# Elimination of *Clavibacter michiganensis* subsp. *michiganensis* from tomato cultures and seeds by highly sensitive detection methods and effective seed treatments

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**D7**

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# Contents

# General Introduction

## Disease history

Bacterial canker of tomato, caused by the bacterium *Clavibacter michiganensis* subsp. *michiganensis* (Smith. 1910) Davis *et al.* 1984 is a serious and destructive disease worldwide. The disease was at first described by Smith who found it in 1909 in Grand Rapids, Michigan (Strider, 1969), after which the pathogen spread into nearly all main tomato production areas world-wide. Recently, the incidence of bacterial canker of tomato increased in Europe and was newly reported in several countries worldwide causing considerable losses. Therfore, a new distribution map of the pathogen (Figure 1) was issued (CABI/EPPO, 2009). The bacterium is considered as a quarantine organism in the European Union and many other countries (Council Directive 2000/29/EC; OEPP/EPPO, 1982).

In **Germany**, the pathogen is known since 1929 (Kotte, 1930; Stapp, 1958), and caused serious losses in 1978, especially in greenhouses (Griesbach, person. commun.). Recently, the disease was transmitted in 1998 into the peninsula Reichenau in South Germany in Baden-Württemberg (Schmidt, 2006, person. commun.) and newly in 2002 into "Knoblauchsland" near Nürnberg in Bavaria (Maeritz, 2006, personal commun.), also in 2006 into North-Rhine-Westphalia (Matthäus-Staack and Eickeln, 2006, personal commun.) and very recently again into new locations of Baden-Württemberg in 2009 (Moltmann, 2009, personal commun.). Recently, the disease also occurred in neighbouring countries of Germany, such as Austria (Weber and Fuchs, 2007, personal observation and commun.), Switzerland (Wasserfallen, 2008, personal commun.), the Netherlands, and was newly reported by EPPO (CABI/EPPO, 2009) in several European and non-European countries.

