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Originalarbeit

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Investigations for improving the detection of *Clavibacter michiganensis* ssp. *michiganensis* in tomato seedlings

Untersuchungen zur Verbesserung des Nachweises von *Clavibacter michiganensis* ssp. *michiganensis* an Tomaten-Jungpflanzen

Abstract

Bacterial canker caused by *Clavibacter michiganensis* ssp. *michiganensis* is one of the most serious tomato diseases, inducing substantial economic losses worldwide. It is considered a quarantine organism in the European Union and in several other countries. There is neither an efficient suppression, nor are there any resistant tomato varieties.

Bacterial canker of tomato is a parasitic vascular wilt disease, the bacterium is located in xylem vessels and spreads at temperatures ranging between 26° and 28°C.

Proving the evidence of *Clavibacter michiganensis* ssp. *michiganensis* is not always possible, especially on tomato seedlings. This slow-growing pathogen is difficult to be detected, with symptoms only appearing as plants approach maturity. As obviously a very low level of bacteria is always difficult to be detected with common ELISA tests, in these tests for detection improvement the intention was to raise bacteria concentration at least to the threshold of common ELISA tests.

At the beginning, tomato seedlings were inoculated with different bacteria dilutions. The plants were 22 to 30 cm high on average. They were inoculated by stabbing the stem at the height of approximately 10 cm. Following this procedure, leaves and stems were tested separately. Leave results were always negative even with control dilution which was highest in these tests (8×10^8 cfu/ml), but stem results were all positive. Therefore, for following tests only stems of tomato seedlings were used.

The experiments were based on 3 different methods, carried out alone or in combination, to enrich bacteria concentration in plant sap. Methods were as follows: incubating at 20° and 26°C, filtering and centrifuging. A direct test on plant sap from artificially inoculated plants served as control. Samples for detection with ELISA test were taken exactly on days 3, 5 and 7 after inoculation. When using the lowest bacteria dilution of $1{,}2 \times 10$ cfu/ml, only 3 days after infection the following variants showed positive results in ELISA testing: plant sap incubated at 20° (16 hours), filtrate of plant sap also incubated at 20° (16 hours), filtrate + centrifugate at 20° (16 hours) as well as pure centrifugate incubated at 20° (16 hours). Thus, bacteria reproduction was increased and made possibly existing bacteria in tomato seedlings more sensitive to ELISA testing.

In addition, *Clavibacter michiganensis* ssp. *michiganensis* was successfully detected directly from young plants of about 30 cm height by PCR. A favourable method to test tomato seedlings is represented by the ELISA testing method.

Key words: *Clavibacter michiganensis* ssp. *michiganensis*, tomato seedlings, detection, ELISA, PCR

Zusammenfassung

Es wurden drei Grundverfahren alleine und in verschiedener Kombination untereinander angewendet, um den

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Gehalt der Bakterien im Presssaft der Pflanzen anzureichern, z.B. Bebrüten bei Temperaturen 20° und 26°C, Filtrieren und Zentrifugieren. Als Kontrolle diente der Test direkt aus dem Pflanzensaft einer künstlich inokulierten Pflanze.

Proben für den Nachweis mittels ELISA wurden 3, 5 und 7 Tage nach künstlicher Inokulation genommen. Von allen Varianten waren bereits bei einer inokulierten Bakterienkonzentration von 1,2 × 10 cfu/ml bei einer Probennahme 3 Tage nach Inokulation und einer Bebrütung des Presssaftes bei 20°C (16 Stunden), ein Filtrat dieses Presssaftes ebenfalls bei Inkubation bei 20°C (16 Stunden) sowie Filtrat + Zentrifugat (das Pellet) bei 20°C (16 Stunden) als auch das Zentrifugat alleine bei 20°C (16 Stunden) im ELISA-Test positiv. Es konnte damit der Bakterientiter von eventuell in Jungpflanzen vorhandenen Bakterien auf die Nachweisgrenze des ELISA-Tests erhöht werden.

Darüber hinaus gelang es auch, einen Nachweis von *Clavibacter michiganensis* ssp. *michiganensis* aus Jungpflanzen des DC- Stadiums 23 mittels PCR direkt aus Pflanzen zu führen.

