pairs designed within the RAPD and U-PCR sequences showed a single amplification product with EPS62e DNA of 177 and 392 bp, respectively. Both SCAR primer pairs were specific for EPS62e, because they did not amplify any of the 162 *P. fluorescens* strains, the 75 strains of other related species, and the 61 field samples analysed.

**Development of Real-time PCR:** Two designs of Real-time PCR were performed within the SCAR markers, showing a good amplification signal with C<sub>T</sub> values between 15 and 16, when 10 ng of EPS62e purified DNA were used. Three calibration curves for EPS62e quantification were developed and compared. Type A standard curve, based on dilution of EPS62e cells in plant extract prior to DNA extraction, was chosen because it showed high efficiency and a good linearity over a five-log range ( $y = 35.25 - 3.12x$ ) with a correlation coefficient ( $R^2$ ) of 0.994.

**Validation of Real-time PCR against cfu cell counting method:** There were no significant differences between Real-time PCR and cfu cell counting ( $P > 0.05$ ) in estimations of EPS62e NaCl population levels on apple blossoms, regardless whether the assay was performed under greenhouse (Table 2) or field conditions (Table 3). The correlation of values obtained from both techniques was plotted for the greenhouse and field trials, and good fit was obtained ( $\log(\text{Real-time PCR}) = 1.64 + 0.72 \log(\text{cfu counts})$ ;  $R^2 = 0.868$ ;  $P < 0.0001$ ). The population level of EPS62e NaCl increased or was maintained high upon inoculation reaching values from 10<sup>7</sup> to 10<sup>8</sup> cfu or cells per blossom.

**Tab. 2** Monitoring of colonisation of apple blossoms by *P. fluorescens* EPS62e under greenhouse conditions

Time days	Trial 1		Trial 2		Trial 3	
	Plating Mean Log cfu ± SD	Real-time PCR Mean Log cells ± SD	Plating Mean Log cfu ± SD	Real-time PCR Mean Log cells ± SD	Plating Mean Log cfu ± SD	Real-time PCR Mean Log cells ± SD
1	7.24 ± 0.04	7.11 ± 0.11	7.27 ± 0.16	7.11 ± 0.11	7.33 ± 0.35	7.11 ± 0.11
3	7.46 ± 0.18	7.29 ± 0.14	7.25 ± 0.25	7.29 ± 0.14	7.08 ± 0.26	7.29 ± 0.14
7	6.84 ± 0.29	6.66 ± 0.17	6.88 ± 0.24	6.66 ± 0.17	6.57 ± 0.13	6.66 ± 0.17

**Tab. 3** Monitoring of colonisation of apple blossoms by *P. fluorescens* EPS62e under field conditions

Trial 4		
Time days	Plating Mean Log cfu ± SD	Real-time PCR Mean Log cells ± SD
1	6.88 ± 0.22	6.65 ± 0.13
6	7.88 ± 0.25	7.99 ± 0.19
17	8.14 ± 0.28	8.11 ± 0.18
55	7.60 ± 0.14	7.62 ± 0.31

## Discussion

The development of *P. fluorescens* EPS62e as a new biological control agent against fire blight has revealed the need to have available monitoring methods to study its environmental fate after field release. Traditionally, culture-based methods for monitoring biological control agents have been developed by means of plating a spontaneous mutant of the wild type strain, resistant to an antibiotic, on selective media, such as EPS62e Nal (2,7). However, an antibiotic resistant genotype is a limitation for the registration and commercialisation of a biological control agent, thus the wild type strain without modifications is preferred. Therefore, molecular monitoring methods targeting natural genomic markers were developed for EPS62e.

RAPD and U-PCR were useful techniques to find polymorphisms within EPS62e genome. Two suitable fragments were selected and converted into SCAR markers which allowed the unambiguous detection of EPS62e from a heterogeneous sample, avoiding the need to culture and isolate the target strain (13). The quantification method was performed by designing a Real-time PCR into the SCAR specific sequences. Even though the SCAR primers were specific for EPS62e amplification, specificity was verified again in Real-time PCR because of the change of one primer. A fluorescent signal was not observed when non-target DNA of different strains was used. The standard curve of type A, based on EPS62e cells mixed with plant extract before DNA extraction, was chosen because it showed high efficiency and good fit. As in the present work, the standard curve made from cells instead of DNA has been commonly used since it involves the DNA extraction efficiency, the possible presence of PCR inhibitors and the quantification of cells is not extrapolated from DNA quantity (1,10).

Real-time PCR was validated against cfu cell counting for monitoring EPS62e in blossoms and immature fruit since no significant differences were observed in the population level estimated by both techniques. Monitoring assays performed in blossoms confirmed that EPS62e efficiently colonised flowers and immature fruit, reaching high population levels even during fruit growth in fields 55 days after inoculation. This is in accordance with the fact that colonisation ability is one of the main factors involved in *E. amylovora* biocontrol (3).

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## Characterization of an inhibitory strain of *Pseudomonas syringae* pv. *syringae* with potential as a biocontrol agent of bacterial blight on soybean

### Abstract

*Pseudomonas syringae* pv. *syringae* 22d/93 (Pss22d) produces syringomycin, syringopeptin and one unknown toxin, which inhibits *Pseudomonas syringae* pv. *glycinea* (Psg) *in vitro*. The unknown toxin was detected by the Psg growth inhibition assay. Since growth inhibition was reversed by arginine but not by argininosuccinate, it was suggested that the unknown toxin could interfere with this step of citrulline/arginine biosynthesis. In order to identify the genes responsible for production of the unknown toxin a random Tn5-mutagenesis was conducted. After screening via inhibition assays we found 20 phenotypes of Pss22d mutants: 10 did not produce the unknown toxin, 8 produced less than the wild type and 2 produced more than the wild type. Furthermore we are planning to analyse the chemical structure of the unknown toxin.

Via marker-exchange mutagenesis in genes coding for non-ribosomal protein synthesis responsible for initial steps in biosynthesis of syringomycin (*syrE*) and syringopeptin (*sypA*), we constructed different toxin-negative mutants. A double mutant will be constructed later. The aim of this study was to determine the role of toxins in the antagonism *in vitro* as well as *in planta*.

### Introduction

The ability of naturally occurring microorganisms to inhibit growth or metabolic activity of pathogens can be used to control plant diseases in agriculture and horticulture (Ji and Wilson 2003; Mascher et al. 2003). Biological control agents of this nature offer an alternative to conventional pesticides.

The focus of our research is the biological control of bacterial leaf spot diseases using resident bacterial epiphytes of the target plants. We investigate the antagonism between the pathogen of bacterial blight Psg and the epiphytic bacterium Pss22d on soybean. Pss22d has a strong potential to control bacterial blight. It has been demonstrated successfully *in vitro*, *in planta*, and under field conditions (Völksch and May 2001). The mechanisms of antagonistic action remains unknown. Three widely recognized mechanisms of microbial control mediated by biocontrol organisms are production of siderophores (Bultreys and Gheysen 2000), production of toxins (Cirvilleri et al. 2005) and competition for nutrients and area on the leaf surface (Figure 1).

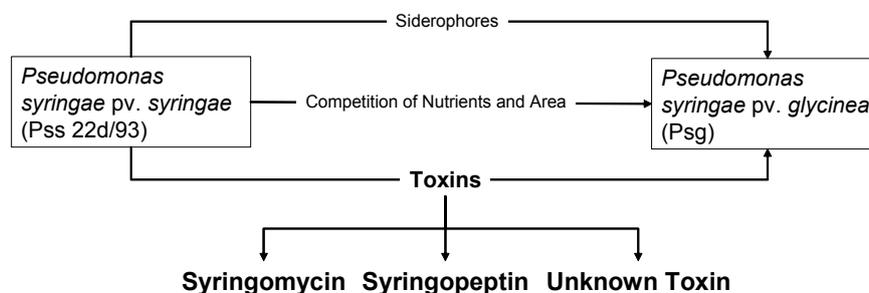


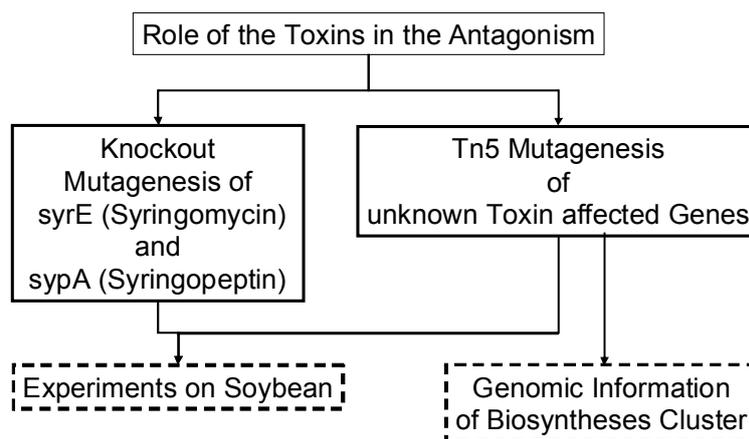
Fig. 1 Three possible mechanisms of antagonism between the biocontrol organism and the pathogen

Herein we studied the toxin production as a potential mechanism in the antagonism between Pss22d and Psg. Simultaneously, siderophore production was being analysed along with the group of M. Ullrich (IU Bremen) as a second plausible explanation for this antagonism. Pss22d produces three different active

substances: syringopeptin, syringomycin and an unknown toxin. Syringomycin and syringopeptin are known to be produced by *Pss* strains and are well described in the literature (Bender 1998; Scholz-Schroeder et al. 2001a). To determine the potential role of each toxin in the antagonism we designed toxin negative mutants to be tested on soybean plants under greenhouse conditions. Furthermore we are planning to elucidate the chemical structure and biosynthetic pathway of the unknown toxin.

## Methods

The gene clusters of syringomycin and syringopeptin are well known (Scholz-Schroeder et al. 2001b); this allowed a knockout mutagenesis of the genes *syrE* and *sypA* (Figure 2). Both genes are involved in the non-ribosomal protein synthesis of syringomycin and syringopeptin, respectively. Primers were derived from the related strain *Pss* B728a (*Pss*B728a). To determine syringomycin and syringopeptin production of *Pss*22d bioassays with *Geotrichum candidum* and *Bacillus megaterium* as indicator strains were used. A random mutagenesis of *Pss*22d was conducted to develop negative mutants of the unknown toxin. It was performed with the suicide plasmid pCAM140 (Wilson et al. 1995) by conjugation in *Pss*22d. Over 5000 *Pss*22d Tn5-mutants were screened in a bioassay with *Psg* as indicator strain to find unknown toxin negative mutants. The genomic information of these unknown toxin negative mutants was obtained via shotgun sequencing. In short, genomic DNA was digested with the enzyme *Sa*II followed by ligation in the vector pBBR1MCS and transformed in *Escherichia coli* DH5 $\alpha$ . For the spectinomycin resistance a gene located in the Tn5 transposon was screened. The plasmids of resistant clones were used to obtain the genes which had been deleted by the Tn5 transposon. All sequences derived were compared with the fully sequenced genome of the related strain *Pss*B728a.



**Fig. 2** To determine the role of each toxin in the antagonism between *Pss*22d and *Psg* toxin-negative mutants were generated using different methods. In addition, genomic information of the biosynthesis cluster from unknown toxin negative Tn5 mutants was obtained. (Dashed boxes represent work in process)

## Results and Discussion

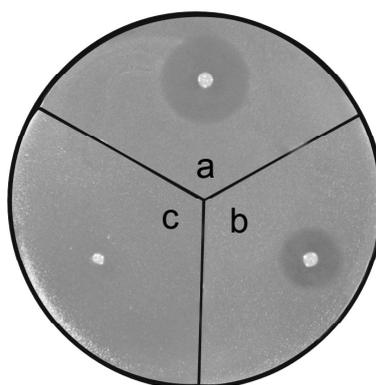
*Pss*22d was screened in growth inhibition assays and compared to the well-characterized related strains *Pss*B728a and *Pss*B301D. The fungus *G. candidum* and the bacterial species *B. megaterium* are known as indicator strains for syringomycin and syringopeptin, respectively. *Pss*22d, *Pss*B728a, and *Pss*B301D showed growth inhibitions in these bioassays. The unknown toxin was identified *in vitro* by bioassays with *Psg* as indicator strain. Of the three strains tested only *Pss*22d inhibited *Psg in vitro*. We, therefore, assume that the unknown toxin is produced only by *Pss*22d.

All generated knockout mutants were verified in these bioassays to control toxin production (Table 1). The single mutants *Pss*22d $\Delta$ *syrE* and *Pss*22d $\Delta$ *sypA* did not produce syringomycin and syringopeptin, respectively. The double mutant *Pss*22d $\Delta$ *syrE/sypA* produced the unknown toxin only.

**Table 1** Phenotype of all knockout mutants, tested with bioassays with (1) *Geotrichum candidum*; (2) *Bacillus megaterium*; (3) *Pseudomonas syringae* pv. *glycinea*

	Syringomycin <sup>1</sup>	Syringopeptin <sup>2</sup>	Unknown Toxins <sup>3</sup>
Pss22dΔsypA	+	–	+
Pss22dΔsyrE	–	+	+
Pss22dΔsypA/syrE	–	–	+

The growth inhibition of *Psg* caused by *Pss22d* was compensated by arginine (Figure 3). Neither the direct precursor of arginine, argininosuccinate, nor any other precursor in the arginine biosynthesis compensated the inhibition. These results suggest that the unknown toxin affects the biosynthesis of arginine. We suppose that the possible target enzyme is argininosuccinase, which converts argininosuccinate to arginine. A similar mechanism is well described for phaseolotoxin (Bender et al. 1999).


**Fig. 3** Bioassays with *Pss22d* and *Psg* as indicator strain; (a) without amino acid, (b) with 1mM argininosuccinate, (3) with 1 mM arginine

**Tab. 2** Analysed Tn5 mutants by shotgun sequencing, sequence information were compared with the related strain *PssB728a*. Toxin production was analysed with bioassays. UT-unknown toxin; SM-syringomycin; SP-spectinomycin

Mutant	Locus <i>Pss 728a</i>	Product	UT	SM	SP
Tn5_21/15	psyr_3708	sensor histidine kinase PhoQ, putative	-	-	+
Tn5_20/34	psyr_3698	histidine kinase, GacS	-	-	+
Tn5_23/2	psyr_5056	regulatory protein, GntR	-	-	+
Tn5_15/17	psyr_5056	regulatory protein, GntR	-	-	+
Tn5_39/15	psyr_4858	Peptidase, S45	-	-	+
Tn5_16/3	psyr_4739	tRNA (guanine-N(7)-)-methyltransferase	-	-	+
Tn5_30/20	psyr_0118	hypothetical protein	-	+	-
Tn5_35/13	psyr_1325	Protein of unknown function DUF548	+/-	+/-	+
Tn5_37/15	psyr_0660	regulatory protein, ArsR	+/-	+	+
Tn5_41/23	psyr_5009	hypothetical protein	++	++	+/-
Tn5_22/20	psyr_1749	Peptidase S16, ATP-dependent protease La	++	++	+/-

The Tn5 mutants were screened in all growth inhibition assays to check if syringomycin, syringopeptin, or the unknown toxin were still being produced (Table 2). Phenotypes of all Tn5 mutants were obtained and analysed by shotgun sequencing. The majority of the unknown toxin negative mutants did not produce syringomycin, but the sequence information showed that, in these cases, regulatory genes were deleted. In mutant Tn5\_21/14 the Tn5-transposon was inserted into the *phoP-phoQ* regulator system. This major regulator controls over 40 genes, including virulence (Miller et al. 1989) and lipopolysaccharide synthesis (Guo et al. 1997). In Tn5\_39/15 the transposon was inserted in a gene of a peptidase of the family S45. This penicillin amidase is involved in the pathway of penicillin (Valle et al. 1991). The relationship between the pathway of syringomycin and the unknown toxin is still unclear. Additionally, we found mutants with increased production of the unknown toxin and syringomycin in

contrast to the wild type *Pss22d* (Tn5\_41/23; Tn5\_22/20). It seems that both deleted genes are negative regulators of syringomycin and the unknown toxin. The results of the phenotypic analyses of the Tn5 mutants suggest that the regulation of syringomycin and the unknown toxin is related. None of the Tn5 mutants showed a phenotype that suggested that only the unknown toxin production was affected. This finding minimizes the chance to get information on the biosynthesis cluster.

It is known that the wild type *Pss22d* effectively suppresses *Psg*, *in vitro*, *in planta*, and under field conditions. *In planta* experiments with the constructed mutants will be carried out under greenhouse conditions. For this purpose leaves of soybean plants will be co-inoculated with toxin-negative mutants and *Psg* by prick-technique (May et al. 1997). Furthermore, the biosynthesis genes and the structure of the unknown toxin will be identified.

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## Influence of siderophore production on biological control among *Pseudomonas syringae* strains

### Introduction

Competition for limiting nutrient resources is a fundamental ecological principle. In biological control systems the pathogen and its antagonistic control agent have to compete for nutrients and space. In this respect the competition for iron is of significant importance (7,11). Although iron is quite abundant on earth, it is not directly available due to the low water solubility of Fe<sup>3+</sup> at neutral pH. Bacteria circumvent this problem by the production of siderophores molecules of low molecular weight that chelate and thus solubilize the iron. Organisms vary in quality and quantity of the siderophores produced, and an antagonist with a siderophore of higher affinity will be able to out-compete a rivaling pathogen. The importance of siderophore production has been proven for many rhizospheric biocontrol organisms, for example for different *Pseudomonas fluorescens* strains capable to control the fungus *Gaeumannomyces graminis* var. *tritici*, the causal agent of take-all disease of wheat (5), or for the control of *Pythium*-induced damping-off and *Botrytis cinerea* infection of tomato by *Pseudomonas aeruginosa* (1,3,4).

In contrast to these rhizosphere biocontrol systems, the biological control of the soybean pathogen *Pseudomonas syringae* pv. *glycinea* (*Psg*) by *P. syringae* pv. *syringae* strain 22d/93 (*Pss22d*) takes place in the phyllosphere (13). It is not known whether, in this completely different setting, control mechanisms like scavenging of iron do apply in a similar way. Prior studies could demonstrate the effectiveness of *Pss22d* against *Psg* in greenhouse experiments and under field conditions (8,12). The stability of this biological control system in various environments provides an optimal setup for further investigations on the underlying principles. It is the aim of this study to investigate the impact of siderophore production on the biological control of *Psg* by *Pss22d*.

### Results and discussion

The antagonist *Pss22d* and the pathogen *Psg* both belong to the group of fluorescent pseudomonads producing the peptide-type siderophore pyoverdinin (2). Nevertheless, they show clearly distinct iron chelating phenotypes. When tested on CAS agar, a general indicator medium for siderophore production (10), the antagonist produces a much larger siderophore halo than the pathogen, in comparison.

A pyoverdinin-negative mutant of the antagonist *Pss22d* was generated by marker exchange mutagenesis, resulting in strain *Pss22dΔpv.sA*. As expected, this strain was lacking the fluorescence of pyoverdinin. However, it still formed a halo on CAS agar, indicating the presence of a second siderophore. In order to identify the second siderophore, a transposon mutagenesis was conducted in *22dΔpv.sA*. Out of 500 Tn5-mutants of *Pss22dΔpv.sA*, three siderophore-negative mutants were obtained (*Pss22dΔsid*). The transposon-flanking region of these mutants was subcloned and sequenced. BLAST analysis showed considerable similarities towards sequences of achromobactin biosynthetic genes. Achromobactin is a citrate-type siderophore produced by *Dickeya chrysanthemi* (9). This implies that *Pss22d* produces a similar citrate-type siderophore beside of pyoverdinin. To investigate the impact of the achromobactin-like siderophore on the epiphytic fitness of *Pss22d*, a marker exchange mutant of *Pss22d* deficient for this siderophore was constructed (strain *Pss22dΔAchr*).

To verify the phenotypes of the respective mutants, siderophore production of *Pss22d* wt and its respective mutants was compared by isoelectric focusing (IEF) and CAS overlay (6). The IEF pattern of *Pss22d* wt showed three CAS-active bands. Two of them corresponded to reference pyoverdinin (courtesy of Prof. J. M. Meyer, Strasbourg, France) and the third corresponded to purified achromobactin. The phenotypes of the siderophore mutants matched their genotypes. Surprisingly, the comparison of the IEF

pattern of antagonist *Pss22d* and the pathogen *Psg* revealed identical siderophore pattern. The pathogen produces the same type of pyoverdins as the antagonist and it also produces the achromobactin-like siderophore.

This result excludes a direct role of competition for iron in the biological control of *Psg* by *Pss22d*. Both strains can cross utilize the siderophores of each other.

Nevertheless, siderophore production might have a significant impact on the epiphytic fitness of the antagonist, it might enable *Pss22d* to colonize the soybean leaf and outcompete other epiphytic bacteria. To assess this question, soybean leaves were inoculated with either the *Pss22d* wt or one of the three siderophore mutants by wound inoculation. The survival rates and population densities of the strains were monitored. Surprisingly, there was no difference between the strains, not in survival and not in growth. Even strain *Pss22d*Δ*sid*, which does not produce any siderophore at all, performed as well as the *Pss22d* wt. This indicates that the bacteria did not suffer iron limitation during this experiment. It is possible that the wound inoculation procedure leads to an iron leakage from the leaf, thus this experiment needs to be confirmed by a different inoculation technique like spray inoculation.

Our results indicate that siderophore production is not a primary determinant for the antagonism between *Pss22d* and *Psg*. Other parameters like toxin production or niche exclusion could provide the basis for the biological control abilities of *Pss22d*. Yet, it is important to determine these parameters. Only the knowledge about the mode of action of a biological control agent can guarantee a predictable and reproducible outcome of its application.

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## Characterization of the inhibitory strain *Pantoea* sp. 48b/90 with potential as a biocontrol agent for bacterial plant pathogens

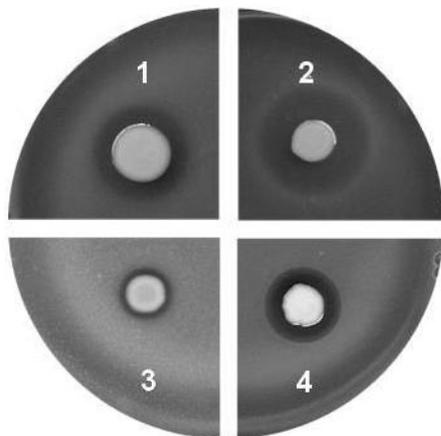
### Introduction

Aerial leaf surfaces are habitats for epiphytic bacteria which are able to grow and survive on plants. Many of these saprophytic bacteria can antagonize phytopathogenic bacteria by inhibition of their growth or metabolic activity. Naturally occurring antagonists can be used to control plant diseases in agri- and horticulture and offer an interesting alternative to conventional pesticides.

*Pantoea* sp. 48b/90 (former *Erwinia herbicola*) is a naturally occurring epiphyte, which was isolated from a soybean leaf. Strains belonging to *E. herbicola* are members of the *E. herbicola-Enterobacter agglomerans* cluster; some have been re-designated *P. agglomerans* or *P. dispersa*, while others did not fall into either of the two species (Gavini et al. 1989). The taxonomic position in the genus *Pantoea* of the former *E. herbicola* strains inhabiting plant surfaces remains problematic.

### Results and discussion

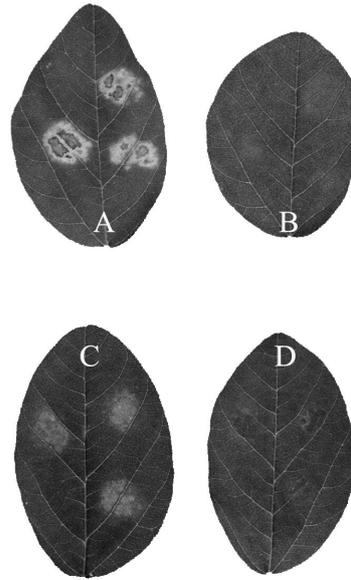
Antagonistic activities: Strain 48b/90 showed clear antagonism against a wide range of bacteria, including a number of plant pathogens in vitro by agar-diffusion assay on a mineral salts medium (MM). It was active against 21 of the 29 bacteria tested. Almost all of the 15 different phytopathogenic species/pathovars tested were inhibited except *Burkholderia gladioli* and *Pseudomonas cichorii*. As a representative example the inhibition zones for 4 selected phytopathogenic strains are shown in Figure 1.



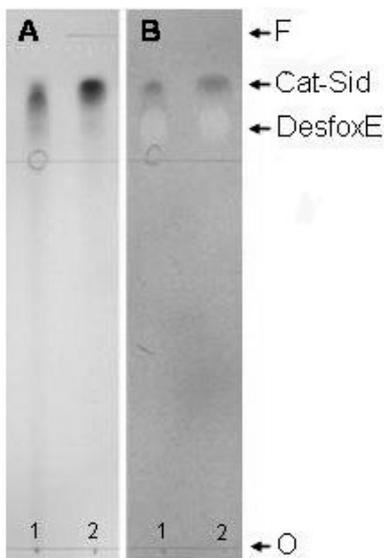
**Fig. 1** Growth inhibition zones on MM-agar caused by *Pantoea* sp. 48b/90 on a lawn of:  
1: *Pseudomonas syringae* pv. *glycinea* 8/83;  
2: *Erwinia amylovora* 273;  
3: *Agrobacterium tumefaciens* 30150;  
4: *Pseudomonas syringae* pv. *morum-prunorum* 886 (48b/90 were spot-inoculated)

An essential prerequisite for bacteria that could be used for biological control is its epiphytic fitness (Lindow 1998). Strain 48b/90 showed good epiphytic fitness. After adaptation it had the ability to establish and maintain stable epiphytic populations on soybean plants (about  $5 \times 10^4$  cfu/g FW) over the entire growing season after a single spray inoculation. Besides, it was able to reduce the development of *Pseudomonas syringae* pv. *glycinea* (*Psg*) in planta and inhibited the appearance of bacterial blight symptoms (greenhouse experiments) (Figure 2).

**Fig. 2** Comparison of symptom development in soybean leaves inoculated with varying ratios of *Psg* (P) and 48b/90 (A) after 7 days:  
**A:** *Psg* 33a/93 control  
**B:** 48b/90 control  
**C:** P:A=1:1  
**D:** P:A=1:9



Possible mechanisms for the antagonistic activities: Strain 48b/90 produced at least two different siderophores, the hydroxamate siderophore ferrioxamine E, a principal siderophore of *E. herbicola* (Berner et al. 1988), and an unidentified catechol siderophore during growth under iron limitation (Figure 3). The level of siderophores at 18 °C was about 3.0-fold in the catechol assay (Rioux et al. 1983) and 1.5-fold in the CAS assay (Schwyn and Neilands 1987) that at 28 °C after 48 h (Figure 4).

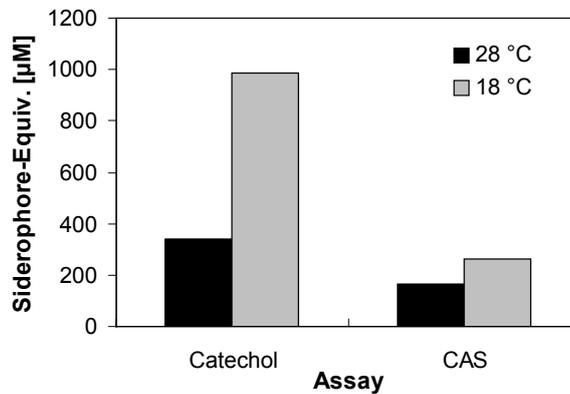


**Fig. 3** Thin layer chromatograms of SP-extracts (LiChrolut-RP18, Merck ) from 48b/90, grown in low-iron 5b medium for 48 h at 28 °C (1) and 18 °C (2). Chromatograms were developed in methanol:chloroform:acetic acid (90:5:5) and analyzed by spraying with 0,1 M FeCl<sub>3</sub> in 0,1 M HCl (A) and CAS-assay-solution (B). (F: solvent front, O: origin of migration, DesfoxE: Desferrioxamine E, Cat-Sid: catecholate siderophore of unknown structure)

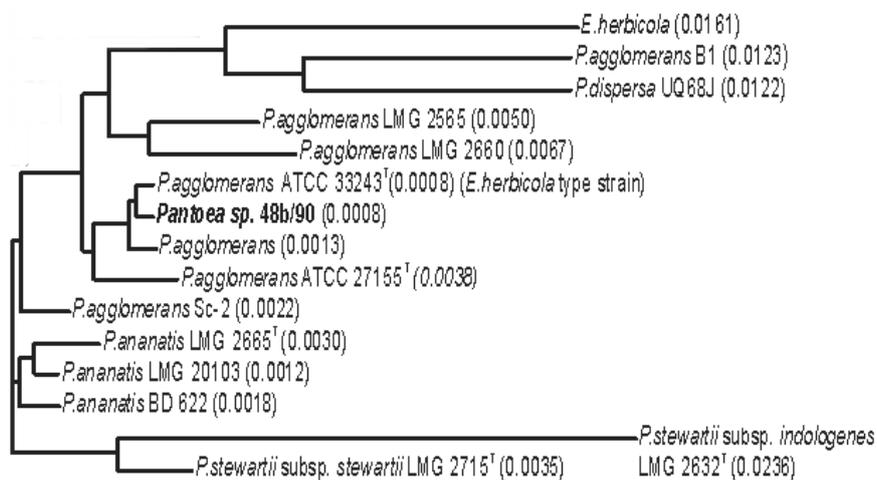
The nonpathogenic bacterium *P. agglomerans* (*E. herbicola*) has been found to produce a family of antibiotics which have different patterns of biological activity and chemical structures. The antibiotics pantocin A, pantocin B and the phenazine antibiotic D-alanyl-griseoluteic acid (AGA) are well-characterized. Pantocin A is inactive in the presence of histidine and pantocin B is inactive in the presence of arginine (Sutton and Clardy 2001); whereas AGA is not inhibited by addition of amino acids (Kearns and Hale 1996). *Pantoea sp.* 48b/90 produced a broad-spectrum antibiotic activity *in vitro* (Figure 1). The antibiotic activity was present in cell-free culture supernatant of late log/early stationary phase cultures of 48b/90 in synthetic media. This antibiotic activity was not inhibited by essential amino

acids (neither by histidine nor by arginine). It was stable to acidic and basic pH and was also stable at high temperature (100 °C). 48b/90 synthesized at least one still unknown toxin that inhibited the growth of a broad spectrum of phytopathogenic bacteria species *in vitro* and inhibited the development of bacterial blight symptoms on soybean plants in glasshouse trials. The antibiotic of 48b/90 is alike AGA. It has a broad spectrum of activity, is pH stable, is not inhibited by amino acids, in contrast to AGA it is heat stable.

**Fig. 4** Siderophore production of 48b/90 in low-iron Pipes medium after 48 h growth at 18 °C and 28 °C. (DFOM-equivalents = CAS-assay; DHBA-equivalents = Catechol-assay)



*P. agglomerans* or *Pantoea dispersa*, also known as *Erwinia herbicola* (Löhnis) Dye are ubiquitous in nature. Strains of this species produce a family of antibiotics in a poorly understood strain dependent fashion (Ishimaru et al. 1988; Wodzinski and Paulin 1994). The identification and characterization of antibiotic(s) produced by strain 48b/90 will provide important information for the development of new biocontrol agents and could constitute a biological agent against phytopathogenic bacteria.



**Fig. 5** Phylogenetic position of *Pantoea sp.* 48b/90 among members of the genus *Pantoea* in a dendrogram based on a comparison of nearly complete 16S rDNA sequences (Neighbor-joining analysis)

**Taxonomic characterization:** The phenotypic characteristics of 48b/90 are typical for *Enterobacteriaceae*. Based on the 16S rDNA sequences it belongs to the genus *Pantoea* (Figure 5). The actual genus *Pantoea* includes 7 described species (*agglomerans*, *ananatis*, *citrea*, *dispersa*, *punctata*, *stewartii*, *terrea*) (Brenner et al. 2005). The type strains of *Erwinia herbicola*, *Erwinia milletiae*, and *Enterobacter agglomerans* were assigned to *Pantoea agglomerans* (Kwon et al. 1997). 48b/90 shows 83 % sequence similarity with the type strain *P. agglomerans* ATCC 27155 and 95 to 99 % with the other strains examined. It differs from the type strains in some phenotypic characteristics (Table 1).

**Tab. 1** Differential phenotypic characteristics of strain 48b/90, the type strains *P. agglomerans* and *P. dispersa*

Characteristics	<i>Pantoea</i> sp. 48b/90	<i>P. agglomerans</i> *ATCC 27155 <sup>T</sup>	<i>P. dispersa</i> *ATCC 14589 <sup>T</sup>
Growth at 4 °C	⊕	(+)	⊕
Growth at 41 °C	+	⊕	+
Growth at 44 °C	+	⊕	⊕
Ornithine decarboxylase	⊕	⊕	+
Utilization of:			
Citrate	⊕	+	+
Inositol	⊕	(+)	+
Melibiose	+	⊕	+
Salicin	+	+	⊕
Hydrolysis of esculin	+	+	⊕
NO <sub>2</sub> production	+	+	⊕

+, positive reaction; (+), slow reaction; -, negative reaction; \* Data from Gavini et al. (1989)

The data presented herein and previously shown in other reports suggest that there are still other groups within the “*Enterobacter agglomerans* complex” which might also be included as additional species in the genus *Pantoea*. More data analysis of more strains is needed to confirm these observations and to clarify the taxonomic position of plant-associated members of the former *Erwinia herbicola* group.

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## VI. Biological control of fire blight

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### Current status of biological control against fire blight

Fire blight, caused by *Erwinia amylovora*, is a devastating disease in most regions where pome fruit are grown. The emergence of pathogen populations that are resistant to the standard control product, streptomycin, has driven the search for effective alternative materials. In response to this as well as to health concerns linked to the overuse of antibiotics, most European countries have banned or strictly regulate the use of streptomycin, making alternatives imperative. The research concerning the use of biological controls to manage fire blight has proven to be both promising and challenging. The field is growing rapidly and new products are being developed at a fast pace. This article provides a brief overview of the general types of biological control methods for fire blight control being studied by numerous international research groups. Representative examples within each group are discussed here, although many new and innovative products and methods are constantly emerging.

#### Antagonistic Bacteria

Antagonistic bacteria have been shown to provide protection against blossom blight infection by competing with the pathogen for space and nutrients on stigmatic surfaces (1). Most of the organisms included in this category were originally found growing naturally on the stigma of apple or pear blossoms. *Pseudomonas fluorescens* A506 ('BlightBan A506', Nufarm, USA) and *Pantoea agglomerans* P10c ('Blossom Bless', Gro-Chem, NZ) are currently registered products. Two strains of *P. agglomerans*, E325 ('Bloomtime Biological', NAP, USA) and C9-1 ('BlightBan C9-1', Nufarm, USA), are in the advanced stages of research. 'Bloomtime Biological' should be available to growers in the USA and Canada for the 2007 growing season. In addition to providing competition, *P. agglomerans* C9-1 produces an antibacterial metabolite which inhibits the growth of *E. amylovora*. In Germany, *Rahnella aquatilis* Ra39, a fresh water bacterial inhabitant, has shown promising efficacy in the field when applied with an aromatic compound (2). Researchers have also had successful disease control in the lab with non-pathogenic *Erwinia* species (3).

Successful disease control depends on maintaining sufficient population levels of the beneficial bacteria on stigmatic surfaces. Researchers are currently evaluating the use of additives and surfactants to optimize population size and stability (4). In regions where the use of streptomycin is permitted, these organisms provide an effective rotation product resulting in the reduction of antibiotic sprays per season.

#### Antagonistic Yeast

Yeast have also been shown to have antagonistic potential against *E. amylovora* when applied to blossoms. 'Blossom Protect' (Bio-Protect GmbH Konstanz, GER) is a mixture of two strains of *Aureobasidium pullulans* (CF10 and CF40) for use on pome fruit and ornamentals in Germany. These strains along with another yeast, *Metschnikowia pulcherrima* MSK1, make up the formulation called 'BPMC 2023' from the same company (5). Pathogen populations were significantly lowered when this formulation was applied to detached blossoms prior to artificial inoculation with the pathogen (5). The mode of action of these organisms may be through competitive exclusion as well as by antibiosis and will be further explored (5,6). Recent efficacy field studies demonstrated statistically similar blossom blight control of the formulation 'BPMC 2023' (two yeast strains reported) to streptomycin (7). Challenges facing the use of antagonistic yeast include 1) possible russet development caused by the biocontrol agents, 2) sensitivity to fungicides used in integrated programs and 3) variability in efficacy among cultivars (5).

### ***Bacillus subtilis* strains**

Three strains of *Bacillus subtilis* are available to growers for fire blight control in the USA and Europe. These organisms originate from soil, therefore, their mode of action is different from the antagonistic bacteria. They produce lipopeptide metabolites that exhibit antimicrobial activity. Coverage is the key to successful disease management with these products, as these metabolites are the most effective component. Some growers currently use *B. subtilis* strain QST 713 ('Serenade', Agraquest, USA) in the USA for blossom blight control. *B. subtilis* BD170 ('Biopro') is registered in Europe, while *B. subtilis* ceppo BSF4 (Agribiotech, ITA) is labeled for use on pear in Italy. Many researchers have demonstrated effective disease control of these products, especially when alternated with an application of an antibiotic. Some strains of this bacterium have been shown to colonize and spread among apple flowers within an orchard (8,9).

### **Bacteriophages**

A number of naturally occurring phage strains were isolated from plant tissue and soil throughout Michigan, identified and evaluated for their effectiveness to lyse *E. amylovora* cells in liquid culture (10). The pathogen was found to be highly sensitive to one particular strain, ΦEa116C. In order to utilize phage as directly applied control products, a suitable carrier host population, such as avirulent *E. amylovora*, must be successfully established prior to the application of the phage (11). Research in Canada is underway to develop a successful method to apply bacteriophage to apple trees for management of fire blight (12).

### **SAR-Inducers**

This family of biological controls work by stimulating the plant's systemic acquired resistance (SAR) pathways. SAR-inducers available include 'BION' or 'Actigard' (Syngenta, USA), acibenzolar-S-methyl, and 'Messenger' (Eden Bioscience, USA), harpin protein. Acibenzolar-S-methyl is not currently registered for use on apple in the USA, however, weekly applications have resulted in reduction of blossom blight statistically similar that from streptomycin (13). Harpin protein is registered for use on apple in the USA. It has shown to provide disease protection in some years (14) but has been ineffective in recent trials (15).

A number of plant products are currently being studied, including etheric oil of *Thymbra spicata*, called 'Biozell 2000B' (16). A second product, 'Physpe' or 'Vacciplant' (Agrimar, FRA), is a glucan from brown algae. There is some evidence of induced resistance from both of these products (17, 18).

### **Current Use of Products in USA**

Biological control agents are mostly used by growers in California, Oregon, Washington and Michigan in the USA. According to the 2003 USDA National Agricultural Statistics Service, the two main products used are 'BlightBan A506' and 'Serenade' (Table 1) (19). For example, 9% of total acres of apples in Washington were sprayed with oxytetracycline, 3% with streptomycin, 4% with 'BlightBan A506', and less than 0.5% with 'Serenade' (Table 2). Similarly, on pears in California, oxytetracycline was sprayed on 59% of total acres, streptomycin on 51% total acres and 'BlightBan A506' on 24% (Table 3). The trend seems to be that biological control agents are being utilized more heavily in areas with pathogen populations resistant to streptomycin, as oxytetracycline does not have the same effectiveness as streptomycin.

**Tab. 1** Reported uses of biological control agents in the USA in 2003\*

Crop	Organism	State			
		California	Michigan	Oregon	Washington
Apple	<i>Bacillus subtilis</i> QST 713		X	X	X
Apple	<i>Pseudomonas fluorescens</i> A506	X		X	X
Pear	<i>Bacillus subtilis</i> QST 713	X		X	X
Pear	<i>Pseudomonas fluorescens</i> A506	X		X	X

\* USDA, NASS, 2003 Fruit Summary

**Tab. 2.** Reported use of biological control agents on apples in Washington, USA in 2003\*

Product	Acres Applied (% of Total)	Total Pesticide Applied (kg)
<i>Bacillus subtilis</i> QST 713	<0.5	--
<i>Pseudomonas fluorescens</i> A506	4.0	953
Streptomycin sulfate	3.0	771
Oxytetracycline	9.0	1542

\* USDA, NASS, 2003 Fruit Summary

**Tab. 3.** Reported use of biological control agents on pears in California, USA in 2003\*

Product	Acres Applied (% of Total)	Total Pesticide Applied (kg)
<i>Pseudomonas fluorescens</i> A506	24.0	680
Streptomycin sulfate	51.0	1316
Oxytetracycline	59.0	2087

\* USDA, NASS, 2003 Fruit Summary

### Future Perspective

The future of biological control organisms looks promising in fire blight management. Sixty-five percent of the EPA-registered organisms were registered within the last 10 years (20). Thirty-six percent were registered over the last 5 years. Continuation of studies and identification of new strains and types of biological control organisms looks promising.

Challenges for further study include but are not limited to overcoming the naturally varying environmental conditions, and optimizing delivery systems and formulations.

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## Biological control of fire blight: an overview of the work carried out in New Zealand

### Abstract

Biological control of fire blight has been the focus of a major research programme in New Zealand for the last 15 years. During that time several strategies for the control of this bacterial disease of apple and pear have been investigated. Today, two commercial products have been generated through this programme. This manuscript presents an overview of the work and the strategies developed during that time.

Keywords: *Erwinia amylovora*, *Pantoea agglomerans* P10c, *Pantoea agglomerans* Eh252, *Pseudomonas fluorescens* A506, honey bees, essential oils, elicitors

### Introduction

Several factors contribute to uphold fire blight as the most devastating bacterial disease of apple and pear (10). Several new cultivars of apple introduced in the world are quite susceptible to this disease and some modern orchard management practices favour its development. But most importantly, few compounds are available for the control of bacterial diseases in general and of fire blight in particular. Most of these compounds are either antibiotics or heavy metals (4). Most countries where fire blight is present do not allow the use of antibiotics for control of plant diseases. Strains of the pathogen resistant to streptomycin have been isolated in countries where the use of this antibiotic is authorised: the USA, Israel and New Zealand (3). Copper containing compounds are widely used for the control of fungal and bacterial diseases; however problems of phytotoxicity and pathogen resistance are limiting the use of these compounds. The use of compounds that act as elicitors has been only a recent addition to the array of chemicals that can be used against bacterial diseases. Acylcyclohexanediones (prohexadione-calcium and trinexapac-ethyl) for example have only recently been shown to reduce incidence of fire blight on shoots (2) and more recently on flowers (5). The prospect of using biological control agents to limit the incidence of fire blight has been a very attractive option for many years, leading to the set up of several research programmes to develop strategies of control, which include the use of biological control agents.

### Fire blight disease

Fire blight is caused by the Gram-negative bacterium *Erwinia amylovora*. In early spring this bacterium is brought by insects, wind and rain to host plant flowers from the cankers where it overwinters (8). Colonisation of the stigmatic surfaces will lead, if climatic conditions are favourable, to blossom infection. Those early infections will induce production of exudate and necrosis. This exudate is mostly made of bacteria and represents the inoculum that will allow further flower or shoot infections during spring and summer. Preventing early blossom infection reduces the inoculum available for further infection and therefore should result in a major reduction of fire blight incidence throughout the growing season. Most experiments using biological control agents aim to reduce the number of early infections on apple or pear flowers.

### *Pantoea agglomerans* Eh252

Early work on biological control was done with several strains of non pathogenic epiphytic bacterium, mostly strains of *Pseudomonas fluorescens* or *Pantoea agglomerans* (ex *Erwinia herbicola*) isolated from apple or pear trees. The first experiments in research orchards in New Zealand were conducted with *P. fluorescens* strain A506 isolated from pear in California (USA) (20) and with *P. agglomerans* Eh252 isolated from apple in New York (USA) (18). The strain Eh252 had previously been shown in experiments conducted in New York and France to be one of the most effective strains to reduce fire

blight incidence (9). However, the level of control and the consistency of control were not as good as that obtained with streptomycin. In order to increase the degree and consistency of control achieved by Eh252, two different approaches were followed. The first approach was to study the mode of action of Eh252 with the view of using this knowledge to increase its efficacy; the other approach was to combine this strain with either another bacterial strain or a natural compound.