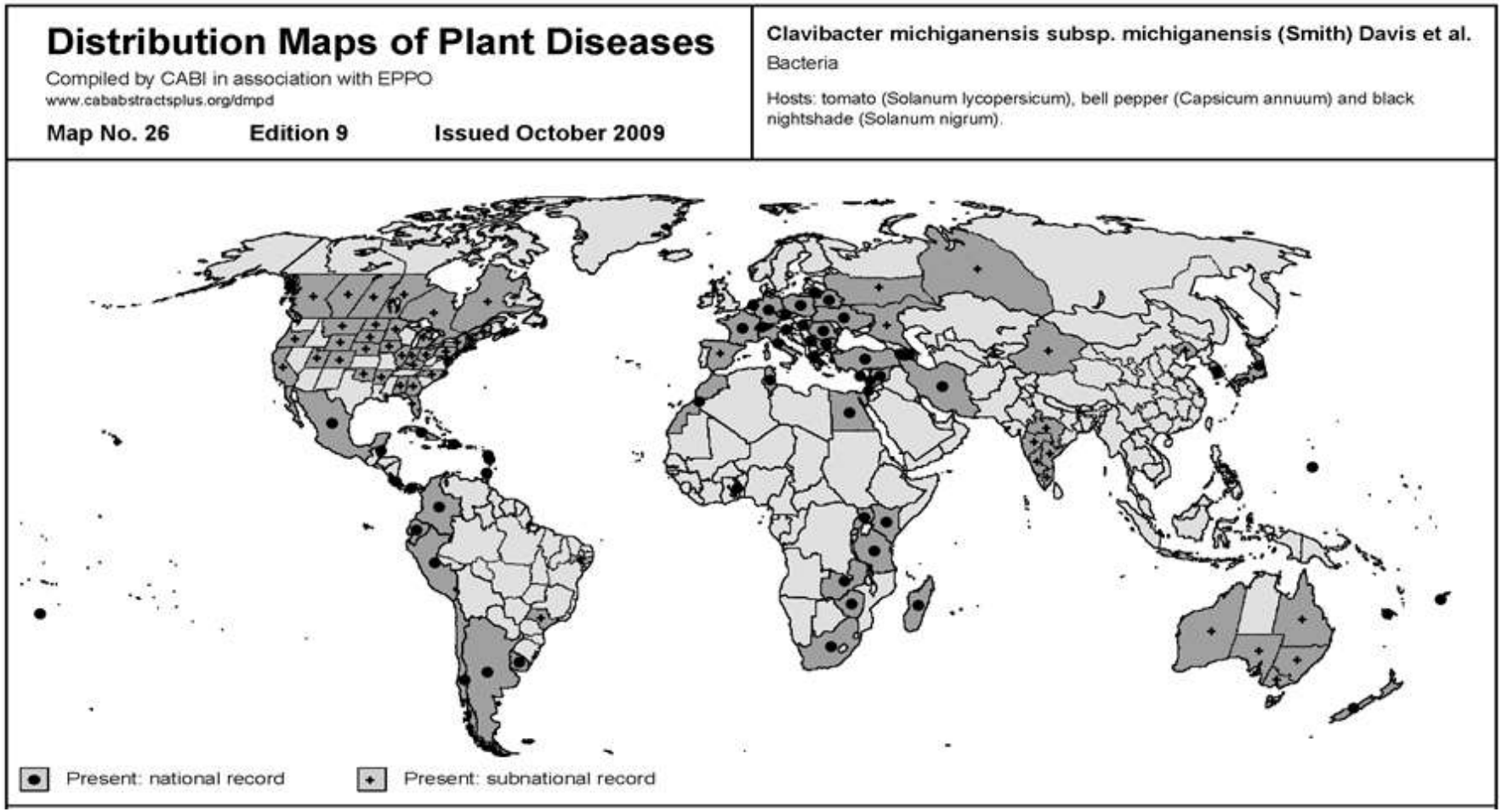


**Figure 1.** New distribution map of *Clavibacter michiganensis* subsp. *michiganensis*, issued by CABI/ EPPO in 2009 (Map no. 26).

## Tomato production in Germany

The total acrage of commercially grown greenhouse tomatoes in Germany ranges between 300 and 400 ha, whereas open field cultivations are little and not important. In 2008, the area of greenhouse-grown tomato was about 308 ha with a total greenhouse number of 2.808 and a production of 65.096 ton (Behr, 2009, personal commun. ZMP, 2009).

The largest greenhouse production areas of tomato are located in Baden-Württemberg (79.81 ha), Bavaria (44.7 ha) and North-Rhine-Westphalia (42.8 ha), and additional tomato cultivation areas exist in all other states of Germany (ZMP, 2009). In Baden-Württemberg, tomatoes are mostly cultivated in classical normal greenhouses in soil, whereas in North-Rhine-Westphalia and Bavaria tomatoes are often grown in hydro cultures in so-called "high-tech" greenhouses using sterile artificial substrates instead of soil, hybrid tomato plants that are grafted onto basic cultivars with resistance against soil-borne fungal and nematode diseases. These tomato cultures require large investments, because of the intensive cultivation, e.g. computerized and mechanized watering, air conditioning and fertilizing (Figure 2). The vegetation period in such high-tech greenhouses lasts 12 months, since two-month-old transplants are planted in the beginning of January and cultivated in the greenhouse until the end of November. The tomato plants reach a final length of 10 to 12 m at season's end, and during December old plants and the substrate are removed, watering systems and greenhouse structures are sterilized and greenhouses are prepared again for the new vegetation period. Some high-tech greenhouses are cultivated with 10,000 to 25,000 plants or more.