Stichwörter: *Clavibacter michiganensis* ssp. *michiganensis*, Tomaten-Jungpflanzen, Nachweis, ELISA, PCR

Introduction

Bacterial diseases of great importance such as the tomato bacterial spot (*Xanthomonas vesicatoria*), the bacterial speck *(Pseudomonas syringae* pv. *tomato*), the pith necrosis (*Pseudomonas corrugata*) and bacterial canker, as well as the disease caused by *Clavibacter michiganensis* ssp. *michiganensis* (Smith) Davis et al. could be found in warm and dry tomato growing areas and in greenhouses.

Bacterial canker is one of the most serious tomato diseases that causes great economic losses (de Leon et al., 2006).

A direct control of the disease has not been possible, in addition no resistant tomato varieties are available (Stüwe and von Tiedemann, 2010). Therefore, in the European Union and in many other countries, quarantine regulations are valid in order to prevent the entry or spread of the disease agent.

The detection procedure of *C. michiganensis* ssp. *michiganensis* is executed by EPPO Standards (2005), especially for seeds and adult plants. It is not always possible to detect the pathogen on young plants because the pathogen can be localized in different plant parts depending on growing conditions during seedling production and can occur in different concentrations (EPPO Standards, 2005). For commercial ELISA Kits the detection limit for *C. michiganensis* ssp. *michiganensis* is currently at about 8×10^4 cfu/ml (De Leon et al., 2006).

The aim of this study was to improve existing procedures, thus creating a simple, fast and inexpensive method for the detection of *C. michiganensis* ssp. *michiganensis* on tomato seedlings.

Materials and Methods

Seedlings

Uncoated tomato seeds were used for infection experiments (cv. 'Mercedes'). The seedlings were transplanted into peat substrate seven days after sowing. During cultivation the day temperatures were about 25°C, night temperatures about 20°C. Irrigation was carried out manually. After 4 weeks, the seedlings were transferred to a vector-proof cabin and infested with *C. michiganensis* ssp. *michiganensis* in different concentrations. After inoculation the plants were grown for 7 days.

Bacterial Culture

For the infection of seedlings a freeze-dried culture of strain No.DSM 46346 from the German Collection of Microorganisms and Cell Cultures (DSZM) was used.

Seedlings Inoculation

The inoculation of the seedlings was carried out in the stage BBCH 13(12); seedlings were about 22 to 30 cm high. The seedlings were inoculated by injection into the stem, 1 to 2 cm above the cotyledons. The stem was pierced with the needle and by removing the needle a drop was left inside the tissue. Afterwards seedlings were grown under normal conditions in the greenhouse. The infected test plants were used 3, 5 and 7 days past infection (dpi), selecting five plants of each variant for ELISA and PCR tests.

ELISA Tests

For the detection of *C. michiganenis* ssp. *michiganensis* a commercial ELISA Kit of AGDIA was used. Tests were carried out according to the instruction sheet of the Kit. A homogenizer (Homex 6, Bioreba) was used to make pressed plant sap from five stems at a time. The following scheme was used (Tab. 1) for sap samples processing.

The tomato seedlings were artificially inoculated with nine differently concentrated bacteria solutions. Afterwards ELISA and PCR tests were performed. In a first part of this study the following concentrations were used: 8×10^8 cfu/ml, 8×10^4 cfu/ml and 8×10^2 cfu/ml. For each testing sample leaves and stems were pressed separately.

About 1 ml of plant sap was immediately frozen without further processing. 1 ml was incubated overnight at 20°C and 26°C for enrichment of bacteria. The incubated variants were frozen after about 16 hours of incubation. Approximately 1 ml of plant sap was filtered through sterile tips and filters (pore size 2.7 µm). The filtrates were, just as the plant sap, frozen immediately and also incubated at 20°C and 26°C, respectively.