Like numerous other epiphytic bacteria, Eh252 inhibits the growth of *E. amylovora in vitro*. To determine whether production of this compound was responsible for the ability of Eh252 to reduce incidence of fire blight, transposon induced mutants which differed from the wild type strain only by their inability to produce this inhibitory compound were selected (18). The ability of these mutants to reduce fire blight incidence was then compared with that of the wild type strain using a modified immature pear fruit assay (16). The mutants were not able to reduce incidence of fire blight as well as the wild type strain (18), although at high concentration they were still able to prevent fire blight. Conversely, at low concentration the wild type strain could not prevent fire blight, suggesting that production of this compound was only one of the factors responsible for reduction of fire blight incidence, and that ability to colonise the infection court was also a very important factor. This was confirmed by repeating the experiment on apple flowers in growth chambers (9) and by comparing the ability to control fire blight of Eh252 with that of the mutants complemented for production of this inhibitory compound (16). This was also confirmed by field experiments carried out in the USA by Stockwell *et al.* (7).

The compound produced by Eh252 was found to be a small molecule with a proteinaceous component whose production was not regulated by the SOS system (15). Those characteristics define a class of compounds called microcins. That Eh252 produces a microcin was confirmed by DNA sequence analysis (15). Other strains of *P. agglomerans* have been found to produce microcin compounds (21). Some strains of *P. agglomerans* produce different microcins and different strains of *P. agglomerans* could produce the same or a similar microcin (21).

Several experiments in research orchards were carried out using *P. agglomerans* Eh252 and *P. fluorescens* A506 separately or in a mixture. These biological control agents were chosen because they respond differently to climatic conditions and were thought to reduce fire blight incidence by different mechanisms. When sprayed as a mixture onto apple flowers, these two strains did not inhibit each other and reduced fire blight to a level similar to the best strain sprayed by itself (19). No synergetic effect was observed. Similar results were obtained using *P. fluorescens* A506 and *P. agglomerans* C9-1 (6). Recently, it has been shown that A506 does produce an extracellular protease which inactivates the antibiotic type compounds produced by different strains of *P. agglomerans* including Eh252 and C9-1 (1). Production of this protease could explain why no synergy has been observed when mixing these strains of *P. agglomerans* and *P. fluorescens* A506.

Several essential oils and other natural compounds were found to inhibit *E. amylovora in vitro* (11). Oxygenated terpenes were particularly good inhibitors. The monoterpenes were more effective than the bicyclic structures and the alcohol or phenolic compounds were more inhibitory than the hydrocarbon monoterpenes (9). Interestingly, a similar situation was encountered when using these compounds against fungal pathogens (17). Derivatives of the fungal metabolite 6-pentyl- $\alpha$ -pyrone were also found to inhibit *E. amylovora*. Unfortunately, almost all of these compounds (essential oils, natural products and 6-pentyl- $\alpha$ -pyrone derivatives) also inhibited Eh252 or A506 (11). This susceptibility ruled out the prospect of using combinations of either of these biological control agents with these natural compounds.

### ***Pantoea agglomerans* P10c**

In parallel to this work, a large screening programme was carried out to find bacterial strains from New Zealand able to reduce fire blight incidence. Among the most effective and most consistent strains isolated was the strain of *P. agglomerans* P10c. Efficacy was determined in apple and pear research orchards and in commercial orchards (14). The ability of this strain to reduce effectively fire blight on apple and pear was confirmed during experiments conducted in Italy. This strain is now commercialised for the control of fire blight in New Zealand, under the name of Blossom Bless™. It is also produced in Italy under the name of PomaVita™.

### Honey bees as a delivery mechanism

Some of our early work focused on using honey bees to deliver *P. agglomerans* Eh252 or P10c onto apple and pear flowers (13). Using a simple device called a pollen dispenser filled with a lyophilised preparation of a biological control agent; it took honey bees only a few days to deliver the biological control agent to every single flower that was opened in the orchard (13). This was shown in apple and nashi (Asian pear, *Pyrus pyrifolia*) orchards. Furthermore, apple trees in an orchard where beehives were fitted with a pollen insert were less infected by fire blight than trees in an orchard where beehives were not fitted with such a device (14). Pollen dispensers are very simple and ingenious devices, but they are not practical enough for growers to use on large orchards. A more sophisticated device called BeeFORCE™ which can dispense any powder automatically to honey bees on their way out of the beehive, is being assessed for its usefulness in delivering biological control agents.

### Control of shoot blight

The focus of biological control has been protection of the flowers. Yet summer infections on shoots can represent a major problem for orchardists. To determine whether the biological control agents selected to prevent flower infection could also be used to protect from shoot infection, the ability of these microorganisms to colonise the surfaces of apple and pear leaves has been determined. None of the biological control agents tested was able to adequately colonise the surface of apple or pear leaves (12). Most of them disappeared after a few days.

Bacterial strains selected from screening programmes conducted in Italy and Germany were also tested in New Zealand for their ability to reduce fire blight on flowers or on shoots. None of the strains tested in New Zealand was significantly better than P10c and none was able to colonise the surfaces of apple or pear leaves.

### Conclusions

The availability of biological control agents for reducing incidence of fire blight has been welcomed by apple and pear growers wherever such products have been commercialised. This simple strategy relies on one microorganism establishing and competing with the pathogen. There is no doubt that in the future more complex strategies will be developed and will lead to greater levels of control.

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## **Chemistry of apple and pear stigma exudates related to bacterial antagonism toward *Erwinia amylovora***

### **Abstract**

In the present study, stigma exudates from apple and pear were chemically analyzed, and results were used in developing a synthetic medium for studying interactions between *Erwinia amylovora* and microbial antagonists. The simulation was tested by comparing pathogen-antagonist interactions in the stigma-based medium (SBM) with those occurring in assays involving detached crab apple flowers. The importance of nutrient components in SBM was evaluated by examining interactions in altered forms of this medium. Finally, acid production by bacteria was investigated as a mode of antagonism toward *E. amylovora* in SBM and stigma exudates.

### **Introduction**

The fire blight disease of apple and pear trees is most commonly initiated by epiphytic populations of *E. amylovora* on blossoms (26). Under typical dry conditions in the western US, bacteria colonize flower stigmas, and subsequent rain or heavy dew facilitates movement to the hypanthium, where infection usually occurs through nectarhodes (25). Reliance on streptomycin to suppress *E. amylovora* on floral parts has led to pathogen resistance (16).

The use of microbial antagonists to suppress *E. amylovora* on flowers is a promising alternative (4,11) or complementary measure to antibiotics (15,24). *Pseudomonas fluorescens* strain A506, the active ingredient in BlightBan A506 (NuFarm Inc., Sugar Land, TX), was initially selected based on its inhibition of an ice-nucleating strain of *Pseudomonas syringae* on corn leaves and later found to reduce fire blight incidence by preemptively excluding the pathogen (4). Another bacterial antagonist of commercial interest is *Pantoea agglomerans* (syn. *Erwinia herbicola*) strain C9-1 (11), which was selected based on in vitro production of antibiotics inhibitory to *E. amylovora* (10). Other microbial antagonists have similarly been identified in screening assays involving artificial media or immature pear fruit (11). Laboratory screening with detached crab apple flowers led to the selection of *P. agglomerans* strain E325, based on its effectiveness in suppressing populations of *E. amylovora* on flower stigmas (19,20). This antagonist is being developed commercially under the product name "Bloomtime Biological" (Northwest Agricultural Products, Pasco, WA).

The flower stigma is the primary site of interaction between microbial antagonists and *E. amylovora* (4,11). Exudates present on the stigma serve as an excellent food and water source for bacteria, as evidenced by much higher population capacities on stigmas than on other aerial plant surfaces (11). Considering the importance of the stigma in pome fruit development and in pathogen-antagonist interactions on apple and pear flowers, surprisingly little information is available regarding its chemistry.

### **Materials and methods**

Sugar and amino acid analyses: Stigma exudates were collected from flowers of pear and apple in 2003 and 2004. Tree cultivars were 'd'Anjou' and 'Bartlett' pear, and 'Fuji,' 'Gala' and 'Golden Delicious' apple, located near Wenatchee, WA. Crab apple, used as a laboratory blossom model (19), was also evaluated. Flowers were sampled at three stages based on stamen dehiscence: non-dehisced, 40-60% dehisced, and 100% dehisced. For each cultivar and stage per year, a minimum of two groups of 50 flowers were sampled. Stigma tips of 50 flowers were submerged one flower at a time in the same 600- $\mu$ l volume of water and sonicated for 10 s. Pollen, debris and microorganisms were removed by centrifugation at 14,000 rpm for 5 min and subsequent filtration of supernatant in syringe-driven unit with PVDF membrane, 4 mm diam. and 0.22  $\mu$ m pore size. Macromolecules were eliminated by filtration in centrifugal devices with 100-kilodalton and 3-kilodalton cutoffs.

Sugars were converted to trimethylsilyl derivatives prior to gas chromatography (GC) analysis according to Mateo et al. (17). Amino acids were converted to derivatives with *o*-phthalaldehyde (OPA) and 9-fluorenylmethoxycarbonyl chloride (FMOC) using the method of Herbert et al. (8), with several minor modifications, before analysis by high-performance liquid chromatography (HPLC).

Stigma exudates collected from 'Fuji' and 'Gala' apple were also analyzed for total carbohydrates, proteins and lipids. Extracts were prepared as already described, except macromolecules were not removed. Carbohydrates were hydrolyzed using the method of Goubet et al. (7) and then analyzed for monosaccharides. Protein quantities were estimated by the Bradford method (2) and lipids were evaluated by GC analysis of fatty acid methyl esters (18).

Stigma-based medium: Information generated from the analyses of sugars and amino acids in stigma exudates was applied toward the development of a liquid defined medium that simulates, in part, the availability of nutrients to microorganisms on the flower stigma. The minimal medium 925 (14) was used as a starting point. Glucose and fructose were incorporated in equal portions totaling 5% sugar. Predominant free amino acids detected at low levels in stigma exudates were also included, but not as the sole nitrogen source. The buffer concentration was very low compared to medium 925; this was not intended at first, but was continued due to encouraging results. Nicotinic acid, a growth factor required by *E. amylovora* (26), was added. Thus, ingredients in SBM, by weight in 1 ml, are as follows: 25 mg glucose, 25 mg fructose, 0.2 mg amino acid mix (proline, asparagine, glutamine and serine in ratio of 3:2:2:1), 0.8 mg NH<sub>4</sub>Cl, 0.15 mg K<sub>2</sub>HPO<sub>4</sub>, 0.05 mg NaH<sub>2</sub>PO<sub>4</sub>, 0.4 mg MgSO<sub>4</sub> and 1.2 µg nicotinic acid. Stock solutions of the chemicals were filter sterilized.

Bacterial strains: The pathogen used was a nalidixic acid derivative of *E. amylovora* strain Ea153 obtained from K. Johnson (12). Antagonist strains used were *P. fluorescens* strain A506 (15) and *P. agglomerans* strains C9-1 (10) and E325 (19). Strain A506 is naturally resistant to rifampicin. Rifampicin-resistant derivatives of C9-1 and E325 were used. Bacteria were cultured on nutrient yeast dextrose agar (NYDA; nutrient broth, 8 g; yeast, 5 g; dextrose, 5 g; agar, 15 g; and deionized water, 1 liter) at 24°C for 24 h. Inoculum suspensions were prepared in 10 mM potassium phosphate buffer (pH 7.0) and 0.03% Tween-20, and adjusted to 0.1 optical density at 600 nm using a spectrophotometer to an approximate cell density of  $1 \times 10^8$  cfu/ml.

Assays with SBM and flowers: Tests were performed to assess whether the relative suppression of *E. amylovora* by antagonist strains in SBM is similar to that on flower stigmas. A 10-µl volume of each antagonist at  $10^7$  cfu/ml was added to 250 µl of SBM in each of three wells of a 96-well microplate. Microplates were incubated at 24°C for 24 h in a closed 4-L plastic container on a rotary shaker at 125 rpm and then inoculated with 10 µl of strain Ea153 at  $10^7$  CfU/ml. After further incubation under the same conditions for 24 or 48 h, populations of both antagonist and pathogen were determined by dilution plating on CCT medium (9) amended with nalidixic acid (100 µg/ml) and Kings medium B (13) amended with rifampicin (25 µg/ml) and cycloheximide (50 µg/ml).

Concurrent with SBM tests, assays were performed with detached crab apple flowers as described previously (19), using trees of 'Manchurian' on M26 rootstock. Flowers were maintained with cut pedicle in 10% sucrose in a 2-ml vial. Approximately 0.1 to 0.2 µl of antagonist suspension was applied per flower by touching aqueous meniscus at micropipette tip to each stigma to form a thin film of moisture. Following incubation at 24°C for 24 h, pathogen strain Ea153 was applied using the same technique. Flowers were incubated at 24°C for another 24 h, and then population sizes of antagonist and pathogen were estimated. Stigmas were placed in microcentrifuge tubes with 1 ml sterile buffer (10 mM potassium phosphate, pH 7.0), vortexed briefly and placed in a sonication bath for 60 s. Samples were again vortexed, and serial dilutions were spread on the same media types used for determining populations in SBM.

Effects of altering SBM components: Tests were performed to study the relationship of SBM constituents to antagonist suppression of *E. amylovora* by (a) replacing sugars with an equivalent percent of total sugar in the form of only glucose, fructose or sucrose; (b) varying the total sugar concentration, using 1, 5 or 15%; (c) replacing the mixture of NH<sub>4</sub>Cl and amino acids with the nitrogen gram equivalent of one or the other; and (d) varying the phosphate buffer concentration, using 0X, 1X, 4X and 20X that in original SBM. In the last test, pH was measured in addition to estimating bacterial population sizes. To

further investigate the role of acid production in antagonism, strain E325 was singled out and cultured in media with alterations indicated for tests a, b and c; pH was measured after 24, 48 and 72 h.

Acid production in stigma exudates: Newly opened 'Gala' flowers from the orchard were inoculated and maintained as described for detached crab apple blossoms. Flowers were non-inoculated, inoculated with strain Ea153, or inoculated with strain E325. Three samples of 50 flowers were used per treatment. After 24 hours incubation, stigma exudates were extracted as described, and pH was recorded. Samples were also dried and reconstituted in smaller aqueous volumes so that solute concentrations were more comparable to that of stigma exudates on flowers. The pH was again measured in 10 X and 50 X concentrations.

Statistical analysis: Experiments were performed two or more times. Data from different trials were tested for homogeneity using Hartley's F-max test, then pooled and subjected to ANOVA or Pearson correlation.

## Results and discussion

Free sugars in stigma exudates from pear, apple and crab apple flowers were predominately glucose and fructose in near-equal proportions. These sugars tended to increase with flower age and ranged from 0.31 to 1.27 µg per flower for pear and from 1.87 to 3.19 µg per flower for apple. Given the volume of liquid required to coat stigmas with a thin film in routine assays, it is speculated that sugar concentrations fluctuate within the range of about 1 to 10%, depending on moisture conditions. Analyses of free amino acids in stigma exudates indicated total amounts of less than one picogram per flower. For all flower types, the predominant free amino acids in stigma exudates were proline, asparagine, glutamine and serine. Total carbohydrates in apple stigma exudates averaged 24.2 µg per flower, with galactose and arabinose being predominant in the hydrolyzed samples. Total protein was estimated at 23.2 µg per flower. The high proportion of protein along with galactose and arabinose may be indicative of arabinogalactan proteins (AGPs), which have been reported for other flowers (3). Also, the dominant free amino acids detected (i.e., asparagine, glutamine and serine) are among those reported as dominant in AGPs of tobacco stigmas (1). Proline, the most common free amino acid in pome samples is a precursor of hydroxyproline, the most prevalent amino acid in tobacco AGPs. No lipids were detected in the aqueous extracts.

When antagonist strains were cultured in SBM 24 h prior to inoculation with *E. amylovora* strain Ea153, and the mixed cultures were incubated for another 24 or 48 h, mean population sizes of all antagonists generally reached about log 9 cfu/ml. Populations of Ea153, however, were consistently smaller in the antagonist-inoculated wells (near or below the starting population of log 5.57 cfu/ml) as compared to control wells inoculated only with Ea153 (between log 8 and 9 cfu/ml). At 48 h after inoculation with Ea153, mean population sizes of the pathogen in response to antagonist treatments were different ( $P = 0.05$ ), with the highest in SBM with A506 (log 5.4 cfu/ml) and the lowest in SBM with E325 (log 3.0 cfu/ml). Results were very similar to blossom assays performed at the same time, and with previous inoculations of flowers in the laboratory (19,22) and field (19,21).

Experiments with altered SBM were helpful in assessing the importance of its components in the pathogen-antagonist interactions observed. Replacement of glucose and fructose (1:1) with an equivalent percentage of single sugars (glucose, fructose or sucrose) affected the efficacy of antagonists relative to each other and the control. In general, separation of mean population sizes was greater for original SBM than it was for media with single sugars. The greatest suppression of the pathogen ( $P = 0.05$ ) occurred in response to strain E325 in the original medium with both glucose and fructose ( $P = 0.05$ ). When sugar composition of SBM was kept the same, but total sugar concentrations varied, results with 1 and 5% sugar were similar though suppression by E325 was greater ( $P = 0.05$ ) at 1% than at 5% sugar. A total of 15% sugar resulted in *P. agglomerans* strains C9-1 and E325 being less effective and not different from each other. We can only speculate about concentrations of free sugar on pomaceous stigmas, but it's conceivable that levels could reach 15% or higher under dry conditions.

When nitrogen in SBM was altered, strains C9-1 and E325 were significantly less effective ( $P = 0.05$ ) in the medium with only amino acids as the nitrogen source, as compared to their performance in original SBM with a mixture of amino acids and NH<sub>4</sub>Cl. In the medium with NH<sub>4</sub>Cl as the sole nitrogen source,

however, both of these strains increased ( $P = 0.05$ ) in effectiveness, and strain E325 caused the greatest suppression of *E. amylovora*.

Consideration of these results and early reports of in vitro acid production by bacterial antagonists of *E. amylovora* (5,6,23) led us to examine the buffering capacity and pH changes in original and modified SBM. As the buffer concentration was increased, the statistical separation of pathogen population means in response to antagonist treatments became smaller; at 20X the buffer concentration in SBM there was no difference among antagonists ( $P = 0.05$ ). Analysis of pH data indicated a very similar pattern. Further, pH and population size of *E. amylovora* were highly correlated; within each buffer concentration,  $r$  values ranged from 0.87 to 0.95 ( $P < 0.0001$ ) and analysis of combined data gave an  $r$  value of 0.78 ( $P < 0.0001$ ).

To determine the relationship of acid production to specific carbon and nitrogen sources, strain E325 was incubated in modified forms of SBM previously used in evaluating changes in antagonist efficacy toward *E. amylovora*. Interestingly, acid production was higher ( $P = 0.05$ ) in original SBM with glucose and fructose than in any of the media with single sugars. Decreasing the sugar concentration in SBM to 1% resulted in an increase in acid production ( $P = 0.05$ ), and increasing sugar to 15% decreased it. Replacement of  $\text{NH}_4\text{Cl}$  and amino acids in original SBM with only  $\text{NH}_4\text{Cl}$  as the nitrogen source resulted in increased acid production and use of only amino acids decreased it. These changes in acid production due to SBM alterations corresponded to changes in the performance of E325; the alterations that induced greater acid production were the same ones that resulted in greater suppression of *E. amylovora*.

Although test results with SBM provided evidence that acid production by *P. agglomerans*, particularly E325, may be an important mode of antagonism toward *E. amylovora*, the question remained whether pH of actual exudates on flowers are in fact altered by these bacteria. Measurements of pH in exudates from inoculated 'Gala' apple stigmas showed that indeed strain E325 lowered the pH on the plant. This was demonstrated with the dilute aqueous samples and with the same samples concentrated by 10X and 50X (~1.2  $\mu\text{l}$  and 0.24  $\mu\text{l}$  per flower, respectively) to obtain solute concentrations more comparable to those existing on flower stigmas. All dilutions of the exudate samples from E325-inoculated flowers had a mean pH close to 5, which was significantly lower ( $P = 0.05$ ) than those from untreated flowers and pathogen-inoculated flowers, which had pH means near 6 and 6.5, respectively. Perhaps early workers (5,6,23) who speculated, based on in vitro studies, that *P. agglomerans* or related bacteria inhibited *E. amylovora* in vivo by producing acid were correct. Further investigation with flowers is necessary to validate this.

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## **Factors affecting efficiency of biological control of fire blight by *Pseudomonas fluorescens* EPS62e**

### **Abstract**

The effect of the virulence of five strains of *Erwinia amylovora* on biocontrol efficacy was studied in immature pear fruits of Passe Crassane cultivar. The efficacy of biological control using *Pseudomonas fluorescens* varied significantly among strains of *E. amylovora*. The effect of cultivar type and plant material was studied in dose-response experiments with the same pathogen strain. The median effective dose  $ED_{50}$  of the pathogen ( $K_x$ ) and biocontrol agent ( $K_z$ ) were estimated by fitting data to a hyperbolic saturation model. Host changes at the level of cultivar or type of plant material had a significant effect in biocontrol efficacy of fire blight. An analysis of  $K_x$  and  $K_z$  was performed with four pear plant cultivars and two types of plant material. The  $K_x$  for the *E. amylovora* EPS101 covered a range of 10<sup>2</sup>-fold (from 1 to 1.1 x 10<sup>2</sup> cfu per site of inoculation),  $K_z$  for the *P. fluorescens* EPS62e varied 10<sup>2</sup>-fold (from 6.7 x 10<sup>2</sup> to 5.0 x 10<sup>4</sup> cfu per site of inoculation) and the efficiency of the biocontrol agent as the ratio  $K_z/K_x$  varied 10-fold (from 3.8 x 10<sup>2</sup> to 3.6 x 10<sup>3</sup> cfu of biocontrol agent/cfu of pathogen).

### **Introduction**

Biological control is the result of complex host, pathogen and biocontrol agent interactions that are affected by environmental conditions. Most efforts have been dedicated to characterize biocontrol agents, study the diverse mechanisms of action operating and improve efficacy. There is a need to better understand general principles operating in the ecological interactions established such as the source of variation given by the host and the pathogen. Several reports have described variation among crop species or cultivars in response to various biological control agents (1,3,5,6,10).

Studies to quantify biocontrol agent efficacy and pathogen susceptibility are complicated by the fact that the effect of biocontrol agent and pathogen on the host depends on the respective concentrations. Considerable progress was made after Johnson's proposal of an epidemiological model to relate disease with the pathogen and biocontrol agent dose (4, 9). The development of tools by Montesinos and Bonaterra (7) to estimate parameters related to the virulence of the pathogen and the efficiency of the biological control agent provided with dose-independent objective means to establish comparisons between biocontrol agents, pathogens, host plants, and diverse environmental conditions (7).

We were interested to extend the knowledge of dose-response relationships to a pathosystem model consisting of the bacterial pathogen *E. amylovora* causing fire blight of several rosaceous plants. The disease is of great economic importance affecting mainly apple and pear production and several woody ornamental plants world wide and was spread through new regions in the past years (12). The restrictions in the use of antibiotics in many countries and the lack of effective means of control have stimulated the research to develop efficient biological control agents (13).

The aim of the present work was: (i) to study the effect of pathogenic strains on biological control of *E. amylovora* by *P. fluorescens* EPS62e (ii) to study the effect of hosts (cultivar and plant material) on biological control of *E. amylovora* by *P. fluorescens* EPS62e.

### **Materials and methods**

**Bacterial strains and inoculum preparation:** A total of five *E. amylovora* strains from different sources were used in the present study (Table 1). The biocontrol agent used was *P. fluorescens* EPS62e, a strain which was isolated from healthy pear of cultivar Conference in a commercial orchard near Girona. The strain is very effective in preventive treatment for controlling fire blight on flowers, whole plants and

immature fruits, and the mechanism of action is by preemptive colonisation of the host, nutrient competition and direct interaction with the pathogen (2).

**Tab. 1** Origin and year of isolation of *E. amylovora* strains used in this study

Strain	Host plant	Source	Country	Year of isolation
Ea273	<i>Malus sylvestris</i>	CUCM	USA	1971
EPS101	<i>Pyrus communis</i>	EPS	Lleida (Spain)	1999
NCPPB2080	<i>Pyrus communis</i>	NCPPB	New Zealand	1968
Ea1185	<i>Crataegus</i> sp.	OMP-BO	Italy	1994
SIA4	<i>Pyrus communis</i>	SIA	Zaragoza (Spain)	2000

EPS, Escola Politècnica Superior-Universitat de Girona (Spain); CUCM, Cornell University Collection of Microorganisms, Ithaca (USA); NCPPB, National Collection of Plant Pathogenic Bacteria, Plant Pathogen Laboratory, Harpenden, Hertfordshire (England); OMP-BO, Università degli Studi di Bologna, Bologna (Italy); SIA, Servicio de Investigaciones Agrarias de Aragón (Spain).

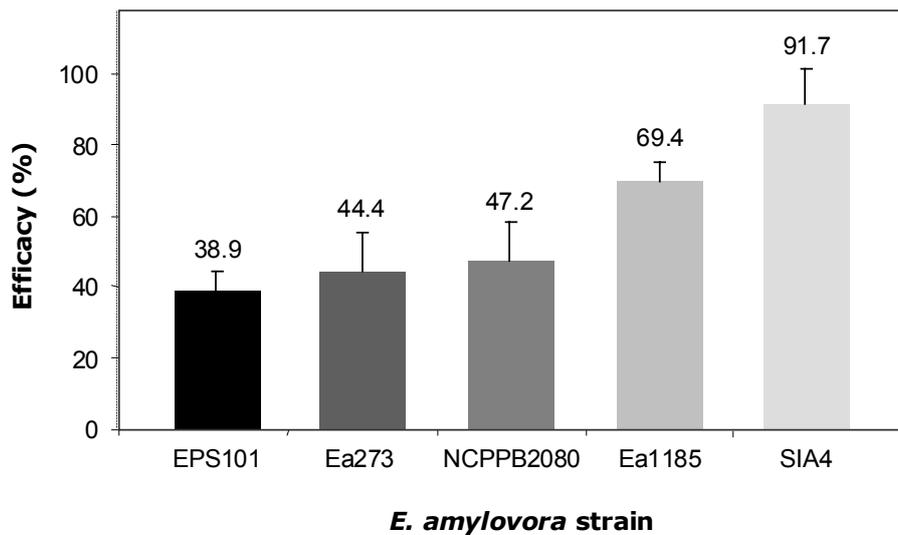
**Plant material and assays:** Immature fruits and flowers of pear used in the experiments were obtained from commercial orchards of Girona (Spain). The pear cultivars used were Doyenne du Comice and Conference. Fruits were surface-disinfected and each fruit was perforated four times. Detached pear flowers were obtained from detached pear branches bearing dormant flower buds that were forced to bloom in the laboratory. The open blossoms were detached from branches and the individual flowers were maintained with the cut peduncle submerged in 1 ml of a 10% sucrose solution (8). *P. fluorescens* EPS62e was applied by introducing 10 µl of the antagonist suspension in each wound in immature fruits or on the surface of the hypanthium of flowers and incubated at 21 °C for 24 h. Then immature fruits and flowers were inoculated with the pathogen by means of 10 µl of a suspension of the corresponding strain and incubated at the same conditions. Fruits and flowers were considered as infected when necrosis and/or ooze were observed in wounds and hypanthia.

**Range of action of *P. fluorescens* EPS62e against different strains of *E. amylovora*:** The spectrum of action of EPS62e against five strains of *E. amylovora* (EPS101, Ea273, Ea1185, SIA4, NCPPB2080) was evaluated on Passe Crassane immature pear fruits. The immature fruit assay was performed as previously described by treatment with EPS62e at 10<sup>8</sup> cfu/ml followed by inoculation with *E. amylovora* strains at 10<sup>7</sup> cfu/ml. Non-treated controls were only inoculated with each pathogen strain. Incidence of infections was determined at 10 days of the pathogen inoculation. Analysis of variance (ANOVA) was performed to test the effect of pathogen strain on the efficacy of EPS62e in the reduction of the incidence of *E. amylovora* infections. Means were separated using the Tukey test at  $P \leq 0.05$ .

**Dose-response experiments:** Immature fruits and flowers of pear were treated with EPS62e at 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup>, 10<sup>8</sup>, and 10<sup>9</sup> cfu/ml and inoculated with *E. amylovora* EPS101 at 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup> and 10<sup>8</sup> cfu/ml. The incidence of infections was calculated after 5 days of pathogen inoculation. Disease incidence data of the dose-response experiments were used to estimate the median effective dose ( $ED_{50}$ ) of the pathogen ( $K_x$ ) and biocontrol agent ( $K_z$ ) using a hyperbolic saturation model (7). Regression and parameter estimation were performed by a non-linear-least-squares method. ANOVA was performed to test the significance of the effect of plant material and cultivar on the efficiency of the pathogen and the biocontrol agent. Means were separated using the Tukey test at  $P \leq 0.05$ .

## Results

**Effect of pathogen strain on biological control:** Figure shows the efficacy of EPS62e against different strains of *E. amylovora* (Table 1). The efficacy was calculated with respect to the incidence in the non-treated control that was of 100% for all the strains. A significant variation of efficacy was observed among *E. amylovora* strains ( $F=24.26$ ,  $P<0.001$ ). The most sensitive strain of *E. amylovora* to EPS62e, SIA4, was completely controlled by the biocontrol agent, with a reduction by 90% of the incidence. Most strains were sensitive or moderately sensitive, with reductions of the incidence between 70 to 44%, while strain EPS101 was the least sensitive with a reduction of about 30%.



**Figure** Efficacy of *P. fluorescens* EPS62e in the reduction of incidence of infections caused by different strains of *E. amylovora* on Passe Crassane immature pears. Biocontrol agent was applied to  $10^8$  cfu/ml 24 h in advance of *E. amylovora* inoculation with  $10^7$  cfu/ml. Fruits were incubated for 10 days at 21°C and high relative humidity. The incidence in non-treated control was 100% for all the strains

Effect of pear cultivar and type of plant material on pathogen and biocontrol agent efficacy parameters: Table 2 summarizes the values of the estimated parameters according to the HS model.  $Y_{max}$  approximates 1.00 in all cultivars and plant material indicating that the pathogen was able to produce maximal disease levels. The  $ED_{50}$  for the pathogen varied 100-fold (from 1 to  $1.1 \times 10^2$  cfu/site) and differences were significant between plant materials and cultivars. The maximum proportion of pathogen inactivated by the biocontrol agent ( $I_{max}$ ) ranged from 0.75 to 0.87, indicating that in some cases (e.g. flowers) a part of the pathogen may be protected from the effect of the biocontrol agent. The  $ED_{50}$  for the biocontrol agent ( $K_z$ ) varied 100-fold (from  $6.7 \times 10^2$  to  $5.0 \times 10^4$  cfu/site) and was significantly different among cultivars and type of plant material. The efficiency of biocontrol in terms of ratio  $K_z/K_x$  varied about 10-fold (from  $3.8 \times 10^2$  to  $3.6 \times 10^3$  cfu/cfu of biocontrol agent/pathogen).

**Tab. 2** Estimated parameters for the hyperbolic saturation model that relates incidence of infections on immature fruits and flowers of different pear cultivars to the concentrations of the pathogen *E. amylovora* EPS101 and biocontrol agent *P. fluorescens* EPS62e

Plant material	Cultivar	df <sup>x</sup>	Parameter <sup>y</sup>				
			$Y_{max}$	$K_x$	$I_{max}$	$K_z$	$K_z/K_x$
Immature fruits	D. du Comice	40	1.00	14.0	0.78	$5.0 \cdot 10^4$	3571
	Conference	34	1.00	$1.1 \cdot 10^2$	0.87	$4.1 \cdot 10^3$	377
Detached pear flowers	D. du Comice	34	1.00	1.0	0.75	$1.2 \cdot 10^3$	1200
	Conference	34	1.00	10.0	0.76	$6.7 \cdot 10^2$	670

<sup>x</sup> Degrees of freedom.

<sup>y</sup>  $Y_{max}$ , maximum disease proportion;  $K_x$ ,  $ED_{50}$  pathogen;  $I_{max}$ , maximum pathogen proportion inactivated;  $K_z$ ,  $ED_{50}$  biocontrol agent. Densities for *E. amylovora* EPS101 and for *P. fluorescens* EPS62e are cfu per site of inoculation (fruit wound or flower hypanthium). Values correspond to the mean of parameters estimations for the three independent experiments performed. Different letters within the same column and parameter show significant differences for the means according to Tukey's test.<sup>z</sup> Goodness of fit of the model was measured as the mean square error

## Discussion

In the present study we have tested the effect of pathogen strains on biocontrol using the biocontrol agent *P. fluorescens* EPS62e. Differences were observed in the efficacy of EPS62e in the reduction of the incidence of infections caused by different *E. amylovora* strains. The fact that the effect of pathogen type on biocontrol operates at a subspecies level (pathogen strain) agreed with the report of a better biocontrol of fire blight in apple seedlings in less virulent than in more virulent strains of *E. amylovora* (11). These results indicate the existence of an important effect of the pathogen strain on the biocontrol, suggesting the importance to test the range of action of a biocontrol agent.

We also used the hyperbolic saturation model for estimating different parameters that provide useful information about the efficiency of the biocontrol agent and the aggressiveness of the pathogen in different plant materials and cultivars. As was proposed by Montesinos and Bonaterra (7,9) low values of  $K_z$  and  $K_x$  for this model are indications of a high efficacy of the biocontrol agent and pathogen, respectively. Significant differences in the efficiency of the biocontrol agent and the aggressiveness of the pathogen were observed in function of plant material and cultivar. Thus, pear flowers were more susceptible than immature pear fruits, and the Doyenne du Comice was also more susceptible than Conference. On the other hand, the best efficiency of a biocontrol agent was observed in immature pear fruits of Conference cultivar.

In conclusion, the finding of an effect of the host on pathogen aggressiveness and biocontrol agent efficiency, and the fact that biocontrol is affected by changes in the pathogenic strains reported here has practical implications, because it predicts difficulties for finding a wide range of action of biological control agents against fire blight.

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## **Biocontrol of fire blight with the antagonist *Rahnella aquatilis* and a natural product of *Thymbra spicata*, BioZell-2000B**

### **Abstract**

As alternative products to the antibiotic streptomycin against fire blight, bacterial antagonists and a natural product were tested for their efficacy in greenhouse and field experiments. The antagonistic strain of *Rahnella aquatilis* Ra39 in combination with aromatic compounds could increase the efficacy up to 68%. The etheric oil compound, BioZell-2000B was able to reduce blossom infection by up to 52% and is proposed as a bioagent for ecological farming.

**Key words:** Fire blight, biocontrol, bacterial antagonists, natural products

### **Introduction**

Research on alternatives to the antibiotic streptomycin for the control of fire blight in pome fruits has become of main interest in German and European fruit growing, since the permission for the use of antibiotics in EEC-countries will be withdrawn in the future. Thus the development of biologicals on the basis of natural products and antagonistic bacteria against the pathogen has been undertaken in Germany, especially by the Federal Research Centre for Agriculture and Forestry (BBA), in cooperation with the plant protection service. Latest results with antagonistic bacteria and a natural product based on an etheric oil of the thyme species *Thymbra spicata*, BioZell-2000B, which was developed together with Turkish colleagues (Yegen et al. 2002), will be presented.

### **Material and Methods**

Studies with bacterial antagonists: In this study the virulent strain of *Erwinia amylovora* Ea 7/74 and the antagonistic strain *Rahnella aquatilis* Ra39 were used. *In vitro* studies, for estimating the effect of aromatic compounds and the antagonistic strain on the growth of *E. amylovora* were carried out in buffered nutrient sucrose (NS) medium. The used benzoate concentration was between 10 and 100 mM; the degradation of Na-benzoate by *R. aquatilis* Ra39 was measured spectrophotometrically (UV) over 9 days after addition of the bacteria to the medium. Field studies were done during full blossom of apple 'Golden Delicious' applying to a spraying schedule with the antagonistic strain Ra39 in different combinations with Na-benzoate and the growth regulator of BASF REGALIS (APOGEE) under natural infection conditions. Infected blossom clusters were counted after blossom time and the efficacy was calculated in %.

#### Studies with natural products

Plant material and application of treatments: As host plants, M26 rootstock apple plants were grown in pots under greenhouse conditions until a shoot length of 20-30 cm. BioZell-2000B was used at a concentration of 0.05 % (diluted with tap water) as inducing agent by spraying on leaves 48 h before inoculation. Control plants were treated with tap water.

Inoculation: After cutting off the tip of the youngest two leaves of the shoots, the leaves were inoculated by dipping into a suspension of  $10^8$  cfu/ml of the virulent strain of *Erwinia amylovora* Ea 7/74 (Zeller and Meyer 1975).

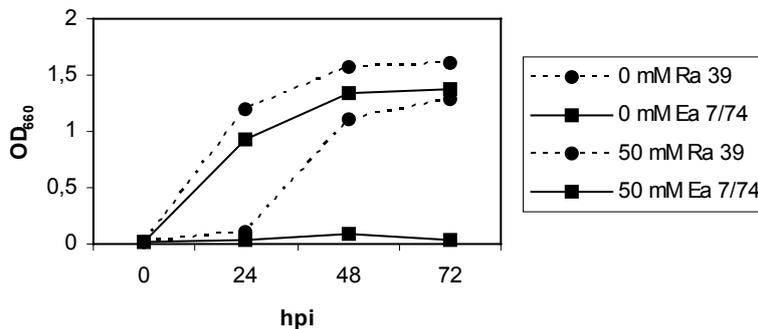
Determination of symptom development: Symptom development was evaluated according to a rating system from 0 (no symptom) to 10 (whole shoot infected). From the data the disease index (%) was calculated according to Baysal and Zeller (2000).

**Results and Discussion**

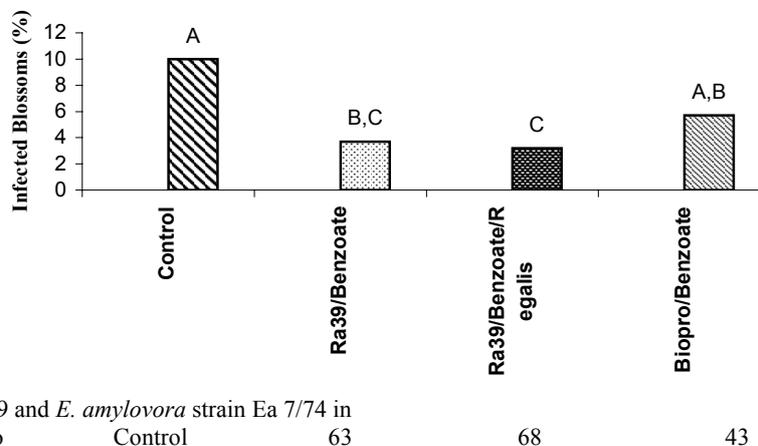
Studies on bacterial antagonists: Screening of several potential antagonists among more than 120 isolates showed inhibitory effects mainly of the species *Pseudomonas fluorescens*, *Pantoea agglomerans* and *Bacillus subtilis* (Zeller and Wolf 1996), later also of the epiphytic bacterium *Rahnella aquatilis* (Laux et al. 2002). Moreover, in field experiments efficacies up to 61% could be observed (Table 1) but without a control effect similar to streptomycin. In order to increase the efficacy of strain Ra39, the antagonist was combined with aromatic compounds, since it was less sensitive to benzoate than the fire blight pathogen *in vitro* and since it was able to use this bactericidal substance as nutritive source (Figure 1). In field experiments the combination of Ra39 and Na-benzoate was nearly comparable in the efficacy to streptomycin with 68 to 77%. Also another combination with the growth regulator REGALIS (APOGEE) showed a high control effect of 68% (Figure 2).

**Tab. 1** Efficacy of bacterial antagonists against blossom blight of *Erwinia amylovora*, 1998-2001

Antagonist	Origin	Efficacy %
<i>Bacillus subtilis</i> BsBD 170 (BIOPRO)		30-60
<i>Rahnella aquatilis</i> Ra39	Apple blossom (Steinbrenner, 1991)	39-53
<i>Pseudomonas</i> spp	Shoot tissue	45-58
<i>Pantoea agglomerans</i> Pa21889	Apple Blossom (Steinbrenner, 1991)	50-61
Control		
Streptomycin	<i>Streptomyces griseus</i>	68-80



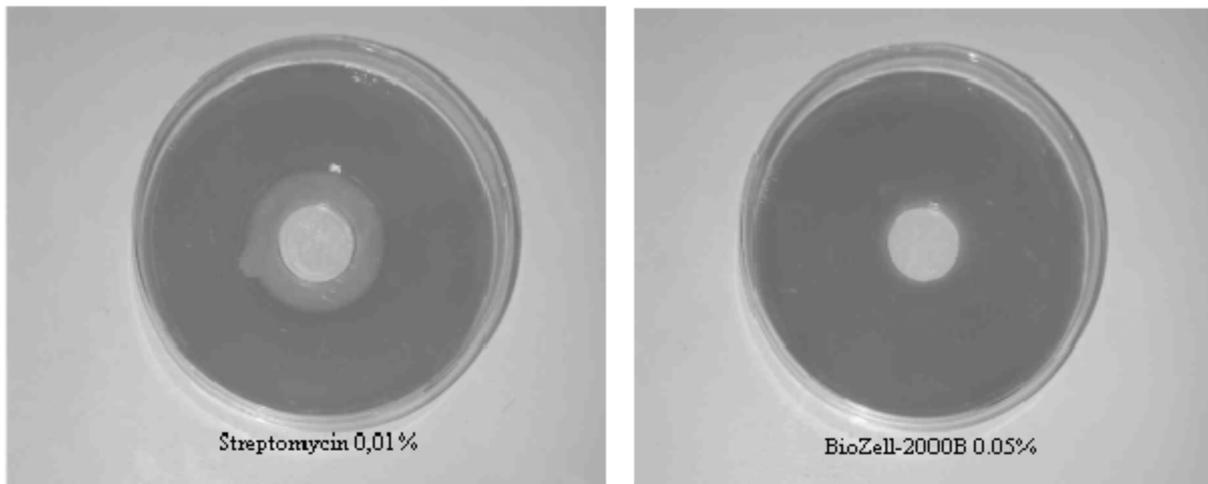
**Fig. 1** Effect of Na-benzoate on the growth of the antagonistic strain



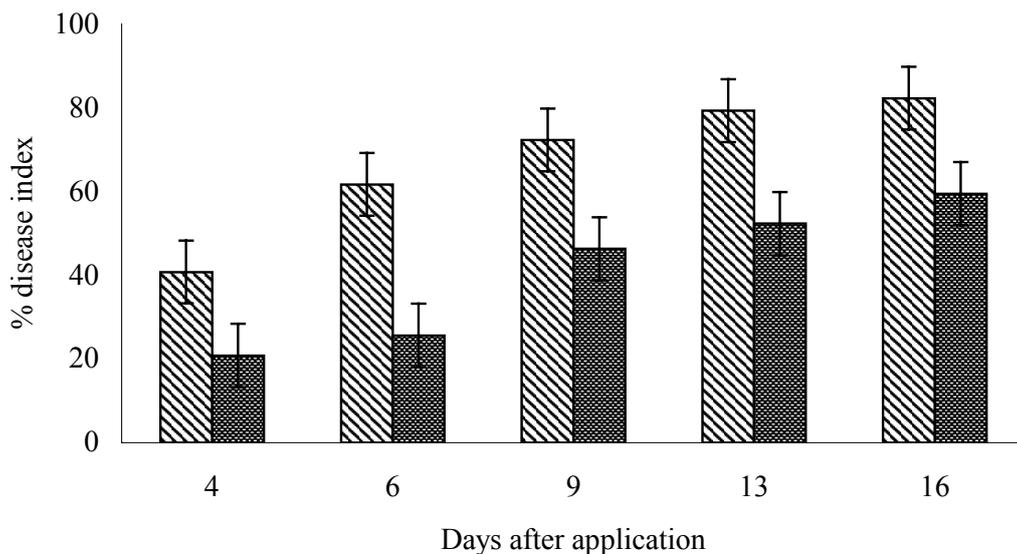
**Fig. 2** Fire blight control with the antagonist Ra39 in different combination on the apple variety 'Golden Delicious' after natural infection, 2003

Studies on natural products: As another biological alternative, the natural product BioZell-2000B, a compound prepared of 70% thyme oil from *Thymbra spicata*. It has been registered in Turkey as a plant protection substance (Yegen et al. 2002) and in Germany as a plant strengthener. Here we tested it for its efficacy against fire blight under greenhouse and field conditions.