Generally, **disease incidence** in greenhouses with hydroponic cultures is higher than in normal greenhouses with soil cultivation, because of the additional infection source by watering and because plants in these greenhouses are more susceptible to diseases (Figure 9B). In Germany, a primary infection with *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) was recorded during 2006 in some greenhouses with 25,000 plants (in Knoblauchsland, Bavaria) or with 13,000 plants (in Straelen, North- Rhine-Westphalia) on only 5 young plants. However, when the hygienic measures were not followed in Straelen (due to first occurrence of the disease), 80% of all plants (13,000) wilted completely after few months and the residual plants showed very strong wilting symptoms (Figure 9B). But when very strict hygienic measures were applied, disease incidence could be kept under 2% in the greenhouse with 25,000 plants (in Knoblauchsland).

**Figure 2.** Intensive hydroponic tomato production in a "high-tech" greenhouse, plants can reach a length of 10-12 m at season's end.

## Symptoms

Disease symptoms are variable and seldom appear concomitantly on one plant or in one field or greenhouse. Typical symptoms include unilateral wilt of leaflets (Figure 3), canker of the stem (Figure 4), necrosis of leaf margins (Figure 5), and wilting of young plants (Figure 6). On fruits "bird's eye spots" may appear (Figure 7 A and B). By cutting the side shoots or the stem, brown discoloration of the xylem which forms "horseshoe" symptoms may be seen (Figure 8). Finally the whole plants wilt, in the field (Figure 9A) as well as in the greenhouse (Figure 9B).

**Figure 3.** Unilateral wilting of leaflets.

**Figure 4.** Canker of a stem.

**Figure 5.** Necrosis on leaf margins.

**Figure 6.** Wilting of a young plant.


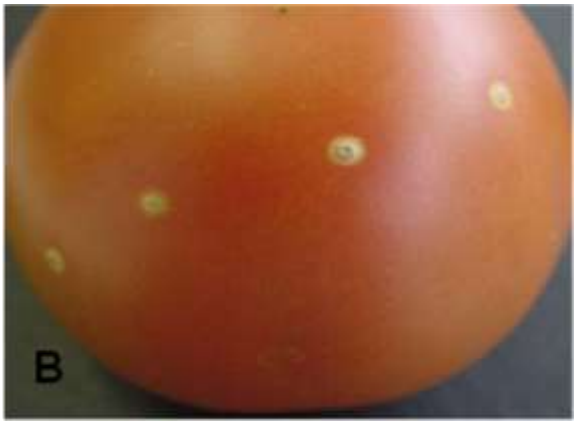


**Figure 7.** Bird's eye lesions on unripe fruit (A) and ripe fruit (B).

**Figure 8.** Horseshoe symptom on a side-shoot section.

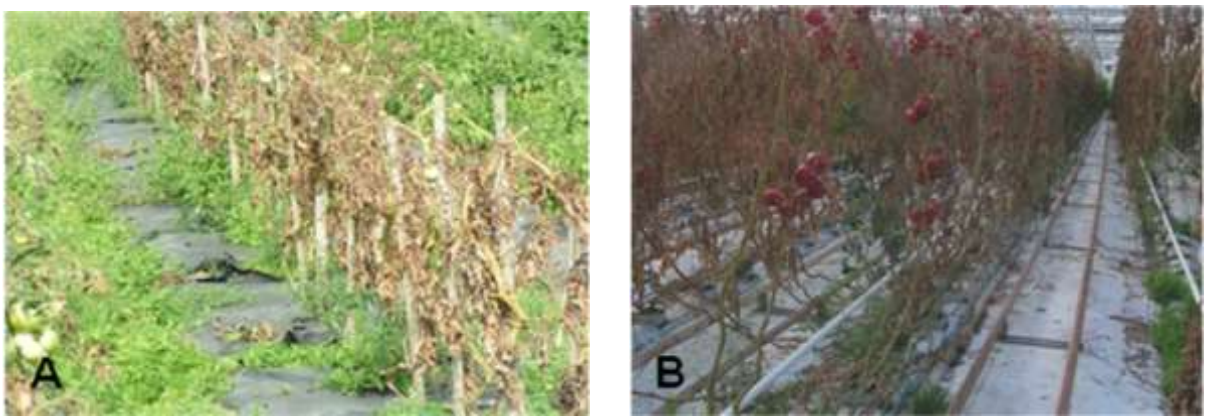


**Figure 9.** Wilting of field tomato plants (A) and all greenhouse tomato plants (B) due to *Clavibacter michiganensis* subsp. *michiganensis*.

