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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. amyolovora*. 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 agebts 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 x 10^7 cfu/ml). 24 h later, the stigma was inoculated with 5 µl Ea1/79Sm culture (1 x 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 x 10^7 cfu/ml were applied to the trees. At day 1, Ea1/79 culture with a concentration 1 x 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, *Candia 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).

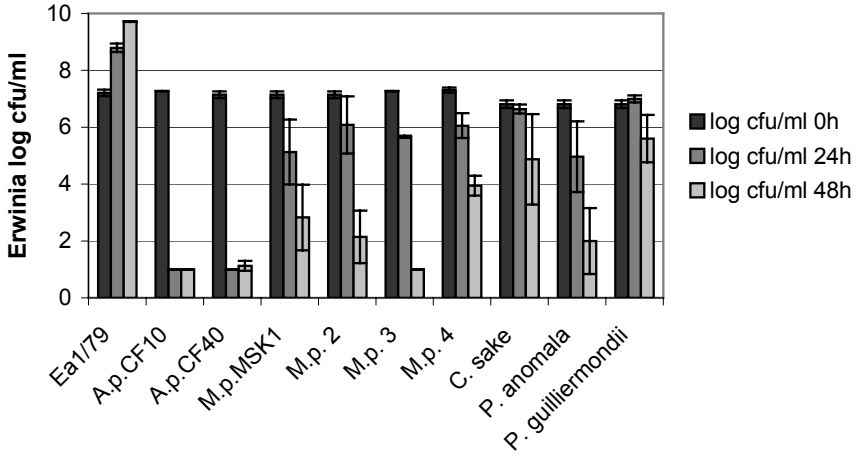

Fig. 1 Growth of *Erwinia amyolvora* Ea1/79 in liquid basal medium at 28 °C together with different species of yeasts and yeast-like fungi, respectively, preinoculated 24 h before Ea1/79. Cell titer of Ea1/79 was determined with dilution plating on Kings B medium containing cycloheximide (100 µg/ml). (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 10^4 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.

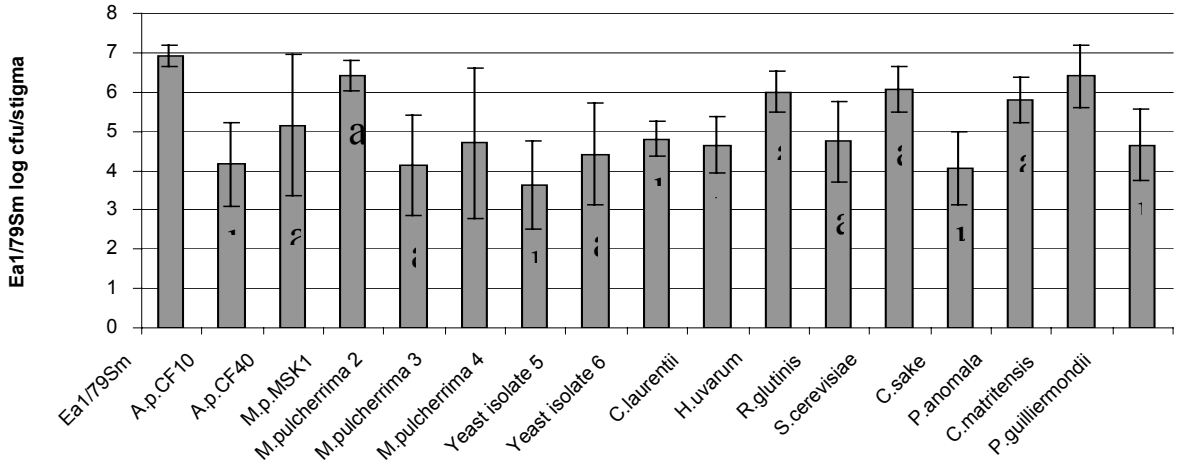

Fig. 2 Repression of the growth of *Erwinia amylovora* Ea1/79Sm by different yeasts and yeast-like fungi on the stigma of detached apple blossoms. One day old Gala 'Royal' apple blossoms were inoculated with overnight yeast culture 24 h before Ea1/79Sm inoculation. Colony forming units of Ea1/79Sm were 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 comparision 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 10^4 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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