Under *in vitro* conditions no direct effect could be observed on the pathogen with BioZell-2000B at a concentration of 0.05% (Figure 3), indicating that the plant strengthener had no direct effect. In the greenhouse, symptom development on leaves of the highly susceptible apple rootstock M26 was markedly reduced after 4 days induction time with BioZell-2000B (Figure 4). Under field conditions a significant reduction of blossom infection of 52% was found on the apple variety Boskoop (Table 2). In a further control experiment with a highly susceptible *Cotoneaster* variety of *C. salicifolius* the control effect could be confirmed with 85% (Zeller and Laux 2002).



**Fig. 3** Test of BioZell-2000-B (0.05%) in comparison to streptomycin (0.01%) in the agar-diffusion-test against *E. amylovora*



**Fig. 4** Disease Index of fire blight on shoots of M26 apple rootstock after treatment with BioZell-2000-B (hatched) and of controls without treatment (dark)

**Tab. 2** Fire blight control with BioZell-2000B on apple variety 'Boskoop' after natural blossom infection, 2001

Treatment	No. of blossom clusters		Infection %	Efficacy %
	Total	Infected		
Inf. control	720	70	9,7 a	-
BioZell-2000B	680	30	4,7 b	52

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## ***Rahnella aquatilis* Ra39 – a bacterial antagonist against fire blight (*Erwinia amylovora*)**

### **Abstract**

The epiphytic bacterial strain *Rahnella aquatilis* Ra39 was shown to reduce fire blight symptoms in field trials during 1998, 1999 and 2000. The isolate was investigated on its mode of action against *Erwinia amylovora*, the causal agent of the disease. The interaction between the populations of the fire blight pathogen *E. amylovora* strain Ea7/74 and *R. aquatilis* Ra39 was studied *in vitro*. In experiments with co-cultures, a direct inhibition of Ea 7/74 by Ra39 *in vitro* was observed at a sucrose concentration of 0.2% but not at a concentration of 2%. Therefore competition for sucrose might be involved in the mode of action of *R. aquatilis* Ra39 against fire blight. Furthermore it could be shown that the lipopolysaccharide of *R. aquatilis* Ra39 is able to inhibit the multiplication of *Xanthomonas campestris* pv. *begoniae*, the causal agent of the Oil Spot Disease of *Begonia tuberhybrida* in leaves of its host plant. From the observations on this model system it was concluded that induced resistance may also play a role in antagonism of *R. aquatilis* Ra39 against *E. amylovora*.

**Key words:** *Erwinia amylovora*, *Rahnella aquatilis*, antagonism, mode of action, induced resistance

### **Introduction**

The epiphytic bacterial strain *Rahnella aquatilis* Ra39, which has shown a significant reduction of fire blight symptoms in field trials during 1998, 1999 and 2000 (Wesche 1998; Laux et al. 1999; Laux and Zeller 2000), was investigated on its mechanism against *Erwinia amylovora*, the causal agent of the disease. No information on the mode of action of *R. aquatilis* against fire blight is available until now.

Mechanisms of bacterial antagonists against the fire blight pathogen include antibiosis (Vanneste et al. 1992), competition for nutrients and sites (Wilson and Lindow 1993; Wright and Beer 1997) and induced resistance. Antagonism against fire blight has been described mainly for strains of the species *Pantoea agglomerans*, *Pseudomonas fluorescens* and *Bacillus subtilis*. While for the majority of *P. agglomerans* strains antibiosis has been assumed as the principle mode of action against fire blight (Vanneste et al. 1992) this was not found for *P. fluorescens* which was suggested to inhibit *E. amylovora* by colonization of the nectaries (Wilson and Lindow 1993). Also for *B. subtilis*, which is producing bacteriocins with activity against a wide range of bacteria (Klaenhammer 1988), no bactericide activity against *E. amylovora* has been described until now.

In this study we report evidence that induced resistance is involved in the mode of action of *R. aquatilis* strain Ra39 against *E. amylovora* strain Ea 7/74.

### **Materials and methods**

Agar diffusion tests were done on Miller-Schroth (MS), nutrient-sucrose (NS) and glucose-asparagine (GA)-medium. Co-culturing of bacterial antagonists and Ea7/74 was carried out in buffered nutrient-sucrose (NS)-medium. The sucrose concentration was varied between 2% and 0.2 %. Liquid cultures were shaken constantly at 250 rpm. For experiments with co-cultures, spontaneous mutants of Ea7/74 and the antagonistic strains Ra39 and R1 resistant against 100 ppm streptomycin (Sm) or rifampicin (Rf), respectively, were used. Plate counts of mixed cultures of Ea 7/74Sm and Ra39Rf were done on solid NS-medium containing 100 ppm streptomycin or rifampicin respectively.

Bacterial strains and culture media: The bacterial strains used in this study are listed in Table 1.

**Tab. 1** Bacterial strains

Strain	Isolated from	Source or reference
<i>Erwinia amylovora</i> Ea7/74	<i>Cotoneaster</i> sp.	Zeller and Meyer 1975
<i>Rahnella aquatilis</i> Ra39	<i>Malus domestica</i>	Steinbrenner 1991
<i>Xanthomonas campestris</i> pv. <i>begoniae</i> Xcb 525	<i>Begonia</i> sp.	Collection of phytopathogenic bacteria Göttingen (GSPB)

Lipopolysaccharides (LPS) were extracted by the phenol-water method (Westphal and Jann 1965). The aqueous LPS solution was dialysed against demineralised water (12000 Da; Serva) until reaching a conductivity below 50 µS and lyophilised.

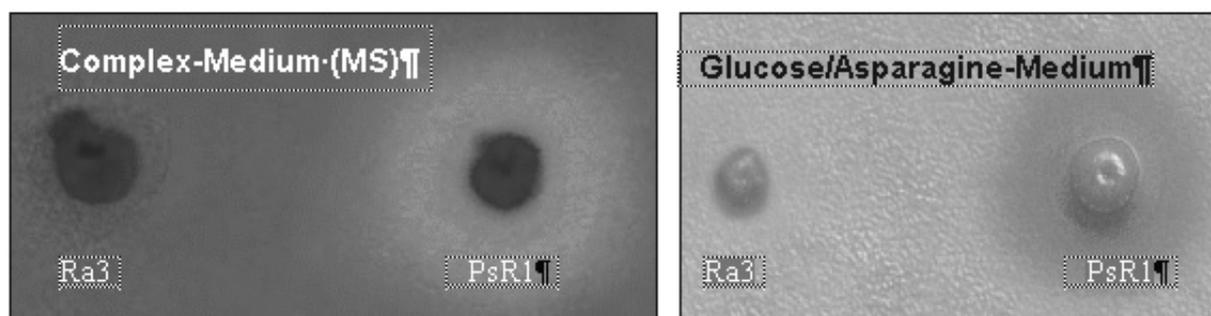
## Results

Field experiments: The bacterial strains Ra39 and R1 were shown to reduce fire blight symptoms in field trials in Egypt and Germany during 1998, 1999 and 2000. The efficacy of both isolates varied between 39 and 53% or 45 and 58%, respectively (Table 2).

**Tab. 2** Efficacy of *Rahnella aquatilis* Ra39 and *Pseudomonas* spec. R1 against fire blight

Antagonistic strain	Efficacy %
<i>Rahnella aquatilis</i> Ra39	39-53
<i>Pseudomonas</i> spec. R1	45-58
Standard (Plantomycin)	68-80

Agar diffusion test: In the agar diffusion test the antagonistic strain *Pseudomonas* spec. R1 caused the formation of inhibition zones. There was no difference in antibiotic activity between MS- and GA-medium. In contrast, *R. aquatilis* Ra39 did not show any antibiotic activity in this test (Figure 1).



**Fig. 1** *In vitro* test of strain Ra39 in comparison to strain PsR1 on two different media

Multiplication of Ea 7/74 and Ra39 in pure and co-culture: When Ra39 was grown in co-culture together with Ea7/74 in NS-medium containing 2% sucrose, no inhibitory effect of the antagonist on the pathogen was observed. The same experiment in NS-medium containing 0.2% sucrose revealed a growth depression of Ea7/74. Beginning from the third day after inoculation when the concentration was 100 fold lower, the difference increased to 10<sup>3</sup> cfu per ml at the fourth and fifth day after inoculation (Figure 2).

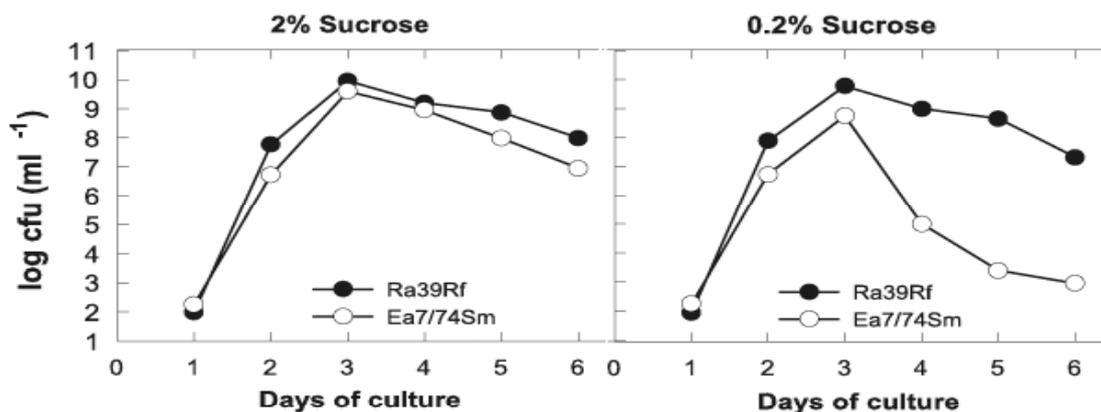


Fig. 2 Influence of Ra39Rf on the growth of Ea7/74Sm co-cultured in NS-medium

Effect of *R. aquatilis* on multiplication of *Xanthomonas campestris* pv. *begoniae* Xcb525 in planta: Multiplication of Xcb 525 in planta was determined by dilution plating on selective medium. After infiltration of leaves from *Begonia tuberhybrida* with a cell suspension, cell fragments or lipopolysaccharide (LPS) of Ra39 multiplication of Xcb was suppressed in comparison to the water treated control (data not shown).

## Discussion

The strain *R. aquatilis* Ra39 never showed any antibiosis in the agar diffusion test independent of the amino acid composition of the medium in this system. In experiments with co-cultures, a direct inhibition of Ea 7/74 by Ra39 *in vitro* was observed at a sucrose concentration of 0.2% but not at a concentration of 2%. In contrast, the strain R1 caused a direct inhibition of Ea7/74 independent of the sucrose concentration. Therefore, competition for sucrose might be involved in the mode of action of *R. aquatilis* Ra39 against fire blight. Until now, this mechanism has not been demonstrated for antagonistic bacteria of *E. amylovora*. Because the strain *Pseudomonas* R1 caused a direct inhibition of Ea7/74 in the agar diffusion test and in co-culture with the pathogen, we assume an antibiotic effect against the pathogen for this strain. For many *P. agglomerans* strains the inhibition of bactericide activity by the presence of amino acids in the medium has been described (Wodzinski et al. 1994). Therefore, amino acid composition of the plant tissue was suggested to affect the efficacy of *P. agglomerans* strains in preventing fire blight symptoms. In contrast, the antibiotic activity of the strains *R. aquatilis* Ra39 and *Pseudomonas* spec. R1 was not dependent on the amino acid composition of the medium. While *Pseudomonas* spec. R1 expressed antibiotic activity on all media tested, *R. aquatilis* Ra39 never showed this effect. Despite these results it cannot be excluded that specific substrates, only available at the flower surface of fire blight host plants, are stimulating a direct inhibition of the pathogen by this strain. The result that LPS of Ra39 suppressed the multiplication of *X.s campestris* pv. *begoniae* in planta indicates that induced resistance might be a mechanism of Ra39 against Ea7/74.

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## Characterization of epiphytic bacteria from Australia and Europe as possible fire blight antagonists

### Abstract

Several epiphytic bacteria were isolated from apple and pear trees at three places in Australia and classified as the novel species *Erwinia tasmaniensis* by microbiological data and DNA/DNA hybridization assays as well as nucleotide sequence analyses of several house keeping genes. They are non-pathogenic for apple and pear, produce levan from sucrose and can induce a hypersensitive response (HR) on leaves of non-host plants. Taxonomically different strains, classified as *Erwinia billingiae*, were isolated in England, and were assayed similarly as *E. tasmaniensis*. Most of the investigated plant-associated bacteria produced the autoinducer AI-2. A *Vibrio harveyi* mutant was used as a sensor for induction of bioluminescence by culture supernatants of the epiphytic bacteria. *E. billingiae* additionally produced an acyl-homoserine lactone (AHL, AI-1), which was monitored by complementation of a *Chromobacter violaceum* mutant for pigment secretion. When slices of immature pears were soaked with *E. billingiae* or *E. tasmaniensis* the bacteria interfered with the growth of *E. amylovora* for formation of ooze and necrotic symptoms. When inoculated in excess to *E. amylovora* into apple flowers, the pathogen propagation was strongly reduced. First trials in an experimental orchard confirmed their ability to prevent symptom formation of fire blight in blossoms of apple trees to a significant extent.

### Introduction

The causative agent of fire blight affecting apple and pear trees is *Erwinia amylovora*. In short distances, the disease is predominantly distributed via bees and other insects visiting flowers. The low numbers of inoculum need appropriate conditions for efficient multiplication of the pathogen within a short time. Asian pear blight of pears in Korea and Japan is caused by bacteria of the related species *Erwinia pyrifoliae*. Strains of these species have been differentiated mainly by PFGE analysis (Jock and Geider 2004). Fire blight and other bacterial diseases can be controlled by application of antibiotics. Streptomycin (Sm) has been the compound of choice for a long time to spray flowers in orchards, when the weather forecast has suggested a high risk of fire blight outbreaks. Repeated applications increased occurrence of Sm-resistant strains either by selection for a spontaneous chromosomal mutation or by selection for a transposon encoding low resistance against Sm (Jones and Schnabel 2000). In countries with a high level of Sm-resistant strains other antibiotics were applied with some success such as oxy-tetracycline and oxolinic acid (Kleitman et al. 2005). Nevertheless, an increasing concern about application of antibiotics in orchards led to restriction for its use especially in European countries. Alternative methods for control of fire blight have been widely proposed such as spraying other chemicals and various microorganisms. Microorganisms include epiphytic bacteria, which can secrete toxic compounds or directly interfere with growth of pathogens. Gram-positive bacteria often produce bacterial growth inhibitors (Emmert and Handelsman, 1999). Application of those cell culture preparations comprises bacilli, spores and toxins. The latter may well survive commercial formulations. Cell growth and sporulation are favored by elevated temperatures on the plant surfaces. Gram-negative bacteria have been applied from freeze-dried cultures as liquid suspensions (Stockwell et al. 2002).

A few reports deal with yeasts or bacteriophages for control of fire blight. Yeasts are often sensitive to fungicides and can cause leaf rust. Bacteriophages are tedious to separate from the pathogenic host cells, they may be unstable in their titer (plaque forming units, PFU) and may not efficiently reach the bacteria on the plant surface for destruction of the pathogen. Spontaneous resistance to bacteriophages is common for bacterial host cells, so several phages with different receptor targets should be preferred.

The release of large amounts of microorganisms could cause environmental imbalances. Application of enriched or purified bactericidal compounds may therefore be often preferred to cell preparations. In this

view, we have isolated and expressed an EPS depolymerase gene from the *E. amylovora* phage  $\phi$ -Ea1h and the His-tagged protein purified on Ni-columns (Kim and Geider 2000). The enzyme cleaves the repeating units of the exopolysaccharide (EPS) amylovoran behind the galactose residue bearing the side chain. Destruction of the capsules exposes *E. amylovora* cells to plant defense mechanisms. Expression of the *dpo* gene in apple (Hanke et al. 2003) and pear (Malnoy et al. 2005) reduced symptom formation of inoculated seedlings.

## Materials and methods

Isolation of epiphytic bacteria from apple and pear at three places in Australia will be described (Geider et al. 2005). The nucleotide sequences of the 16S rRNA and the "house keeping genes" *recA*, *gapdh* will be also reported. The levansucrase encoding genes (*lsc*) of the Australian epiphytes were sequenced and differed from the *E. amylovora lsc* gene. *Erwinia billingiae* strains had been isolated in English orchards from various plant tissues by Eve Billing as "white herbicolos" and were recently placed into a novel species (Mergaert et al. 1999). The antagonistic effects of the bacteria were determined on green pear slices and on apple flowers. The pear slices were soaked with cells of *E. tasmaniensis* or *E. billingiae*, air dried and diluted suspensions of *E. amylovora* added. Flowers were removed from trees, inoculated with a mixture of an antagonist and *E. amylovora* and further incubated in a moist chamber for 5 days. The amount of *E. amylovora* was determined by titration of a streptomycin-resistant strain and by real-time PCR.

## Results and discussion

Characterization of *Erwinia tasmaniensis* for control of fire blight: Bacteria on plant surfaces do not damage the plant tissue to a visible extent. The epiphytes use secreted plant compounds as nutrients. In flowers, the bacteria will find carbohydrates in the nectar, which are a source to support growth of many microorganisms. On the other hand, high sugar concentrations favor osmotolerant bacteria.

A novel species of epiphytic bacteria from pear and apple flowers and apple bark, named *Erwinia tasmaniensis*, was characterized for microbiological and molecular properties (Table). Selected as white, levan-producing bacteria, they cause a hypersensitive response (HR) on tobacco after induction in a starvation medium. HR could increase their fitness on plant tissue, levan synthesis their growth in high sugar concentrations found in nectar of flowers. The epiphytic strains isolated at three different places on Australia seem to contain a plasmid in the size range of 50 kb and are very similar to each other for nucleotide sequences of the 16S rRNA, the "house keeping" genes *recA* and *gapdh*. They can be distinguished from the fire blight pathogen *E. amylovora* and the Asian pear pathogen *Erwinia pyrifoliae* not only by the lack of virulence on apple and pear, but also by sequence alignments of these genes. They can also be separated from other related bacteria such as the species *Pectobacterium*, *Pantoea* or *Brenneria*. Levansucrase genes of *E. tasmaniensis* strains show nucleotide changes compared to *E. amylovora*. The strains are non-pathogenic when inoculated on green pear slices or into apple seedlings. By microbiological assays (together with B. Völksch, Jena) and by DNA/DNA hybridizations (together with G. Auling, Hannover), they were classified as *Erwinia tasmaniensis*, a novel species in the genus *Erwinia*.

**Table** Biochemical properties of two antagonistic bacterial species and of *E. amylovora* and their interaction with plants

Species	levan	AHL-synthesis	AI-2 production	virulence*	HR**
<i>Erwinia tasmaniensis</i>	yes	no	yes	no	yes
<i>Erwinia billingiae</i>	no	yes	yes	no	no
<i>Erwinia amylovora</i>	yes	no	yes	yes	yes

\*on apple and pear; \*\*on tobacco

Autoinducers of the epiphytic bacteria for cell/cell communication: Another species of epiphytic bacteria, *Erwinia billingiae*, does not produce levan and does not induce HR on tobacco. These bacteria produce acyl homoserine lactone (AHL), a small molecule secreted for cell/cell communication, called quorum sensing. AHL accumulates at high cell densities in the medium and interferes with gene

expression. Selectivity can result from the chain length of the acyl-residues, which is supposed to discriminate its binding to LuxR-proteins of bacteria. Another type of molecules for quorum sensing is the autoinducer 2 (AI-2), a furanosyl borate diester (Federle and Bassler 2003). In *Vibrio harveyi*, AI-2 and AHL (AI-1) induce the expression of the *lux* operon at high cell densities. Although most bacteria do not display bioluminescence, the autoinducers were shown to interfere with bacterial gene expression which can be activation or reduction of transcription. *E. amylovora* produces AI-2 (Mohammadi and Geider 2005) and may be affected by this compound for growth, also after secretion of AI-2 by other bacteria in the environment. A key enzyme for AI-2 synthesis is encoded by *luxS*, which is highly conserved for *E. amylovora* strains, but not identical to *luxS* of *E. tasmaniensis*.

Controversial reports exist about synthesis of AHL by *E. amylovora*. Sensor strains for AHL were described to respond to culture supernatants of *E. amylovora* (Venturi et al. 2004; Molina et al. 2005), but we could not confirm these findings. PCR primers proposed for amplification of *luxI/R* analogous genes of *E. amylovora* did not result in DNA amplification in our hands, nor were the nucleotide sequences indicated in the reports found in blast searches with the "shotgun/contig" library of *E. amylovora*, provided by the Sanger Institute, Cambridge, UK. Quorum sensing of *E. amylovora* may only depend on AI-2.

Antagonistic effects of epiphytic bacteria against fire blight: In growth assays with slices of immature pears or with apple flowers, application of *E. tasmaniensis* or *E. billingiae* strains resulted in growth inhibition of *E. amylovora* (Jakovljevic et al. 2006). An excess of the antagonist was essential to interfere with growth of *E. amylovora*. The titer of the pathogen was not reduced by the epiphytic bacteria after application together with *E. tasmaniensis* or *E. billingiae* into apple flowers, so the epiphytic bacteria may compete by their initial high cell density. Strong growth retardation may eventually result in autolysis of *E. amylovora*, which can grow, when undisturbed, to a density above  $1 \times 10^7$  cfu per flower. A reduction of typical fire blight symptoms was found in the experimental orchard applying the antagonistic bacteria in advance to the challenge by *E. amylovora* (unpublished).

Gram-positive bacteria, classified as *Bacillus megaterium* and *Bacillus pumilus*, can also interfere with growth of *E. amylovora* on cell lawns (Jock et al. 2002). Growth inhibition is caused by the release of inhibitors, which could explain the antagonistic effect of *B. pumilus* in apple flowers, which prevented growth of *E. amylovora* as well as *E. tasmaniensis* and *E. billingiae*.

## Conclusions

Two antagonistic bacterial species, *E. tasmaniensis* and *E. billingiae*, were assayed for their interference with growth of *E. amylovora* on plant tissue and in apple flowers. An excess to the pathogen reduced or even eliminated growth of *E. amylovora* and the formation of fire blight symptoms. A common feature of the antagonistic bacteria and *E. amylovora* is synthesis of AI-2, which may restrict growth of the pathogen by also sensing the concentration of a foreign autoinducer and cause growth retardation at high autoinducer levels. The application of the antagonists in orchards still needs further experiments about their growth and persistence in flowers, storage properties after cultivation in liquid media and toxicological studies about their interaction with other organisms including humans.

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## **Fire blight control in organic fruit growing – systematic investigation of the mode of action of potential control agents**

### **Abstract**

Effective control agents are needed to prevent blossom infections by the fire blight pathogen *Erwinia amylovora* in organic fruit growing. In this study 18 preparations of potential control agents were compared for their efficiency against *E. amylovora*. In shaken cultures twelve control agents inhibited the growth of *E. amylovora* completely. In this system different mechanisms of action were found. Six control agents shifted the pH of the cultures to values not suitable for bacterial growth (<5 or >8). Three agents act by copper, a known bactericide. The control agent Elot-Vis has high ethanol content, which was responsible for the high efficiency *in vitro*. On detached apple blossoms only four control agents led to a symptom reduction of more than 50%. Only two of them (Blossom-Protect fb, BPASc) exhibited a high efficiency in field trials. Our results suggest that the control agents, which led to a high efficiency in shaken cultures, are not sufficient for good performance in the field.

### **Introduction**

Fire blight caused by *E. amylovora* is the most serious bacterial disease in apple trees. The economic importance of fire blight is considerable. During the last three decades it has spread throughout Europe. Since pruning of diseased material and other sanitation methods are not sufficient to stop the spread of the disease, efficient control agents are needed. Primary infection occurs in the blossom, where the pathogen enters through natural openings after multiplying on the stigmas. To prevent blossom infections in organic fruit growing orchards several potential control agents were evaluated.

In this study 18 preparations were tested for their ability to suppress the multiplication of *E. amylovora* in shaken cultures and to reduce the symptom development on detached blossoms. The mode of action of effective preparations has been evaluated.

### **Materials and methods**

Test preparations: The preparations were tested at doses in accordance with the manufacturer's recommendations (Table 1). Slaked lime was purchased from Sigma Aldrich. Streptomycin sulfate (Sigma Aldrich) was used as a positive control in the laboratory experiments.

Reduction of *E. amylovora* growth in shaken cultures: *E. amylovora* strain Ea385 was subcultured on NBS-A (8 g/l nutrient broth, 50 g/l sucrose, 20 g/l agar) at 27°C. Inoculum suspensions of Ea385 were standardised by measurement of their optical density at 660 nm.  $1 \times 10^7$  cells/ml of Ea385 were added to 25 ml NBS (8 g/l nutrient broth, 50 g/l sucrose) in a 100 ml Erlenmeyer flask containing the test preparation. Four hours and 24 h after incubation on a rotary shaker at 27°C, the Ea385 concentration in the culture was calculated by dilutions plated on Petri-dishes with NB-A (8 g/l Nutrient Broth, 20 g/l agar). If the test preparations contained *Bacillus subtilis* or *Aureobasidium pullulans* the culture was plated on MacC-A (40g/l MacConkey broth, 20g/l Agar) or NBAC-A (8 g/l nutrient broth, 0,05g/l actidione, 20 g/l agar) respectively. Preparations which changed the pH value in the NBS below 5 or above 8 were also tested in NBS-pH7 (NBS amended with 5.4 g/l  $\text{KH}_2\text{PO}_4$  and 10.6 g/l  $\text{K}_2\text{HPO}_4$ ).

Reduction of fire blight symptoms on detached blossoms: An *in vivo* test system with detached apple blossoms was established according to Pusey (10). Apple trees ('Gala') were stored at 2°C in the dark from January to August. Every week a group of trees was transferred to the greenhouse to force them to bloom. The blossoms were cut and maintained with the pedicel submerged in 10 % sucrose in plastic racks (23°C, 100% RH). Blossoms were sprayed with a suspension of Ea385 ( $10^6$  cfu/ml) in water until

run-off. Treatments were applied 1h after inoculation. The number of blossoms with bacterial ooze at the pedicel was counted 6 days following inoculation (Table 1).

**Tab. 1.** Name of tested preparations, main ingredients and providers

Name	Ingredients	Provider
Cutisan	Clay mineral	Biofa AG
Kaolin Tec.	Clay mineral	Biofa AG
Phyto-Vital	Lignin derivatives	Ligmeda Consult
Quassia extract	Quassin	Biofa AG
Biplantol Erwinia	Homoeopathic	Bioplant Naturverfahren GmbH
Fungend	Essential oil of <i>Thymbra spicata</i>	Biofa AG
BioZell2000B	Essential oil of <i>Thymbra spicata</i>	Gisela Zeller
DoMoF/Lysozym	Lysozyme and milk proteins	Novaprot GmbH
Elot-Viss	Alcoholic extract of plants	Biofa AG
Mycosin	Acidic stone meal	Biofa AG
Funguran	Fungicide, Copper oxychloride	Biofa AG
Protex-Cu	Fertiliser, Copper sulfate	MAC-GmbH
Copper-Protein	Copper chloride, protein-complex	Proagro GmbH
Serenade WPO	<i>B. subtilis</i> QST713	GAB Consulting GmbH
Lime sulphur	Calcium polysulphide	Biofa AG
Blossom-Protect	<i>A. pullulans</i> and buffer P	Blossom-Protect
BPASc	<i>A. pullulans</i> and buffer C	Bio-Protect GmbH

Efficiency of the preparations in field experiments: The results from the laboratory studies were compared to efficiencies found in field experiments conducted from 1997 to 2004 in Germany in accordance with EPPO guideline PP1/166(3). In the test orchards one tree per lot was inoculated with the pathogen. From this tree *E. amylovora* was spread over the entire orchard by natural vectors. (2) Only the results from trees which had not been inoculated were taken into account ((5) and literature therein).

## Results

Efficiency of control agents: 18 preparations were tested in shaken cultures and on detached apple blossoms for their efficiency against *E. amylovora*. The use of Cutisan (15 g/l), Kaolin Tec (15 g/l), Phyto-Vital (20ml/l), Quassia extract (2g/l), Biplantol Erwinia (2ml/l) or Fungend (0.25 ml/l) neither reduced the growth of *E. amylovora* in shaken cultures nor prevented symptom development on detached blossoms. Kaolin Tec, Biplantol Erwinia and Fungend were also tested in field experiments and did not reduce disease incidence (6).

**Tab. 2** Efficiency of control agents for fire blight control in shaken cultures, on detached blossoms or in field experiments.

Preparation	Dose (%)	Shaken culture % reduction of Ea385 after 24h ( pH)	Symptom reduction on detached blossoms (%)	Field trials in Germany 1997-2004 [5]	
				efficiency (%)	No.
Plantomycin	0,06			84 ± 7	9
Streptomycin sulfate	0,025	100 (7.2)	84 ± 15		
Blossom-Protect	1.2	100 (4.0)	70 ± 18	72 ± 10	4
BPASc	1.2	100 (3.7)	78 ± 13	83	1
Serenade WPO	1.0	100 (6.4)	46 ± 17	45 ± 26	6
Mycosin	1.0	100 (3.8)	78 ± 11	38 ± 18	7
Funguran	0.3	100 (6.2)	73 ± 15	-	
	0.03	100 ( - )	47 ± 15	-	
Protex -Cu	0.1	100 (4.7)	34 ± 10	49	1
Copper protein	1.0	100 (6.9)	40 ± 21	-	
Slaked lime	2.0	100 (12.4)	14 ± 20	48	1
Lime sulphur	1.5	100 (9.2)	-5 ± 10	28	1
Elot-Vis	10.0	100 (7.3)	54 ± 26	19	1
DoMoF/Lysozym	2.0	100 (6.9)	-36 ± 47	-	
BioZell 2000B	0.05	100 (6.7)	7 ± 18	-	

All other preparations completely inhibited the growth of the pathogen in shaken cultures. Elot-Vis, Funguran, Mycosin, BPASc and Blossom-Protect also exhibited a high efficiency on detached blossoms (Table 2). Elot-Vis and Mycosin however showed no or only slight disease reduction in the field. Only BPASc and Blossom-Protect exhibited a high efficiency in field trials comparable to streptomycin sulfate.

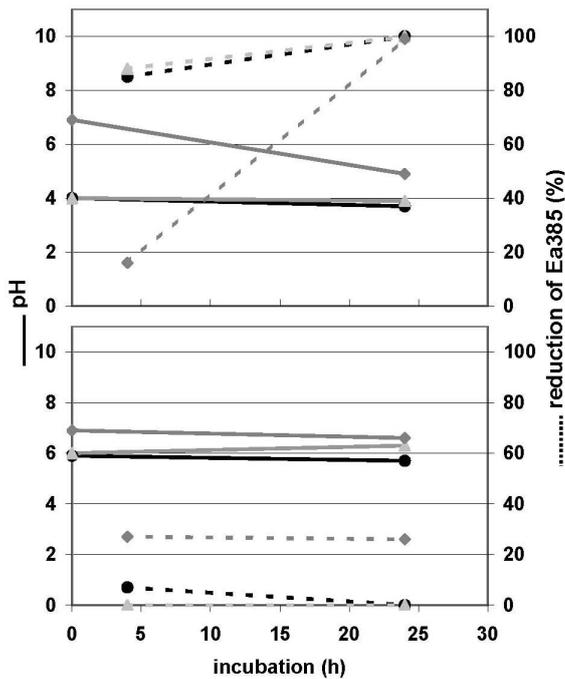
Mode of action: The efficiency in shaken cultures was necessary but not sufficient to predict a high efficiency on detached blossoms or in the field. The following information with regard to the mode of action in shaken cultures helps to explain the discrepancy between high *in vitro* activity and bad field performance.

Some of the preparations contain substances with known bactericidal effects, which explain the high efficiency in liquid medium. BioZell2000B contains essential oil from *Thymbra spicata* which is described as a bactericide (1). Elot-Vis is a plant extract with an alcohol content of more than 90%. 10 % alcohol was also effective in the shaken culture. After evaporation of the alcohol from Elot-Vis, the remaining extract was resolved in water. This solution had no effect in shaken cultures. This indicates that the efficiency of Elot-Vis in shaken cultures can be attributed to the alcohol content. DoMoF/Lysozym consists of milk proteins and lysozyme from chickens eggs. Lysozymes have a potential as anti-microbial proteins because of their lytic activity against bacterial cell walls (11). Funguran, Protex-Cu and Copper-Protein contain copper ions as active ingredients. The bactericidal activity of the copper ion is already well documented (9) and all three copper agents completely inhibited Ea385 in the tested concentrations. Serenade WPO contains spores of *Bacillus subtilis* strain QST713 and substances produced by this strain during fermentation. After re-suspending Serenade WPO (10 g/l) the *B. subtilis* spores were separated from the supernatant. Both the spore pellet and the supernatant inhibited the growth of Ea385 in shaken cultures. This indicates that Serenade WPO acts by bactericidal substances which are contained in the formulation and by the antagonistic behaviour of the *B. subtilis* strain.

The second group of preparations shifted the pH of the medium to values not suitable for *E. amylovora*. Lime sulphur and slaked lime increased the pH of NBS (Table 2). Lime sulphur was also efficient in NBS-pH7 although the pH was maintained at 7. This indicates that a second mechanism was involved. Slaked lime increased the pH to a level above 8 even when NBS-pH7 was used. Therefore CaCl<sub>2</sub> was tested for its efficiency against Ea385 at a concentration corresponding to the Ca-concentration in 20g/l slaked lime. This CaCl<sub>2</sub>-concentration (30 g/l) inhibited the growth of Ea385 completely, indicating that slaked lime acts by both, an increase of pH and by the high salt concentration. A lower concentration of 15 g/l CaCl<sub>2</sub> however was totally ineffective.

Mycosin, BPASc and Blossom-Protect decreased the pH of the medium to or below pH 4. At pH 4 Ea385 was not able to multiply. This explains the high efficiency of these preparations in shaken cultures. Mycosin or Blossom-Protect were also added to NBS-pH7. In NBS-pH7 the pH value did not decrease below 6 and the two preparations did not inhibit the growth of Ea385. This indicates that the shift in pH is the mode of action for these preparations.

Blossom-Protect consists of two components (4). For further examinations the two components were tested separately in shaken cultures (Figure). Component A showed the same results as Blossom-Protect. Component B did not lead to a reduction of Ea385 concentration after 4h, but the growth of Ea385 was completely inhibited when measured after 24h (bacteriostatic). Interestingly the Component B did not inhibit the growth of Ea385 in NBS-pH7 (Figure). This indicates that the efficiency of component B is also pH dependent. As component B decreased the pH of NBS from 6.9 to 4.9 during 24h of incubation, it is likely that *A. pullulans* acts by acidifying the medium.



**Figure** Change in pH and reduction of Ea385 concentration in shaken cultures by Blossom-Protect (●), Component A of Blossom-Protect (▲) and Component B of Blossom-Protect (◆) in NBS (above) or in NBSpH7 (below)

## Discussion

Many control agents are under discussion to prevent blossom infections by the fire blight pathogen *Erwinia amylovora*. In a systematic evaluation 18 preparations were tested in different test systems in comparison to streptomycin sulfate. Twelve preparations inhibited *E. amylovora in vitro*, demonstrating their bacteriostatic or bactericidal potential. However, only two of them, Blossom-Protect and BPASc, have a high efficiency in field experiments (5). This shows that the *in vitro* activity is a necessary criterion but not sufficient to predict a high effectiveness on detached blossoms or in the field.

The mode of action was evaluated for the preparations with high *in vitro* activity. Some of them contain known bactericidal substances, which explains the high efficiency *in vitro*. Higher doses of the active ingredient are needed for a high efficiency in the field than in *in vitro* experiments because of dilution effects on the plant surface. For example 0.3 g/l or 3 g/l Funguran inhibited Ea385 in shaken cultures completely. On detached blossoms the efficiency of Funguran increased from 47% to 73% when the dose was increased from 0.3 g/l to 3 g/l. Streptomycin sulfate inhibits *E. amylovora in vitro* at doses of 1 mg/l (8). In the field a dose of 123 mg/l is recommended. Therefore the dose of an active ingredient should be 10 to 100 times higher in the field than its lowest dose efficient *in vitro*.

None of the preparations with bactericidal ingredients tested in this study can be sprayed in the field in such high doses. Copper is phytotoxic at high concentrations (9). The others have already been tested at high concentrations (e.g. Elot-Vis 100 ml/l; DoMoF/Lysozyme 20g/l), or are too expensive to use 10 fold higher concentrations.

The use of antagonists, able to produce antibiotics on the plant surface, could be a possibility to bring high concentrations of active ingredients to the stigma. Once the antagonist has been sprayed on the flower it should produce its antibiotic and thus prevent growth of *E. amylovora*. Serenade WPO contains spores of *B. subtilis* QST713 and it contains antibiotics produced by the antagonist during the fermentation process. The fact that Serenade WPO has only a moderate efficiency on detached blossoms and in the field (5) indicates that the *B. subtilis* spores need too much time for germination and subsequent antibiotic production on the blossom surface.

All preparations which act by altering the pH of the medium have the same problem as the bactericides. It is easy to alter the pH in the spray solution but after spraying it on the blossom the pH will be neutralised due to dilution or reaction with e.g. atmospheric CO<sub>2</sub>, or substances on the blossom. Blossom-Protect acts by reducing the pH to approximately 4 and has two mechanisms to prevent

neutralisation. Component A is a strong buffer which decreases the pH on the surface immediately after application and *A. pullulans* is able to hold this low pH during growth on blossom surfaces.

The two preparations BPASc and Blossom-Protect showed the best results on detached blossoms and in field experiments. Therefore the yeast preparations are promising tools for fire blight control in organic apple growing. Both preparations contain blastospores of *A. pullulans*. Despite reports that *A. pullulans* causes fruit russet (7,12), no increase in russeted fruits was found in field trials after the application of Blossom-Protect, carried out over nearly 20 ha by organic farmers in 2004 and 2005 (3).

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## Antagonistic activity of different yeast spp. against *Erwinia amylovora*

### Abstract

Epiphytic yeast-like fungi and yeast strains show antagonistic activity against *Erwinia amylovora*, the fire blight pathogen, in field experiments and symptom reduction on detached apple blossoms. In this study, sixteen yeast-like fungi and yeast strains including five new isolates from the apple phyllosphere, and seven yeast strains, antagonistic against postharvest diseases, were investigated for their antagonistic potential against *E. amylovora*. Results of co-culture experiments, experiments on detached apple blossoms and population studies indicated the best antagonistic effects by the yeast-like fungus *Aureobasidium pullulans* and the two yeast strains *Candida sake* DSM 70763 and *Metschnikowia pulcherrima* strain 4 against *E. amylovora*. It is not clear, which modes of action are involved. After testing the most effective strain combination in the greenhouse, their antagonistic activity has to be confirmed in the field.

### Introduction

Fire blight, caused by the bacterium *Erwinia amylovora*, is a serious disease of pome fruits in the southern part of Germany. Except for the antibiotics streptomycin and oxytetracycline as well as other compounds such as Starner, copper compounds and Blightban, which are not registered in Germany, there is no efficient agent to control the disease. On this account, we are searching for alternative biocontrol agents against fire blight.

Yeasts are suitable as biocontrol antagonists in the phyllosphere. They colonize leaf and fruit surfaces rapidly and produce extracellular polysaccharides which help them to survive under dry environmental conditions (Janisiewicz 1991). Different yeast strains have shown antagonistic properties against postharvest diseases on apple (McLaughlin et al. 1990; Piano et al. 1997; McCormack et al. 1994; Qin et al. 2003; Schena et al. 1999; Spadaro et al. 2002; Usall et al. 2000). The knowledge of the mode of action of many antagonists of postharvest diseases is poorly understood. In the absence of the production of antibiotics, it appears that the mode of action comprises a complex mechanism which could involve one or several of the following processes: nutrient competition, site exclusion, induced host resistance, and direct interaction between the antagonist and the pathogen (Wilson and Wisniewski 1994).

Epiphytic yeast strains of *Aureobasidium pullulans* and *Metschnikowia pulcherrima* have also shown antagonistic activity against *Erwinia amylovora* in laboratory-, greenhouse-, and field experiments (Seibold et al. 2004, 2005). The biological control agent "Blossom Protect", based on two strains (CF10, CF40) of the yeast-like fungus *Aureobasidium pullulans*, resulted in symptom reduction on detached apple blossoms (Kunz 2004). Biocontrol yeast agents based on yeasts showed efficacies of 0-20 % below streptomycin in field experiments (Seibold et al. 2004).

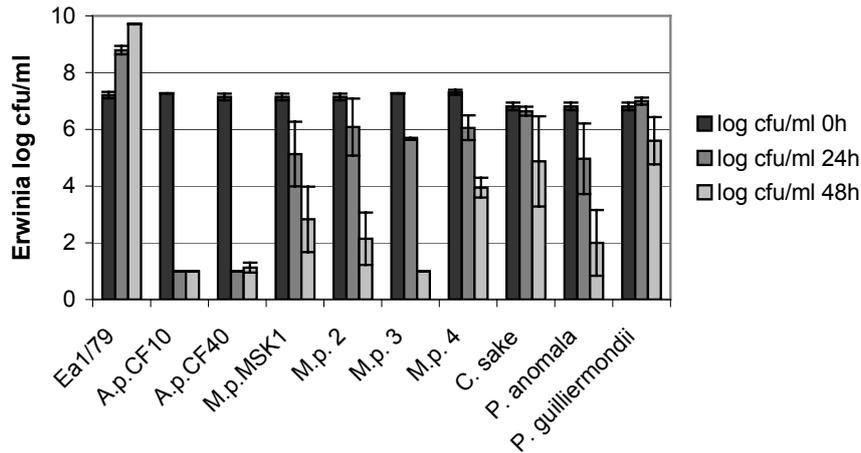
With the aim of increasing the efficacy of yeast agents in the field, sixteen yeast-like fungi and yeast strains including five isolates derived from the apple phyllosphere and seven strains described as antagonistic against postharvest diseases on apples (McCormack et al. 1994; McLaughlin et al. 1990; Pujol et al. 2004; Qin et al. 2003; Schena et al. 1999; Spadaro et al. 2002; Usall et al. 2000) and citrus (Droby et al. 1998) were investigated for their antagonistic potential against *E. amylovora*.