## Disease epidemiology

The pathogen can survive in the soil in plant debris (Fatmi and Schaad, 2002; Ftayeh, 2004; Ftayeh *et al.*, 2004; Strider, 1969) and on greenhouse structures (Strider, 1969), but the main transmission of the disease takes place by contaminated or infected tomato seed or plants. Disease resistance is known (Coaker *et al.*, 2004; Poysa, 1993; van Steekelenburg, 1985), but has not been incorporated into commercial tomato cultivars to our knowledge.

The disease can be very destructive, and disease control is not possible once disease appears, because bactericides for control are not available. The incubation period can last up to 5 or 6 months (Ftayeh *et al*., 2008a). Therefore, infected and neighbouring plants must be destroyed immediately when disease symptoms appear, and very strict hygienic measures should be applied after disease appearance (Strider, 1969).

Thus, the use of pathogen-free seed, whether obtained naturally or by treating seeds with chemical eradicants, could eliminate a potential source of inocula (Fatmi *et al.*, 1991) and is considered to be the best strategy for controlling canker disease. Although seed transmission is less than 1% (Grogan and Kendrick, 1953), already 0.01 to 0.05% of infested seeds can cause an epidemic in suitable conditions (Chang *et al.*, 1991).

**Infested seeds** and young plants are responsible for primary infection and disease transmission into new locations, This may happen even when very strict quarantine measures are used to control import and export and all kinds of movements of tomato seed, and

although commercial tomato seed is normally sold together with seed-health certificates proving that the tomato seed had been certified as pathogen-free according to internationally standardized testing methods.

## Seed health certification

Certification of commercial tomato seeds as pathogen-free can be fulfilled according to the European Commission Directives 2000/29/EC when:
Tomato seeds are gained from healthy-appearing plants, which did not show any disease symptoms until the date of seed extraction, and if one of the following conditions is fulfilled:

1) Seeds have been extracted by diluted acids, or

2) Seeds have been tested according to internationally accepted laboratory methods.

However, these directives were insufficient to prevent further spread of the disease in Europe recently because:

- Healthy appearing plants may be latently infected and the incubation time may extend more than 5 months (Ftayeh *et al*., 2008a).
- An internationally accepted standardized seed extraction method by acids is not available although this method has been required by the European Community and also recommended by EPPO (Council Directive 2000/29/EC; Petter, 2009, personal commun.).
- Seed extractions by acids do not ensure an effective and absolute eradication of the pathogen which is required by the international quarantine regulations for *Cmm* that restrict import and export to zero tolerance for Canada, the USA, the EU (Council Directive 2000/29/EC; Bach *et al.*, 2003) and several other countries in order to prevent the outbreak of bacterial canker of tomato. Even one contaminated seed in 10,000 must be detectable. Thus, Anwar *et al*. (2004) and Gitaitis and Walcott (2007) proved the presence of *Cmm* in certified commercial tomato seed indicating the need for more sensitive detection methods.

The recent **outbreaks** of bacterial canker in the European Community resulted in increased attention of the national and international plant quarantine and plant protection authorities as well as the European Plant Protection Organization (EPPO) and the International Seed Health Initiative (ISHI), concerning the source of inocula in newly infected areas and the reliability of detection protocols described for *Cmm* by EPPO (OEPP/EPPO, 2005) and by ISHI (ISHI,

2008) that are normally used for detecting *Cmm* and for issues of "Seed-Health Certifications". Thus, a European collaborative study was organized and started at the end of 2008 with eight laboratories from six countries, including Lithuania, Czech Republic, Spain, The Netherlands, Slovenia, France to show the strengths and weaknesses of the currently used protocols and to open perspectives for development of alternative methods (Olivier *et al.*, 2009). This collaborative project was confirming the aims and objectives of our research project which started in June 2006 and which was based on our previous observations concerning the potential source of infections with *Cmm* as well as the lack in sensitivity and in reliability of the detection protocols that were suggested several years ago by EPPO and by ISHI and were recently updated in 2005 and 2008 by both, EPPO and ISHI, respectively.