Furthermore, one part of plant sap was centrifuged with a laboratory centrifuge (Heraeus Sapatech) for 10 minutes at 9°C and 14,000 RCF. The centrifugates (pellets) were resuspended into buffer or nutrient solution (YPG) and processed according to the scheme above. Also, the centrifugates were frozen or mixed with nutrient solution (NS) and incubated overnight at 20°C and

Tab. 1. Sample scheme

No.	for each concentration
1	pressed sap
2	pressed sap 20°C
3	pressed sap 26°C
4	filtrate
5	filtrate 20°C
6	filtrate 26°C
7	filtrate + centrifuge
8	filtrate + centrifuge + NS 20°C
9	filtrate + centrifuge + NS 26°C
10	filtrate + centrifuge supernatant
11	filtrate + centrifuge supernatant 20°C
12	filtrate + centrifuge supernatant 26°C
13	centrifugate + buffer
14	centrifugate + NS 20°C
15	centrifugate + NS 26°C
16	centrifugate supernatant
17	centrifugate supernatant + NS 20°C
18	centrifugate supernatant + NS 26°C

26°C. The last version was a combination of filtration and centrifugation with the samples processed as above.

PCR

For DNA extraction and PCR REDExtract-N-Amp Plant PCR Kit™ was used (Sigma-Aldrich, Steinheim, Germany).

The plant stems were cut about 1 cm above the inoculation site and a 3 mm-long stem section was taken into a sterile Eppendorf tube. The DNA extraction was done according to the description of the REDExtract-N-Amp Plant PCR Kits™.The PCR tests were performed with some modifications according to the method of STÖGER and RUPPITSCH (2004) and the EPPO Diagnostic Standard PM 7/42 (EPPO Standards, 2005).

Specific primers were used Cmm5 (1) and Cmm6 (1) with primer pairs Cmm5 (5′ – GCGAATAAGCCCATATCAA – 3′) and Cmm6 (5′ – CGTCAGGAGGTCGCTAATA – 3′) by DREIER et al. (1995).

Results

Growth Curve

To determine the growth curve, the three different nutrient solutions (NB, YPG and Kings B) were compared. In 2 ml of nutrient solution the bacteria cultures were shaken overnight at 26°C.

The measurement of optical density (OD) by photometer at 600 nm was done the next day. For that purpose the overnight cultures (ONC) were diluted 1:10 with the respective nutrient solution and measured against pure nutrient solution as a blank sample. 30 ml of each nutrient solution were filled into 100 ml Erlenmeyer flasks and autoclaved. After cooling down the nutrient solutions were inoculated with *C. michiganensis* ssp. *michiganensis* from ONC- cultures, so that the optical density was at 0.05 at the beginning.The flasks were shaken at 26°C. Every hour 100 µl of the bacterial suspensions were taken out and their optical density was measured at 600 nm to determine the growth curve. After carrying out 12 measurements during one day, the flasks were placed in the fridge overnight and further incubated the next day. They were measured until bacteria reached the death phase. From these values, generation time and division rate were calculated (Fig. 1).

ELISA Tests

After all samples from leaves sap came out with negative results, as a consequence only stem saps were tested furtheron. During the first part of testing the following three concentrations were used: 8×10^8 cfu/ml, 8×10^4 cfu/ml and 8×10^2 cfu/ml. As soon as pure plant sap started to