## Materials and methods

All experiments were performed with the *Erwinia amylovora* strain Ea1/79 (Falkenstein et al. 1988). For the experiment with detached apple blossoms we used a spontaneous streptomycin resistant mutant of this strain. As antagonistic strains, the two yeast-like fungal *Aureobasidium pullulans* strains CF10, CF40 and the yeast *Metschnikowia pulcherrima* MSK1 strain from Bio-Protect GmbH Konstanz (Kunz 2004) were used. Other yeasts included in the tests were *Metschnikowia pulcherrima* strains 2-4 and two unidentified isolates, isolated from the phyllosphere of apple trees in the southern part of Germany. *Rhodotorula glutinis* was provided from the Univ. Kaiserslautern (Germany). The yeast strains *Candida sake* DSM 70763, *Pichia anomala* DSM 6766, *Pichia guilliermondii* DSM 6381, *Cryptococcus laurentii* DSM 70766, *Hanseniasporum uvarum* DSM 2768, *Citeromyces matritensis* DSM 70187, and as a control, *Saccharomyces cerevisiae* DSM 70499 were obtained from the German culture collection. Ea1/79 was grown in Kings B medium, the yeasts and yeast-like fungi were grown in YM medium (Sigma), at 28 °C. To evaluate the antagonistic potential of different yeast strains against *E. amylovora*, antagonistic effects were investigated in three different experiments. Co-culture experiments in liquid media were performed with all yeast-like fungi and yeast strains as described previously (Seibold et al. 2005). The experiment with detached apple blossoms was performed with one day old 'Gala' apple blossoms. We used three blossoms per trial and inoculated the stigma with 5 µl yeast culture ( $1 \times 10^7$  cfu/ml). 24 h later, the stigma was inoculated with 5 µl Ea1/79Sm culture ( $1 \times 10^4$  cfu/ml). After 96 h the colony forming units of Ea1/79Sm were determined with dilution plating on Kings B medium, containing 500 µg/ml streptomycin. The population studies were performed according to Stockwell et al. (1998) with the antagonistic strains *Aureobasidium pullulans* CF10, *Metschnikowia pulcherrima* 4, and *Candida sake* DSM 70763. One year old Gala 'Royal' apple trees were incubated in a climate chamber with 80 % relative humidity, 13 h day with 22 000 lux, 22 °C and 11 h night at 15 °C. One day old blossoms were labelled and at day 0 yeast cells from overnight cultures in 10 mM potassium phosphate buffer with a concentration  $1 \times 10^7$  cfu/ml were applied to the trees. At day 1, Ea1/79 culture with a concentration  $1 \times 10^4$  cfu/ml was applied. At the day 2, 4, and 6, samples were taken. Therefore 10 blossoms were removed from at least two apple trees. The determination of colony forming units of Ea1/79 and the yeast on stigma and the hypanthium was performed with dilution plating on both YM-agar containing streptomycin (500 µg/ml) and Kings B medium containing cycloheximide (100 µg/ml).

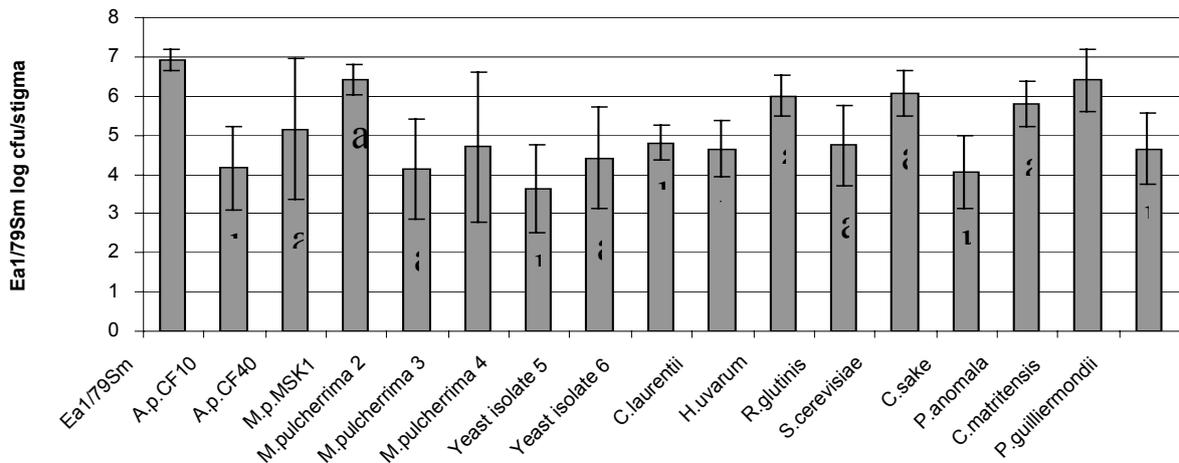
## Results and discussion

In the experiments, both, *Aureobasidium pullulans* strains, all *Metschnikowia pulcherrima* strains, *Candida sake*, *Pichia anomala* and *Pichia guilliermondii* showed growth suppression of the fire blight pathogen 48 h after its inoculation (Figure 1). The other yeast strains had no effect against Ea1/79. The pH of the medium seemed to be partially involved in the growth suppression of Ea1/79. In all samples where suppression occurs, it decreased to 5 or lower within 48 h after Ea1/79 inoculation (results not shown). A low pH of the medium alone did not result in such a strong growth inhibition. Therefore additional effects must be responsible for the decrease of the pathogen. One reason for the additional suppression could be the production of antimicrobial compounds. *Metschnikowia pulcherrima* strains are known to produce several volatile alcoholic compounds (Clemente-Jimenez et al. 2004). Maybe these compounds have an antagonistic effect on *E. amylovora*. *Aureobasidium pullulans* was described to produce three mycotoxins (Schrattenholz and Flesch 1993). It is not clear if they could have an antimicrobial effect, too. Antibiosis as a mode of action was not described for *Candida sake*, *Pichia anomala* and *Pichia guilliermondii* against postharvest diseases. However, production of antibiotics in culture media may not be necessarily indicative of their production at the site of action in the blossom. Some antibiotics have been shown to be produced in culture only (Wilson and Wisniewski 1994). Information on the mechanisms of action for most of the antagonists investigated is still incomplete (Spadaro and Gullino 2004).



**Fig. 1** Growth of *Erwinia amylovora* Ea1/79 in liquid basal medium at 28 °C together with different species of yeasts and yeast-like fungi, respectively, preinoculated 24 h before Ea1/79. Cell titer of Ea1/79 was determined with dilution plating on Kings B medium containing cycloheximide (100 µg/ml). (A.p. = *Aureobasidium pullulans*; M.p. = *Metschnikowia pulcherrima*; C. sake = *Candida sake*; P. anomala = *Pichia anomala*; P. guilliermondii = *Pichia guilliermondii*) Bars represent the standard error of the mean of three experiments

The colonisation of the stigma and the multiplication of *E. amylovora* on the stigmatic surface are crucial steps in the infection process of blossoms (Thomson 1986). In this site, bacterial control agents must interact with *E. amylovora* and successfully antagonise the pathogen (Hattingh et al. 1986; Thomson 1986; Wilson et al. 1989; Vanneste 1995). Suppression of the increase in population size of *E. amylovora* on stigmatic surfaces reduces the probability of floral infection and spread of the pathogen to other blossoms (Johnson and Stockwell 2000). Experiments on detached apple blossoms were performed to screen the yeast strains and yeast-like fungi for their ability to suppress the growth of Ea1/79Sm on the stigmatic surface. *Aureobasidium pullulans* CF10, *Metschnikowia pulcherrima* strain 4, yeast isolate 6, *Cryptococcus laurentii*, *Candida sake* and *Pichia guilliermondii* suppressed the growth of Ea1/79Sm on the stigma of detached apple blossoms significantly after four days (Figure 2). They reduced Ea1/79Sm to a cell number of around 10<sup>4</sup> cfu per stigma. Compared to the growth of Ea1/79Sm on the stigma alone, the yeast-like fungi and yeast strains suppressed the pathogen by the factor 100 to 1000 significantly. For some antagonists the standard deviation was high, but this experiment was only used to screen the antagonistic effect of the yeast strains on the stigma.



**Fig. 2** Repression of the growth of *Erwinia amylovora* Ea1/79Sm by different yeasts and yeast-like fungi on the stigma of detached apple blossoms. One day old Gala 'Royal' apple blossoms were inoculated with overnight yeast culture 24 h before Ea1/79Sm inoculation. Colony forming units of Ea1/79Sm were determined after 96 h with dilution plating on Kings B medium containing cycloheximide (100 µg/ml) and streptomycin (500 µg/ml). Bars represent the standard error of the mean. The significant differences according to t-test (P = 0,05) are indicated by letters in comparison to the control Ea1/79Sm

Population studies were conducted with three most effective strains against *E. amylovora* of the co-culture experiment and the experiment on detached apple blossoms, *Aureobasidium pullulans* CF10, *Metschnikowia pulcherrima* strain 4 and *Candida sake*. The three strains decreased the cell number of Ea1/79 on the stigma over a period of 6 days on intact apple blossoms by the factor 100-1000 significantly after 6 days to a population size of around 10<sup>4</sup> cfu per stigma (results not shown). Suppression of Ea1/79 on the hypanthium was not measurable, because of the low cell number of the pathogen on this blossom part.

In conclusion our results indicated various effects of these three yeast-like fungi and yeast species against *E. amylovora*. Until now, it is not clear, which modes of action are involved. Therefore further investigations are needed. Furthermore, these strains have to be tested for the most effective combination in the greenhouse and at last in the field to confirm their antagonistic activity against fire blight in the natural habitat.

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## Control of the fire blight pathogen with bacteriophages

### Abstract

Field trials were conducted with bacteriophages that were selected for their broad host range on *Erwinia amylovora* and their ability to control the pathogen in forced pear blossom bioassays. *Pantoea agglomerans* was used to deliver and sustain phages on the blossom surface. The lytic ability of bacteriophages and the additional biological control activity of *P. agglomerans* provided effective and stable control of the fire blight pathogen with efficacy comparable to streptomycin.

**Keywords:** bacteriophages, fire blight, biological control

### Introduction

Rosaceous spp. infected with the fire blight pathogen, *E. amylovora*, served as a source of wild type bacteriophages. Soil samples were collected from geographically different locations that were situated below the canopies of diseased *Malus*, *Pyrus* and *Sorbus* species. Phage isolation and enrichment techniques were as described by Adams (1959). The isolated phages were initially characterised by PCR (Schnabel and Jones 2001), restriction endonuclease digestion (Gill 2002), plaque size, transmission electron microscopy and host range (Gill et al. 2003). Based on their molecular characterisation profiles the *Erwinia* phages in the collection were placed into six distinct groups (Gill et al. 2003). The phages from each group were further characterised based on their ability to infect the orchard epiphyte, *P. agglomerans*. *In vitro* forced pear blossom assays and field trials were conducted to test the efficacy of the phages and the phage-carrier system against *E. amylovora* during the blossom period. A multiplex real-time PCR protocol was developed to simultaneously follow the pathogen, carrier and phage populations in the orchard.

### Materials and methods

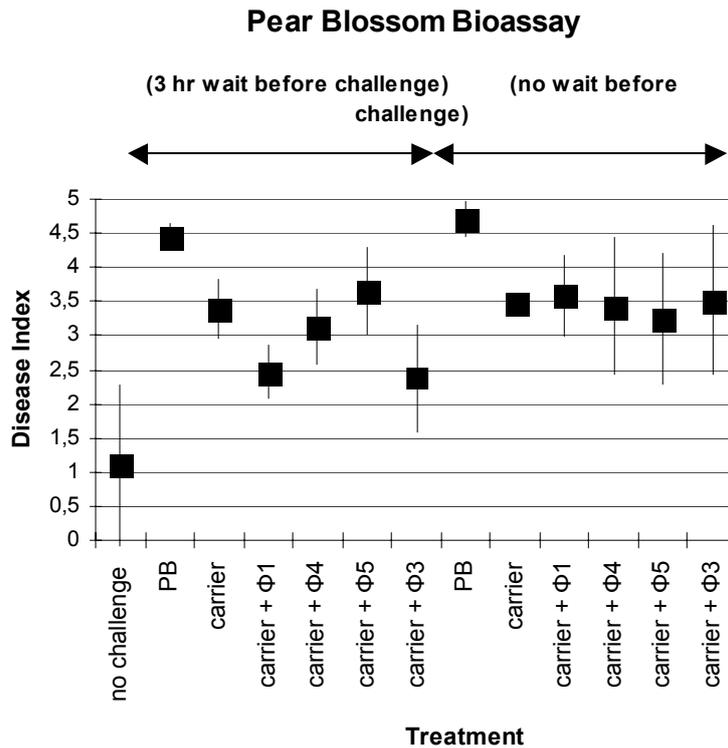
Overnight phage cultures were prepared by inoculating the original *E. amylovora* host with phages in 10L culture flasks. Bacteria were removed by continuous flow centrifugation and filtration through 0.22 µm Millipore membranes. Phage solutions were concentrated by ultrafiltration on 100 kd membranes. *P. agglomerans* cultures at  $1 \times 10^8$  cfu/ml were directly prepared by removing cells from 48 h cultures on 2% Nutrient Agar (Difco). The phages plus carrier strains were mixed just prior to application.

Experimental orchards consisted of 100 trees/orchard and were composed of pear (Bartlett) and apple (Gala) trees. The experimental treatments were arranged in a randomized complete block design. The treatments consisted of mixtures of carrier bacteria (*P. agglomerans*,  $10^6$ - $10^8$  cfu/ml) and phages (various multiplicities of infection), BlightBan® C9-1, BlightBan® A506, streptomycin (100 µg/ml a.i.), and water control. Biological control agents were applied at 25-50% and 50-100% bloom and the pathogen was applied at 50-100% bloom.

Multiplex real-time PCR protocol uses unique probes and primers to detect each microorganism on the hypanthial surface without DNA isolation steps. Collected samples were sonicated with direct plant extraction buffer (DPEB) for 2 min. The supernatant was concentrated to 100 X and a 2 µl aliquot was mixed directly with 23 µl of multiplex reaction buffer for PCR operation. Based on multiplex standard curve analysis, the technique can detect introduced bacteria to the minimum level of 20 cfu/ml.

**Results and discussion**

*In vitro* forced pear blossom assays tested the efficacy of various combinations of bacteriophages and bacteriophages plus carrier system (Figure). In separate experiments, 100 wild type isolates of *P. agglomerans* were screened for their ability to be infected with the lytic bacteriophages. Field trials in pear and apple orchards tested the efficacy of 4 phage isolates and associated carrier bacteria. The biological activity of the phages was compared to BlightBan® A506 and BlightBan® C9-1 formulations and streptomycin. The carrier-phage and the carrier alone treatments decreased fire blight incidence ( $p < 0.05$ ) in the field.



**Figure** Severity of fire blight symptoms (mean  $\pm$  SD) in forced blossom bioassay. Phages were applied with the carrier (m.o.i.=2). *E. amylovora* was applied either immediately after inoculation with the biocontrol agents, or three hours after inoculation with the biocontrol agents. Disease severity was assessed after four days. Pre-treatment with the carrier reduced disease severity. Any additional effect of each phage was only noticeable in the case where three hours elapsed between inoculation with the biocontrol agent and application of the pathogen.

The multiplex PCR system has all of the advantages of conventional PCR minus the need for gel analysis of products. The system is sensitive, reliable and reproducible and with the capacity to monitor three target populations in the orchard. This method eliminates tedious DNA extraction steps. Three primer/probe sets were designed based on chromosomal DNA sequence with the size of the amplicon at 70 bp to 150 bp range. No cross reaction was found among three sets of primer/probe and each set showed high specificity. Consequently, this method saved time and cost while it maintained comparable sensitivity to DNA isolation.

Host ranges have been established for each phage in the collection on 13 *E. amylovora* hosts and 245 *P. agglomerans*. Based on the forced pear blossom bioassays, a single carrier was chosen, and approximately 10 phages are being tested in field trials. First season data shows that several phage-carrier combinations controlled the fire blight pathogen under field conditions. The multiplex Q-PCR protocol monitors the pathogen, carrier and phage populations under field conditions. The multiplex PCR data show that the carrier supports a replicating phage population, and that the phage may replicate preferentially on the pathogen when it is present.

Work continues on the optimisation of the phage-carrier system *in planta* and the development of technologies essential for the scale up production of phages and carrier bacteria.

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## *In vitro* inhibition of growth of *Erwinia amylovora* by plant oils

### Abstract

Previous studies have demonstrated the antimicrobial properties of essential oils and their constituents from a wide variety of plants being effective against different microorganisms. The aim of this investigation was to assess the antibacterial activities of test oils against *E. amylovora*. Thirty four essential oils from medicinal plants grown in Hungary were investigated using the agar diffusion method.

Experiments were carried out *in vitro* to determine the magnitude of antibacterial activity of essential oils (Aromax Rt., Budapest) towards different antibiotic sensitive and resistant strains of *E. amylovora*: Ea1 [Strep<sup>S</sup> Hungary (H), apple], Ea 110 (Rif<sup>R</sup> USA, apple), Ea 88 (Strept<sup>R</sup> USA, pear) and Ca 11 (Strept<sup>R</sup> USA, apple). Other species such as *Pseudomonas syringae* pv. *syringae* P53 (H, pepper), *P. savastanoi* pv. *phaseolicola* E1356 (H, bean), *Xanthomonas vesicatoria* XV73 (H, pepper), XVS03 (H, tomato) served as test bacteria.

In the first selection ten different oils displayed a very effective inhibition of bacteria. From this group three oils of *Mentha*, *Thymus*, and *Tagetes* spp. displayed the most comprehensive inhibitory properties even in a dilution of 1:4. Gas chromatography analysis showed that the most effective components are carvone, dihydrocarvone, thymol, and carvacrol. There was no difference between the antibiotic sensitive (Hungarian) and resistant (USA) *E. amylovora* strains in their reaction to essential oils.

### Introduction

Previous studies showed the antimicrobial properties of essential oils and their constituents from a wide variety of plants (Maiti et al. 1985; Deans and Ritchie 1987; Dorman and Deans 2000). There are indications that extracts of *Rhus typhina*, *Juglans nigra*, *Berberis vulgaris* (Mosch et al. 1990) *Allium sativum*, *Hedera helix*, *Origanum vulgare*, *Sambucus nigra*, *Salvia officinalis*, and *Satureja hortensis* were effective against *E. amylovora* (Scorticini and Rossi 1991). Besides direct *in vitro* bactericide effects there are indications that induction of defence mechanisms in plants may also occur with plant oils. *H. helix* induced defence reactions in the plants through phenol metabolism and by increasing peroxidase, polyphenol oxidase-phenyl alanine ammonia lyase activities (Zeller and Brulez 1987; Mosch et al. 1996; Mosch and Zeller 1999).

The aim of the present investigation was to assess the *in vitro* antibacterial activities of medicinal plant oils to different phytopathogenic bacteria, especially to *E. amylovora*, with application of the agar diffusion method and determination of the active components by gas-chromatography.

### Materials and Methods

Bacteria were grown in solid King's medium B (King et al. 1954) or nutrient agar (Table 1), incubated at 26 °C during 24 h, and then washed with sterile distilled water. For standardization of the bacterial cell concentration a spectrophotometer (model 55 65-05 COLE PARMER, USA) was used at  $\lambda=460$ . The virulence test bacteria were preserved at -18 °C or lyophilized for long-term storage.

Antibacterial activity of the plant oils (Table 2) was determined by standard agar diffusion method that has been used primarily in medical microbiology measuring the size of inhibition zones. 15 ml nutrient agar medium was poured in a Petri dish. Actively growing indicator bacteria ( $100 \mu\text{l}/10^7 \text{ cfu ml}^{-1}$ ) were mixed with 3 ml 45 °C soft (0.7%) agar, then poured over the surface of the nutrient agar base. 5 holes per Petri dish (1 cm in diameter) were cut with a cork borer and filled with plant oils at different dilutions (1:2-1:16). Streptomycin sulfate, the antibiotic most commonly used for spray application against different bacterial diseases, was as control at a concentration of  $200 \mu\text{g ml}^{-1}$ . Plates were kept in a

refrigerator at 5 °C for 6 h for diffusion of the materials. After 24 h incubation at 26 °C the sensitivity of the species or strains were evaluated by measuring the diameter of the inhibition zones surrounding the holes.

**Tab. 1** Phytopathogenic bacterial species and strains

Bacteria	Code	Host plant
<i>Xanthomonas vesicatoria</i>	Xv 73	pepper
<i>Xanthomonas vesicatoria</i>	*S 03	tomato
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	P 53	apricot
<i>Pseudomonas savastanoi</i> pv. <i>phaseolicola</i>	E 1356	bean
<i>Erwinia amylovora</i>	Ea1	apple
	** Ea 110 <sup>Rif R</sup>	apple
	*** Ea 88 <sup>Strept R</sup>	pear
	***Ca 11 <sup>Strept R</sup>	apple

Test organisms were isolated and determined by M. Hevesi (Hungary), Z. Klement\* (Hungary), M. Keck\*\*(Austria), A.L. Jones, Michigan, (USA)\*\*\*

**Tab. 2** Genera of plants of the commercially prepared essential oils

<i>Achillea</i>	<i>Basilicum</i>
<i>Capsicum</i>	<i>Cinnamomum</i>
<i>Citrus</i>	<i>Cymbopogon</i>
<i>Eucalyptus</i>	<i>Eugenia</i>
<i>Hippophae</i>	<i>Hyssopus</i>
<i>Lavandula</i>	<i>Matricaria</i>
<i>Melaleuca</i>	<i>Mentha</i>
<i>Ocimum</i>	<i>Origanum</i>
<i>Pimpinella</i>	<i>Pinus</i>
<i>Rosmarinus</i>	<i>Salvia</i>
<i>Sambucus</i>	<i>Satureja</i>
<i>Tagetes</i>	<i>Thymus</i>
<i>Zingiber</i>	

Essential oils were chromatographed using a Shimadzu GC-14B gas chromatograph equipped with a SPB<sup>TM</sup>-1 capillary column (30 m x 0.2 mm id x 0.25 µm film thickness). The carrier gas was nitrogen and the injector temperature was 220 °C. The program temperature of analysis was 110 °C (for 3 min) and was gradually increased at a rate of 8 °C/min up to 220 °C (till 5 min). For detection flame ionization was used at a temperature of 250 °C. The essential oil components were identified by comparing their relative retention time with those of authentic samples.

## Results and discussion

We evaluated 34 essential oils. Three were selected as “very effective” with inhibition zones between 21-31 cm in diameter around the holes. Oils of *Mentha*, *Thymus*, and *Tagetes* spp. were remarkable in all respects (wide antibacterial spectra and effectivity in dilution 1:8 v/v). *E. amylovora* strains, originating from Hungary and USA, were not different in sensitivity to the selected essential oils.

Table 3 shows the main components of the oils analyzed. According to literature data the bactericidal effect is most probably due to the ketones carvone and dihydrocarvone, the two isomeric phenols thymol and carvacrol, and alcohol linalool. (Sivropoulou et al. 1996; Cosentino et al. 1999). Quantitative differences between the concentrations of these active components varied in the oils.

**Tab. 3** Principal components of essential oils determined by gas chromatography analysis

thymol	p-cimol
$\beta$ -terpinene	carvacrol
linalool	$\beta$ -pinene
cineol	camphene
$\beta$ -caryophyllene	borneol
$\alpha$ -pinene	limonene
dihydro-carvon	carvon
$\beta$ -ocimene	$\gamma$ -terpinene
p-cymene	carvacrol acetate

The potential utility of these essential plant oils as biopesticides should be confirmed *in vivo* by future experiments.

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## Influence of polyphenols on fire blight resistance in apple trees

### Introduction

*Erwinia amylovora*, the causal agent of the fire blight disease, is a Gram-negative, rod-shaped bacterium belonging to the family Enterobacteriaceae and infects most members of the subfamily Pomoideae, such as pear and apple. The economically most important hosts are apple and pear (Vanneste 2000). Despite all efforts of restriction, there is a progressive spread of the disease. *E. amylovora* may infect flowers early in the season (primary fire blight) and later invades shoots (secondary fire blight). The disease threatens developing fruits and may also affect fruit load of the following years by killing fruit spurs, branches and, even worse, the whole tree. Fire blight is currently controlled mainly by pruning and rooting out of affected trees (Vanneste 2000). These practices are very costly and can result in substantially reduced fruit production. Therefore, antibiotics have been used against this pathogen in some countries. However, this poses the risk of inducing resistance to antibiotics in bacteria, which are also pathogenic to humans and livestock (McManus et al. 2002). One of the most promising approaches for a sustainable and low-input agriculture is the cultivation of resistant varieties. This minimises the need for plant protectants and thus the potential risks to the environment, consumers and fruit growers.

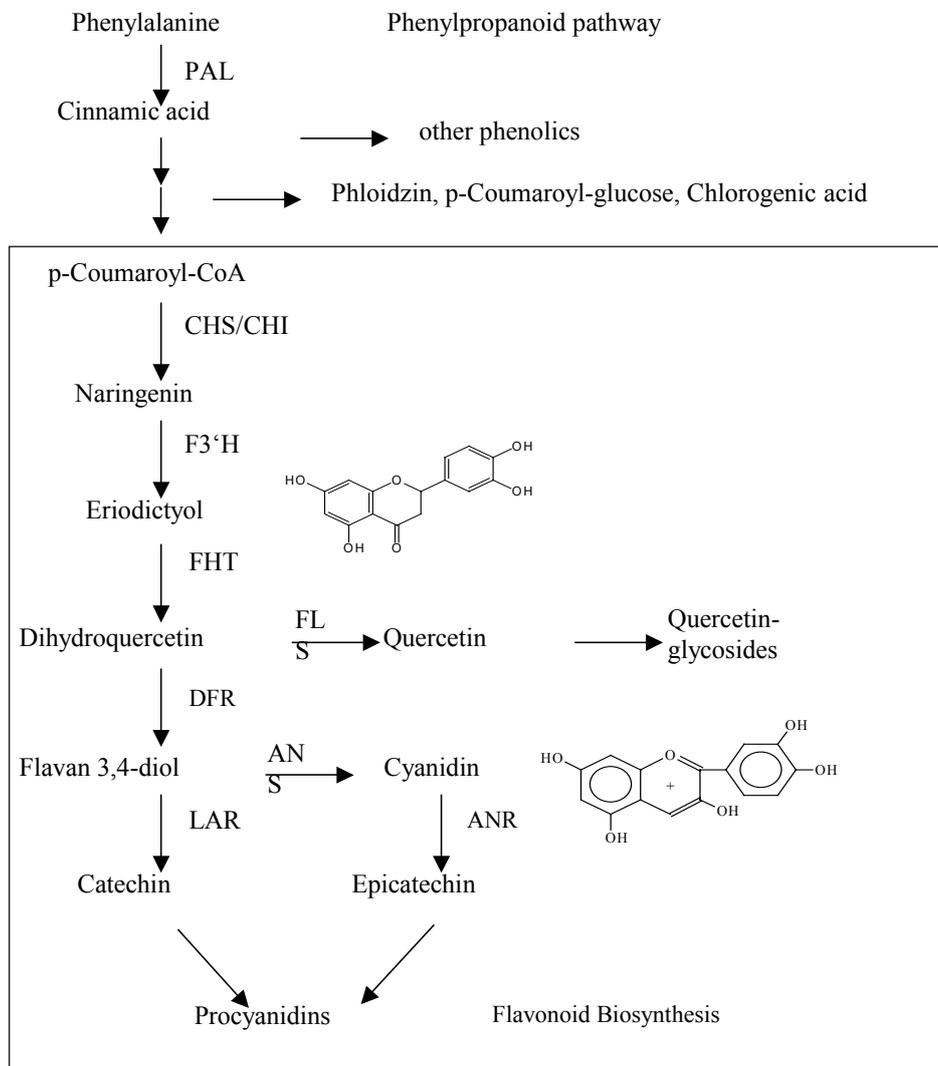
In many host plants, resistance against a wide range of fungi and bacteria is connected to the presence of phenolic compounds, in particular flavonoids (Snyder and Nicholson 1990; Feucht et al. 1998). The natural formation of 3-deoxyflavonoid phytoalexins represents a principle of resistance which can be found only in a few plant species (Lo et al. 1999; Viswanathan et al. 1996). In *Sorghum bicolor* the 3-deoxyanthocyanidins apigenidin and luteolinidin act as phytoalexins and mediate resistance to the fungus *Colletotrichum graminicola* (Snyder and Nicholson 1990). Meanwhile it could be shown that formation of 3-deoxyflavonoids can be induced by transient inhibition of flavanone 3-hydroxylase in many crops and that this is correlated to improved resistance against various plant diseases (Gosch et al. 2003; Roemmelt et al. 2003; Bazzi et al. 2003; Spinelli et al. 2005). For an in-depth biochemical and molecular biological investigation of the influence of polyphenols on fire blight resistance, we are performing a research programme which focuses on different resistant and susceptible varieties.

### Materials and methods

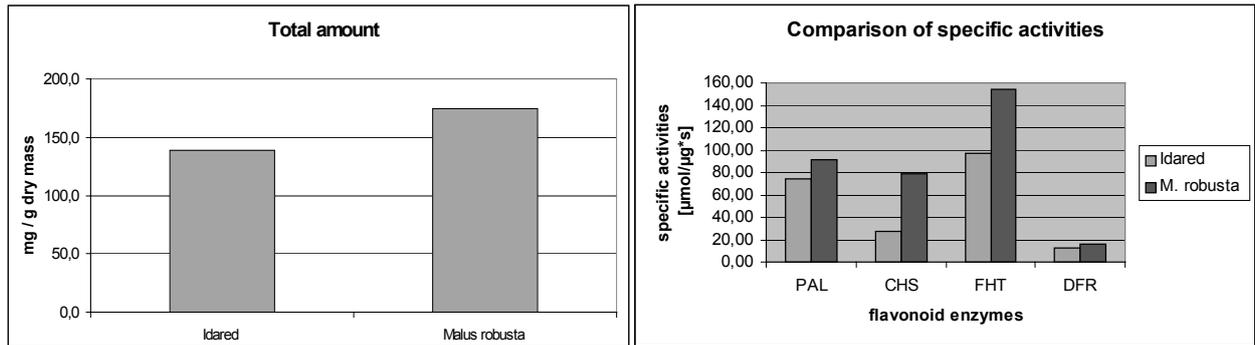
Young leaves of *Malus domestica* cv. Idared and *Malus robusta* were supplied by the Institute of Fruit Breeding (BAZ, Dresden-Pillnitz). The leaves were harvested in May 2004, frozen immediately with liquid nitrogen and stored at -80°C until use. Determination and identification of phenolic compounds were performed according to Strissel et al. (2005). Quantification was performed as follows: Total phenolic content was obtained as a sum of all compounds detected at 280 nm. Catechin and epicatechin were available as standard, procyanidins were calculated as procyanidin B2, and flavonols as rutin. Standard enzyme assays for apple leaves were carried out as previously described (Halbwirth et al. 2002). Specific activities were calculated based on the protein content, which was determined by a modified Lowry procedure (Sandermann and Strominger 1972) using bovine serum albumine as a standard. The infection experiment was performed in the greenhouse on one year old plants on *M. domestica* cv. Idared, cv. Rebella and *M. robusta*. 30 plants of each variety/species were infected by pricking the shoots with toothpicks whereas the 30 control plants were treated with the culture medium. The youngest leaves (leaf 1 and 2) were harvested before (0h) and at 4 times after infection (6h, 1d, 2d, 3d). Each sample contained 5 leaves from different trees.

**Results and discussion**

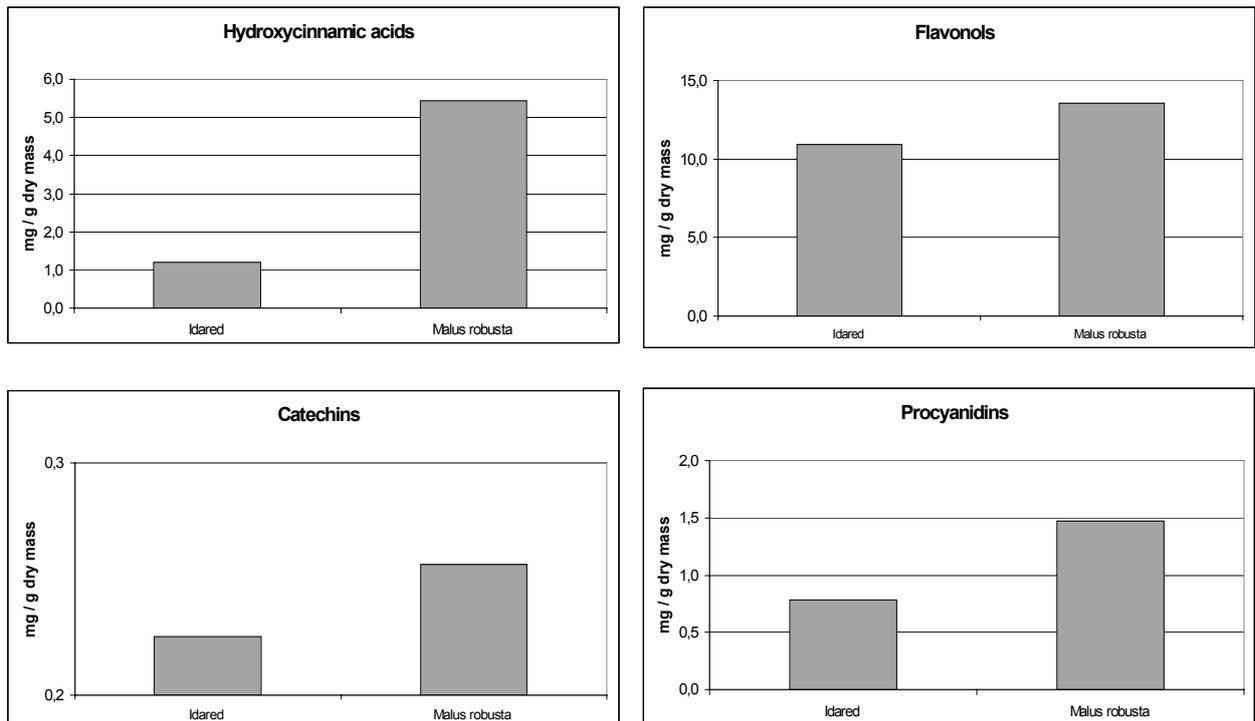
Apart from phloridzin and other phloretin derivatives, the main phenolic compounds in *Malus* leaves are a great variety of polyphenols, such as flavonol glycosides, anthocyanins, flavan 3-ols (catechins) and flavan 3-ol polymers (proanthocyanidins) (Figure 1). The resistant *M. robusta* showed enhanced total amounts of phenolic compounds compared to the susceptible cultivar Idared (Figure 2). Disease resistance of apple trees has been shown to be connected with the amount of phenylpropanoids present in the leaves (Feucht et al. 1998; Rühmann et al. 2002; Leser et al. 2005). Thus, an infection with the apple scab fungus *Venturia inaequalis* causes an increase of phenylpropanoids as a defence mechanism (Mayr et al. 1997; Picinelli et al. 1997). With regard to the different polyphenol classes, greatest differences were observed at the level of hydroxycinnamic acids, but also at the level of catechin and procyanidins considerable differences were found (Figure 3). In accordance with the analytical data, higher specific activities of the key flavonoid enzymes phenylalanine ammonia lyase (PAL), chalcone synthase/chalcone isomerase (CHS/CHI), flavanone 3-hydroxylase (FHT) and dihydroflavonol 4-reductase (DFR) were determined with enzyme preparations of *M. robusta*. However, as a rule, it is not the constitutive situation alone which decides about resistance or susceptibility. Often the velocity in activating defence reactions, once a plant is infected, is decisive.



**Fig. 1** Main flavonoid pathway in pome fruit (apple and pear) trees

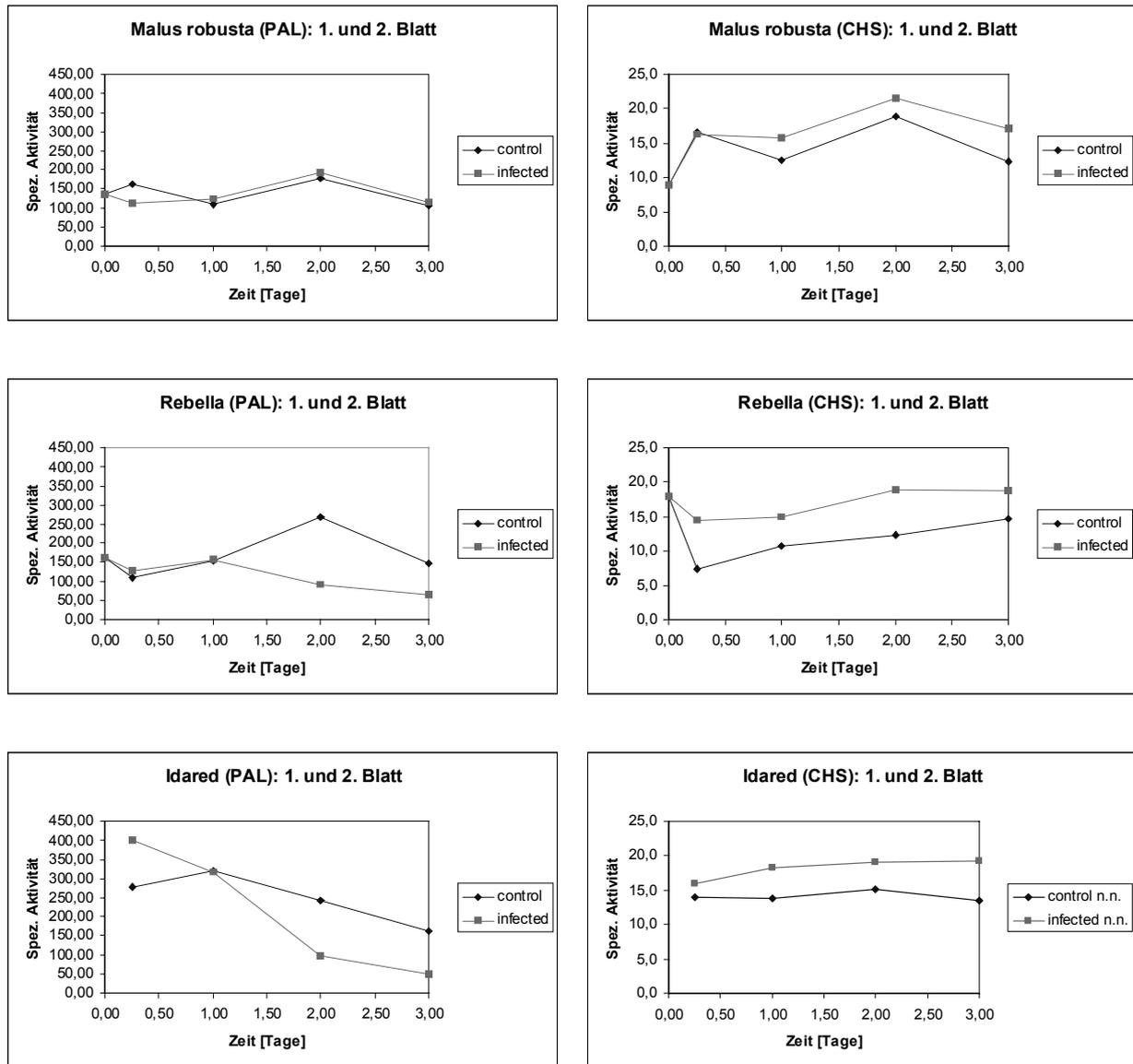


**Fig. 2** Comparison of the resistant *Malus robusta* and the susceptible *Malus domestica* cv. Idared. Left: Total amount of phenolic compounds, Right: Specific activities of key flavonoid enzymes



**Fig. 3** Differences in the concentration of different polyphenol classes in the resistant *Malus robusta* and the susceptible *Malus domestica* cv. Idared

During the infection experiment, the apple plants showed different reactions to the infection. Whereas *M. robusta* was not at all affected by the disease, Idared and Rebella developed clear symptoms and the experiment had to be stopped after the third day due to complete damage of the infected Idared trees. Regarding PAL activity, the enzyme located at the interface between primary and secondary metabolism, we observed almost no changes in the youngest leaves of the resistant wild type whereas in Rebella and Idared the activity clearly increased from the first day on. (Figure 4). Regarding CHS/CHI, all three apple cultivars/species showed a clear induction of CHS after infection, indicating an involvement of the flavonoid pathway in the defence reaction. However, the resistant apple species showed the latest and lowest induction.



**Fig. 4** Time course of two key enzymes of the flavonoid pathway: PAL (left) and CHS (right), after infection with *Erwinia amylovora* in three apple varieties showing different susceptibility. First row: resistant *Malus robusta*, second row: tolerant *Malus domestica* cv. Rebella, third row: susceptible *Malus domestica* cv. Idared

### Acknowledgement

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### **Characterization of epiphytic bacteria originating from quince and medlar trees and their antagonistic activity against *Erwinia amylovora* *in vitro***

*Erwinia amylovora* has been reported as the causal agent of fire blight in Serbia since 1989. Quince (*Cydonia oblonga*) and medlar (*Mespilus germanica*) had a high incidence of fire blight symptoms during the past few years and these fruit trees were considered very susceptible. Although quince and medlar trees are not economically significant in fruit production in Serbia, the infected trees represent a very important inoculum source for commercial apple and pear orchards. In order to detect the presence of epiphytic bacteria and to study their antagonistic effects against *Erwinia amylovora*, samples of symptomless quince and medlar flowers and leaves were collected from different regions in Serbia. Samples were taken three times during 2004-2005. First samples were collected during the flowering period, the second one month later and third two months later. During the flowering period in both years, Gram-negative, anaerobic, yellow-pigmented bacteria were predominant. According to their phenotypic characteristics they belonged to *Pantoea agglomerans*, *Pseudomonas fluorescens*, *Bacillus* sp., and *Erwinia amylovora* strains were isolated later on. Some of these strains showed antagonistic effects against *Erwinia amylovora* *in vitro*, especially a few *Pantoea agglomerans* strains and one *Bacillus* sp. strain. Further investigation is in progress.

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## **Evaluation of epiphytic bacteria for control of the fire blight pathogen**

### **Abstract**

As promising bacteria for biological control to manage fire blight Gram-variable endospore-forming rods were tested from epiphytic micro-flora of host plants. Three of ten bacteria might be effective against *Erwinia amylovora*. Their inhibitory properties were shown using diffusion tests on Emmerson-Nutrient agar medium in Petri dishes. They were additionally tested on host plants with high susceptibility to *E. amylovora* in field conditions. The test results were particularly promising. Spraying the test bacteria before was more effective than spraying them after application of *E. amylovora*. Antagonistic bacteria were not shown to provide protection against blossom blight infection.

### **Introduction**

In the Czech Republic (CR), fire blight of rosaceous plants, caused by *E. amylovora*, was first observed in 1986 (Kúdela 1988). Nineteen years later, in 2005, the disease occurs in nearly all fruit-growing areas. The use of antibiotics against the fire blight bacterium is not allowed for application in the CR, as in many other European countries. The use of copper compounds is restricted because of phytotoxicity. Therefore, biological control would be a welcome alternative to streptomycin and other antibiotics. In a survey of pear orchards in USA, several indigenous culturable bacteria, including *Bacillus* sp., were detected on pear blossoms (Stockwell et al. 1996). The purpose of our investigation was to isolate micro-organisms from epiphytic microflora that are antagonistic to *E. amylovora*.

### **Materials and methods**

Blighted plant parts of *Malus domestica*, *Pyrus communis* and *Crataegus x monogyna* were used as the source of bacterial strains with potential antagonistic activity against *E. amylovora*. The bacteria were isolated from plant tissue using 5% sucrose nutrient agar (SNA). The primary screening of the antagonistic behaviour of isolated bacterial strains against *E. amylovora* was carried out using Emmerson-Nutrient agar medium (ENA) in Petri dishes. In the test, the pathogen was incorporated into the medium and drops of cell suspension of potential antagonists were placed on the surface of the medium in spots of 5-10 mm in diameter. After 24 h incubation at 27 °C, growth inhibition of the fire blight pathogen was determined. The Biolog microplate system was used for identification of bacterial isolates. To select the most active antagonists for the orchard test, a pear fruit test (Beer and Rundle 1983) was used.