## International requirements

According to our knowledge, there are many aspects that need to be further investigated in order to improve the possibilities of eliminating the pathogen, to reduce disease incidence, and to develop new strategies for disease detection and disease control, such as:

1) A standardized acid extraction method is not yet available in order to be internationally applied uniformly by the seed industry. Thus, seed companies are applying non-uniform and different processing methods for seed extraction which may be not effective enough for eradicating the pathogen. The previously described methods for seed treatments were either not effective enough against *Cmm* or effective but severely reducing seed germination. Therefore, new treatments have to be established.

2) The previously recommended detection protocols for *Cmm* by EPPO (OEPP/EPPO, 2005) and ISHI (ISHI, 2008) are not suitable for a sensitive detection of *Cmm* from latently infected seed and plant samples. Therefore, advanced and more sensitive detection methods must be developed.

3) The virulence of *Cmm* is not well understood yet.

4) There is a lack in research concerning the mechanisms of resistance against *Cmm* in tomato cultivars as well as the incorporation of resistance into commercial breeding lines.

5) Seed transmission is not well understood, some reports described surface seed infestation with *Cmm*, and others reported internal seed infections with *Cmm.* This

was the same in old and new reports issued by EPPO. Therefore the exact location of *Cmm* on or under the seed coat should be carefully investigated.

## Objectives

The objectives of our study were to develop more effective methods in order to eliminate the pathogen from tomato cultures. These new methods include:

1) Development of a new selective and highly sensitive nutrient medium for *Cmm.* The current available semiselective media for detecting *Cmm* are the main weaknesses of the applied detection protocols that are based on plating assays, because these media often revealed false negative results.

2) Testing the previously used primers on their specificity for *Cmm* and searching for more specific ones which could be used in combination with a potentially developed new selective medium (Bio PCR).

3) Selection of the best suited disinfection methods for eradicating *Cmm* from infected seeds.

## Outcomes

The results of this work can be specified as the following points:

1) A highly sensitive selective medium for detection of *Clavibacter michiganensis* subsp. *michiganensis* has been developed (Chapter 1; Ftayeh *et al.*, 2008c).

2) A Bio-PCR assay for a highly sensitive detection of *Cmm* was established, based on utilizing newly adapted primers and a new PCR protocol in combination with the new selective medium BCT (Chapter 2; Ftayeh *et al.*, 2010b).

3) Numerous seed treatment methods for eradication of *Cmm* from systemically infected seeds were investigated, resulting in selection of and very effective methods which absolutely eradicated the pathogen from seeds without a significant reduction in seed germination were recorded (Chapter 3, Ftayeh *et al.*, 2008d).

4) The current situation of bacterial canker of tomato in the Syrian Mediterranean strip provinces and in different locations in Germany was investigated and documented. 50 new *Cmm* strains were isolated from different German and Syrian locations. Reports

about disease occurrence in Syria were published (Table 1 of Chapter 2; Chapter 4; Ftayeh, *et al.*, 2008b; Ftayeh *et al.*, 2010a).

5) Furthermore, many aspects dealt with other investigations which are not included in this thesis, such as isolation of 45 different antagonists with high efficiency against *Cmm in vitro* that could be a potential object for further studies. Other investigations were carried out on the epidemiology of the pathogen under field and greenhouse conditions, incubation time of *Cmm* in tomato plants and its relation to temperatures and inocula densities, survival of the pathogen in seeds and in binding strings, population dynamics and spread of the bacterium *in planta*, as well as the impact of soil microorganisms on infections *via* infected seeds (Ftayeh 2004).