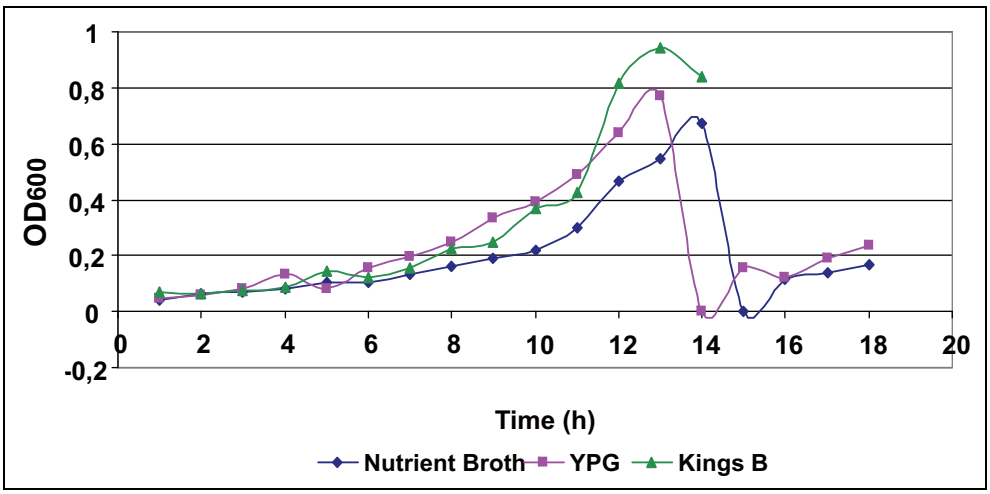

Fig. 1. Growth curve of *Clavibacter michiganensis* ssp. *michiganensis*.

Tab. 2. Results from ELISA-tests at the lowest concentration (1.2×10 cfu/ml)

No.		3 dpi	5 dpi	7 dpi
1	pressed sap	–	–	+
2	pressed sap 26°C	–	+	+
3	pressed sap 20°C	+	+	+
4	filtrate	–	+	+
5	filtrate 20°C	+	+	+
6	filtrate 26°C	+	+	+
7	filtrate + centrifugate	–	–	+
8	filtrate + centrifugate + NS 20°C	+	+	+
9	filtrate + centrifugate + NS 26°C	+	+	+
10	filtrate + centrifuge supernatant	–	–	–
11	filtrate + centrifuge supernatant 20°C	–	–	–
12	filtrate + centrifuge supernatant 26°C	+	+	+
13	centrifugate + buffer	–	–	+
14	centrifugate + NS 20°C	+	+	+
15	centrifugate + NS 26°C	+	+	+
16	centrifugate	–	+	+
17	centrifugate supernatant + NS 20°C	–	–	–
18	centrifugate supernatant + NS 26°C	–	+	+

show positive reaction in ELISA, lower concentrations of bacteria solutions were used in subsequent testings:

8×10^2 cfu/ml, 4×10^2 cfu/ml, 2×10^2 cfu/ml, 1×10^2 cfu/ml, 5×10 cfu/ml, 2.5×10 cfu/ml, 1.2×10 cfu/ml.

All experiments were performed twice. The sampling for ELISA tests was performed 3, 5 and 7 days past infection (Tab. 2).

PCR Tests

The PCR tests were performed as a control for the ELISA test. The following concentrations were tested as well:

8×10^8 cfu/ml, 8×10^4 cfu/ml, 8×10^2 cfu/ml, 4×10^2 cfu/ml, 2×10^2 cfu/ml, 1×10^2 cfu/ml, 5×10 cfu/ml, 2.5×10 cfu/ml and 1.2×10 cfu/ml.

All concentrations, including the lowest, were positive in PCR testing after 3 dpi.

Conclusion

The detection threshold of standard ELISA testing is currently at 8×10^4 cfu/ml (De Leon et al., 2006). By a conventional PCR the detection threshold can be reduced to 5×10^2 cfu/ml (Dreier et al., 1995), and by Bio-PCR to 4×10^2 cfu/ml (Burokiene, 2006). His studies have improved the sensitivity of the Bio-PCR using the primer sets CMM5 and CMM6. The tests were performed on five-week-old seedlings, about 40 cm high. The stem parts were taken 8 cm above the inoculation site.

A young plant in size of 40 cm does not correspond to the time of delivery from seedling companies to the protected cultivation in central-European practice.

Moreover, it seemed to be important to inoculate tomato seedlings with lower concentrations of bacteria, subsequently to enrich the bacteria in the pressed plant sap and detect them by ELISA and PCR.

The common smears of sap on nutrient media, followed by ELISA testing of suspicious bacteria colonies are not appropriate due to their long study duration.

Due to the enrichment of bacteria by methods such as incubation, filtering and centrifuging – alone or in combination –, the titer of bacteria could be increased and the detection limit of standard Elisa tests in stems of young plants can be raised. Detection by PCR directly from fresh plant material also succeeded.

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