The ability of antagonistic strains to prevent fire blight was tested in a screen house. Shoots of hawthorn (*Crataegus x monogyna*) and pear trees (*Pyrus communis* cv. *Amphora*) were sprayed with the suspension of the test strains (ca. 10<sup>6</sup> cfu/ml) or with streptomycin (500 ppm), copper preparations, Kocide 2000 (0.3%) and Kupricol 50 (0.3%). The next day the shoots of hawthorn and pear were inoculated with *E. amylovora* (ca. 10<sup>7</sup> cfu/ml). Control plants were treated with distilled water.

To examine the quantitative changes in population of *E. amylovora* after the treatments, individual leaves were harvested (30 – 40 leaves per one experimental variant) 14 and 28 days after inoculation. Hawthorn and pear leaves were shaken in sterile water (10 leaf blades/100 ml) at 24 °C for 1 h, held still for 1 h, and then shaken again for 10 min. The obtained suspensions were both streaked on Miller and Schroth (1972) agar medium and deposited in wells of immature pear fruits, cut in halves. The ability of test strains to suppress the development of water soaking and necrosis of tissue was regarded as an index of efficiency of antagonism against *E. amylovora* in screen house and orchard tests.

## Results and discussion

Among isolated epiphytic micro-organisms, 10 isolates of Gram-variable, endospore-forming bacteria (GVS bacteria) with antagonistic activity against *E. amylovora* were collected from blighted host plants (apple, pear and hawthorn trees). An attempt to identify strains of GVS bacteria using the Biolog system failed. Biochemical reactions and additional tests were used to clarify their identity (growth on medium King B, ENA; on 75% NaCl and medium growth on 5% sucrose nutrient agar (SNA) containing ampicilin; pH from 6.6 to 8.6; temperature 6 °C – 40 °C).

In agar plate tests (ENA in Petri dishes), two strains of GVS bacteria (K 3Y, L1) inhibited *E. amylovora* as well as copper compounds and one strain (K 11) inhibited *E. amylovora* similar to streptomycin. Streptomycin prevented growth of *E. amylovora* in zones an average of 1.3 cm. Kocide 2000 (0.3%) and Kupricol 50 (0.3%) showed no inhibition zones and our bacteria K 11: 1.2 cm of eliminating zone with 0.6 cm of inhibiting zones, bacteria K 3Y: 0.8 cm of eliminating zone with 0.4 cm of inhibiting zones and bacteria L 1: 1.1 cm of eliminating zone with 0.4 cm of inhibiting zones.

In the screen-house and orchard tests, potential antagonistic strains and chemicals were applied on hawthorn and pear shoots, followed by inoculation of shoots with fire blight bacteria. Fourteen and 28 days later, leaves were harvested to examine the quantitative population changes of *E. amylovora*. The leaves were shaken in sterile water and immature pear fruits were inoculated with the obtained suspension (Table 1). Again strain K 11 showed the most pronounced inhibitory effect on *E. amylovora* and compares with the activity of streptomycin (500 ppm).

The biological activity was verified on hawthorn as medium resistant against *E. amylovora*. Results were very promising and surprising. The biological activity of the examined bacteria was higher than that of streptomycin. But these results must be confirmed.

**Table** Comparison of biological activity of examined bacteria with copper compounds and streptomycin

<b>2004</b>	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>E</b>	<b>F</b>	<b>Sum</b>	<b>Index</b>
H <sub>2</sub> O	10	0	0	0	0	0	0	0
Streptomycin (500 ppm)	3	5	2	0	0	0	9	0.9
K 11	2	6	2				10	1
Kocide 2000 (0,3%)	4	3	1	2	0	0	11	1.1
Kuprikol 50 (0,3%)	2	5	1	2	0	0	13	1.3
K 3Y	2	3	3	2			15	1.5
L 1	1	4	4	1			15	1.5
Ea 11/95	0	0	5	5	0	0	25	2.5
<b>2005</b>	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>E</b>	<b>F</b>	<b>Sum</b>	<b>Index</b>
H <sub>2</sub> O	10	0	0	0	0	0	0	0
K 11	4	4	1	1			9	0.9
Streptomycin (500 ppm)	0	10					10	1
L1	3	3	3	1			12	1.2
K 3Y	2	4	3	1			13	1.3
Kocide 2000 (0,3%)	3	3	2	2	0	0	13	1.3
Kuprikol 50 (0,3%)	1	5	2	2	0	0	15	1.5
Ea 11/95						10	50	5

Reactions of immature pear fruits (10 cut fruits) were inoculated with suspension, evaluative scale: 0 without symptoms; black margin; ooze inside of pear socket; ooze outside of pear socket; penetration of bacteria *E. amylovora* outside of pear socket; black lesion of whole fruit with ooze of *E. amylovora*; Sum = 0\*A + 1\*B + 2\*C + 3\*D + 4\*E + 5\*F; Index = Sum/10

## Conclusions

Several rod-shaped, Gram-variable, endospore-forming bacteria with antagonistic activity against *E. amylovora* were isolated from blighted apple, pear and hawthorn trees. The ability of test strains and chemicals to suppress the development of *E. amylovora* was shown in agar plate and pear fruit tests. The strain K 11 showed the most pronounced inhibitory effect on *E. amylovora* comparable to the activity of streptomycin.

## Acknowledgement

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## Two years of research on biological control of fire blight in New York

### Introduction

Biological control of the blossom blight phase of fire blight can be achieved by treating the open flowers with an antagonistic organism, usually a bacterium, to open flowers that then colonize the stigmatic surfaces (4,9,10). Under conducive environmental conditions, the antagonist reaches population levels sufficient to prevent floral infection of the pathogen through preemptive competitive exclusion. One such antagonistic bacterium, *Pseudomonas fluorescens* strain A506, is currently labeled as a microbial pesticide under the name of BlightBan A506 (NuFarm Americas Inc., Houston, TX, USA). A second, *Pantoea agglomerans* strain C9-1 (*PaC9-1*), also naturally produces antibiotics (herbicolin O and I) that inhibit the growth of *Erwinia amylovora* (3). A third organism, *P. agglomerans* strain E325, is currently being developed as a product called Bloomtime Biological (Northwest Agricultural Products, Pasco WA, USA). This organism provides protection through preemptive competitive exclusion.

Another product available to growers is the biological fungicide, Serenade (*Bacillus subtilis* strain QST 713). This spore-forming bacterium produces lipopeptides that exhibit antimicrobial activity. It is naturally found in soil and is sensitive to UV light. It is not a natural colonizer of stigma surfaces and is most effective when applied to open blossoms close in time to a predicted infection event.

The objective of this study was to determine the efficacy of three biological antagonists and a biological fungicide to manage the blossom blight phase of fire blight on apple. We evaluated the products in two consecutive years.

### Materials and methods

The experiments were conducted in a research orchard in Geneva, NY to evaluate the efficacy of three biological control agents, BlightBan A506, *PaC9-1*, and Bloomtime Biological; two formulations of a biological fungicide, *Bacillus subtilis* strain QST 713 (Serenade and Serenade Max) and one antibiotic formulation, streptomycin (Agrimycin 17). Individual plots consisted of two adjacent trees within a row (one inoculated and one non-inoculated), and were arranged in a randomized complete block design with five replications. Two blocks included alternately planted 'Idared' (inoculated) and 'Empire' (non-inoculated) trees and three blocks included only 'Idared' trees, all on M.7 rootstock. 'Idared' trees were inoculated at full bloom (13 May, 2004; 17 May, 2005) with *E. amylovora* strain Ea 273 at  $1 \times 10^7$  cfu/ml using a Solo backpack sprayer. The products were applied to runoff to entire trees, at timings depending on their modes of action (Tables 1 and 2), with a single nozzle handgun sprayer at 10.3 kg/cm<sup>2</sup> pressure. Blossom blight incidence was evaluated by counting the number of infected clusters out of a maximum of 200 clusters per tree 4 wk after inoculation. The percentage of the surface of 20 fruits per tree that became russeted was determined 6 wk after inoculation. Data were analyzed with the glm procedure and the Waller-Duncan *k*-ratio *t* test using SAS statistical software (SAS Institute, Cary, NC).

### Results

In 2004, non-treated, inoculated trees had 86.6% blossom clusters blighted (Table 1; Figure 1). Among the inoculated trees, Agrimycin alone reduced the number of blighted blossoms by 44% relative to the non-treated control. Serenade provided 21% control and was significantly better than the non-treated control, but not as good as the Agrimycin treatment. *PaC9-1* provided 34% control, did not differ significantly from Agrimycin, and was significantly better than BlightBan A506 alone (8% control). The efficacy of Bloomtime Biological and BlightBan A506 alone or in combination with *PaC9-1* were not significantly different from the non-treated control. Among the non-inoculated trees all treatments were significantly better than the non-treated control, yet not significantly different from each other. Fruit

russet averaged less than 4% per fruit and was not significantly different among the treatments (data not shown).

**Tab. 1** Effect of treatments on incidence of blossom blight of apple trees in New York in 2004

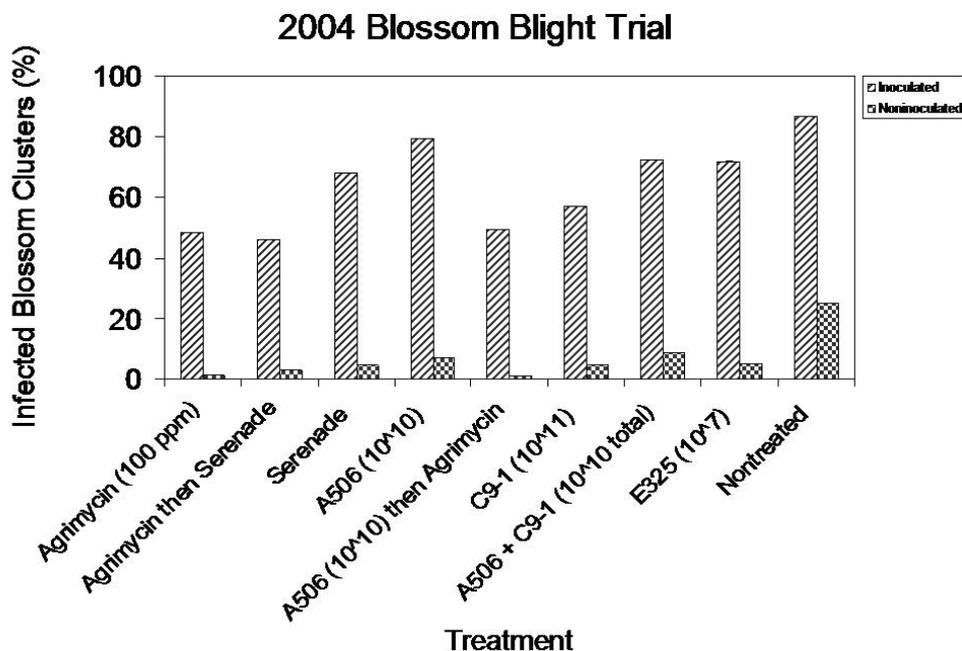
Material(s), rate/L	Spray timings <sup>z</sup>	% blossom clusters blighted <sup>y</sup>			
		I <sup>x</sup>		N <sup>w</sup>	
Agrimycin 17WP 6.0 g	4,6,7	48.4	a	1.4	a
Agrimycin 17WP 6.0 g then Serenade 10WP 28.0 g	4,6 7	46.2	a	3.1	a
Serenade 10WP 28.0 g	4,6,7	68.1	cde	4.5	a
BlightBan A506 (1x10 <sup>10</sup> cfu/g) 7.0 g	1,3,5	79.3	ef	7.1	ab
BlightBan A506 (1x10 <sup>10</sup> cfu/g) then Agrimycin 17WP 6.0 g	1 3,5	49.4	ab	0.9	a
<i>Pantoea agglomerans</i> C9-1 (1 x 10 <sup>11</sup> cfu/g) 7.0 g	1,3,5	57.2	abc	4.6	a
BlightBan A506 (5x10 <sup>9</sup> cfu/g) + <i>P. agglomerans</i> C9-1 (5 x 10 <sup>9</sup> cfu/g) 7.0 g	1,3,5	72.3	cdef	8.6	ab
Bloomtime Biological (1x10 <sup>7</sup> cfu/ml) 14.2 g	1,2	71.9	cdef	5.0	ab
nontreated	--	86.6	f	25.2	c

<sup>z</sup>Spray timing designations: 1=20-30% bloom (10 May), 2=-48 hr inoculation & 70-80% bloom (11 May), 3=-24 hr inoculation (12 May), 4=full bloom & inoculation (13 May), 5=+24 hr inoculation (14 May), 6=+4 days inoculation (17 May), 7=+11 days inoculation (24 May).

<sup>y</sup>Results are per tree and represent mean values from five replicates per treatment. Means within a column followed by a common letter are not significantly different (P>0.05) as determined by Waller-Duncan *k*-ratio *t* test.

<sup>x</sup>Blossoms for all treatments were inoculated 13 May with *E. amylovora* strain Ea 273 at 1 x 10<sup>7</sup> cfu/ml.

<sup>w</sup>Noninoculated tree within the row and adjacent to inoculated tree of the same treatment.



**Figure 1**

In 2005, non-treated, inoculated trees had 33% blossom clusters blighted (Table 2; Fig. 2). Among the inoculated trees, Agrimycin provided 80% control of blossom blight. *PaC9-1* provided 3% control and did not differ significantly from the non-treated control. Bloomtime Biological provided 28% control at the high rate, no control at the label rate, and did not differ from the non-treated control. All other treatments were statistically similar to the non-treated control. The natural disease pressure was too low

to provide sufficient mean separation between treatments of non-inoculated trees. The amount of russet on fruit of trees treated with A506 and Breakthru averaged 13.7% on inoculated, and 17.3% on non-inoculated trees and was statistically greater than all other treatments and the control.

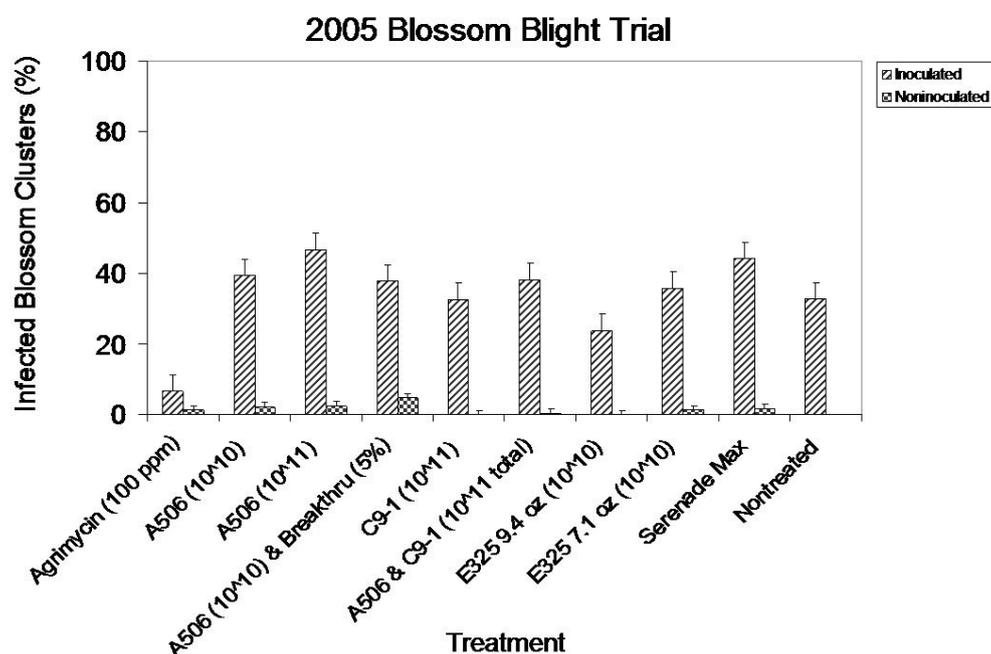


Figure 2

Tab. 2. Effect of treatments on incidence of blossom blight of apple trees in New York in 2005

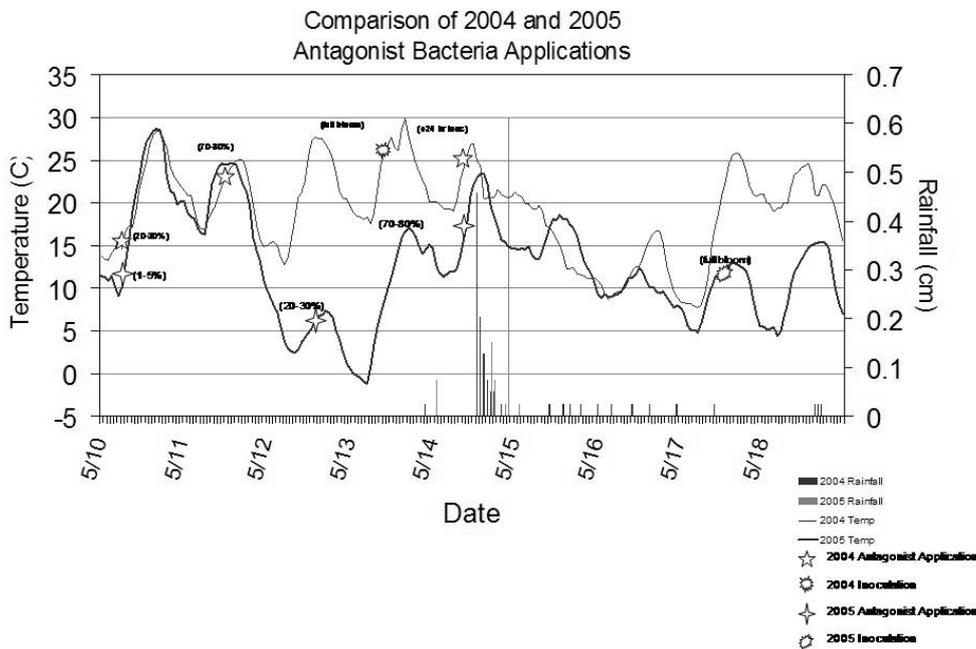
Material(s), rate/L	Spray timings <sup>z</sup>	% blossom clusters blighted <sup>y</sup>		% russet per fruit <sup>y</sup>					
		I <sup>x</sup>	N <sup>w</sup>	I <sup>x</sup>	N <sup>w</sup>				
Agrimycin 17WP 6.0 g	4,5	6.5	a	1.2	ab	3.8	a	1.3	a
BlightBan A506 (1x10 <sup>10</sup> cfu/g) 7.0 g	2,3	39.5	de	2.2	ab	3.6	a	1.9	a
BlightBan A506 (1x10 <sup>11</sup> cfu/g) 7.0 g	2,3	46.8	e	2.5	ab	3.4	a	1.5	a
BlightBan A506 (1x10 <sup>10</sup> cfu/g) 7.0 g + Breakthru 0.5% (only Timing 1)	1,2,3	37.9	de	4.7	b	13.7	b	17.2	b
<i>Pantoea agglomerans</i> C9-1 (1 x 10 <sup>11</sup> cfu/g) 7.0 g	2,3	32.7	cd	0.0	a	3.9	a	1.6	a
BlightBan A506 (5x10 <sup>10</sup> cfu/g)+ <i>P. agglomerans</i> C9-1 (5 x 10 <sup>10</sup> cfu/g) 7.0 g	2,3	38.2	de	0.3	ab	5.2	a	1.1	a
Bloomtime Biological (1x10 <sup>10</sup> cfu/ml) 7.0 g	2,3	23.7	bc	0.0	a	4.0	a	1.6	a
Bloomtime Biological (1x10 <sup>10</sup> cfu/ml) 5.3 g	2,3	35.8	cde	1.2	ab	3.3	a	1.2	a
Serenade Max 10.0 g	2,3	44.2	de	0.7	ab	3.6	a	2.5	a
nontreated	--	32.8	cd	1.7	ab	4.5	a	1.5	a

<sup>z</sup> Spray timing designations: 1=1-5% bloom (10 May), 2=20-30% bloom (12 May), 3=70-80% bloom (14 May), 4=24 h inoculation (16 May), 5=+24 h inoculation (18 May), 6=extended bloom (25 May).<sup>y</sup> Results are per tree and represent mean values from five replicates per treatment. Means within a column followed by a common letter are not significantly different ( $P>0.05$ ) as determined by Waller-Duncan  $k$ -ratio  $t$  test.<sup>x</sup> Blossoms for all treatments were inoculated 17 May with *E. amylovora* strain Ea 273 at 1 x 10<sup>7</sup> cfu/ml.<sup>w</sup> Non-inoculated tree was within the row and adjacent to inoculated tree of the same treatment

**Discussion**

In 2004, *PaC9-1* provided an effective alternative to antibiotics, while BlightBan A506 and Serenade provided effective rotation materials with antibiotics for the management of blossom blight. Data collected in 2004 agree with prior findings in New York when *PaC9-1* provided between 33% to 48% control of blossom blight and was more effective than BlightBan A506 (1,2,6,8). Studies in the Pacific Northwest and California have shown *PaC9-1* to reduce blossom infection from 50% to 80%, which was more effective than A506 which reduced blossom blight by 40% to 60% (4).

The efficacy of the antagonistic bacteria treatments in 2005 was much lower than in 2004, which may have resulted from the much lower temperatures during bloom of that year (Figure 3). Optimal growth temperatures were estimated for *P. fluorescens* A506, *P. agglomerans* C9-1 and *P. agglomerans* E325 as approximately 25 °C, 28 °C and 27 °C, respectively (7). After the 20-30% bloom application of the antagonists, the temperature reached below 0 °C. This event likely reduced population levels below effective levels. After the 70-80% bloom application, temperatures were closer to optimal growth temperatures, yet dropped down to 4 °C prior to inoculation.



**Figure 3**

These two years of research illustrate the high degree of variability of control of blossom blight with antagonistic bacteria. Integrated programs used by growers include the use of forecasting models, such as MaryBlyt and Cougarblight. As designed, these models predict the risk level of infection and prescribe the optimal time to apply antibiotics. Johnson et al. 2004 (5) recently adapted the Cougarblight model and developed a decision matrix for optimizing applications of BlightBan A506 and *PaC9-1*. Similar adaptation of the MARYBLYT forecasting model would be beneficial to growers in areas where this program is used. Replications of the field experiments are planned.

**Acknowledgements**

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## **BACTOFRUCT – Development of a biological pesticide against fire blight**

Fire blight is a major disease of apple and pear trees in many fruit producing areas of the world. The disease is most commonly initiated by epiphytic populations of the bacterium *Erwinia amylovora*. Microbial biocontrol of fire blight has been demonstrated as an alternative to antibiotics. In November 2004 the Bactofruct-project was started, funded by the European Community under the Sixth Framework Program. The aim of the project is the development of biocontrol agents for use in fire blight control. An extensive collection of *Bacillus subtilis* and related non-pathogenic *Bacillus*-species is available for the project. Screening by the University of Konstanz has shown that the bacterium *Bacillus subtilis* has potential to be an effective antagonist against fire blight. In laboratory and field trials in Belgium, Germany, Hungary, the Netherlands and Spain the efficacy of selected *Bacillus subtilis* strains will be evaluated.

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## Field experiments for fire blight control by artificial and non-artificial infection of apple trees in 2005

Fire blight, caused by the bacterium *Erwinia amylovora*, is one of the most dangerous infectious diseases of apple and pear trees. The main route of infection is during bloom via natural openings in the blossom. At present there is no efficient method to prevent infection without using antibiotics. Field experiments were arranged at a site in South-West Germany (Kirschgartshausen) to test alternatives to control fire blight. The experimental design has been evaluated over eight years [2] and follows EPPO standard PP 1/166(3). The guideline is based on artificial inoculation of single trees as source of high disease pressure for non-artificially inoculated neighbouring trees.

The results of non-artificially infected trees in the year 2005 fulfilled the EPPO guideline of 5 % infected blossoms in the untreated control but the required minimal number of blossom clusters (200) was not attained in every plot. Therefore most efficacies were not significantly different from the untreated control but confirm the general trend of previous years [1]. Strepto with the active ingredient streptomycin achieved the best control with an efficacy of 81 % followed by Blossom Protect. Blossom Protect contains two antagonistic yeast strains and had an efficacy about 60 % when applied according to fire blight prediction models or when combined with the yeast-incompatible fungicide Dithianon (Delan). We also demonstrated that fewer applications of Blossom Protect with timing based on fire blight prediction systems were as effective as applications made according to bloom phenology [3]. In combination with the fungicide Dithianon (Delan), the tested strategy seems to be practical because the efficacy of the yeast agent was not impaired. With this strategy it should be possible to fight apple scab (*Venturia inaequalis*) during bloom. It is not known if these yeast strains cause russetting. Two new antagonists *Erwinia tasmaniensis* and *Erwinia billingiae* showed medium efficacies about 50%. The efficacy of two Serenade formulations with *Bacillus subtilis* strains as active agents displayed a more or less constant level of control of around 50 %. Another yeast strain combined with a buffer (BPMSK) or resistance inducers (Phytovital and FZB 42/FZB 24, a *Bacillus subtilis* strain) as active agents, had no effect on fire blight at all.

The results obtained in this study indicate that the effectiveness of the alternative disease control products was less than streptomycin, whose efficacy and reliability is unmatched. Blossom Protect provided good disease control as reported in previous years [3], even when the number of applications was reduced from a maximum of four to a minimum of two. Progress also was made with the application of Blossom Protect in combination with the yeast-incompatible fungicide Dithianon (Delan) to fight apple scab (*Venturia inaequalis*). The effect of yeast species on fruit russetting, however, is the subject of further investigations. Serenade displayed a constant control at around 50 % and new antagonists included in this study showed promising results. Further experiments are needed to confirm and to improve the efficacies of these agents.

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## ***In vitro* studies on fire blight control by bacterial antagonists**

### **Introduction**

Both biological and chemical control agents for management of fire blight (*Erwinia amylovora*) are applied during blossom blight phase, which is considered as a key point in the management of this destructive disease of pome fruits. Prevention of blossom infections should lead to interruption of the disease cycle [2,7].

Biological control methods as application of antagonistic microorganisms [1,6,10] are alternative methods to the use of the controversial antibiotic streptomycin sulfate. *Pantoea agglomerans* (*P.a.*) is the agent of the biological control product Blossom Bless. The objectives of the present studies were 1) the isolation of *E. amylovora* suppressive bacteria, especially *P. agglomerans*, from fire blight host plants in Austria and 2) the comparison of four agar diffusion tests for their suitability for rapid *in vitro* screening for antagonistic bacterial isolates.

### **Material and methods**

Comparison of 4 agar diffusion tests: A streak assay, overlay assay [8], live assay, and chloroform assay [9] were the four agar diffusion assays evaluated. With each assay, suppression of *E. amylovora* strain 295/93 [3] by some *P. agglomerans*-isolates from bees and bee-products was observed. We also evaluated the influence of four media: King's medium B, glucose-asparagine-medium, *E. coli*-minimal medium and glycerol-ammonium medium [8,9] as the first layer in the agar diffusion tests on inhibition of *E. amylovora*. The tests were replicated four times.

Screening and identification of antagonistic bacteria isolated from fire blight host plants: Yellow pigmented bacteria were recovered from different fire blight host plants (*Cotoneaster*, *Crataegus*, *Cydonia*, *Malus*, *Pyrus* und *Sorbus* spp.) and tested for their ability to inhibit growth of *E. amylovora* 295/93 [3] in the live assay. In the live assay a first layer of a solid medium was covered with an overlay medium containing *E. amylovora* 295/93 cells. One loop of fresh suspension (~10<sup>8</sup> cfu/ml) of the test strains was streaked onto the gelled surface of the overlay. After 24 and 48 h incubation time plates were examined for clear inhibition zones in the *E. amylovora*-seeded overlay the zone around the growth of test strains.

After observing suppression of *E. amylovora* in the agar diffusion test, the ability of 12 bacterial isolates to inhibit growth of *E. amylovora* was tested further in an immature apple slice assay on apple cv. 'Gloster' [4, mod.]. Surface sterilized apple slices were placed into a suspension of test strains (10<sup>6</sup> cfu/dip). After 40 min, the treated apple slices were dipped into the suspension of *E. amylovora* (10<sup>6</sup> cfu/dip). The apple slices were incubated at 26 °C in a humid chamber and observed for *E. amylovora* ooze during a period of 10 days. As controls, apple slices were inoculated with suspensions of only the test strains or *E. amylovora*, respectively.

Antagonistic isolates, which had caused the widest inhibition zones in agar diffusion tests, were further tested [5] for phenotypical characteristics like Gram-staining, oxidase, catalase, nitrate reduction, motility, substrate utilization (BIOLOG) and a genomic fingerprinting method (REP-PCR).

## Results and discussion

*E. amylovora* suppressive strains were isolated from different fire blight host plants in Austria. Some of the most suppressive strains were isolated from *Pyrus* sp., *Malus* sp., *Cotoneaster* sp., *Sorbus* sp. and *Cydonia* sp. from different Austrian provinces. The live assay was selected as the most rapid, simple and reproducible agar diffusion test for *in vitro* screening for potentially antagonistic bacteria. In the live assay and using the media EcMM and GA as first layers, inhibition zones in the *E. amylovora* layer near the antagonistic bacteria appeared after 24 h incubation time. The size of inhibition zones often was variable in the agar diffusion test; the medium and some bacterial strains produced inhibition zones only on a single medium or with single assay methods. Around some tested isolates even a zone of greater growth in the *E. amylovora* layer could be observed.

Of selected bacteria isolated from fire blight host plants, 45% (n=130) produced inhibition zones in the live assay; inhibition zones of 21 isolates were  $\geq 5$  mm wide. Nine of 12 antagonistic strains completely suppressed ooze production by *E. amylovora* in the immature apple slice assay. Four of the antagonistic strains were identified as *P. agglomerans* by biochemical tests and the BIOLOG-system. In addition to matching the phenotypic characteristics, seven of the nine most suppressive strains share the same amplification products (0.65 kb, 1.7 kb, 1.75 kb, 2.25 kb) as the *P. agglomerans* type strain in REP-PCR and thus are most likely *P. agglomerans*.

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## Fire blight control by application of fungal antagonists

### Introduction

Since 1993 fire blight has spread towards many parts of Austria and is an increasing problem to pome fruit orchards and extensive plantations in many parts of the country. Searching for control measures as alternatives to the antibiotic streptomycin sulfate, the efficacy of two fungal antagonistic products for reducing incidence of fire blight blossom infections was investigated under controlled conditions.

### Material and methods

Trials were arranged under controlled conditions in quarantine glasshouses according to EPPO standard PP 1/166(3) [2] unless otherwise specified. For details see Tables 1, 2.

The preparations Blossom Protect Fb and BPMC<sub>p</sub> were sprayed to run-off according to the manufacturer's recommendations at BBCH growth stages 61 (~10 % of flowers opened), 64 (~40 % of flowers opened), and 65 (~70 and ~90 % of flowers opened). The reference product Plantomycin (streptomycin sulfate) was applied as specified for the test products.

Opened flowers were inoculated individually by placing two drops of *E. amylovora* suspension (strain 329/98): one drop placed on stigmas and one drop on the hypanthium next to the filaments, 4 h after application of the test and reference products.

**Tab. 1** Elements of the trial

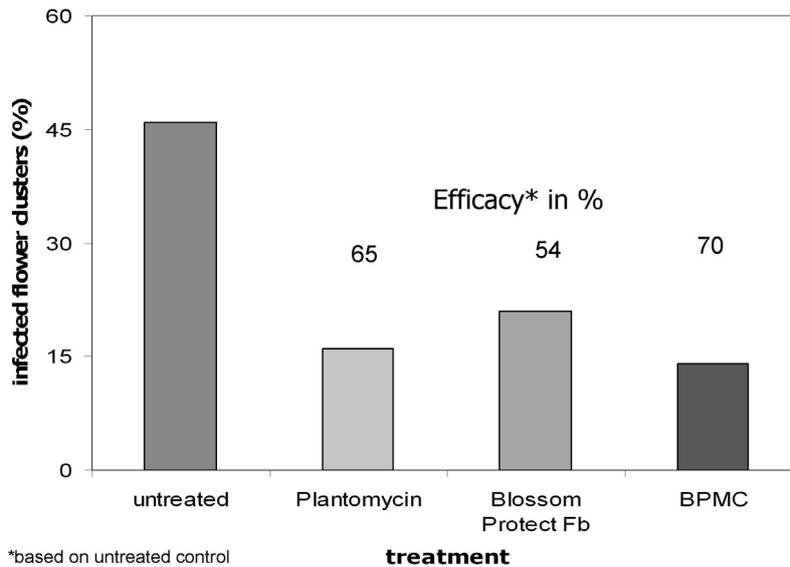
	agents	concentration
untreated control	-	-
Plantomycin <sup>®</sup>	streptomycin sulfate 21,2%	0,06 %
Blossom Protect Fb	<i>Aureobasidium pullulans</i> (= yeastlike fungus) 2x10 <sup>10</sup> blastospores/g	agents 0,15 % adjuvans 1,05 %
BPMC <sub>p</sub>	<i>Metschnikowia pulcherrima</i> (= yeast) 5x10 <sup>10</sup> blastospores/g + <i>A. pullulans</i> 1x10 <sup>10</sup> blastospores/g	agents 0,15 % adjuvans 1,00 %

**Tab. 2** Data of test conditions

location and climate	quarantine glasshouses; day: 27 °C; night: 15 °C; 80 % RH
test plants	one year old container-grown apple trees cv. Golden Reinder's <sup>TM</sup> , M9
replications	3
number of trees per treatment group	10
type of application	spray application with a simple hand-held atomizer
artificial inoculation	<i>E.a.</i> 329/98; flowers inoculated individually via micropipette (~2x10 µl suspension/flower)
other control measures	none
type of assessment	percentage of infected flower clusters

### Results

Average blossom infection rates of the tested products were 20% for Blossom Protect Fb and 15% for BPMC<sub>p</sub> respectively. In the untreated control more than 48 % of the flowers showed the typical symptoms of infection. The efficacies of the tested products were calculated 54 % for Blossom Protect Fb, 70% for BPMC<sub>p</sub> and 65% for the reference product Plantomycin.



**Figure** Efficacy of biocontrol products against fire blight on artificially inoculated apple trees “Golden Reinder’s” on M9 in greenhouse trials 2005

### Discussion

Under controlled conditions and after artificial inoculation incidence of blighted flower clusters was significantly reduced after application of BPMC<sub>p</sub> and Blossom Protect Fb. Both products showed efficacies of over 50% when applied 4 times according to the manufacturer’s recommendations. Efficacies of BPMC<sub>p</sub> and Blossom Protect Fb were comparable to streptomycin sulfate in these trials. These findings show the same tendency as results of field trials conducted in Germany [1,3,4]. Further trials are needed to confirm these results under field conditions.

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- Dispersal of the biocontrol agent *Aureobasidium pullulans* for fire blight control using honeybees (*Apis mellifera carnica*).

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## **Dispersal of the biocontrol agent *Aureobasidium pullulans* for fire blight control using honey-bees (*Apis mellifera carnica*)**

### **Abstract**

The effectiveness of using honey-bees to disperse mixture of two strains of *Aureobasidium pullulans* CF10 and CF40, active ingredients of Blossom Protect™ from hive-mounted dispenser to apple trees in controlled greenhouse experiments as well as to apple and pear trees in two orchards in Vorarlberg and Upper Austria was evaluated during spring 2005. In addition, in greenhouse trials on plant-to-plant transport of *Erwinia amylovora* and the potential of dispersed *A. pullulans* strains to reduce fire blight incidence was studied. Samples of freshly stored nectar and honey were collected from hives in two orchards and were tested for the presence of distributed strains. In greenhouse experiments, beehives with approx. 1500 bees and in orchard experiments commercial honey bee colonies were used. All hives were equipped with dispensers. Populations of *A. pullulans*-like colonies on flowers collected from honey-bee-delivered treatment in greenhouse trials were higher than those on flowers collected from control treatment. *E. amylovora* was detected on the majority of control flowers, which indicated a high transfer rate from inoculated to control trees by honey-bees. Dispersed antagonistic strains showed significant potential to reduce the incidence of fire blight. After dispersal of *A. pullulans* CF10 and CF40 in orchard trials from both locations, none of these strains could be re-isolated. Only a few viable antagonists were re-isolated from freshly stored nectar and honey. Our data demonstrate that the dispersal of the antagonistic fungi *A. pullulans* CF10 and CF40 using honey-bees in greenhouse experiments represents a promising alternative to control fire blight. For an effective use of honey-bees in dispersal of antagonistic microorganisms in orchards, some improvements have to be made on dispenser construction and formulation of antagonistic material. Specific primers for the rapid identification of *A. pullulans* CF10 and CF40 should be developed.

**Key words:** *Aureobasidium pullulans*, honey-bees, *Erwinia amylovora*, fire blight, biological control

### **Introduction**

The cosmopolitan dermatiaceous fungus *A. pullulans* (de Bary) Arnaud is one of the most distributed and well-adapted saprophytes in the phyllosphere and carposphere (2). Some *A. pullulans* strains show antagonistic activity against a number of phytopathogenic microorganisms (6,7). Two strains of *A. pullulans* CF10 and CF40, active ingredients of Blossom Protect™, showed antagonistic potential against *E. amylovora*, causative agent of fire blight (7). The control of fire blight pathogens still needs to be improved, especially in extensive plantations in many parts of Austria. The use of honey-bees to disperse antagonists of *E. amylovora* to apple and pear flowers could be an alternative control strategy. It has been previously shown that honey-bees are able to disperse biocontrol agents (4) as well as *E. amylovora* (1). The aim of this study was the assessment of the role of honey-bees in dispersion of fungal antagonists of fire blight. We investigated if honey-bees under controlled greenhouse conditions were able to transfer antagonistics from a hive-mounted dispenser to surrounding apple trees. In addition, we studied plant-to-plant transport of *E. amylovora* strain 329/98 during foraging activity, as well as the influence of dispersed fungal strains in the incidence of fire blight. Additionally, the dispersal of the fungi on apple and pear trees was monitored in two orchards. Freshly stored nectar and honey were tested for the presence of distributed strains.

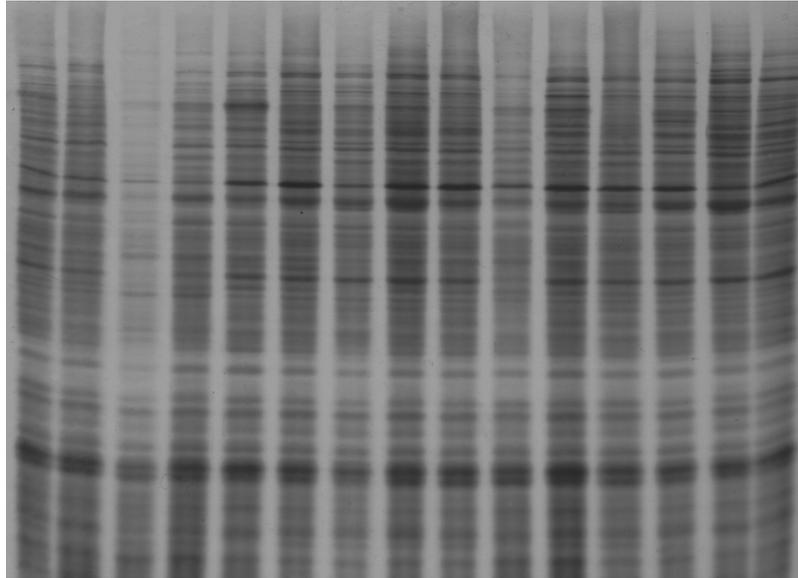
## Material and methods

Greenhouse experiments (4 series) were arranged in two separate quarantine cabins under controlled conditions: day: 27 °C; night: 15 °C; 80 % RH. Each quarantine cabin was equipped with 16 one-year-old container-grown apple trees cv. Golden Reinder's™, M9 and a beehive with approx. 1500 honey-bees equipped with a dispenser. When the bloom started, one dispenser was filled with a mixture of *A. pullulans* CF10 and CF40. The dispenser was filled with antagonistic strains for 4 days. In the other quarantine cabin a dispenser without antagonists was used as control. In each quarantine cabin flowers of 6 trees were inoculated with the local strain of *E. amylovora* 329/98 (using a micropipette, 2x10 µl/flower, approx. 10<sup>8</sup> cfu/ml) for 3 days and re-inoculated with honey-bees during foraging activity. Flowers of four other trees were inoculated only by honey-bees used as a control from which samples were collected. After 3 days, beginning 24 h after filling the dispenser with antagonists 10 flowers, 2 days old, were collected. Flowers without petals were washed in a 2 ml-microcentrifuge tube, containing 1 ml 10 mM phosphate buffered saline (PBS). Dilutions (0.1 ml) were plated on KB-agar and on SAB-dextrose (SAB) agar plates. KB-agar plates were incubated for 48 h to isolate *E. amylovora* and SAB agar plates were incubated for 72 h to isolate *A. pullulans*. The identity of representative colonies of each sample on KB-agar was subsequently determined by PCR (3). To quantify the fungal-vectoring ability of honey-bees, the number of cfu of colonies similar to *A. pullulans* were enumerated. To identify *A. pullulans* CF10 and CF40 among isolates on SAB, colonies with obvious similarities in colony morphology and pigmentation to *A. pullulans* CF10 and CF40, were randomly picked and subjected to SDS-PAGE for comparison of their protein pattern (6). Disease incidence was rated 14 days after the appearance of the first symptoms in the control quarantine cabin. For statistical analyses only trees with more than 10 flower clusters were evaluated. The percentage of infected flower clusters from the treated quarantine cabin was compared with that of the control cabin.

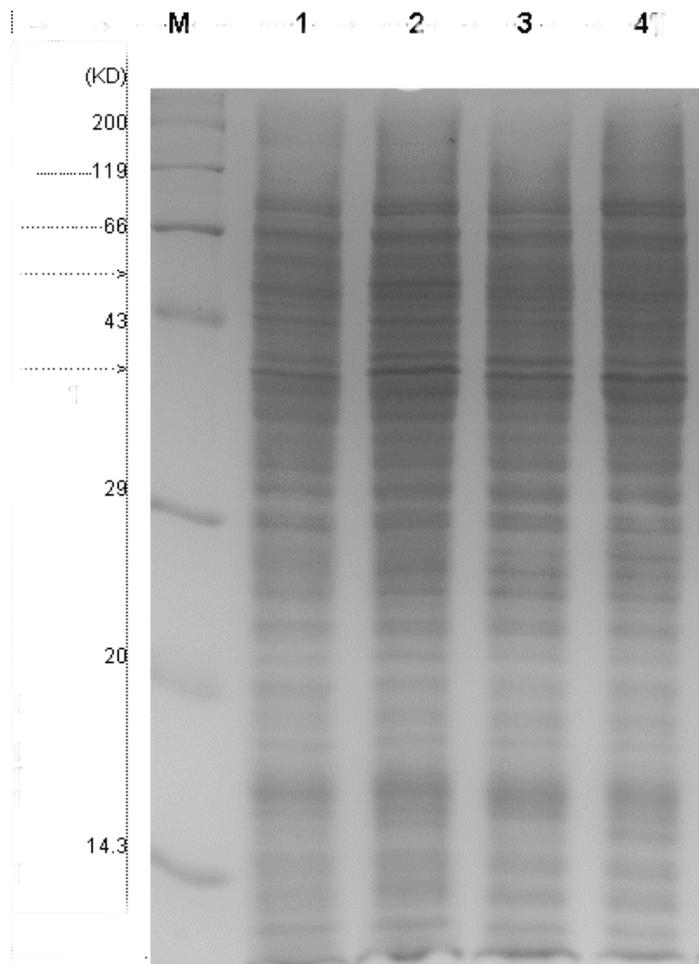
To determine the effectiveness of honey-bees in delivering *A. pullulans* CF10 and CF40 to apple and pear flowers, orchard trials were carried out in 2 orchards (Vorarlberg and Upper Austria). In each orchard, 5 commercial honey-bee colonies were equipped with a dispenser filled with antagonistic strains. Dispensers were filled with *A. pullulans* CF10 and CF40 every morning during the whole bloom period (14 days). Five apple and 5 pear trees (distances: next to beehive, approx. 50 m, 100 m, 150 m and 200 m) from each orchard were selected for evaluation 100 pear flowers (20/tree) were collected before application of the antagonists and 100 apple and 100 pear flowers (also 20/tree) after application of *A. pullulans* CF10 and CF40 were collected. Identification of *A. pullulans* CF10 and CF40 was made as described for greenhouse experiments. On the last day of dispersal, samples of freshly stored nectar were collected from each beehive equipped with a dispenser. One sample per hive was monitored for the presence of distributed strains. A total of 2 ml of freshly stored nectar was sampled by using a micropipette. Freshly stored nectar was diluted with the same amount of sterile aqua bi-dist. and dilutions (0.1 ml) were plated on SAB agar. Identification of *A. pullulans* CF10 and CF40 was identified as described for greenhouse experiments. Honey samples were collected during extraction and tested for the presence of distributed strains. 2 g of honey was mixed with 2 ml of sterile aqua bi-dist and dilutions (0.1 ml) were plated on SAB agar. Identification of *A. pullulans* CF10 and CF40 was made as described for greenhouse experiments.