The present work may open new ways in understanding, detection, elimination and management of bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis*.

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# Chapter 1

## Development of new selective and highly sensitive nutrient media for *Clavibacter michiganensis* subsp. *michiganensis* and other subspecies


## Summary

All published semiselective media for *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) proved to be not satisfactory for a sensitive detection of *Cmm* in infected tomato plants and seeds. Therefore new selective media for *Cmm* were developed in three steps: 1) Selection of a basic medium allowing good growth of *Cmm* but excluding or slowing down several other bacterial species; 2) screening a wide range of antibiotics and other inhibitors for selective inhibition of often accompanying bacterial or fungal species; 3) optimizing the composition of inhibitors and nutrient components.

Initial tests for selection of antibiotics which did not inhibit *Cmm* were conducted with 30 strains of accompanying pathogenic and non-pathogenic bacterial species isolated from tomato seeds and plants that were obtained from different locations. For these experiments, tomato plants were cultivated in the field and artificially inoculated with very low concentrations of a rifampicin and streptomycin resistant strain of *Cmm.* These tomato plants did not develop disease symptoms but were latently infected with the pathogen. On the other hand, homogenates from leaves, stems or tomato fruits were heavily contaminated with various microorganisms (bacteria and fungi). The exact concentration of *Cmm* cells contained in the homogenates was determined by dilution plating on NGY agar medium supplemented with rifampicin, streptomycin and a fungicide. Parallely, dilution plating assays from the same homogenates were conducted on many newly designed compositions for a potential semiselective medium. The best suited new media were then tested for isolation of *Cmm* from naturally infected plants obtained from different locations in Germany, Syria and Austria, in order to enlarge the diversity of naturally occurring microorganisms on or in tomato plants.

Compared with all previously recommended semiselective media for *Cmm,* the new media (BCT and BCT-2) proved to be well suited for sufficient and fast growth of a wide range of *Cmm* strains.On the other hand, the new media inhibited growth of naturally occurring microorganisms to an extent of 98 to 100%. By testing tomato seeds and plants which were

latently infected with *Cmm* and highly contaminated with different saprophytic bacteria, the *Cmm* population was always detected on the new media, whereas all published semiselective media revealed false negative results under these conditions.

Additional tests revealed that the new selective media were also well suited for isolation and identification of the *Clavibacter michiganensis* subspecies *nebraskensis*, *insidiosus* and *tessellarius*, but neither for *C. m.* ssp. *sepedonicus* nor for *Curtobacterium flaccumfaciens* pv. *flaccumfaciens.*

## Conclusions

The new media BCT and BCT-2 are superior to all published semiselective media for *Cmm* and are denoted as selective media because:

- the mean plating efficiency amounted up to 89%, all the 30 *Cmm* strains from a wide range of different origins grew on the new media (one exception for BCT-2),
- high selectivity, accompanying bacterial species occurring on tomato plants and seeds or obtained from culture collections were inhibited to an extent of 98 to 100%, and
- remarkable detection sensitivity. Thus, very low *Cmm* populations occurring in plant and seed material in the presence of high concentrations (thousand-fold more) of non-target accompanying bacteria were detected on the new media but never on the published semiselective media.

# Introduction

*Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) (Smith, 1910) Davis *et al.*, 1984 can cause a very destructive wilt disease of tomato plants, especially in greenhouses. Therefore, the pathogen has been classified as an $A_2$ quarantine organism by the European Plant Protection Organization (OEPP/EPPO, 2005; Council Directive 2000/29/EC). The disease may result in serious losses, and very strict hygienic measures must be applied once it appears (Strider, 1969). Infested seeds and transplants are responsible for disease transmission into new areas (Chang *et al.*, 1991; Strider, 1969; Werner *et al.*, 2002), whereas transmission by soil appears to be of minor importance (Ftayeh, 2004; Ftayeh *et al*., 2004; Strider, 1969). Thus, indexing of tomato seed for the canker pathogen is a key for disease control (Biggerstaff *et al.*, 2000).