## Results

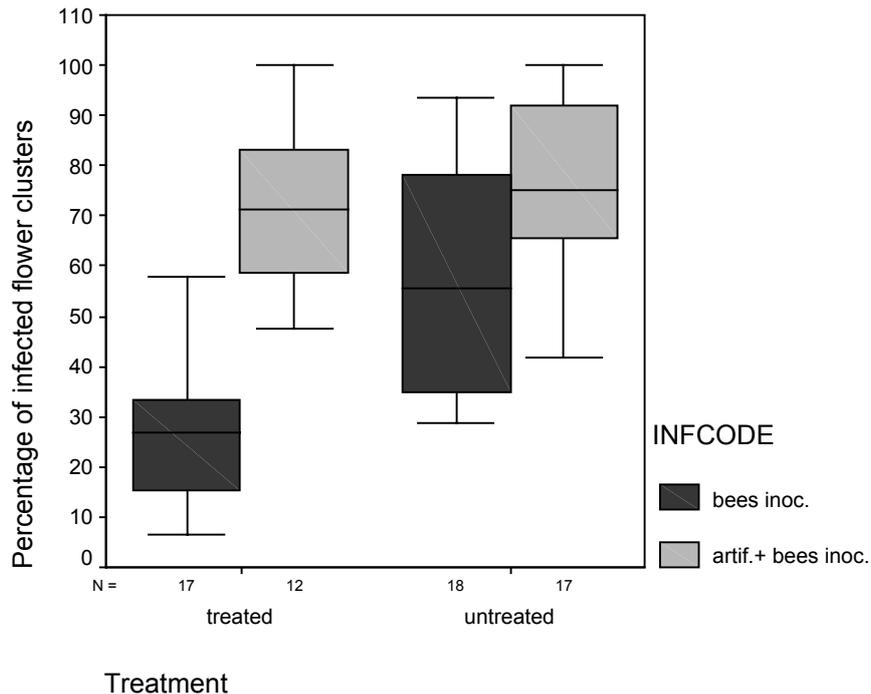
After dispersal of *A. pullulans* CF10 and CF40 in all greenhouse trials, the populations of *A. pullulans*-like colonies were higher (approx. 10<sup>4</sup> cfu/blossom) compared to the control (less than 10 cfu/blossom). Analysis of protein patterns revealed that 68.9% of representative strains had the same protein pattern as *A. pullulans* CF10 or CF40 (Figure 1). Protein gels are shown in Figures 1 and 2. *E. amylovora* was detected on 93.6% of the control flowers, which indicated a high transfer rate from inoculated to control trees by honey-bees. The influence of dispersed strains on the reduction of fire blight incidence is shown in Figure 3.



**Fig.1** SDS-PAGE of diverse naturally occurring *A. pullulans* strains



**Fig. 2** SDS-PAGE of *A. pullulans* strains. The lanes show: M, protein weight marker, 1, *A. pullulans* CBS 584.75, 2, *A. pullulans* CF40, 3, *A. pullulans* CF10, and 4, wild type of *A. pullulans*. The largest differences in protein patterns are marked by arrowheads. In both locations of orchard trials the antagonistic strains *A. pullulans* CF10 and CF40 were not found prior to their dispersal. After dispersal of *A. pullulans* CF10 and CF40 by honey-bees in orchards none of these strains could be re-isolated from apple or from pear flowers



**Fig. 3** Influence of *Aureobasidium pullulans* dispersal by honey-bees on fire blight incidence in greenhouse trials (4 series)

In freshly stored nectar, 1 out of 41 representative isolates showed the same protein pattern as *A. pullulans* CF40. In honey, 2 out of 17 representative isolates showed the same protein pattern as those from the distributed strains.

### Discussion

In this study it was shown that honey-bees are able to disperse two antagonistic strains of the fungus *A. pullulans* from hive-mounted dispenser to surrounding apple trees under controlled conditions in the greenhouse. These results are in agreement with observations in some outdoor experiments using bacterial antagonists (9). Application of a mixture of strains *A. pullulans* CF10 and CF40 increased the population sizes of *A. pullulans*-like colonies on the flower surface. In contrast, populations of *A. pullulans* in control treatments were the same during the period of sample collecting. The population sizes of *A. pullulans* CF10 and CF40 on flowers required to provide satisfactory control of fire blight is not known. On the basis of our greenhouse-experiments, however, we demonstrated that the strains *A. pullulans* CF10 and CF40 dispersed by honey-bees showed a significant potential to reduce the incidence of fire blight. In orchard trials antagonists dispersed by honey-bees were not isolated from apple or pear flowers. During orchard trials weather conditions were unfavourable for an effective dispersal of antagonists. Rain limited the amount of dispersed antagonists and decreased bee activity. Honey-bees also were attracted by other flowers (e.g. dandelion, cherry and colza). The SDS-PAGE technique allowed us to distinguish strains *A. pullulans* CF10 and CF40 from each other and from other naturally occurring *A. pullulans* strains. Other techniques such as RAPD-PCR (5) were time-consuming and costly. Specific primers for strain-specific identification of *A. pullulans* CF10 and CF40 have not been developed yet.

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## **The essential oil of thyme as a natural plant extract for fire blight control**

### **Abstract**

Plant extracts including essential oil (EO) of *Thymus vulgaris* were assessed for control of *Erwinia amylovora* both *in vitro* and *in vivo*. The antibacterial effects of different extracts were screened in dual cultures against *E. amylovora* in agar-well diffusion method and immature pear fruit assays. Phytotoxicity of the extracts was tested on intact pear blossoms. Among different extracts, thyme EO isolated by hydro-distillation showed a high level of bacteriostatic effects producing 20-26 mm inhibition zones on SNA medium and suppressing bacterial exudates on pear slices. Neither crude water/organic mixtures nor soxhlett extracts could exhibit distinct antibacterial properties. It was only found that the alcoholic extracts obtained by soxhlett extraction gave some degree of bacterial growth inhibition (2 mm) without bacterial exudation. Efficiency of the alcoholic phase decreased within 48 h. This result seems to be promising when compared with the efficacy of some other recommended chemicals such as 0.3% copper oxychloride, 1% Bordeaux mixture (1:3:100) and 1% Serenade inducing traces of <1 mm and >1mm inhibition zones, respectively.

**Keywords:** *Thymus vulgaris*, essential oil, fire blight control

### **Introduction**

Fire blight was initially observed as endemic incidence from the countryside of Karaj in 1989 (Zakeri and Sharifnabi 1991). Since then two epidemic outbreaks were reported in the main pome fruit growing areas of the country; about 20,000 ha. pomaceous fruit trees mainly quince, pear and apple were seriously affected by fire blight disease (Hassanzadeh 2002). Among different disease managements, application of certain bioagents including antagonistic bacteria and plant extracts are promising (Mosch et al. 1990; Scortichini and Rossi 1993; Mosch and Zeller 1999; Rezaee and Hassanzadeh 1998; Zeller 2004). In this study the efficacy of thyme essential oil on *E. amylovora* was compared with some natural, chemical and biological compounds.

### **Materials and methods**

Extraction of essential oil (EO): 60 g of spring thyme plant samples, grown in experimental fields of the Pesticide and Fertilizer Industrial Research Center, were collected, shade dried and distilled in 1200 ml of D.H<sub>2</sub>O for 4 h. About 1.5 ml essential oil was obtained from each sample.

Other extracts: Other extracts were obtained by mixing 20 g of powdered leaf and flower samples in flasks containing 600 ml of different organic solvents (methanol, ethanol, acetone and petroleum ether) and distilled water as a control. The flasks were then shaken at 150 rpm for 4 h. The mixtures were evaporated to 50 ml and the soxhlett mixtures were heated at 70 °C for 4 h.

Bioassays and field assays: Inhibitory effects of different extracts were assessed in dual cultures against *E. amylovora*. 4-mm holes were made in the center of levan medium, each was filled with 6 µl aliquots of thyme plant extracts, copper oxychloride (0.3%), Serenade (1%) and 1% Bordeaux mixture (1:3:100). After two h of incubation period and soon after the drops were absorbed, the plates were sprayed uniformly with a suspension of *E. amylovora* at ca 10<sup>8</sup> cfu/ml. The immature pear fruit test (IFB) was assessed with the bacterial suspension added equally in the same wells. The apple, pear and quince blossoms were sprayed with few natural and chemical compounds without an artificial inoculation.

**Results and discussion**

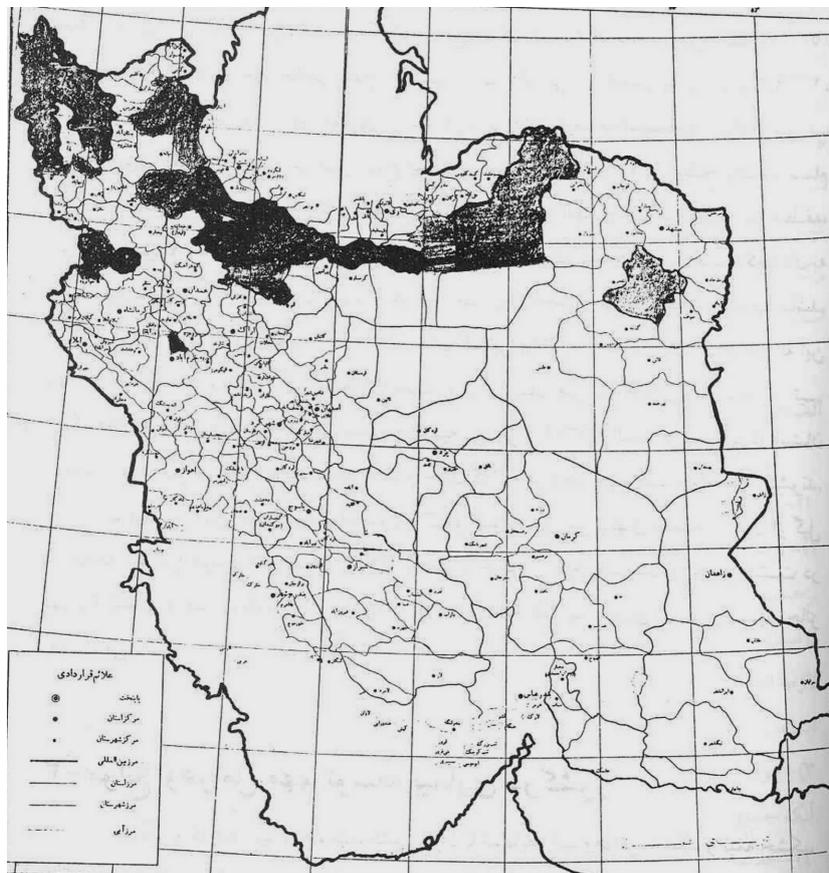
Thyme EO isolated by the hydro-distillation method showed high levels of bacteriostatic effects producing 20-26 mm inhibition zones on SNA medium and suppressing bacterial exudates on pear slices (Table 1). Neither the crude water/organic mixtures nor soxhlett extracts could exhibit distinct antibacterial effects. On the other hand, ethanolic soxhlett extracts exhibited 2 mm inhibition zones with no distinct bacterial exudation and blossom side-effect.

**Tab. 1** *in vitro* assays of different natural and chemical compounds against *E. amylovora*

Extract/compound	Inhibition zones (mm) on SNA		Exudation on IPF test	Blossom phytotoxicity
	24h	48h		
Thyme EO	20-26	20-26	-	++
Distillate extract	trace	-	+	-
Boiled extract	trace	-	+	-
Ethanolic Soxhlett	1.5	-	-	W
Crude extracts	trace	-	NT	-
Serenade 1%	1-2	1	+	-
Bordeaux mixture 1%	0.5-1	0.5	-	W
Copper oxychloride 0.3%	trace	-	+	-

++, highly phytotoxic -, no reaction, NT, not tested, W, weak reaction

The main disadvantage of plant EOs including thyme EO is their phytotoxic effect. When thyme EO was diluted at 10<sup>-3</sup> level with a mixture of ethylene glycol and soprophor FL (1:1), the mixture on the culture media induced 1-1.5 mm inhibition zones, leaving trace amounts of phytotoxic effects on blossoms. Based on these results the application of plant essential oils against *E. amylovora* seems to be promising.



**Figure** Map of Iran and current fire blight diseased areas

## Acknowledgements

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## Evaluation of the diversity of *Erwinia amylovora* in Bulgaria

Fifty one strains of *Erwinia amylovora* were isolated from various host plants (pear, apple, quince, chokeberry, strawberry, hawthorn, *Cotoneaster* and *Pyracantha*) growing in distant regions of Bulgaria. The strains were characterized and identified using API 20E, BIOLOG and PCR-amplification of specific regions in chromosome and pEA29 plasmid. The diversity among the strains was studied by different approaches: determination of antibiotic resistance, total cellular proteins, metabolic activity, and macro-restriction analysis of chromosome DNA. The strains showed very similar reactions to antibiotics tested and all were sensitive to streptomycin. The protein profiles obtained after SDS-PAGE revealed high similarity among the strains studied. At 75% similarity they were grouped in four clusters. Cluster I included 24% of the strains, cluster II, the type strain of *E. amylovora* and 61% of Bulgarian isolates, cluster III, 13% of the isolates, and cluster IV consisted of only one strain. The determination of the metabolic fingerprints of the isolates was performed with the BIOLOG system. At 75% similarity the strains were grouped in three clusters. The patterns obtained by *Xba*I digest after PFGE allowed us to distinguish five groups of Bulgarian *E. amylovora* strains. The major group included 79% of the strains and their profile is type Pt2. We established a new PFGE profile, unknown in the literature and typical of strawberry strains. The PFGE patterns obtained by *Spe*I digests were similar.

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## Identification of resistance donors for fire blight in *Malus*

Fire blight is a dangerous disease in pome fruit production. The cultivation of resistant apple varieties can prevent losses caused by *Erwinia amylovora* thereby avoiding spraying of chemicals. Some resistant varieties (eg. 'Reanda', 'Remo', 'Rewena') were bred at Dresden-Pillnitz. The donor of resistance was *Malus x floribunda*. To enlarge the genetic basis of resistance to fire blight, a wide range of accessions of wild species, present in the gene bank in Dresden-Pillnitz, was tested for resistance to fire blight in the greenhouse. Some accessions were tested up to 14 times in the last 14 years to find durable resistance donors independent of the *E. amylovora* strain used.

Highly virulent strains of *E. amylovora* were inoculated into growing shoots grafted on rootstocks. Eight weeks after inoculation the ratio between length of necrosis and shoot length was calculated. Accessions of *M. x atrosanguinea*, *M. x dawsoniana*, *M. fusca*, *M. x prunifolia*, *M. x robusta*, *M. sieboldii* and *M. x zumi* were found to be highly resistant to fire blight, whereas accessions of *M. tschonosky*, *M. arnoldiana* and *M. trilobata* were very susceptible to the pathogen. Accessions of *M. baccata* revealed large differences in the reaction to fire blight, some of them were very highly resistant and some highly susceptible.

The wild species showing stable resistance to fire blight provide a valuable tool for breeding of fire blight resistant varieties.

**Table** Wild species of *Malus* as a tool for fire blight resistance breeding

<i>Malus</i> wild species	Accession number	% shoot blighted	Years tested
Idared (susceptible variety)		72,3	12
<i>M. x arnoldiana</i>	19	91,3	1
<i>M. x arnoldiana</i>	516	86,8	1
<i>M. x artosanguinea</i>		3,3	2
<i>M. baccata</i>	43	29,5	1
<i>M. baccata</i>	183	96,3	1
<i>M. baccata</i>	42	37,3	1
<i>M. baccata</i>	373	3,3	1
<i>M. baccata</i>	60	35,8	2
<i>M. baccata</i>	328	0,5	1
<i>M. baccata</i>	396	80,1	1
<i>M. baccata</i>	55	82,3	1
<i>M. baccata</i>	4	0,2	5
<i>M. baccata</i>	324	0,9	1
<i>M. baccata</i>	458	99,5	1
<i>M. baccata</i>	467	4,7	1
<i>M. baccata</i> var. <i>jackii</i>	419	9,8	1
<i>M. baccata</i> 'Lady Northcliff'	156	91,1	1
<i>M. baccata</i> var. <i>mandshurica</i>	777	2,1	1
<i>M. baccata</i> var. <i>mandshurica</i>	778	81,7	1
<i>M. baccata</i> Nr. 9 Pa	783	63,3	1
<i>M. baccata</i> <i>pulchella</i>	779	95,1	1
<i>M. baccata</i> var. <i>sachalinensis</i>	782	81,9	1
<i>M. baccata</i> var. <i>sibirica</i>	769	1,1	1

<b>Malus wild species</b>	<b>Accession number</b>	<b>% shoot blighted</b>	<b>Years tested</b>
<i>M. baccata</i> 'Tanner'	421	74,2	1
<i>M. coronaria</i>	363	65,6	1
<i>M. coronaria</i>	723	46,1	1
<i>M. x dawsoniana</i>	121	19,7	3
<i>M. domestica</i> 'Gibbs Golden Gage'	135	100,0	1
<i>M. x floribunda</i>	54	7,1	3
<i>M. x floribunda</i> 821		4,6	5
<i>M. x floribunda</i> 821		9,1	1
<i>M. x floribunda</i>	401	5,3	2
<i>M. x floribunda</i>	359	5,7	3
<i>M. x floribunda</i>	12	6,4	2
<i>M. fusca</i>	768	29,6	1
<i>M. fusca</i>	45	0,6	5
<i>M. fusca</i>	289	0,5	3
<i>M. fusca</i>	200	13,0	4
<i>M. fusca</i>	357	1,1	1
<i>M. x halliana</i>	572	14,3	2
<i>M. x micromalus</i>	158	64,0	1
<i>M. x micromalus</i>	531	76,8	1
<i>M. x prunifolia</i>	58	80,7	1
<i>M. x prunifolia</i>	108	2,9	3
<i>M. x prunifolia</i>	109	2,6	1
<i>M. x prunifolia</i>	322	5,3	1
<i>M. x robusta</i> 5		5,7	14
<i>M. x robusta</i>	711	10,2	2
<i>M. x robusta</i>	595	10,1	1
<i>M. x robusta</i> Alnarp		7,9	1
<i>M. x robusta</i> var. <i>persicifolia</i>	205	9,4	4
<i>M. x robusta</i> USA		2,9	1
<i>M. sargentii</i>	333	88,5	1
<i>M. sargentii</i>	708	65,7	1
<i>M. sargentii</i>	710	71,1	1
<i>M. sieboldii</i>		9,1	1
<i>M. 'Baskatong'</i>	594	57,2	1
<i>M. x spectabilis</i>	376	57,9	2
<i>M. x soulardii</i>	593	83,9	1
<i>M. x sublobata</i> 'Cashmere'	547	91,8	1
<i>M. trilobata</i>	463	95,8	1
<i>M. trilobata</i> x <i>M. baccata</i>	764	30,3	1
<i>M. tschonoskii</i>	240	100,4	1
<i>M. xanthocarpa</i>		79,0	1
<i>M. x zumi</i>	248	7,4	1
<i>M. x zumi</i> var. <i>calocarpa</i>	400	34,6	2

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## Control of fire blight (*Erwinia amylovora*) using epiphytic bacteria with known activity against postharvest diseases of apples

### Abstract

Strains B90 of *Pantoea agglomerans* and B262 of *Pseudomonas* sp., isolated from apple leaves, were used in studies on biological protection of apple and pear tissue against fire blight. Our earlier experiments had shown that both strains were highly effective in protecting apples against gray mold (*Botrytis cinerea*) and blue mold (*Penicillium expansum*) during storage. Apple flowers on trees cv. Idared/M9, growing in pots in the greenhouse, sprayed with water suspension of B90 and inoculated after 2 h with a highly virulent strain of *Erwinia amylovora* (Ea 659), were significantly less infected than those non-treated. However, treatment of flowers with B262 was not effective. Both strains, applied as water suspension sprays on wounded apple terminal shoots of cv. Idared/M9 trees kept in a greenhouse, were moderately effective against fire blight up to 13 days after inoculation. On the other hand, the disease symptoms on pear fruitlet slices cv. Conference, dipped in water suspension of both strains and after 6 h inoculated with *E. amylovora*, were very mild up to 7 days of the experiment. The efficacy of both strains was comparable with that of copper oxychloride.

### Introduction

Fire blight control consists of integration of various activities aiming at the elimination of disease source, protection of plants against infection by *E. amylovora* and decreasing the susceptibility of plants to this disease (Steiner 2000; Sobiczewski et al. 2004). The possibility of application of resistance inducers and methods alternative to conventional ones, including the use of beneficial microorganisms and plant genetic transformation, is also considered, and in some cases already recommended in practice (Hevesi et al. 2006; Norelli et al. 2003; Sobiczewski and Buban 2004; Vanneste et al. 2002; Zeller 2006).

### Materials and methods

Two bacterial strains, B90 (*P. agglomerans*) and B262 (*Pseudomonas* sp.), were evaluated with respect to their efficacy for control of fire blight on apple flowers, apple terminal shoots and pear fruitlets. Both strains were isolated from apple leaf surfaces. They were highly effective in protecting apples against gray mold (*B. cinerea*) and blue mold (*P. expansum*) during storage (Bryk et al. 1998; 2004; Sobiczewski et al. 1999).

Apple flower assay: Flowers on 'Idared/M9' trees (at full bloom) growing in pots in the greenhouse were sprayed with water suspension of both strains at a concentration of  $10^{10}$  cfu/ml. After 2 h they were inoculated with the highly virulent strain of *Erwinia amylovora* (Ea 659) at a concentration of  $10^7$  cfu/ml. Treatment with Miedzian Extra 350 SC (copper oxychloride), Bordoflow (Cu-Bordeaux mixture) and Aliette 80 WP (phosetyl Al) were included for comparison. After 5 and 8 days the diseased flowers as well as the total number of flowers were counted on each tree. Each treatment was represented by 12 trees.

Terminal shoot assay: Green, vigorously growing shoots of 'Idared/M9' trees growing in pots in the greenhouse were injured by cutting off their tips below the first undeveloped leaf using scissors and immediately sprayed with water suspension of both strains at a concentration of  $10^{10}$  cfu/ml. After 2 h they were inoculated with the highly virulent strain of *Erwinia amylovora* (Ea 659) by spraying with water suspension of bacteria at a concentration of  $10^7$  cfu/ml. One or 2 shoots were inoculated on each tree. After 5, 9, 13 and 17 days the presence of fire blight was assessed using a scale: 0 - no symptoms; 1 - trace of necrosis up to 1 cm around wound; 2 - necrosis length of 1-3 cm + ooze; 3 - necrosis up to 1<sup>st</sup> leaf; 4 - necrosis up to 1<sup>st</sup> leaf + ooze; 5 - necrosis up to more leaves. Each treatment was represented by 12 trees.

**Pear fruitlet assay:** The pear fruitlet slices of cv. Conference were cut and immediately dipped into a water suspension of B90 and B262 ( $10^9$  or  $10^{10}$  cfu/ml) and then placed on moist filter paper in Petri dishes. After 6 h the slices were inoculated by spraying with water suspension of strain *E. amylovora* Ea 659 ( $10^6$  or  $10^7$  cfu/ml). For comparison Miedzian 50 WP (copper oxychloride) was included. The occurrence of fire blight on the slices was evaluated on the 7<sup>th</sup> day of incubation at room temperature. The following scale was used: 0 – no symptoms, 1 – trace of bacterial ooze, 2 – about half of the slice with ooze and small necrosis, 3 – more than half of slice with ooze and necrosis, 4 – total necrosis of slice. Each treatment was tested in four replications; 10 fruitlet slices represented one replication.

### Results and discussion

Apple flowers protected by strain B90 were significantly less infected by *E. amylovora* than those not treated; treatment of flowers with B262 was not effective (Table 1).

**Tab. 1** Percentage of blighted flowers on 'Idared' apple trees after protective spraying with suspensions of B90 and B262 bacteria.

Treatment	Observations	
	5 days	8 days
Untreated/ Inoculated	47.0 c	77.5 c
B90/ Inoculated	33.0 bc	46.8 b
B262/ Inoculated	65.0 d	71.0 c
B90 only	0.0 a	0.0 a
B262 only	0.0 a	0.0 a
Miedzian Extra 3 l/ha/ Inoculated	31.2 b	33.0 b
Bordoflow 6 l/ha/ Inoculated	34.5 bc	37.2 b
Aliette 80 WP2.5 kg/ha/ Inoculated	28.2 b	32.2 b

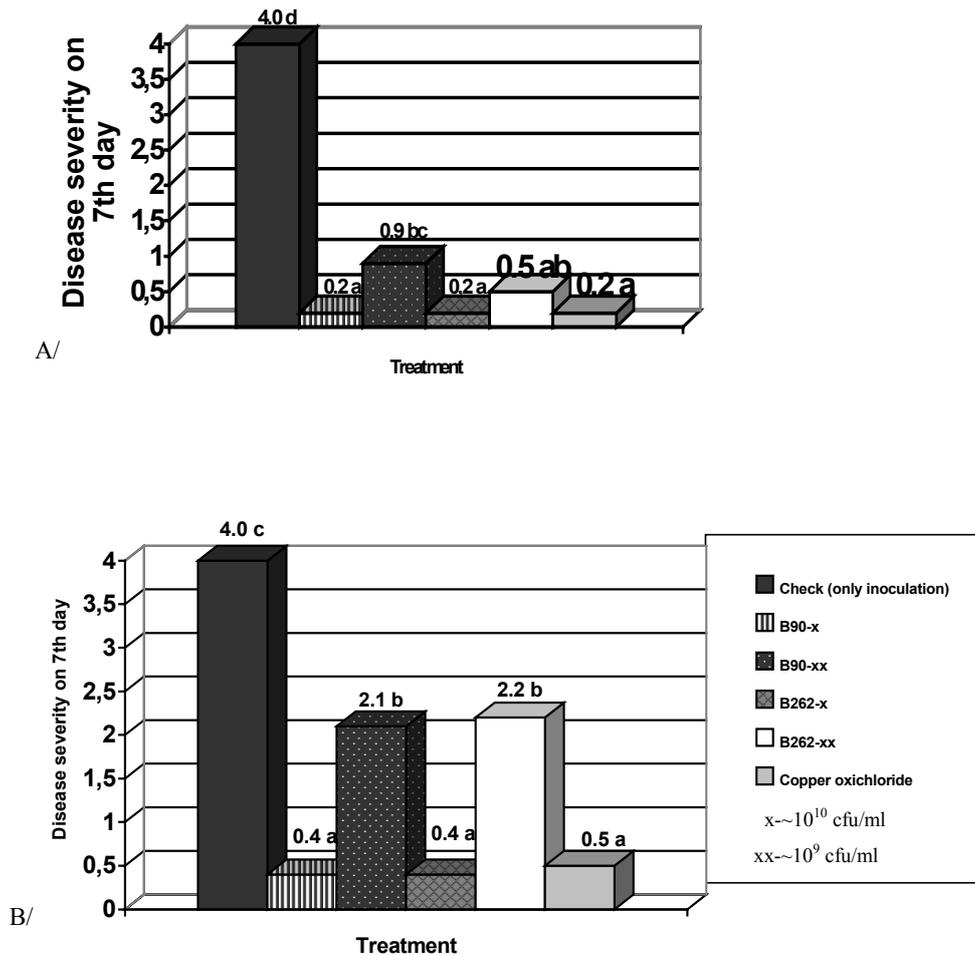
Statistical analysis was made separately for each observation; Values followed by the same letter(s) do not differ significantly at  $p=0.05$

Both strains applied as water suspension sprays on wounded apple terminal shoots were moderately effective (Table 2). Fire blight severity on pear fruitlet slices protected with both strains was very mild compared to treatment with copper oxychloride (Figure).

**Tab. 2.** Severity of fire blight on terminal shoots treated with suspensions of B90 and B262 bacteria

Treatment	Observations			
	5days	9 days	13 days	17 days
Untreated/ Inoculated	1.5 c	3.3 e	4.2 b	5.0 c
B90/ Inoculated	0.6 b	1.5 bc	2.1 a	3.5 ab
B262/Inoculated	0.1 a	0.8 ab	1.7 a	3.7 ab
Bordoflow 6 l/ha/ Inoculated	0.0 a	0.5 a	1.0 a	3.2 a
Miedzian 50 WP 3 kg/ha/ Inoculated	0.8 b	2.4 d	3.3 b	4.6 bc
Aliette 80 WP 2.5 kg/ha/ Inoculated	0.7 b	2.2 cd	3.4 b	4.2 abc

Rating scale: 0 – 5; Statistical analysis was made separately for each observation; Values followed by the same letter(s) do not differ significantly at  $p=0.05$



**Figure** Protective activity of B90 and B262 bacteria against fire blight on pear fruitlets; Inoculation after treatment with water suspension of Ea 659: A – inoculum: 10<sup>6</sup> cfu/ml, B – inoculum: 10<sup>7</sup> cfu/ml; statistical analysis was made separately for each observation; values followed by the same letter(s) do not differ significantly at p=0,05

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## ***Pantoea agglomerans* HIP32: a potential new antagonist of *Erwinia amylovora***

### **Abstract**

*Pantoea agglomerans* strain HIP32 was isolated from leaves of the apple cultivar ‘Starking’ by M. Hevesi and K.F. El-Arabi in Hungary. The efficacy of this strain was tested in detached apple flowers under controlled conditions.

In the first year of investigations (2004) detached flowers of ‘Sampion’ and ‘Gala Must’ apples were pre-treated with HIP32 (spraying  $10^7$  cfu/ml), incubated for 24 h followed by the inoculation with *Erwinia amylovora* strain Ea1 ( $5 \times 10^7$  cfu/ml). In 2005, ‘Idared’ apple trees were sprayed with HIP32. The initial population size of the antagonist was  $>10^4$  cfu/flower. Detached flowers were treated and investigated as in 2004, except that *E. amylovora* Ea1 Kan<sup>R</sup> ( $5 \times 10^4$  cfu/flower) was used for inoculation and its population size was measured by plating on selective medium.

HIP32 treatments resulted in a decrease of blighted blossoms by about 50% for ‘Sampion’ and even more for ‘Gala Must’ and ‘Idared’. Both disease severity and the index of infection in flowers treated with HIP32 were reduced nearly to the half (‘Sampion’) and to one third (Gala Must’). In the ‘Idared’ flowers the disease severity and the index of infection were about 50% and 40% of the control values, respectively. The PCR-detectable population (involving both viable and dead cells) of *E. amylovora* was not decreased in 2004, however, while measuring viable cells only in 2005, the population size of the pathogen was reduced by about two orders of magnitude, due to the treatment with HIP32.

**Key words:** fire blight, biological control, biocontrol agents (BCAs)

### **Introduction**

Fire blight caused by *E. amylovora* (Burr.) Winslow et al. is the most dangerous bacterial disease of pome fruit trees. The presence of the pathogen, as of 2004, is reported from 46 countries (van der Zwet 2004). The costs of the physical control measures like sanitation, pruning and/or eradication of the infected trees, as well as the expense of spraying antibiotics, amount to enormous economic losses. Recent publications quote precedents as follows.

Austria: in 2002 eradication costs in three provinces amounted to 1.72 million € and in 2003 costs for pruning and eradication of another 6000 infected trees came to 1.8 million € (Keck 2004). Czech Republic: the average compensation/year to growers for property loss between 1998-2002 was 54 679 €, and 109 375 € estimated for 2003 (Behalová 2004). Hungary: by the end of 1996, the year of the first appearance of fire blight, as much as 64 900 pome fruit trees were eradicated at the cost of \$1.1 million (Fishl 1997; Bonn and van der Zwet 2000). Italy: during the epidemics of 1997 and 1998, more than one million pear plants were destroyed (Finelli et al. 2004). Slovenia: in 2003 the damage due to the destruction of infected host plants (mainly apples, pears and quinces) was 476 200 € (Knapic et al. 2004). USA: there were economic losses of \$ 68 million in the major pome fruit production areas of Washington and northern Oregon (1998) and two years later \$ 42 million in Michigan (Stockwell et al. 2002).

Antibiotics have been used since the 1950s to control certain bacterial diseases of fruit trees, vegetable and ornamental plants. Today, the most commonly used are oxytetracycline and streptomycin. For example, the use of active ingredients between 1991-1999 in the USA was in apple orchards: oxytetracyclin 3 674 kg, streptomycin 40 914 kg, and in pear orchards: oxytetracyclin 26 400 kg,

streptomycin 22 589 kg (McManus et al. 2002). As for Hungary in 2003 streptomycin was applied to 11 319 hectares (Németh 2004). The use of streptomycin and other antibiotics, however, is banned in most countries of the European Union, e.g. in the year 2004 streptomycin against fire blight was allowed only in Germany, Greece and The Netherlands (Spinelli et al. 2005).

Because of the lack of effective, non-phytotoxic and publicly-acceptable chemicals to combat fire blight, novel control strategies like biological control have gained much interest recently. One of the possibilities is the application of chemicals which are presumed to trigger plant defence mechanisms and possess favourable eco-toxicological features (reviewed by Bubán et al. 2003).

Biological control of fire blight can be achieved by applying bacterial antagonists on the flowers before a sizable epiphytic population of *E. amylovora* is established. Mainly selected strains of *Pseudomonas fluorescens* and *P. agglomerans* are used. Over the past 20 years, trials carried out with container-grown pear trees (Wilson and Lindow 1993) or with a crab apple blossom model (Pusey 1999b) proved to be highly successful. Furthermore, even under field conditions in apple and pear orchards, a distinct suppression of the proportion of flowers with detectable *E. amylovora* population was found (Johnson et al. 1993), and the incidence of fire blight was reduced by about 50% (e.g. Lindow et al. 1996; Johnson and Stockwell 1998). These biological control agents, however, could not provide absolute disease control (Stockwell et al. 2002).

As a potential antagonist of the pathogen, *P. agglomerans* strain HIP32 was isolated from the leaves of the apple cultivar 'Starking Delicious' in Hungary (Hevesi and El-Arabi 1999). This study was devoted to test the effectiveness of this strain in detached apple flowers under controlled conditions.

## Materials and methods

Experiment 1 on detached flowers (2004): King flowers within the inflorescence of the apple cultivars 'Sampion' and 'Gala Must' were sampled at their balloon stage (just before anthesis) and a set of them (48 flowers, 8 from 6 trees, each) was used for each treatment carried out in the laboratory. Detached flowers were placed into 5% sucrose and the vials holding individual flowers were incubated in transparent plastic boxes at a day/night temperature of 22/19 °C, 85% relative humidity and a diurnal lighting of 16/8 hours. Temperature and RH% were monitored by a micro-sensor placed into one of the plastic boxes.

By the next day all flowers were open. A set of flowers was sprayed with the suspension of *P. agglomerans* strain HIP32 ( $10^7$  cfu/ml), incubated for 24 h followed by the inoculation with *E. amylovora* strain Ea1. Inoculation was carried out by touching at least 3 stigmas within a flower with a capillary tube holding a  $5 \times 10^7$  cfu/ml suspension of the pathogen. Another set of the flowers was treated with streptomycin (100 ppm) 2 h before inoculation. Positive and negative check flowers (without treatment by HIP32, inoculated and non-inoculated with *E. amylovora*, respectively) were used for comparison.

The 3rd day after inoculation the population size of *E. amylovora* was estimated by semi-quantitative PCR in a subset of 12 flowers (2 from each tree, Dorgai and Bubán 2002), and on the 5th day the remaining 36 flowers were scored for disease development, using a modified rating scale after Pusey (1999a). Details of evaluation are given in the footnote to Table 1.

Experiment 2 on apple trees (2005): 'Idared'/M.9 slender spindle trees, 5 replications and 3 trees in each plot, were sprayed with the bacterial suspension (near runoff, about 0.5 liter/tree) by a backpack sprayer fitted with a hand-directed spray wand. While spraying a single tree, a plastic foil (4 by 2 meters) fixed on two bamboo-canes was held around the tree to avoid the contamination of other trees due to the spray-drift. Fermented culture of *P. agglomerans* HIP32 was stored for 2 days at 3 to 4 °C and just before spraying was centrifuged and diluted with phosphate buffer (PBS) to a concentration of  $4.3 \times 10^9$  cfu/ml, providing an initial population size of  $>10^4$  cfu/flower. Check trees (5 by 3 = 15) were sprayed with PBS. Between treatments and/or replications there were a number of buffer trees.

Two hours after spraying, king flowers of 5 flower clusters from each tree were collected and placed immediately into individual wells of surface-disinfected 24-well plastic microtiter plates to transport them to the laboratory. *P. agglomerans* HIP32 possesses no resistance to antibiotics but has heat

tolerance (grows quickly at 37 °C), therefore estimation of its population size by dilution plating on King B medium should be done after 16 rather than 24 hours.

Five hours after spraying, two flower clusters of each tree were collected and transported to the laboratory. Two king flowers from three trees (= 6 flowers) were taken as a replication. All the detached flowers (four sets of 6 flowers by 5 replications in each treatment, altogether 4 by 30 = 120 flowers/treatment) were treated and investigated as in 2004, except that a kanamycin resistant variant of the pathogen (Dorgai, unpublished) was used for inoculation ( $5 \times 10^4$  cfu/flower). The 1st, 2nd and 4th day after inoculation the pathogen's population size was measured by plating on Miller-Schroth medium amended with 50 ppm kanamycin.

## Results

Pre-treatment of detached flowers with streptomycin or HIP32 failed to decrease the established population size of *E. amylovora* (except streptomycin/‘Gala Must’, Table 1). The presence of a natural *E. amylovora* population in the sampled flowers (see: Check negative) and the method used to measure the population size (PCR detects both dead and living cells) could explain this result.

**Tab. 1** Disease development in apple flowers (2004)

Cultivars and treatments	<i>E. amylovora</i> population <sup>1</sup>	Diseased flowers % <sup>2</sup>		Disease severity <sup>3</sup>	Index of infection <sup>4</sup>
		1 to 4	2 to 4		
<b>‘Sampion’</b>					
Streptomycin <sup>5</sup>	$10^5$ – $10^6$	44	31	0.75	1.17a,b
HIP32 <sup>6</sup>	$10^6$	61	52	2.10	1.69b
Check+ <sup>7</sup>	$10^5$ – $10^6$	100	97	3.50	3.33c
Check- <sup>8</sup>	$10^3$ – $10^4$	11	3	0.00	0.14a
<b>‘Gala Must’</b>					
Streptomycin <sup>5</sup>	$10^3$ – $10^4$	25	11	0.08	0.42a
HIP32 <sup>6</sup>	$10^4$ – $10^5$	83	6	1.00	0.89a
Check+ <sup>7</sup>	$10^4$ – $10^5$	94	83	3.08	2.78c
Check- <sup>8</sup>	$10^5$ – $10^6$	72	58	1.67	1.78b

<sup>1</sup> genome equivalent = GE/flower, estimated by PCR on the 3rd day after inoculation; <sup>2</sup> percentage of diseased flowers scored by rating scale of 1 to 4 or by a tightened rating scale of 2 to 4 (Pusey, 1999a); <sup>3</sup> scored by the rating scale of 1 to 4, median values, n = 36; <sup>4</sup> calculated by an equation (Bertrand and Gottwald, 1978), considering both the incidence and severity of symptoms in flowers scored by the rating scale of 1 to 4. Analysis of variance for each cultivar carried out with SPSS 9.0 program package mean separation by Tukey's. Values followed by the same letter did not differ significantly (p < 0.05); <sup>5</sup> 100 ppm, 2 h before inoculation; <sup>6</sup> *Pantoea agglomerans* strain HIP32,  $10^7$  cfu/ml, 24 h before inoculation; <sup>7</sup> untreated but inoculated flowers; <sup>8</sup> untreated flowers, without inoculation

The incidence of diseased flowers, scored by the whole rating scale (from 1 to 4), was reduced more distinctly by streptomycin than by HIP32. The first grade in the rating scale, however, is a hard-to-interpret discoloration localised to the surface of the hypanthium only. Based on the tightened, more reliable rating scales of 2 to 4, the occurrence of HIP32 treated and then blighted blossoms were diminished by about 50% for ‘Sampion’ and even more for ‘Gala Must’

Data regarding the percentage of diseased flowers, gained by using the modified rating scale of 2-4, were confirmed by the disease development, i.e. disease severity and the index of infection (Table 1). While calculating the later data the whole rating scale (1-4) was used. Regardless of the less stringent evaluation, both disease severity and the index of infection in detached flowers treated with HIP32 were reduced by 40 to 45% in the case of ‘Sampion’, and to about one-third in the ‘Gala Must’ flowers.

According to the the results in the year of 2005 on apple trees, the population size of viable *E. amylovora* in the inoculated flowers of ‘Idared’ apple, measured by dilution plating, was reduced by about two orders of magnitude due to the pre-treatment with HIP32 (Table 2). It is worth mentioning that the population size of the strain HIP32 was steadily high:  $1.17 \times 10^6$ ,  $1.71 \times 10^6$  and  $1.16 \times 10^6$  cfu/flower on the 1st, 2nd and 4th day after inoculation with Ea1 Kan<sup>R</sup>. Disease severity and the index of infection were about 50% and 40% of the check values, respectively.

**Tab. 2** Disease development in apple flowers (2005)

Cultivar and treatments	<i>E. amylovora</i> population <sup>1</sup>	Diseased flowers % <sup>2</sup>		Disease severity <sup>3</sup>	Index of infection <sup>4</sup>
		1 to 4	2 to 4		
<b>'Idared'</b>					
HIP32 <sup>5</sup>	3.75x10 <sup>4</sup>	63	7	1	0.73a
Check+ <sup>6</sup>	1.00x10 <sup>6</sup>	93	57	2	1.97b
Check- <sup>7</sup>	1.67x10 <sup>5</sup>	23	3	0	0.33a

<sup>1</sup> cfu/flower, estimated by dilution plating, on the 4th day after inoculation. Data of the *E. amylovora* population were *In* transformed by one-a-way analysis of variance;  $p < 0.01$ ; <sup>2</sup> percentage of diseased flowers scored by rating scale of 1 to 4, or by tightened rating scale of 2 to 4 (Pusey, 1999a); <sup>3</sup> scored by the rating scale of 1 to 4, median values,  $n = 30$ ; <sup>4</sup> calculated by an equation (Bertrand and Gottwald, 1978), considering both the incidence and severity of symptoms in flowers scored by the rating scale of 1 to 4. Analysis of variance for each cultivar carried out with SPSS 9.0 program package mean separation by Tukey's. Values followed by the same letter did not differ significantly ( $p < 0.05$ ); <sup>5</sup> flowers of treated trees with an initial population  $> 10^4$  cfu/flower (2 h after spraying); <sup>6</sup> untreated but inoculated flowers; <sup>7</sup> untreated flowers, without inoculation

## Discussion

*P. agglomerans* is a constituent of natural microbial communities in foliage of apple trees in Hungary. Under controlled conditions favourable to growing *E. amylovora*, the disease development was considerably suppressed by *P. agglomerans* strain HIP32 (Table 1). These results are further supported by the data of our similar experiments in 2005: the population size of viable *E. amylovora* in the inoculated flowers of 'Idared' apple, measured by dilution plating, was reduced by about two orders of magnitude due to the pre-treatment with HIP32. Disease severity and the index of infection were reduced by 50% or more, compared to the check flowers inoculated with *E. amylovora* only (Table 2). Field trials with *P. agglomerans* HIP32 are in progress.