As few as 0.01-0.05% contaminated seeds or transplants can cause an epidemic in suitable conditions (Chang *et al.*, 1991). New outbreaks of canker diseases of tomato (*Solanum lycopersicum* L) caused by *Cmm* were recently reported in several locations in Europe, including Austria, Belgium, Czech Republic, France, Germany, Netherlands, Serbia, Slovakia, Slovenia and Spain (CABI/ EPPO, 2009), as well as in Syria (Chapetr 4; Ftayeh *et al*., 2008b), and several countries worldwide. The disease occurred in some locations for the first time, although infected plants were originally obtained from tomato seeds and transplants that were certified as pathogen free. Since health certification documents had been issued according to international standard detection and testing methods, many questions arose on the reliability of the presently used diagnostic and detection protocols for *Cmm.* Due to obvious insufficiencies of these protocols, the here presented research project was started at the University of Göttingen in 2006. At the end of 2008, an external evaluation by a European collaborative study was organised between research institutions as well as seed companies in several European countries to determine the weaknesses of diagnostic methods and "to open perspectives for the development of alternative methods" (Olivier *et al.*, 2009).

Protocols for detection of *Cmm* in tomato seeds and symptomless plant tissues, recommended by EPPO, the European Plant Protection Organization (OEPP/EPPO, 2005) and by ISHI, the International Seed Health Initiative (ISHI, 2008) are based on isolation by dilution plating of seed extracts and tissue homogenates on semiselective media, confirmed by identification tests of pure bacterial cultures by a pathogenicity test. According to the EPPO protocol, the identity of the pathogen must be also confirmed by at least one other test, such as biochemical characteristics, SA-agglutination test, IF test, ELISA, PCR, genomic fingerprinting or SDS-

PAGE.

Semiselective media are valuable and essential tools in phytobacteriology for disease diagnosis, indexing and epidemiological studies (Roy and Sasser, 1990). Direct isolations and plating assays onto semiselective media remain the most widely used detection methods and have several advantages for detecting bacterial diseases. Plating onto semiselective media is easier to do, less expensive and results in recovery of viable bacterial cultures that can be used to determine pathogenicity (Schaad, 1982; Schaad *et al.*, 1997).

Semiselective media are based on knowledge of the nutritional requirements and physiological tolerances of the target bacterium. This includes choosing suitable carbon and nitrogen sources that allow growth of the target organism but that are not readily used by other bacteria, minimizing the growth of non-target organisms. After optimizing carbon and nitrogen concentrations, inhibitors such as antibiotics and dyes can be incorporated to enhance selectivity (Gitaitis and Walcott, 2007). Other methods which could increase selectivity of semiselective media include pH levels (Burbage *et al.*, 1982), osmotic concentrations imposed by extremely high concentration of sucrose (Crosse and Goodman, 1973) and incubation temperatures (Gitaitis *et al.*, 1997) that allow growth of the target bacterium but inhibit growth of the background microflora.

Development of semiselective media for coryneforms is difficult because of their fastidious nature and inherent susceptibility to antibiotics and inhibitors (De la Cruz *et al.*, 1992). Semiselective media developed for *Cmm* differ in basal components and in inhibitors added. Inhibitors contailned in previously used semiselective media for *Cmm* include cycloheximide, polymyxin B sulfate, nalidixic acid, nicotinic acid, nystatin, lithium chloride, boric acid, potassium tellurite and sodium azide. Inhibitors may differ in mode of action and in their interactions with components of the basic media, thus effecting selectivity, plating efficiency and growth speed of the target bacterium and as a result sensitivity and reliability for detection of *Cmm*. However, the protocols recently recommended by EPPO and ISHI (OEPP/EPPO, 2005; ISHI, 2008) for detection of *Cmm* in tomato seeds and plants are not sensitive enough, because the suggested semiselective media proved to be not satisfactory.