Besides weather-related circumstances (temperature above 15 °C, no rainfall after spraying) the success of the application of bacterial antagonists in the field depends on several other factors, too. Spraying all trees in an orchard likely will be superior to other possible strategies of antagonist introduction, such as alternative row spraying or getting honey bees to disseminate the biological control agents (Johnson et al. 2000). Antagonists like *P. fluorescens* A506 and *P. agglomerans* Eh318 should be applied at the beginning of bloom time, because they are saprophytic in nature, behave similarly to the pathogen and are unable to multiply exponentially on pistils older than 4 days (Thomson and Gouk 2003).

Lindow et al. (1996) reported that in field trials *P. fluorescens* A506 reduced the incidence of fire blight significantly, but it was most effective in an integrated programme including streptomycin or oxytetracycline. It is interesting that streptomycin did not reduce the incidence of recovery or population size of the antagonists *P. fluorescens* A506 and *Erwinia herbicola* C9-1S. Contrasting it, oxytetracycline application should be delayed until an epiphytic population of antagonists has become established on flowers (Stockwell et al. 1996).

Nowadays there are products registered and commercialised for control of fire blight: BlightBan with *P. fluorescens* A506 (Johnson and Stockwell 1998; Lindow and Suslow 2003), or Blossom Bless with *P. agglomerans* P10c sold in New Zealand (Vanneste et al. 2004). The Bloomtime Biological FD with *P. agglomerans* E325 may be registered in the U.S. in the near future (Pusey and Curry 2004).

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## VII. Biological control of phytopathogenic fungi with antagonistic bacteria

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### Production of hydrogen cyanide (HCN) and lytic enzymes by rhizobacteria isolated from different plants

#### Abstract

Bacterial strains isolated from wheat, maize, cotton, pea, tomato, melon, and alfalfa grown in serozem arid soils from Uzbekistan and from wheat and pea grown in loamy sand from Germany. Strains used for this study belong to *Pseudomonas*, *Bacillus*, *Kocuria*, *Mycobacterium*, *Mycoplana*, *Cellulomonas* and *Microbacterium*. The production of lytic enzymes and hydrogen cyanide (HCN) by rhizosphere bacteria are characterised in this report. The results show that bacterial strains produce enzymes such as cellulase, lipase, pectinase, amylase, protease and hydrogen cyanide (HCN) and were antagonistic to the plant pathogenic fungus *Fusarium oxysporum* in *in vitro* experiments.

#### Introduction

Characterising the ecology of bacteria associated with plant roots is important to understand the impact new agricultural technologies will have upon soil ecology, nutrient transformations and plant succession (Germida and Siciliano 2001). The association of bacteria with a plant root has been related both to their antagonistic activities towards pathogens and to their ability to colonise and produce plant growth promoting compounds within the rhizosphere (Cook et al. 1995; De Weger 1995). Plant growth-promoting rhizobacteria (PGPR) competitively colonise plant roots and stimulate plant growth and/or reduce the incidence of plant disease. The mechanisms that are involved in this process can include nitrogen fixation, phosphate solubilisation, and the production of phytohormones. These mechanisms presuppose a direct contact between bacteria and the root surface and an active status of the introduced bacteria. The beneficial rhizosphere bacteria have been shown to increase plant growth and nutrient uptake of maize, wheat and legumes (Kloepper and Beauchamp 1992; Höflich et al. 1994) and also protect plants from pathogens. Mechanisms of biological control of plant pathogens by plant growth-promoting bacteria involve production of bacterial metabolites such as iron-chelating siderophores, hydrogen cyanide (HCN) and extracellular lytic enzymes. The objective of the present study was to test *in vitro* production of extracellular lytic enzymes and hydrogen cyanide (HCN) by rhizobacteria associated with different plants and soils.

#### Material and methods

The bacterial strains were isolated from the rhizosphere of melon, corn, cotton, tomato, wheat, alfalfa, pea and wheat grown on calcareous serozem soil, a field site of the semi-arid region of Uzbekistan and also from loamy sand from Germany. The identification scheme based on a combination of conventional tests like morphological, physiological and chemotaxonomic characterisation (Holt et al. 1994) and the use of the commercial identification systems like Biolog (GN, GP) from the Biolog Inc. and API 20E or API 20NE (bioMérieux) (Behrendt et al. 1997).

Plant roots were chopped into 10-mm sections and three replicates of the same crop were used for microbiological analysis. Root samples (1 g) were shaken well with 9 ml of sterilised distilled water and  $10^{-1}$  to  $10^{-7}$  serial dilutions were made in 0.2% saline. From each dilution 0.1 ml of suspension was spread in triplicate onto glycerin-peptone-agar plates. The inoculated plates were incubated at 28°C for 2-

3 days. After incubation, the isolated strains were purified by twice streaking on glycerol-peptone agar (GPA), incubated in the same way. The pure cultures were grown on GPA at 28°C for 24 h and the colonies were collected with nutrient solution for further examination.

Pectinase activity of bacterial strains was determined as described by Smibert and Krieg (1994). The plates were incubated at 28°C and zones of starch cleaning recorded after 5 days. Lipase activity was determined on modified sierra agar, containing 10 g peptone, 3 g meat extract, 5 g NaCl, 0.2 g Fe-citrate, 0.1 g CaCl<sub>2</sub> x H<sub>2</sub>O and 15 g agar in 1 L distilled water. Ten ml sterile Tween 20, 60 and 50 ml 0.067 % (w/v) Victoria Blue B solution were added to the medium after autoclaving. Hydrolysis of Tween was recorded as white precipitation around the colonies. The presence of pectinase and cellulase activity was determined according to Jajasanka and Graham (1970).

The plant pathogenic fungus *Fusarium culmorum* was used as indicator strain for the observation of potentially antagonistic strains using simple plate assay methods. This involved pre-incubation of the putative antagonist on yeast malt agar at 28°C for 2 days. Plates were then seeded with individual fungal strains, incubated for further 5 days in a sealed container, zones of inhibition were recorded.

## Results

Bacterial strains were isolated from cotton, wheat, tomato, melon, and alfalfa grown in the semi-arid region of Uzbekistan. Representative isolates were identified as *P. alcaligenes*, *P. mendocina*, *P. rathonis*, *P. aeruginosa*, *B. amyloliquefaciens*, *B. laevolacticus*, *B. latvianus*, *B. megaterium*, *B. polymyxa*, *B. cereus*, *Mycobacterium phlei*, *Mycoplana bullata*, *Arthrobacter globiformis*, *A. simplex*, *A. tumescens*, *Acanitobacter* spp., *Staphylococcus* sp., *Pseudomonas denitrificans*, and *P. rathonis*. The bacterial strains isolated from the loamy sand belong to following species: *Cellulomonas* sp. *Bacillus lentus*, *Bacillus cohnii*, *Bacillus subtilis*, *Kocuria varians*, *Bacillus halodurans*, and *Microbacterium* sp.

**Table 1.** Characterisation of bacterial strains isolated from loamy sand (Germany)

Origin	Bacterial strains	Antagonist against <i>F. oxysporum</i>	HCN	Lipase	Amylase	Protease	Pectinase	Cellulase
Pea	<i>Cellulomonas</i> sp.10/2	-	-	+	+	+	-	+
Pea	<i>Cellulomonas</i> sp. 21/2	-	-	-	-	-	+	-
Wheat	<i>Cellulomonas</i> sp. 22	-	-	-	-	-	+	-
Wheat	<i>Bacillus lentus</i> 17	+	+	-	+	+	+	+
Pea	<i>Bacillus cohnii</i> 19	+	-	-	+	+	-	+
Wheat	ni 23	+	-	+	-	-	-	-
Wheat	ni 16	-	-	-	-	-	-	+
Wheat	<i>Bacillus subtilis</i> 4	+	+	+	+	+	-	+
Wheat	<i>Bacillus subtilis</i> 1	+	-	-	-	-	+	-
Wheat	<i>Kocuria varians</i> 13	-	-	-	-	+	+	-
Wheat	<i>Bacillus halodurans</i> 12	+	+	+	+	+	+	-
Pea	<i>Bacillus</i> sp. 41/1	-	-	-	-	-	+	-
Wheat	<i>Cellulomonas</i> sp.43	-	-	+	+	-	+	-
Pea	<i>Microbacterium</i> sp. 44	-	-	-	-	-	-	-
Pea	<i>Bacillus</i> sp. 37/1	+	+	+	+	+	-	+
Pea	<i>Microbacterium</i> sp. 46	-	-	+	+	+	+	-
Pea	ni 48	-	-	-	+	-	-	+
Wheat	<i>Bacillus</i> sp. 31	-	-	-	+	-	-	-
Pea	<i>Bacillus lentus</i> 28	+	-	-	+	+	-	-
Wheat	<i>Cellulomonas</i> sp. 32	-	-	-	+	-	+	-

The production of hydrogen cyanide and different enzymes such as protease, lipase, cellulose, pectinase and amylase by bacterial strains are shown in Tables 1 and 2. Most bacterial strains isolated from loamy sand produced pectinase and amylase. The bacterial strains isolated from semi-arid soils produced amylase and lipase. Some bacterial strains were able to produce hydrogen cyanide (HCN) and were antagonistic against the fungal pathogen *Fusarium oxysporum* (Tables 1, 2)

**Tab. 2** Characterisation of bacterial strains isolated from serozem soil (Uzbekistan)

Origin	Bacterial strains	Antagonist against <i>F. oxysporum</i>	HCN	Lipase	Amylase	Protease	Pectinase	Cellulase
Maize	<i>P. alcaligenes</i> PsA15	+	+	+	+	-	+	-
Maize	<i>P. mendocina</i> PsM13	-	-	-	-	-	-	-
Alfalfa	<i>P. rathonis</i> PsR20	+	+	+	+	-	-	+
Wheat	<i>P. aeruginosa</i> UW145	+	+	+	+	+	+	+
Melon	<i>B. amyloliquefaciens</i> BcA12	-	-	+	+	-	+	+
Cotton	<i>B. laevolacticus</i> BcL28	-	-	-	+	+	-	-
Melon	<i>B. latvianus</i> BcLt29	-	-	+	+	+	+	-
Tomato	<i>B. megaterium</i> BcMg33	-	-	-	-	+	+	-
Cotton	<i>B. polymyxa</i> BcP26	+	+	-	+	+	+	+
Wheat	<i>B. cereus</i> UW80	-	-	+	+	+	+	-
Maize	<i>Mycobacterium phlei</i> MbP18	-	-	+	+	-	+	+
Wheat	<i>Mycoplasma bullata</i> MpB46	-	-	+	+	-	-	-
Tomato	<i>A. globiformis</i> ArG 1	-	+	+	+	+	-	-
Wheat	<i>A. simplex</i> ArS 43	-	-	+	-	-	-	-
Alfalfa	<i>A. tumescens</i> ArT 16	+	+	+	+	+	+	-
Wheat	<i>Acenitobacter</i> spp. UW132	-	-	+	+	+	+	-
Wheat	<i>Staphylococcus</i> sp. UW 415	-	-	-	-	-	-	-

## Discussion

Rhizosphere bacteria are able to use a wide variety of carbon sources as nutrients, can be plant growth-promoting (PGPR), and may act as biological control agents (BCA). They exhibit a wide range of metabolic activities and are able to use a wide range of low molecular mass organic compounds, and some more complex compounds as carbon and energy sources (Misko and Germida 2002). In this study, the isolated bacteria produced various hydrolytic enzymes. There are many reports that note the production of biologically active compounds including different enzymes, and also siderophores by rhizosphere bacteria (Höflich et al. 1994)

The bacterial strains that produce different hydrolytic enzymes such as protease, lipase, and pectinase were antagonistic towards soil fungi. Abd Rahman et al. (2005) and Caballero et al. (2001) reported that among studied pseudomonads *P. aeruginosa* produced several proteases that were implicated in its pathogenicity. Nielson and Sørensen (1999) demonstrated that isolates of *P. fluorescens* antagonistic to *R. solani* and *Pythium ultimum* produced lytic enzymes. Bastos (2005) reported about strains of *Crinipellis pernicioso* isolated from cocoa (*Theobroma cacao*) and cupuassu (*T. grandiflorum*) that were tested for their ability to produce extracellular enzymes which degrade cellulose, starch, lipids and lignin. All isolates displayed high proteolytic activity.

Some tested bacteria produced hydrogen cyanide (HCN) and were antagonistic against the fungal pathogen *Fusarium*. Deshwal et al. (2003) reported that the biocontrol effect of rhizobia is due to the secretion of secondary metabolites such as antibiotics and HCN. In their work the strains also produced HCN and were antagonistic against fungal pathogens. Our results showed that, independent of the origin,

bacterial strains produced various enzymes and HCN. The bacterial strains that produced hydrogen cyanide were antagonistic against fungal pathogens.

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## ***Bacillus subtilis* as biological control agent of phytopathogenic fungi**

### **Abstract**

Laboratory and on-site farm research revealed that the *Bacillus subtilis* strain B-5009 had antagonistic properties against the phytopathogenic fungi *Monilia fructigena*, *Botrytis cinerea* (Pers.), *Rhizopus nigricans* (Ehrenb.), Honey, *Penicillium claviforme* (Bain), and *Penicillium expansum* (Link.), which affect fruits and berries during storage.

**Key words:** Alternaria, Aspergillus, Botrytis, Cladosporium, Monilia, Mucor, Rhizopus, Penicillium, Fusarium, Bacillus subtilis

### **Introduction**

Long-term storage of fruits and berries in warehouses is quite problematic in Ukraine. It is well known that phytopathogenic fungi and bacteria cause putrefaction or decomposition, affecting the products kept in storage, which leads to significant product losses.

The fungal microflora of fruits frequently consists of the following fungal genera: *Alternaria*, *Aspergillus*, *Cladosporium*, *Monilia*, *Mucor*, *Rhizopus*, *Penicillium*, *Fusarium* and others. Among them the species that are especially pathogenic are: *Monilia fructigena* (Schroet.), *Botrytis cinerea*, *Rhizopus nigricans*, Honey, *Penicillium claviforme*, and *Penicillium expansum*. Also the storage methods are in question of products in containers of refrigerators with regulated gas environment or regulated atmosphere (RGE), chemical antiseptics and pesticides that are generally applied post-harvest on fruits and berries with the purpose of improving storage quality. The selection of antagonistic *Bacillus* strains and the formulation of biological preparations for the protection of storage products are an actual challenge.

Scientific research work on the formulation and implementation of biological preparations into manufacturing processes on the basis of aerobic spore-forming bacteria of the *Bacillus* species is under way at the Institute of Microbiology and Virology of the National Academy of Sciences (NAS) of Ukraine. Laboratory investigations and on-site farm research of biological products are continuing on the basis of *Bacillus subtilis* B-5009 bacteria.

### **Materials and methods**

Our investigations includes the characterization of antagonistic activity of the aerobic spore-forming bacterium *Bacillus subtilis* B-5009 as basis for biopreparation against *Monilia fructigena*, *Botrytis cinerea*, *Rhizopus nigricans*, Honey, *Penicillium claviforme*, and *Penicillium expansum* causing rot in stored fruits and berries.

The trials with the *Bacillus subtilis* strain were performed by means of delayed antagonism according to Yegorov (7) and Kudriasheva (4). Strains of *Bacillus*-antagonists were inoculated onto the center of Petri-dishes with potato dextrose agar (PDA). Macro-colonies were incubated at 28 °C for 3 days. Spores were obtained from 3-day-old *M. fructigena* grown on PDA at 28°C and suspended into sterile water (10<sup>9</sup> spores/ml). The fungal test-cultures were then inoculated in Petri-dishes with *Bacillus*-antagonists by the microbiological loop to the edge of the macro-colonies and left for 3-day-incubation at 28 °C. Length of the growth suppression zones was measured in mm.

## Results

We isolated natural antagonists from soil in Kyivska oblast and identified the strain by its basic morphological and biochemical properties according to Bergey's Manual (8) as *Bacillus subtilis*. The strain was stored in the Institute of Microbiology and Virology, Natl. Acad. Sci. Ukraine (2). We grew the strain in nutrient media: MPA (meat-peptone agar), Gromyko medium (MPA + malt extract), Gause medium №2 and others. The strain has the following cultural, morphological, physical and biochemical properties: Gram-positive, aerobic, spore-forming bacilli, producing catalase. It grows best on MPA, malt extract and Gromyko medium, forming mat folded beige colonies. Strains from colonies grown on MPA for 18 h show bacillus-like cells of 1.9 x 2.1 µm, as single cells, seldom in chains. The cells do not swell during spore formation; when growing on glucose agar vacuoles do not form in the protoplast. The cell culture forms film-like layers on MPA and does not grow in anaerobic conditions and with 10% NaCl.

In addition, we extended the spectrum of our research to pathogens causing grey rot in strawberries, *B. cinerea* and to phytopathogenic fungi, *Penicillium expansum*, *Penicillium claviforme*, and *Rhizopus nigricans*, causing rot in fruits and berries (Table 1).

**Tab. 1** Antagonistic properties of *B. subtilis* B-5009 strain

N	Pathogen	Zone of inhibition of pathogens (mm)
1	<i>Botrytis cinerea</i>	20-21
2	<i>Monilia fructigena</i>	12-16
3	<i>Rhizopus nigricans</i>	14-16
4	<i>Penicillium expansum</i>	10-13
5	<i>Penicillium claviforme</i>	13-15

Depending on environmental factors (temperature and relative air humidity), which have critical importance for *M. fructigena* development, the intensity of rot development changes, therefore it is of particular importance to maintain certain regimes of these factors. We investigated this in 5-fold tests (with 4 apples for each repetition). We assessed the development of the disease by the ratio of rot size to the general fruit surface in percent (%). Table 2 presents the results of assessment of rot development in apples under various storage regimes. To further assess the efficiency of *B. subtilis*, we tested the concentration of the suspension ( $5 \times 10^9$  cfu/ml) and used fitosporin (strain *B. subtilis* 26 D) as standard.

**Tab. 2** Development of apple rot in different storage conditions (var. Snizhny Kalvil; % rel. humidity, RH)

Shelf life (days)	T +2+3 °C, RH 80-85%	T +8+12 °C, RH 80-85%	T +18+24 °C, RH 80-85%	T +2+12 °C, RH 65-70%
1	0	0	0	0
2	0	0	3-4	0
3	0	2-4	11-14	2-3
4	2-5	6-12	15-19	4-9
5	4-8	10-15	18-24	11-16
6	10-13	16-21	34-39	17-21
7	14-17	21-26	49-54	24-31
8	18-21	26-31	79-84	31-36
9	22-24	31-33	99	31-36
10	22-24	33-38	100	31-36

We tested the antagonistic efficiency against fruit rot on disinfected apples by inoculation with rot agents through damaged apple skin, and treatment for 24 h with a suspension of  $5 \times 10^9$  cfu/ml of antagonistic bacilli. The results are presented in Table 3. We measured the disease development by rot growth, which we assessed over 24 h. The results revealed that a culture of *B. subtilis* B-5009 has high antagonistic activity against *M. fructigena*. Fungal growth was almost absent in inoculated sites, while in the controls the diseased zones increased 4 to 5 times.

**Tab. 3** Antagonistic activity of *B. subtilis* B-5009 against rot agents through inoculation of damaged skin (T +24 °C, relative humidity 85%)

Variant	Pathogen	Progress of disease (mm); Shelf life (days)			
		1 d	2 d	3 d	4 d
<i>B. subtilis</i> B-5009	<i>M. fructigena</i>	1-2	2-3	3-5	5-6
Standard	<i>M. fructigena</i>	1-2	2-3	2-3	2-4
Control	<i>M. fructigena</i>	5-6	6-8	9-11	10-12

We performed test series to determine the optimum dose (1, 3, 5 or  $10 \times 10^9$  cfu/ml) of the antagonistic strain, which stops rot development. We disinfected fruits, then inoculated them with a suspension of 1, 3, 5 and  $10 \times 10^9$  cfu/ml and then with the fruit rot agent; the results are presented in Table 4.

The experiments demonstrated that a  $5-10 \times 10^9$  cfu/ml suspension of *B. subtilis* inhibits fruit infection by *M. fructigena*; fruits were stored without disease. Therefore, we conclude that *B. subtilis* prevents the development of the fruit rot agent and completely inhibits its growth after application of suspensions with  $5-10 \times 10^9$  cfu/ml.

**Tab. 4** Antagonistic activity of *B. subtilis* B-5009 strain against *M. fructigena* by artificial inoculation (T +8-10 °C, relative humidity 80-85%)

Variant	Dose ( $10^9$ cfu/ml)	Progress of disease %; Shelf life (days)						
		1 d	7 d	14 d	21 d	30 d	60 d	90 d
<i>B. subtilis</i> B-5009	10	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0
Standard	10	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
	3	0	0	22	45	65	-	-
	1	0	35	65	85	95	-	-
Control	-	0	30	50	100	-	-	-

We performed experiments using the natural infection on the fruit surface, with further damage of the fruit skin and fruit inoculation with suspension of antagonistic bacilli. Each test included 50 fruits. The used suspensions of spores and vegetative cells of *B. subtilis* had a titer  $10 \times 10^9$  cfu/ml (Table 5), resulting in the inhibition of fruit rot growth. The third day, in the control the rot growth of a *Penicillium* was visible, while inoculated apples did not show rot. By the 5<sup>th</sup>, 7<sup>th</sup> and 10<sup>th</sup> day all test variants were affected by rot, while in fruits inoculated with antagonistic bacilli the fruit rot grew more slowly. From 10 days on the rot developed rapidly and was not inhibited by antagonistic bacilli, since the mycelium established inside the fruit pulp. The effect on fruit rot occurs at the beginning of the test, there resulting in a great difference between test and control (Table 6). The suspension prepared from spore cultures of antagonistic bacilli was the most efficient rot inhibitor for 1-3 days compared with the control.

**Tab. 5** Efficacy of *B. subtilis* B-5009 against fruit rot compared with natural infection (T +18-24 °C, relative humidity 85-90%)

Variant Dose (10 <sup>9</sup> cfu/ml)	Progress of disease %; Shelf life (days)				
	3 d	6 d	9 d	12 d	15 d
<i>B. subtilis</i> B-5009 (spores)	0	0	15	37	50
<i>B. subtilis</i> B-5009 (cells)	0	11	23	45	55
Standard (spores)	0	9	19	42	60
Standard (cells)	0	9	30	42	55
Control	6	22	46	65	75

**Tab. 6** Efficiency of *B. subtilis* B-5009 against fruit rot (T +18-24 °C, relative humidity 85-90%)

Variant	Dose (10 <sup>9</sup> cfu/ml)	Progress of disease %; Shelf life (days)					
		1 d	3 d	5 d	7 d	10 d	15 d
<i>B. subtilis</i> B-5009 (spores)	10	0	5	11.4	28.7	55.0	75.0
<i>B. subtilis</i> B-5009 (cells)	10	0	0	0	5.6	29.4	58.0
Standard (spores)	10	0	0	8	21.0	37.0	66.0
Standard (cells)	10	0	0	0	9.6	36.4	61.7
Control	-	0	0	5	9.9	34.4	57.4

## Discussion

The *B. subtilis* strain has antagonistic properties against phytopathogenic fungi, in particular against *M. fructigena*, which affects fruits and berries in gardens, during packing, and causes rot during storage. It is known that *M. fructigena* grows more slowly than *Penicillium*. Often *Penicillium* spores inhibit growth of *M. fructigena*. This pathogen causes great damage to fruits in central and southern parts of the European region of the former USSR (3).

Under laboratory conditions we isolated *M. fructigena* from infected apples. The species is characterized by formation of empty sclerotia in the fruits of the Rosaceae family. The fungal life cycle includes a conidium stage of the *Monilia* type with long, often branched conidium chains on the pads of short conidium-carriers. The fungus penetrates into fruits through injuries. The spots of dead tissue are formed on infected fruits. Concentric rings appear as yellowish pads of sporogenic conidia. The conidia are spread by wind, and very often by the *Rhynchites bacchus* beetle. The globe-shaped sclerotia are formed under the epidermis and the fruits turn black. Sclerotia hibernate and in spring they again produce conidia. This species rarely develops apothecia (1). The strain produces a complex of bioactive substances, including polypeptide antibiotics (5,6).

Application of suspensions of the *B. subtilis* strain B-5009 against *M. fructigena* will allow inhibiting its growth by 3 to 4 times during storage. In case of spraying fruits with antagonists, rot development is inhibited for 1 to 4 days, which proves their high biological activity. Application of spore cultures of antagonistic bacilli will allow the decrease in fruit rot by 23.6 to 66.6 %.

During storage of apples, when inoculated before storage with a suspension of  $5 \times 10^9$  cfu/ml of *B. subtilis* B-5009, fruits could be stored much longer and the quality of inoculated apples was higher than the controls. Thus this biopreparation allows to protect stored fruits and berries against rot.

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## **A novel bacterium for biocontrol of broad spectrum of pathogens**

### **Abstract**

Strains of fluorescent pseudomonads are applied on seed and planting materials to control plant disease caused by soil-borne pathogens or to promote plant growth by suppressing deleterious rhizosphere microorganisms. Beneficial pseudomonads antagonize pathogens by producing one or more metabolites that induce antibiotics, siderophores and other substances such as cyanide. In the present study a novel bacterium, *Pseudomonas chlororaphis*, isolated from rice fields, was characterized. The bacteria suppressed the growth of many important plant pathogens. This bacterium was also an efficient spermosphere and rhizosphere colonizer, an additional advantage for a potential biocontrol agent. We studied the genetic basis of the antagonistic potential and finally its *in vivo* efficiency by field experiments using rice plants as model system.

### **Introduction**

It was observed that rice plants (ADT.36 and IR.20) treated with *P. chlororaphis* as seed treatment followed by foliar application was protected against all pathogens. Intense colonization is one of the reasons for the reduction in blast, sheath blight, bacterial blight and brown spot of rice. Present results confirm those of colonization of chili and tomato and bring additional support to the current concept that rhizosphere bacteria are active inducers of plant disease resistance. The inhibition of pathogens *in vitro* and *in vivo* is due to plasmid (pKL 1) mediated antibiotic production.

There is growing concern, both in developed and in developing countries about the use of hazardous fungicides for controlling plant diseases. Whereas accumulation of hazardous pesticides in tropical soils is debatable, one should accept biological control as a global method to reduce pollution in the environment and as an approach that is distinct from other control methods. It is becoming an urgently needed component in the Integrated Pest and Diseases Management in agriculture.

Strains of fluorescent pseudomonads are applied on seed and planting material to control plant diseases caused by soil-borne pathogens or to promote plant growth by suppressing deleterious rhizosphere microorganisms [1,2]. Beneficial pseudomonads antagonize pathogens by producing a variety of metabolites that induce antibiotics [3], siderophores [4] and other substances such as cyanide [5].

Numerous bacteria and fungi have been studied for use as potential biocontrol agents but only a few have been stably established under field conditions due to their variable results in the field conditions. The fluorescent pseudomonads have emerged as the largest and potentially most promising group of plant growing-promoting rhizobacteria involved in the biocontrol of plant diseases [6,7,8].

In the present study a novel *P. chlororaphis* strain, isolated from a rice field, was characterized, and found to suppress the growth of many important plant pathogens. This bacterium was also an efficient spermosphere and rhizosphere colonizer, an additional advantage for a potential biocontrol agent. We studied the genetic basis of antagonistic potential and finally its *in vivo* efficiency by field experiments using rice plants as model system.

*P. chlororaphis*, isolated from field soils, showed a broad spectrum of antagonistic activity similar to that of *P. chlororaphis* MA 342, a biocontrol agent of barley seed-borne neck blotch caused by *Drechslera teres*, isolated from green house soil.

## Results

Growth kinetics and characterization of the bacterium: The pseudomonad was able to grow on a wide range of carbon sources like xylose sucrose, dextrose, acetate, ethanol, glycerol, etc., but not on citrate, ascorbic acid, lactose, mannose, and sorbitol. *P. chlororaphis* was able to grow at pH values ranging from 5-10 and in temperatures ranging from 20 to 45 °C with maximum antibiotic production at pH 8.0 and 35 °C. In dual plate assays the inhibition of pathogens was not reversed by FeCl<sub>3</sub> amendment in PDA medium, supporting the fact that the inhibition of pathogens was due to the production of siderophores. The pseudomonad showed resistance to a wide range of heavy metals, antibiotics and fungicides as shown in Tables 1-3.

**Tab. 1** Resistance/sensitivity of pathogens to heavy metals

Metals	Concentration (mM)				
	1.0	2.5	5.0	7.5	10.0
Arsenate	+	+	+	+	+
Cadmium	+	+	+	+	+
Cobalt	+	+	-	-	-
Copper	+	+	+	-	-
Iron	+	+	+	-	-
Lead	+	+	+	-	-
Nickel	+	+	+	+	+
Zinc	+	+	+	+	-

**Tab. 2** Resistance/sensitivity of pathogens to antibiotics

Antibiotics	Concentration (µg/ml)				
	10	25	50	75	100
Ampicillin	+	+	+	+	+
Benzyl penicillin	+	+	+	+	+
Chloramphenicol	+	+	+	+	+
Ciproflox	+	+	+	+	+
Gentamycin	-	-	-	-	-
Kanamycin	+	+	-	-	-
Rifampicin	+	+	-	-	-
Streptomycin	+	-	-	-	-
Tetracycline	+	-	-	-	-

**Tab. 3** Resistance/sensitivity of pathogens to fungicides

Fungicide	Concentration (mM)			
	10	25	75	100
Iprodione	+	+	+	+
Bavistin	+	+	+	+
Morpholine	+	+	-	-

The bacterium showed a typical sigmoidal growth pattern in the nutrient medium. The organism was able to grow at 4 °C but was unable to grow at 42 °C, a characteristic feature of *P. chlororaphis*. The pseudomonad grew on a wide range of carbon sources and was resistant to various groups of antibiotics and heavy metals as stated by Papavizas et al. (9). They also suggested that mutants resistant to fungicides, are useful the integration of present biological and chemical methods for improvement over the commercial control methods currently in use.

In the present study, we report on antimicrobial antibiotics produced by *P. chlororaphis*, which was found to be active against a broad spectrum of pathogens. The biocontrol efficacy of *P. chlororaphis* is definitely not mediated by siderophores, because the assay medium supplemented with iron in the form of FeCl<sub>3</sub> did not reverse inhibition of the test organism.

Assay for antibiotics: *P. chlororaphis* showed distinct antibiotic crystals when grown on YDC agar plates. The crude antibiotic, assayed against test pathogens, exhibited distinct inhibition of *Bipolaris oryzae*, *Pyricularia grisea* and *Rhizoctonia solani*. Spectrophotometric analysis of the antibiotics showed prominent absorption peaks at 220 nm, 360 nm and 420 nm. TLC analysis revealed three (2 UV absorbing and 1 UV quenching) spots; the first spot having an  $R_f$  value of 0.085 showing absorbance at 220 nm, a second spot with an  $R_f$  value of 0.512 showed absorbance at 360 nm and a third spot with an  $R_f$  value of 0.850 showed absorbance at 420 nm.

Spermosphere and rhizosphere colonization: *Pseudomonas chlororaphis* was found to colonize the surface of seeds of rice, chili and tomato plants (Table 4). The colony forming units (cfu)/seed increased up to 8 days in all plants, indicating the effective spermosphere colonization by the bacterium without affecting germination; after 8 days the seed germinated, hence a maximum of 8 days was used. The results showed that spermosphere colonization ability of *P. chlororaphis* increased linearly and reached a maximum on day 8, in the case of chili and tomato; whereas, for tomato the colony formation had a marginal increase after 5 days. The seed colonization ability is an asset to the pseudomonad for being used for seed and soil treatment.

**Tab. 4** Spermosphere colonization by *P. chlororaphis*

Plant	Number of colony forming units/seed			
	1 <sup>st</sup> day	3 <sup>rd</sup> day	5 <sup>th</sup> day	8 <sup>th</sup> day
Rice	$1.2 \times 10^7$	$4.5 \times 10^{12}$	$5.2 \times 10^{12}$	$5.7 \times 10^{12}$
Tomato	$3.0 \times 10^9$	$2.2 \times 10^{12}$	$2.7 \times 10^{12}$	$3.0 \times 10^{12}$
Chili	$1.1 \times 10^6$	$8.0 \times 10^{11}$	$6.2 \times 10^{12}$	$3.1 \times 10^{13}$

The rapid colonization of seeds and roots of rice IR50 variety by *P. chlororaphis* agrees with the reports of effective colonization of roots and other parts by *P. putida* and *P. fluorescens* [10], as the restriction of pathogens infection of bacterized plants is attributed to the blocking of penetration of pathogens to the outer root tissues by the root colonizers [11].

The performance of *P. chlororaphis* was similar to *Erwinia herbicola* [12] when used as seed treatment; it was detectable in the germinating seeds, indicating its efficiency to colonize roots. A positive correlation of the population size and biocontrol activity was shown [13] in the case of *P. fluorescens* strain 2-79 towards *Gaeumannomyces graminis* var. *tritici*. The soil-borne bacterium also has the ability to colonize the above ground parts of plants and has biocontrol activity as reported in the case of fire blight of pear trees and serve as good commercial herbicides.

From colonization studies on the outer surfaces of sugar beet and cotton seeds Tombolini et al. [12] hypothesized that disease suppression is favoured by co-localization of *P. chlororaphis* MA342; a similar effect was found in this study with *P. chlororaphis*. As a matter of fact, biocontrol agents should grow and persist or colonize the surface of the plant to be protected since colonization is widely believed to be essential for biocontrol [14,15,16]. Unsuccessful biocontrol field experiments are often correlated with poor colonization of the root system by biocontrolling bacteria (1). In the current research, *P. chlororaphis* grew and colonized the seeds and roots of rice, tomato and chili plants. The suppression of disease is also correlated with colonization of roots by *P. fluorescens*, as analyses for their bacterial population revealed. It was found that the strain of *P. chlororaphis* was able to survive in the rhizosphere of rice, tomato and chili roots with an average of  $3.1 \times 10^5$  cfu/g of root in rice  $3.8 \times 10^4$  cfu/g of root in chili plants (Table 5), indicating that the bacterium can compete and survive in the presence of the other rhizobacteria.

**Tab. 5** Rhizosphere colonization by *P. chlororaphis*

Plant*	Root base <sup>#</sup>		Root tip <sup>#</sup>	
	Initial count	Final count	Initial count	Final count
Rice	$4 \times 10^9$	$4 \times 10^{14}$	$2 \times 10^9$	$4 \times 10^{14}$
Tomato	$4 \times 10^9$	$3 \times 10^{12}$	$3 \times 10^9$	$4 \times 10^{12}$
Chili	$3 \times 10^7$	$4 \times 10^{14}$	$2 \times 10^6$	$5 \times 10^8$

\*Number of colony forming units per g of root; <sup>#</sup>Number of CFU/g root after 20 d of incubation.

The results of the root colonization, obtained in the present study with a simple gnotobiotic system, indicate that both the root base and the root tip were colonized resulting in the uniform distribution of bacteria in the rhizosphere. In contrast with the earlier data on *Pseudomonas* sp. in root colonization [18], the data of the present study indicate a uniform distribution of *P. chlororaphis* in the rhizosphere due to exuded nutrients. The rhizosphere colonizing bacteria are ideal candidates for soil inoculation because of their potential for rapid and dominant colonization [19], necessary for disease control mechanisms to prevent the invasion of detrimental soil microorganisms on the root surface.

Plasmid curing and transformation experiments were carried out to verify the role of a plasmid in antibiotics production, because it is hypothesized that plasmids are involved in the production of some antibiotics, especially because catabolic pathways are plasmid determined in many pseudomonads [20,21]. The mega plasmid (pKL1) of 140 kb harboured by *P. chlororaphis* could not be cured by various agents as ethidium bromide (10 mg/ml), acridine orange (10 mg/ml) nalidixic acid (15 µg/ml), mitomycin C (20 µg/ml), elevated temperature (42 °C for 12h) and by continuous subculturing in stress free medium. But successful transformation of the plasmid was obtained in plasmid-free cells of *P. putida* cells DSM 2112 (MTCC). Transformed *P. putida* cells exhibited the plasmid pKL1 and were also resistant to antibiotics such as benzyl penicillin, ampicillin, rifampicin and chloramphenicol, similar to the parent strain. Furthermore, the *P. putida* transformants exhibited distinct inhibition of the growth of the test pathogen *R. solani*.

*P. chlororaphis* harbours a mega plasmid pKL 1, being transferable to strains of *P. putida* without plasmids. The wild strain of *P. chlororaphis* and the pKL1 transformed *P. putida* strain showed similar biocontrol activities. The antibiotic production and benzene extract peaks were similar in both strains, suggesting that the antibiotic production in *P. chlororaphis* is plasmid mediated and easily transferable [22,23,24]. This is similar to previous reports on the involvement of plasmids in the production of 8-endotoxin from *Bacillus thuringiensis*, agrocin from *Agrobacterium radiobacter* K84 and K1026 and an antibiotic from *Erwinia herbicola* EH1087.

Transposon mutagenesis and PCR analysis: The transposon mutants were isolated on LBA medium amended with chloramphenicol (30 µg/ml) and gentamycin (20 µg/ml) and screened for excitation at 422 nm and emission at 509 nm in a luminescence spectrophotometer LS 50B. The mutants showed green fluorescence due to the presence of GFP; the growth and colony morphology of the tagged strain was similar to that of the parent strain. PCR analysis confirmed the presence of the Tn5 insert. The Tn5::GFP mutants did not inhibit the growth of fungal mycelia. These mutants, when complemented by transformation with plasmid pKL1 (from *P. chlororaphis*), showed biocontrol activity similar to the parent strain, confirming plasmid mediated antibiosis.

*P. chlororaphis* was sensitive to kanamycin and gentamycin; the Tn5 derivatives were used for generalized mutagenesis. The genetic analysis of mutants revealed that each of them contained Tn5::GFP insertions. Similar transpositional mutagenesis experiments were done with *P. fluorescens* 2-79, producing the antibiotic PCA active against take-all disease of wheat. The *phz* mutants show a single Tn5 insertion and they were much less suppressive to take-all disease 2-79 [25]. The *ant* mutants of *P. fluorescens* strain 7-14 also contain a mini Tn5km. Mutants, transposon defective in antibiotic production, do not have antifungal activity. The Tn5 insertion caused changes in the phenotype and some strains, deficient in antibiotic production, demonstrated that antibiotic production is a major factor contributing to disease control. The results in the present study were similar, the mutants with Tn5 insertions showed no antifungal activity.

Field trials: Field experiments carried out to evaluate the potential biocontrol efficiency of *P. chlororaphis* against rice pathogens showed distinct control and maximum reduction of disease incidence, as was observed in combined seed and foliar treatment, which was superior to other treatments. All treatments successfully reduced the disease incidence, showing disease protection from 56-80% upon seed treatment followed by two foliar sprays (Table 6).

**Tab. 6** Disease control of rice pathogens by different treatment procedures.

Treatment	Pathogens		<i>Pyricularia oryzae</i>		<i>Bipolaris oryzae</i>		<i>Rhizoctonia solani</i>	
	<i>Xanthomonas oryzae</i>		%DI	%DC	%DI	%DC	%DI	%DC
Seed	17.1	82.9	34.1	65.8	24.1	75.9	39.2	60.8
Root tip	38.8	61.2	42.1	57.9	31.3	68.7	40.8	59.2
Foliar spray	40.2	59.8	44.4	55.9	34.1	65.9	45.5	54.5
Seed + Foliar spray	18.2	81.8	29.3	70.7	23.5	76.5	30.7	69.3

Percentage of disease incidence (DI) = disease in treatment/disease in control x100; percentage of disease control (DC) = 100 - disease incidence

## Discussion

The results of the current study demonstrate that *P. chlororaphis* persisted in the rice fields and in the crop providing effective control against rice pathogens. It is essential that biocontrol agents are applied as foliar spray in addition to seed treatment as done in the current research work, because only a limited bacteria migration occurs, though they effectively persist in the rice fields. This is in agreement with results obtained from various other field trials [26-30]. In addition to antibiosis, induction of systemic resistance might be another possible mechanism involved in the mechanism of action of *P. chlororaphis*.

The present results demonstrate that rice plants treated with *P. chlororaphis* as seed treatment followed by application of pathogens protected rice plants against the pathogens. Intense colonization is one of the reasons for the reduction in blast, sheath blight, bacterial blight and brown spot of rice. The present results confirm the colonization of chili, tomato and rice roots by *P. chlororaphis* and additionally support the current concepts that rhizosphere bacteria are active inducers of plant disease resistance. The inhibition of pathogens *in vitro* and *in vivo* is due to plasmid (pKL 1) mediated antibiotics. Inoculation of rice with *P. chlororaphis* significantly promoted plant growth, which is associated with the suppression of deleterious micro flora. The results showed that *P. chlororaphis* is a very good root colonizer and foliar antagonist, and with its broad spectrum of antifungal activity, offers a general biocontrol system that works against the pathogens of both root and foliar tissues. Formulation and mass production of the organism is under progress and will provide a challenging control of disease using *P. chlororaphis*.

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## **Biocontrol and plant growth promoting potential of *Pseudomonas* sp. MML2212 from the rice rhizosphere**

### **Abstract**

A total of 460 fluorescent pseudomonads were isolated from rice rhizosphere soils collected from Tamil Nadu State, India and screened against *Rhizoctonia solani*, the sheath blight pathogen of rice by dual culture. Due to superior antagonistic efficacy against the fungal pathogens over the other strains, an efficient bacterial strain (MML2212) was selected and identified as *Pseudomonas* sp. The culture filtrate of *Pseudomonas* sp. MML2212 significantly inhibited the mycelial growth, conidial and sclerotial germination of many fungal pathogens. The strain *Pseudomonas* sp. MML2212 produced two antifungal compounds with the Rf values of 0.67 and 0.36. The production of hydrogen cyanide and siderophores was determined. The whole culture of *Pseudomonas* sp. MML2212 significantly improved the plant growth parameters in rice as compared to culture filtrate and medium control both in non-sterile and sterile soils.

### **Introduction**

Fluorescent pseudomonads are ubiquitous soil microorganisms and common inhabitants in the rhizosphere of crop plants. Many strains suppress plant diseases by protecting seeds or roots from infection caused by fungal and bacterial plant pathogens (Defago and Hass 1990). The application of microorganisms such as *Pseudomonas* spp. in biological control of root disease in agriculture is becoming an important alternative or supplement to chemical fungicides. Diseases caused by plant fungal pathogens such as *Gaeumannomyces graminis* var. *tritici*, *R. solani* and *Pythium* spp., have been effectively controlled by fluorescent pseudomonads in greenhouse and field trials (De Freitas et al. 1991). Plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978) are root colonizers that promote plant growth and control plant diseases in several crop plants. In the present study we investigated the biocontrol and growth promoting potential of a *Pseudomonas* sp. MML2212 isolated from the rice rhizosphere soil.

### **Material and Methods**

Isolation of fluorescent pseudomonads from rice rhizosphere soils: A total of 415 rice rhizosphere soils were collected from the farmers' fields in southern districts of Tamil Nadu state, India. The soil samples were serially diluted up to  $10^{-9}$  and plated on King's B agar medium (King et al. 1954). The plates were incubated at room temperature ( $28 \pm 2$  °C) for 48 h and observed under UV light at 360 nm. Distinct single fluorescent colonies were picked and streaked on KB agar medium to check the purity. The working cultures were maintained on KB agar slants/plates and the permanent cultures were stored in 30% glycerol at  $-20$  °C.

Screening of fluorescent pseudomonads: All 460 fluorescent pseudomonads were screened by dual plate technique (Huang and Hoes 1976) against *R. solani*, the sheath blight pathogen of rice. A total of 52 isolates was short listed after primary screening and documented. They were again screened for their wide spectrum of antagonistic activity against *Macrophomina phaseolina*, *Fusarium udum*, *Pyricularia grisea*, *Alternaria alternata*, *Bipolaris oryzae* and *Curvularia lunata*.

Identification of selected strain: An effective fluorescent pseudomonad designated as MML2212 was selected for further studies due to its efficacy against a wide range of fungal pathogens. Different physiological tests suggested in Bergey's Manual of Determinative Bacteriology (Williams et al. 1994) were carried out to identify the isolate as MML2212.

Effect of culture filtrate of *Pseudomonas* sp. MML2212 on fungal pathogens: *Pseudomonas* sp. MML2212 was grown in KB broth at 150 rpm in a rotary shaker at room temperature. After 24 h, the culture was harvested and centrifuged at 10,000 rpm for 15 min. The cell free culture filtrate and concentrated culture filtrate were tested against the above mentioned fungal pathogens with the agar diffusion method.

Thin layer chromatography: Thin layer chromatography of the culture filtrate was performed using pre-coated TLC plates (E-Merck) to detect the compounds produced by *Pseudomonas* sp. MML2212. The plates were eluted with different solvent systems, visualized using UV and iodine and the R<sub>f</sub> value of each spot was calculated.

Production of siderophores and hydrogen cyanide (HCN): The production of siderophores by *Pseudomonas* sp. MML2212 was determined with the standard methods (Loper 1988). The HCN production was determined according to Lorck (1948) using nutrient sucrose agar medium. *Pseudomonas fluorescens* CHAO and *Pseudomonas chlororaphis* were used as positive controls for HCN and siderophore production, respectively.

Plant growth promotion of *Pseudomonas* sp. MML2212: The rice seeds of cv. IR-50 were surface sterilized with sodium hypochlorite, washed in sterile distilled water and air dried. They were soaked for 6 h in 24 h old culture (0.5 OD at 600 nm) and culture filtrate of *Pseudomonas* sp. MML2212. Sterile nutrient broth and distilled water were used for controls. The seeds were then air dried and 10 seeds placed per Petri dish, lined with moistened filter paper. The plates were incubated at room temperature and the germination was observed after 3 days. The germinated seedlings from each treatment were grown in plastic pots containing sterile and non-sterile garden soil. After ten days, the root and shoot lengths and dry weight of the seedlings were measured. The vigour index of the seedlings in each treatment was calculated according to Baki and Anderson (1973).

## Results

Isolation and screening of antagonistic bacteria: A total of 460 fluorescent pseudomonads was isolated from rice rhizosphere soils and screened for antifungal activity against *R. solani*. Among them, 52 fluorescent pseudomonads exhibited antagonistic activity (Table 1) and produced inhibition zones towards *R. solani*, ranging from 0.2 cm to 2.5 cm. Further screening of all 52 isolates resulted in selection of a fluorescent pseudomonad strain designated as MML 2212 due to its high antifungal activity against a broad spectrum of phytopathogens such as *M. phaseolina*, *F. oxysporum*, *A. alternata*, *B. oryzae*, *P. grisea* and *C. lunata*. The inhibition zones ranged from 2.7 cm to 3.5 cm.

**Tab. 1** Isolation and screening of fluorescent pseudomonads

Location	No. of sample	No. of fluorescent pseudomonads	No. of antagonists
Thirunelveli	70	82	7
Madurai	95	102	15
Viruthunagar	60	78	8
Theni	115	110	12
Dindugal	75	78	10
Total	415	460	52

Identification of MML2212: The selected strain MML2212 was identified as *Pseudomonas* sp. based on the physiological tests.

Effect of culture filtrate of *Pseudomonas* sp. MML2212 on fungal pathogens: The culture filtrate and concentrated culture filtrates of *Pseudomonas* sp. MML2212 effectively inhibited conidial and sclerotial germination of fungal pathogens. The inhibition was more pronounced by the concentrated culture filtrate in which 80-100% inhibition was recorded as against 65-90% by the culture filtrate. Among the fungal pathogens, *A. alternata* and *C. lunata* were more sensitive than other pathogens (Table 2).

**Tab. 2** Inhibition of conidial and sclerotial germination by culture filtrate of *Pseudomonas* sp. MML2212

Pathogen	Conidial/sclerotial inhibition (%)	
	Culture filtrate	Concentrated culture filtrate
<i>R. solani</i>	65	80
<i>F. oxysporum</i>	75	95
<i>B. oryzae</i>	75	90
<i>A. alternata</i>	80	100
<i>C. lunata</i>	90	100

Effect of partially purified compounds of *Pseudomonas* sp. MM2212 on fungal pathogens: The presence of two major compounds with the Rf value of 0.76 and 0.36 was detected by analytical TLC and partially purified by preparative TLC. These partially purified compounds of *Pseudomonas* sp. MML2212 markedly inhibited the conidial and sclerotial germination of all tested pathogens. The inhibition ranged from 50 to 80% by compound 1 and 20-30% by compound 2, and from 80 to 100% (Table 3).

**Tab. 3** Inhibition of conidial and sclerotial germination by the partially purified compounds of *Pseudomonas* sp. MM2212

Pathogen	Inhibition (%)		
	Compound-1	Compound-2	Compound 1 + 2
<i>R. solani</i>	50	30	80
<i>F. oxysporum</i>	65	20	85
<i>B. oryzae</i>	60	30	90
<i>A. alternata</i>	75	25	100
<i>C. lunata</i>	80	20	100

Production of siderophores and HCN: *Pseudomonas* sp. MML2212 produced both siderophores and HCN.

Plant growth promotion: Seed germination of rice was significantly increased due to treatment with whole culture and cell free culture filtrate of *Pseudomonas* sp. MML2212 as compared to treatment with the control medium. However, maximum germination was observed in seeds treated with the whole culture. Similarly the treatment with the whole culture of *Pseudomonas* sp. MML2212 effectively improved the root and shoot lengths, dry weight and vigour index as compared to treatments with culture filtrate and medium (Table 4).

**Tab. 4** Effect of culture and culture filtrate on growth promotion in rice

Treatment	Germination (%)	Root length (cm)	Shoot length (cm)	Dry weight (g)	Vigour index
<b>Non-sterile soil</b>					
Culture	100	13.74	10.87	0.3352	2461
Culture filtrate	100	13.38	10.56	0.3023	2394
Control	90	10.7	6.85	0.2196	1580
<b>Sterile soil</b>					
Culture	100	23.29	15.17	0.335	3846
Culture filtrate	100	15.31	10.01	0.4028	2532
Control	85	11.82	11.56	0.206	1988

## Discussion

In the present study, a total of 460 fluorescent pseudomonads were isolated from the rice rhizosphere, of which 52 strains exhibited antifungal activity against plant pathogens. Among the 52 antagonists, a strain designated as MML2212 was selected because of its broad spectrum of activity against various fungal phytopathogens. It has been established that the fluorescent pseudomonads occur predominantly in the rhizosphere of crop plants. Many plant growth promoting rhizobacteria including fluorescent pseudomonads have already been isolated from the rhizosphere of rice and sugarcane (Ramesh Kumar et al. 2002). The use of plant growth promoting pseudomonads for control of plant diseases and the mechanisms involved have been reported recently (Compant et al. 2005).

The selected strain MML2212 was identified as *Pseudomonas* sp. based on the physiological tests. Further, 16S r RNA sequencing analysis is in progress to identify the species. The culture filtrate and concentrated culture filtrates of *Pseudomonas* sp. MML2212 effectively inhibited conidial and sclerotial germination of fungal pathogens indicating that the antifungal principles are extracellular in nature. The presence of two major compounds with the Rf values of 0.76 and 0.36 was detected by analytical TLC. These partially purified compounds markedly inhibited the conidial and sclerotial germination of all tested pathogens. The activity significantly increased when these two compounds were combined. This indicated that these compounds act synergistically and exerted more antifungal activity towards plant pathogens. The production of siderophores and HCN is a common phenomenon among fluorescent pseudomonads and their production is one of the important mechanisms in disease suppression.

All growth parameters were significantly more increased by treatment with whole cultures than with the culture filtrate of *Pseudomonas* sp. MML2212 and control treated with the medium. Similarly, maximum increase in plant biomass of groundnut was observed following treatment with a rhizosphere isolate of *Bacillus firmus* and two phylloplane isolates of *Bacillus megaterium* and *Pseudomonas aeruginosa* (Kishore 2005). According to Negi et al. (2005) cold tolerant fluorescent pseudomonads isolated from Himalayas improved the plant growth in pea. The above findings suggest that the rhizosphere bacterium *Pseudomonas* sp. MML2212 can be employed as biocontrol agent for the management of plant diseases and also for plant growth promotion.

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## Biocontrol potential of a growth promoting soil bacterium against phytopathogenic fungi

### Abstract

A number of bacterial strains were isolated from soil samples collected from different parts of Tamil Nadu State in South India. *In vitro* screening resulted in selecting a highly efficient bacterial strain designated as MML2501, which was later identified as *Bacillus firmus*. It effectively inhibited the mycelial growth of a wide range of fungal phytopathogens in dual plate assays. The culture filtrate and autoclaved culture filtrate of *B. firmus* MML2501 inhibited the mycelial growth and spore germination of all test pathogens but failed to exhibit antifungal activity against *Rhizoctonia solani* and *Phytophthora infestans*. The proteins of *B. firmus* MML2501 did not exhibit any antifungal activity against pathogenic fungi even at higher concentrations. Therefore, the production of secondary metabolites in the culture filtrate was analyzed and the crude metabolites significantly inhibited spore germination and mycelial growth of fungal phytopathogens. The plant growth promoting activity of *B. firmus* MML2501 was assessed in rice, green gram and tomato. The seeds treated with whole culture of *B. firmus* MML2501 exhibited enhanced growth parameters, such as seed germination, root and shoot lengths, dry weight and seedling vigour in all the crops as compared to seed treated with cell-free culture filtrate and sterile water. Analysis of culture filtrate indicated that *B. firmus* MML2501 was able to produce indole acetic acid (IAA) at high concentrations in the presence of L-tryptophan. The above findings suggest that the soil bacterium *B. firmus* MML2501 can be exploited as biocontrol agent for the management of a wide range of fungal pathogens and the growth promoting ability is an additional advantage of this bacterium.

### Introduction

Bacterial strains isolated from the rhizosphere are promising as seed inoculants in innovative agriculture to promote plant growth and crop yield and reduce various plant diseases. Although many microorganisms have been identified as biocontrol agents against plant pathogens, most research and development efforts have focused towards members of three genera namely, *Bacillus*, *Pseudomonas* and *Trichoderma* (Brain et al. 2004). At present several products based on plant growth promoting rhizobacteria (PGPR) are available commercially world wide and many of them contain strains of *Bacillus*. Treatment with *Bacillus*-based biological control agents (BCAs) of disease resistant hosts has proven to be effective in management of plant diseases. It has been reported that *Bacillus* spp. effectively reduced the development of *Fusarium* wilt of chickpea (Hervas et al. 1998) and bean rust (Baker et al. 1983) diseases. The mechanisms of biological control exerted by the members of *Bacillus* include antibiosis (Chan et al. 2003), parasitism and induced systemic resistance (Bargabus et al. 2002). With this background the present study has been initiated to explore the antifungal activity of *B. firmus* MML2501, isolated from the Indian soil, against major root pathogens. In addition, its plant growth promoting ability and IAA production have also been determined.

### Materials and methods

**Organisms:** The bacterial strain MML2501 was isolated from Indian soil and maintained on nutrient agar (NA) for working culture and stored at -20 °C in glycerol vials. All fungal phytopathogens were obtained from the culture collection of Microbial Metabolites Lab, CAS in Botany, University of Madras, Chennai, India and maintained on potato dextrose agar (PDA).

**Identification of the culture:** The bacterial strain MML2501 was identified as *Bacillus firmus* based on physiological characteristics suggested in the Bergey's Manual of Determinative Bacteriology (Williams et al. 1994).

Antifungal activity in dual plate assay: *B. firmus* MML2501 was screened for its antifungal activity against *Macrophomina phaseolina*, *Fusarium oxysporum*, *Fusarium udum*, *Bipolaris oryzae*, *Pyricularia grisea*, *Alternaria alternata* and *Curvularia lunata* by dual plate assay (Skidmore 1976).

Bioassay of culture filtrate: *B. firmus* MML2501 was grown in NB for 48 h at room temperature ( $28 \pm 2^\circ\text{C}$ ) on a shaker at a speed of 150 rpm. The culture was harvested and centrifuged at 10,000 rpm for 10 min. The cell-free autoclaved and unautoclaved supernatants were used for conidial germination assays.

Bioassay of proteins and crude metabolites: The proteins from the cell-free supernatant of *B. firmus* MML2501 were separated by salting out using ammonium sulfate at 85% saturation followed by centrifugation at 10,000 rpm for 15 min. The pellet was suspended in double distilled water, dialysed and freeze dried. The remaining supernatant was concentrated to 10 fold (crude metabolites) and stored for further studies. The freeze dried protein was dissolved in sterile distilled water to a concentration of 10 mg/ml. The crude metabolites were dissolved in a small quantity of DMSO and mixed with sterile distilled water to a concentration of 10 mg/ml. Both solutions were filter sterilized using 0.2  $\mu\text{m}$  filters and bioassayed using the agar diffusion test with 100  $\mu\text{l}$  in each well. Plates were incubated at room temperature and the zone of inhibition of fungal growth around the well was observed after 5-7 days.

Growth promotion activity: Seeds of rice, green gram and tomato were soaked with bacterial culture (0.1 OD at 640 nm), culture filtrate and sterile water. Then the seeds were placed on moist filter paper in Petri dishes and incubated at room temperature. After 10 days, the germination, shoot and root length, fresh and dry weight were recorded and the seedling vigour index was calculated.

Production of indole acetic acid (IAA): *B. firmus* MML2501 was grown in both NB and minimal medium supplemented with L-tryptophan at concentrations of 5 to 25 mM. Cultures were incubated in total darkness and shaken at  $25^\circ\text{C}$  for 3 days. The production of IAA was colorimetrically estimated and further detected by thin layer chromatography according to Loper and Schroth (1986).

## Results

Identification of the culture: The physiological tests showed that the strain MML2501 consists of rod shaped bacteria, is Gram-positive, motile, and endospore forming. It showed lactose fermenting colonies on Macconkey agar with the production of brownish pigment on PDA. It further showed acid butt/alkaline slant in TSI and was able to hydrolyze starch and gelatine. It showed positive reaction for catalase, oxidase, urease and nitrate and negative reaction for cellulase production and MR/VP. The carbohydrate fermentation showed that MML2501 was able to ferment maltose and lactose. The above biochemical characters were the same as for *Bacillus circulans*, *Bacillus lentus* and *Bacillus firmus*. However, cellulase -ve, no acid production during carbohydrate fermentation and production of brownish pigment in PDA are the specific characteristics of *B. firmus*. *B. circulans* is cellulase +ve and *B. lentus* produces acid during carbohydrate fermentation.

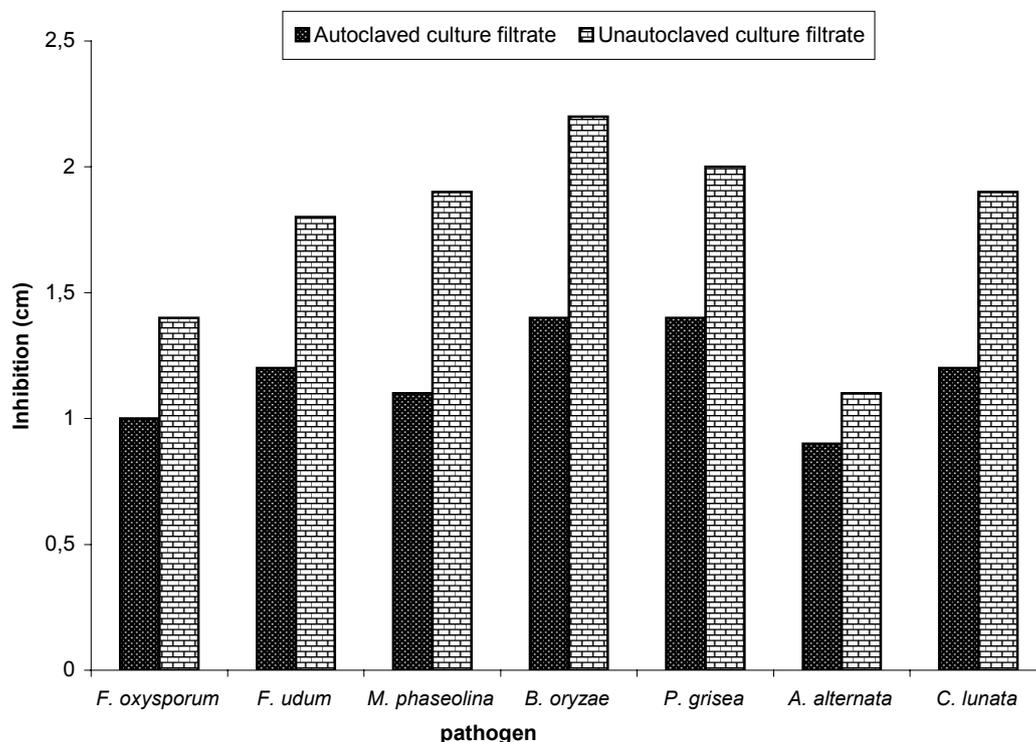
Antifungal activity in dual plate assay: *B. firmus* MML2501 effectively inhibited the mycelial growth of a wide spectrum of plant pathogens. The activity was estimated as 3 in the 0-4 scale towards most pathogens, however, it was 2 on the scale against *F. oxysporum* and *A. alternata* (Table 1).

**Tab. 1** Antifungal activity of *B. firmus* MML2501 against plant pathogens

Pathogen	Growth inhibition (0-4 scale)*
<i>F. oxysporum</i>	2
<i>F. udum</i>	3
<i>M. phaseolina</i>	3
<i>B. oryzae</i>	3
<i>P. grisea</i>	3
<i>A. alternata</i>	2
<i>C. lunata</i>	3

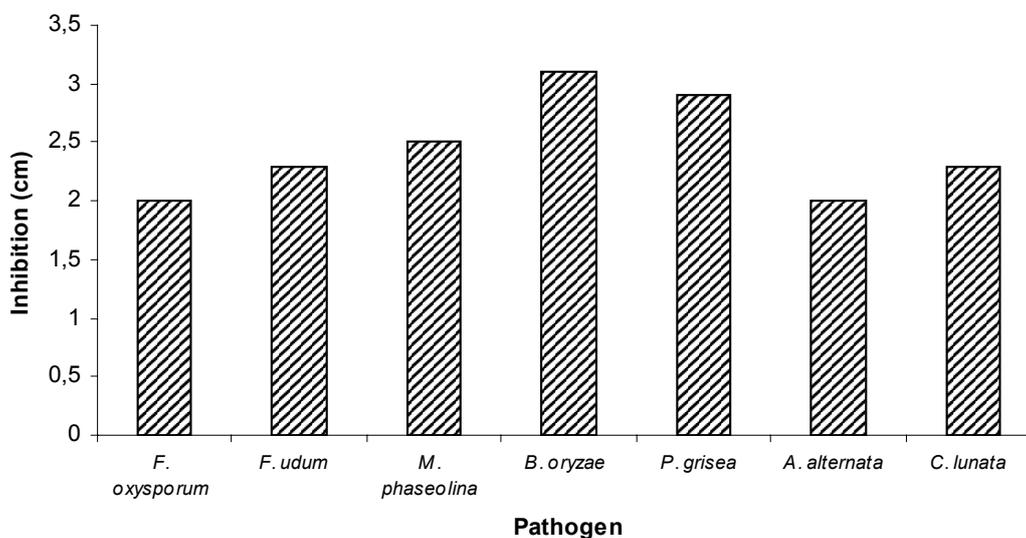
\*0: no inhibition; 1: 1 - 25%; 2: 26 - 50%; 3: 51 - 75%; 4: 76 - 100%

Bioassay of culture filtrate: Both the autoclaved and unautoclaved culture filtrates of *B. firmus* MML2501 exhibited good inhibitory activity on all test pathogens with inhibition zones of more than 1.5 cm. However, the unautoclaved culture filtrates showed higher inhibitory activity than the autoclaved culture filtrate (Figure 1).



**Fig. 1** Effect of autoclaved and unautoclaved culture filtrates of *B. firmus* on fungal pathogens

Bioassay of proteins and crude metabolites: The protein preparation of *B. firmus* MML2501 did not exhibit antifungal activity on any of the test pathogens. But the crude metabolites effectively inhibited all test pathogens with more than 2 cm zones of inhibition (Figure 2). In addition, the crude metabolites completely inhibited the conidial germination of most of the pathogens, against more than 90% germination in the controls.



**Fig. 2** Effect of protein and crude metabolites of *B. firmus* on fungal pathogens

**Growth promotion activity:** All growth parameters such as seed germination, root and shoot lengths, seedling dry weight and vigour index were markedly improved following a treatment with the whole culture and culture filtrate of *B. firmus* MML2501 as compared to the water-treated controls. The activity was more pronounced in whole-culture treated seeds than upon culture-filtrate treatment (Table 2).

**Tab. 2** Effect of *B. firmus* MML2501 and its culture filtrate on seed germination of rice, green gram and tomato

Treatment	Germination (%)	Root length (cm)	Shoot length (cm)	Dry weight (g)	Vigour index
<b>Rice</b>					
Culture	100	6.3	4.96	0.48	1126
Culture filtrate	90	5.0	4.53	0.26	858
Control	90	3.5	4.45	0.15	716
<b>Green gram</b>					
Culture	100	7.23	18.3	0.28	2553
Culture filtrate	100	7.19	17.8	0.23	2499
Control	90	6.59	17.3	0.18	2150
<b>Tomato</b>					
Culture	90	4.04	6.9	0.68	985
Culture filtrate	80	3.67	6.3	0.4	798
Control	40	2.4	5.3	0.36	308

**Production of IAA:** Highest production of IAA (23 µg/ml) was found in 72 h old cultures of *B. firmus* MML2501 in comparison to other ages independent of the concentration of L-tryptophan. Among different concentrations 15 mM L-tryptophan induced the maximum production (Table 3).

**Tab. 3** Production of IAA by *B. firmus* MML2501 at different concentrations of L-tryptophan

L-tryptophan (mM)	IAA (µg/ml)			
	48 h	72 h	96 h	120 h
5	4	7	4	3
10	5	9	5	4
15	8	23	5	5
20	4	8	5	3
25	4	6	4	3

**Thin layer chromatography:** The presence of IAA was detected on TLC as a blue band by spraying Ehrlichs reagent. The RF value of the IAA produced by *B. firmus* MML2501 was calculated as 0.82.

## Discussion

The present study demonstrated the biocontrol potential of *B. firmus* MML2501 against a broad spectrum of plant pathogens such as *F. oxysporum*, *F. udum*, *M. phaseolina*, *A. alternata*, *C. lunata*, *B. oryzae* and *P. grisea*. Autoclaved and unautoclaved culture filtrates showed inhibitory activity against all test pathogens, indicating that the active principles might be heat stable. Hence, these metabolite preparations could be applied in field conditions at different temperatures. The proteins separated from the culture filtrate of *B. firmus* did not exhibit antifungal activity, indicating that the active principles would belong to secondary metabolites. Inhibition of mycelial growth and conidial germination by the crude metabolites also confirmed the nature of the active principles. Such metabolites mediating biological control of fungal pathogens have already been reported (Baker et al. 1983; Stab et al. 1994; Silo-Suh et al. 1994).

The plant growth promoting ability of *B. firmus* MML2501 has been demonstrated in the present study by effectively enhancing all growth parameters in rice, green gram and tomato. This could be due to secretion of plant growth promoting substances such as IAA. Hence, the production of IAA was investigated and it was found to be produced at 23 µg/ml of IAA in MSM medium supplemented with 15 mM of L-tryptophan. This concentration seems to be significantly high because earlier reports reported a maximum production of only 12 µg/ml by *Azospirillum brasilense* (Omay et al. 1993) and 13 µg/ml by *Pseudomonas syringae* (Louise et al. 1988). The overall analysis of the present findings indicates that *B. firmus* is an efficient biocontrol strain of a wide spectrum of fungal phytopathogens and, in addition, it also produces the phytohormone IAA. *Bacillus* spp. is known for its long-term viability with endospores and therefore this strain can be developed as commercial product for field application.

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## VIII. Safety and regulatory aspects of biological control agents (BCA)

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### Biocontrol agents and their potential risk for human health

Biological control using naturally occurring antagonists offers an environmentally friendly and sustainable possibility to control fungal plant pathogens. One natural reservoir of antagonists is the rhizosphere, the zone around roots that is influenced by the plant. Due to a high content of nutrients, this habitat is a “microbial hot-spot”, where bacterial abundances including those with strong antagonistic traits are enhanced (Berg et al. 2005). Various bacterial genera, including *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Ochrobactrum*, *Pseudomonas*, *Ralstonia*, *Staphylococcus*, and *Stenotrophomonas* contain root-associated strains that can encounter bivalent interactions with both plant and human hosts. In general, antagonistic bacteria show a beneficial interaction with their host plants. They are able to promote plant growth and/or to suppress pathogens. Unfortunately, some of them belong to the group of facultative human pathogenic bacteria which cause diseases but only in patients with a strong predisposition to illness, particularly in those who are severely debilitated, immunocompromised or suffering from cystic fibrosis or HIV-infections. During the last years, the number of human infections caused by opportunistic pathogens has increased dramatically.

Mechanisms responsible for colonisation of the rhizosphere and antagonistic activity against plant pathogens are similar to those responsible for colonisation of human organs and tissues, and pathogenicity. Multiple resistances against antibiotics are not only found with clinical strains but also with strains isolated from the rhizosphere. High competition, the occurrence of diverse antibiotics in the rhizosphere, and enhanced horizontal gene transfer rates in this microenvironment appear to contribute to the high levels of natural resistances.

An interesting example for a bacterium with such a bivalent interaction is *Stenotrophomonas*, a very heterogeneous group within the  $\gamma$ -subclass of *Proteobacteria*. So far, all validly described *Stenotrophomonas* species have shown a high intrageneric diversity and heterogeneity at the phenotypic and genotypic level. *Stenotrophomonas* species play an important role in nature, especially in the global element cycle, and they are frequently used in applied microbiology and biotechnology, e.g. in biological plant protection. Recent interest has focused on the *Stenotrophomonas*' capability to degrade xenobiotic compounds and their potential for decontaminating soil (bioremediation). Furthermore, over the last decade multidrug-resistant *S. maltophilia* has become increasingly significant causing case/fatality ratios in certain patient populations, particularly in those who are severely debilitated or immuno-suppressed. *S. maltophilia* is associated with a broad spectrum of clinical syndromes, e.g., bacteremia, endocarditis, respiratory tract infections etc. *Stenotrophomonas* isolates occur ubiquitous and cosmopolitan, and were preferentially isolated from the rhizosphere of diverse plants all over the world especially those of *Brassicaceae*. In addition, the bacterium has been isolated from a wide range of nosocomial sources, e.g. contact lens care systems, dialysis machines, ice-making machines, nebulizers and inhalation therapy equipment. Presently, the virulence factors responsible for the pathogenicity of these organisms are not well understood but several traits have been implicated in antagonism/pathogenicity. *Stenotrophomonas* strains of environmental and clinical origin are able to adhere to many surfaces, e.g. to human epithelial respiratory cells and they are equally able to colonise the rhizosphere of strawberry. Furthermore, it was reported that many strains have the capability to produce extracellular enzymes, e.g. DNase, RNase, protease, lipase, chitinase and elastase are produced. Antibiotics synthesized by *Stenotrophomonas* are involved in pathogen suppression on plants whereas their importance for pathogenicity is still unclear. *S. maltophilia* strains are often highly resistant to multiple antibiotics. Results obtained by Minkwitz and

Berg (2001) indicated that the antibiotic resistance profile of *S. maltophilia* isolates was not associated with their origin (e.g., clinical and environmental especially from the rhizosphere). Such findings would suggest that strains of *S. maltophilia* did not acquire their antibiotic resistance during antibiotic therapy in the clinic/hospital environment. Although a general method to differentiate between clinical and environmental *S. maltophilia* strains could not be established, functional genes were used to differentiate between plant-associated strains of *S. rhizophila* and potentially human-pathogenic strains of *S. maltophilia* (Ribbeck-Busch et al. 2005).

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## **Biosafety regulations in Germany**

With the advance of biotechnology and genetic engineering in the 1970s, it became evident that a legal concept for this area of research was completely missing. 30 years afterwards, we may say that this gap has been filled from several aspects of view.

The first aspect is public and environmental health: to prevent the spread of harmful organisms into the environment resulting in epidemic diseases and plant deterioration. The new act on the prevention of infectious diseases (Infektionsschutzgesetz; BGesBl. 2000) with its extensive reporting system and the necessity to obtain work permits with dangerous pathogens tries to limit access only to trained personal with properly equipped laboratories. It does not, however, give a concise definition of an ‘infectious organism’ or references to other acts in this area. The protection of livestock and useful plants also falls into this chapter, with national regulations much more influenced by international (especially EU) guidelines (Pflanzen-/Tierseuchenerreger-Verordnung). Plant pathogens are listed in 2 groups according to their potential hazard and presence in the area, but the necessary work permit is not issued in general but for individual species only. Special state authorities are responsible for supervision. Guidelines for safe national and international transport and shipment also have their place here (ADR, IATA, postal regulations).

The second field is the prevention of development and use of agents of biological warfare (biosecurity). Here the national regulations are based on international treaties and aim to control the development and spread of material and knowledge necessary for biological (and chemical) attack. Restricted items are listed in extensive annexes to the military weapons control act; biological agents and toxins may be found in section II. Export restrictions also apply to certain goods that per se are harmless but may be used during the process of developing weapons (foreign trade act; annex: dual-use list).

Last but not least aspects of occupational health and safety must be considered and have the most pronounced influence on handling biological agents.

The legislation in Germany and all other EU member states is based on a guideline (90/679 and annexes; codified as 2000/54/EG) that defines the common minimal standards for work with natural biological agents. It is called Biostoffverordnung (Ordinance on safety and health at work involving biological agents) and is supplemented by technical rules (TRBA), which are formulated by a specific advisory committee (ABAS) and published in the ‘Bundesarbeitsblatt’, the official journal of the Ministry of Labour, which is responsible for this area. Online versions of all texts are available from the Federal Institute of Occupational Safety and Health ([www.baua.de](http://www.baua.de)).

The central aspect is to minimize possible contacts with a biological agent by increasing containment with increasing risk for workers and people outside.

Thus, the central point is the meaning of ‘risk’ under the special circumstances of working with biological agents. In general, risk is defined as the probability of a hazard to occur; here it is seen as the danger to come into contact with an agent that may cause infectious diseases in healthy people. It is evident that some grouping is necessary to cope with the wide variety of organisms and the guideline adopted the WHO model of four risk (or hazard) groups from nearly no to very high risk. The lowest risk group 1 is defined by both guideline and ordinance but both are not valid for organisms included in this group.

An extensive risk assessment determines the specific safety measures to be implemented at the workplace before starting the actual work. Besides considering the operations planned and the resulting possibilities and magnitudes of exposure against the agents, the identity and risk grouping of the organism involved is of major importance for this process. It is, therefore, highly important to know the criteria which influence the grouping of biological agents.

Even before the process of legislation had begun, a working group 'Biotechnology' of the German Trade Association of the Chemical Industry started to formulate a guideline for the prevention of accidents in this new field. In due course, the problem of risk grouping was assigned to several groups of experts for a certain taxonomic field, e.g. bacteria, fungi or viruses and included the risk group 1 organisms, too. The results of these discussions were published as short leaflets and were legally binding to all member companies within this association.

Grouping was the result of an extensive literature search and expert discussion with little standardisation between the groups, but the results were highly welcomed by all responsible for health at work. These leaflets have been updated in regular intervals and it was attempted to harmonize their contents as much as possible. Latest example is the new edition of the leaflet on 'Prokaryotes' of July 2005 with nearly 150 pages of text and 250 pages with the complete grouping of all known prokaryotes (except cyanobacteria) based on the 'Approved List of Bacterial Names' (for details see [www.bacterio.cict.fr/](http://www.bacterio.cict.fr/) or [www.dsmz.de/microorganisms/main.php?contentleft\\_id=14](http://www.dsmz.de/microorganisms/main.php?contentleft_id=14)).

Thus German legislation could make use of existing lists and they have been partially integrated into the ordinance as technical rules (TRBA 46X series). Partially, as they only give the lists for organisms of risk group 2 or higher and omit most of the explanatory text. Members of each group of experts form one of the subcommittees of the main advisory group (ABAS) reporting to the ministry. In this way, both groups are firmly linked and the continuous exchange of information is assured.

The close interactions of all groups made it necessary to establish a common basis of decisions in printed form and several TRBA's have been published to this purpose. Most important and worldwide singular is the TRBA 450, which lists the general criteria for the assessment of risk groups to species:

- Systematic position (name and taxonomic position; criteria for identification)
- Metabolic properties (auto-/heterotrophy; tolerance/sensitivity for temperature, pH,  $a_w$ , etc.)
- Natural habitat/mode of living (saprophytic, parasitic, symbiotic; occurrence)
- Pathogenicity for humans (virulence factors, clinical picture, diagnosis, prevention)
- Interactions with other agents (synergistic infections)
- Possibilities of exposure (air-borne, oral, percutaneous, trauma)
- Epidemiology (natural reservoirs, frequency, disease distribution, etc.)
- Following these criteria, it is possible to collect the data facilitating a consistent assessment of any new species to the correct risk group.

There will, however, remain some cases, where the four risk-group concept is too strict and it could be argued to classify a species in two groups depending on the personal weighting of some characteristics. This is the case for many of the so called 'opportunistic' pathogens, which may cause infection only under special conditions like a diminished immune response of the host organism. An additional index (+) was introduced to designate species within the risk group 1, that may be infectious in cases of severe immunosuppression or similar conditions but completely harmless to healthy adults.

A similar situation was found within the risk group 3, where it would have been wasteful to introduce the full program of laboratory safety measures for such organisms that could not infect via the air. A special index (\*\*\*) was introduced to designate such species and a list of the necessary safety precautions published as TRBA 105.

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## ***Pantoea agglomerans*, a biocontrol agent and ubiquitous microorganism – friend or foe?**

### **Introduction**

Strains of *Pantoea agglomerans* (syn. *Erwinia herbicola* and *Enterobacter agglomerans*) are ubiquitous in nature, inhabiting diverse environments, such as plants, water, soil, humans and animals (Gibbins 1978). The bacterium is considered an opportunistic pathogen of humans and animals, a direct cause of septicaemia and an allergen. *P. agglomerans* has been isolated from internal organs of deer, human throats (Murashi et al. 1965), from stool specimens of patients with typhoid fever, but also from excreta of healthy individuals (Dresel and Stickl 1928). In fire blight lesions on rosaceous plants, these bacteria occur as saprophytes. Often they have been isolated from lesions in conjunction with the fire blight pathogen, *Erwinia amylovora* (Rosen 1928; Billing and Baker 1963). They are Gram-negative, rod-shaped and form yellow or white colonies on growth media (Cruickshank 1935; Billing and Baker 1963). *P. agglomerans* is known as a biocontrol agent of several plant pathogens, but it also causes gall-forming diseases in certain plant hosts.

*Pantoea agglomerans* as a biocontrol agent of plant diseases: *P. agglomerans* has been used as a biocontrol agent for many plant pathogens of numerous crops. One of the earliest reports is from 1935, when Adam and Pugsley found that coinoculation of *P. agglomerans* with the halo blight pathogen in beans slowed the rate of development of lesions (Adam and Pugsley 1935). *P. agglomerans* was found not to be pathogenic to bean. During the past three decades, a number of bacterial and fungal diseases of plants have been reported as controlled by *P. agglomerans* (Table 1). Because *P. agglomerans* is easily isolated from many environments, and from diseased plants, and it grows readily on most laboratory media at 22-37 °C, it often has been mistaken for a plant pathogen (Billing 1976). *P. agglomerans* is able to prevent fire blight infection when applied to blossoms or pear fruit in advance of the pathogen (Riggle and Klos 1972; Billing and Hutson 1974). Strains of *P. agglomerans* have been used as protectants against fire blight in the laboratory and in orchards (Beer et al. 1984; Wilson et al. 1990; Johnson et al. 1993).

**Tab. 1** Examples of plant pathogens for which *P. agglomerans* has been used as biocontrol agent

<b>Pathogen controlled</b>	<b>Plant species</b>	<b>Reference</b>
<i>Alternaria solani</i>	tomato	Sujkowski et al. 1994
<i>Fusarium culmorum</i>	wheat	Kempf and Wolf 1989
<i>Leptosphaeria maculans</i>	oilseed rape	Chakraborty et al. 1994
<i>Puccinia recondita</i> f. sp. <i>tritici</i>	wheat	Kempf and Wolf 1989
<i>Pythium</i> sp.	cotton	Nelson 1988
<i>Sclerotinia sclerotiorum</i>	dry bean	Yuen et al. 1994
<i>Erwinia amylovora</i>	pear, apple haw-thorn	Johnson et al. 1993; Beer et al. 1984; Wilson et al. 1990
<i>Pseudomonas syringae</i> pv <i>syringae</i>	barley	Braun-Kiewnick et al. 2000
<i>Xanthomonas albilineans</i>	sugar cane	Zhang and Birch 1996
<i>Xanthomonas campestris</i>	cotton, mung beans, onions	Randhawa et al. 1987; Bora et al. 1993; Paulraj and O'Garro 1993
<i>Xanthomonas oryzae</i>	rice	Hsieh and Buddenhagen 1974

*Pantoea* species as pathogens of humans and plants: *P. agglomerans* has been reported as a human pathogen and an allergen. It is considered to be a strong allergen (Dutkiewicz 1997), especially for farm workers, and strains of *P. agglomerans* were found in seed dust. The bacterium is the probable cause of airborne contact allergic dermatitis (Spiewak et al. 2001). *P. agglomerans* was also the probable cause of

septic arthritis in a knee of a healthy 14-year-old boy, who had been infected by contact with a palm tree thorn (Kratz et al. 2003). The infection caused swelling and pain. A foreign object (the thorn) was found inside the knee, and *P. agglomerans* was isolated from the fluid of the joint. *P. agglomerans* has sometimes been reported to be pathogenic to plants. Some strains of *P. agglomerans* induce galls on different, but specific, plant species. Depending on the plant species to which a strain of *P. agglomerans* is pathogenic, it is given a specific pathovar (pv) epithet (Table 2). For instance, the strain that forms galls on baby's breath is designated *P. agglomerans* pv. *gypsophilae*. In addition to *P. agglomerans* other species of *Pantoea* are pathogenic to plants, for instance, *Pantoea stewarti*, which causes Stewart's wilt on corn, *Pantoea citrea*, which causes pink core disease in pineapple (Cha et al. 1997) and *Pantoea ananatis*, which causes onion seed center rot (Walcott et al. 2002).

**Tab. 2** Pathovars of *P. agglomerans* that attack specific plant hosts

Host plant	Pathovar	Reference
Baby's breath	<i>gypsophilae</i>	Cooksey 1986
Beet	<i>betae</i>	Burr et al. 1991
<i>Wisteria sinensis</i>	<i>milletiae</i>	Opgenorth et al. 1994
Cranberry	None assigned	Best et al. 2004
Douglas fir	None assigned	De Young et al. 1998

Antibiotic biosynthetic operons of *P. agglomerans* are often in mobile genetic elements: Many strains of *P. agglomerans* produce antibiotics that belong to diverse chemical classes. The antibiotics produced by strains that are active against *E. amylovora* have been fairly thoroughly investigated. Several are peptide-based compounds. Their antibiotic activity to *E. amylovora* often is apparent only when *P. agglomerans* and the fireblight pathogen are grown on minimal media; antibiotic activity is inhibited by the presence of specific amino acids usually present in general media. Strain Eh318 inhibits the growth of *E. amylovora* on plates of minimal agar medium through the production of two peptide-based antibiotics; pantocin A (Jin et al. 2003a) and pantocin B (Brady et al. 1999). Strain Eh351 produces A351, another small peptide (Jin 2003). Pantocin B and A351 lose their activity in the presence of arginine, and pantocin A loses its activity in the presence of histidine (Wright et al. 2001). In contrast, andrimid, from strain Eh335, and AGA, from strain Eh1087, are produced and remain inhibitory on complete growth media (Jin 2003; Giddens et al. 2002). Andrimid, is a non-ribosomally synthesized peptide-polyketide hybrid antibiotic (Jin 2003) and AGA is a phenazine (Giddens et al. 2002). Herbicolin A, produced by strain CHS1065, is a glycopeptide antibiotic (Greiner and Winkelmann 1991); whether it requires minimal medium for its production and inhibitory activity is not known.

Molecular genetic evidence suggests that the biosynthetic genes for many *P. agglomerans* antibiotics reside on mobile elements (Giddens et al. 2002; Jin 2003; Jin et al. 2003b; Tenning et al. 1993; Wright et al. 2001; Wright et al. 2006). The genes for herbicolin A reside on a plasmid, and the genes for pantocin A, pantocin B, andrimid and AGA have different G+C ratios than the rest of their respective genomes. Remnants of transposable elements flank the biosynthetic genes for AGA, andrimid and pantocin B (Giddens et al. 2002; Jin 2003; Wright et al. 2006). The presence of DNA of different G+C content in biosynthetic gene clusters, relative to the rest of the bacterial genome, suggests the mobility of these genes across species boundaries. Lateral gene transfer is an important mechanism operative during the evolution of bacteria. Remnants of transposable elements, origins of transfer of phages and phage attachment sites often are seen next to the genes that have been transferred (Lan and Reeves 1996).

The relationship between pathogenic and non-pathogenic strains: *P. agglomerans* is a common epiphyte, but it also inhabits numerous other environments. It is not known what might make a non-pathogenic strain of *P. agglomerans* pathogenic, or how long it might require to become pathogenic. If we consider other species of the Enterobacteriaceae, there is some evidence for the evolution of pathogenic strains from non-pathogenic strains. For example, pathogenic *Shigella* sp. may have arisen from benign strains of *Escherichia coli* by acquiring virulence over time. Pathogenicity entails the acquisition of two chromosomally encoded regions and a virulence plasmid, and, more remarkably, the loss of two genes, *ompT* and *cadA*. [The *OmpT* protein interferes with the expression of the *VirG* protein, which is

involved in intercellular spread. The presence of *cadA* inhibits fluid secretion, one of the symptoms of the disease (Ochman et al. 2000)]. Pathogenic strains of *P. agglomerans* pv. *gypsophilae* (Cooksey 1986) and *P. agglomerans* pv. *betae* (Burr et al. 1991) cause galls on baby's breath and beet, respectively. Determinants of pathogenicity for these strains are carried on a plasmid, of approximately 150 kb, in a region called a pathogenicity island that includes numerous insertion sequences. The pathogenicity island carries *hrp* genes that encode type III effectors and genes involved in the biosynthesis of IAA and cytokinin. In the absence of this plasmid, the strains are not pathogenic.

### Final remarks

In conclusion, *P. agglomerans* frequently has been isolated from healthy and diseased plants, animals and humans. It is rarely a plant pathogen and very rarely a human pathogen. For plant pathogenicity, *P. agglomerans* requires the presence of a 150-kb plasmid. The bacterium has been used in biological control of plant diseases, where it has been effective against several diseases of many crops. More research is needed regarding the relationship of pathogenic to non-pathogenic strains, means to determine if a strain is a potential pathogen of plants or humans, and means to determine biocontrol effectiveness. How readily a strain might gain or lose determinants for these abilities should also be investigated. In addition, easy and reliable tests to determine whether any particular strain of *P. agglomerans* has characteristics of "friend" or "foe" would be highly desirable. The presence of a type III secretion system, the ability to induce a hypersensitive response in model plants and to possibly cause a virulent or allergic reaction in a model animal might help to establish the nature of new strains.

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