Therefore, the aim was to develop a new selective and highly sensitive medium that can be used for routine seed testing and for a reliable isolation and detection of *Clavibacter michiganensis* subsp. *michiganensis* in infested seeds and latently infected plants.

# Materials and Methods

## Bacterial species and strains

For evaluating the plating efficiency, detection sensitivity and selectivity of semi selective media, 72 bacterial strains were tested. These included 30 *Cmm* strains that originated from different countries and were in part self-isolated from different locations in Germany and Syria or obtained from other bacterial collections (Table 1).

In addition, 42 other pathogenic and non-pathogenic bacterial species or strains were tested. Pathogenic bacterial species related to *Cmm* included *C. m.* subsp. *insidiosus, nebraskensis, sepedonicus,* and *tessellarius,* as well as *Curtobacterium flaccumfaciens* pv. *flaccumfaciens.* As reference, 3 well identified strains of saprophytic or epiphytic bacterial species [*Bacillus subtilis, Pantoea agglomerans* (*Erwinia herbicola*) and *Pseudomonas fluorescens*] and five phytopathogenic bacterial species which may occur on tomato plants [*Pectobacterium carotovorum* subsp. *carotovorum* (*Erwinia carotovora* subsp. *carotovora*), *Pseudomonas syringae* pv. *syringae, Pseudomonas syringae* pv. *tomato, Xanthomonas arboricola* pv. *julandis* and *Xanthomonas campestris* pv. *vesicatoria*] were included. In addition, several accompanying saprophytic bacterial isolates (S-1 to S-23, listed in Tables 1 and 3) were obtained from tomato seeds and plants of different origin. Most of these saprophytes were antagonists of *Cmm* and were taxonomically identified by gas chromatographic analysis of their whole cell fatty acid methyl esters (FAME)* as shown in Table 3. The FAME-profile was achieved by the Hewlett-Packard HP5898A Microbial Identification System (MIS) using versions 3.80 and 4.01 of the Aerobic Library (TSBA 40) (MIDI Inc., Newark, DE, USA) and according to the procedure as specified by the manufacturer (Hewlett-Packard, Avondale, PA, USA).

Organisms not identifiable by FAME analysis were coarsely characterized on the basis of biochemical or colony morphology features, Gram reaction, and reaction to antibiotics. The species of these saprophytic bacteria included: *Bacillus cereus, B. coagulans, B. licheniformis, B. pumilus, Microbacterium lacticum, Microbacterium* sp., *Pantoea agglomerans, Pantoea* sp., *Pseudomonas putida, Pseudomonas syringae* and *Rahnella aquatilis*. Even different isolates of the same species differed in susceptibility to antibiotics.

*) FAME, Fatty Acid Methyl Esters analysis were kindly carried out by Dr. Dieter Felgentreu, Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection, Julius-Kühn-Institute, Berlin, Germany.

**Antibiotic-resistant mutant of *Cmm***

In order to determine the detection sensitivity of different variants of selective media, it was necessary to know the exact number of the target *Cmm* cells existing in plant homogenates. Therefore, a spontaneous antibiotic-resistant *Cmm* mutant was selected. This was achieved by culturing a selected *Cmm* strain in several passages in NGY liquid medium (see NGY medium below) containing increasing concentrations of antibiotics. The *Cmm* strain BO-RS (Table 1) with resistance to 100 ppm rifampicin and 600 ppm streptomycin was obtained in this way.

**Media and growth conditions**

All *Cmm* strains and other bacteria used in this study were cultivated on NGY agar medium [0.8% nutrient broth (Roth, Karlsruhe, Germany), 1% glucose (AppliChem, Darmstadt, Germany), 0.3% yeast extract (Roth), pH was adjusted to 7.2; according to Mavridis, person. commun.].

Only the *Pseudomonas* spp. were cultivated on NGY or on King's medium B (King *et al.*, 1954).

For isolation of the mutant strain BO-RS from seeds and plant samples as well as for determining its population in infected samples we used the NGY agar medium, supplemented with 50 ppm rifampicin (25 mg/ml MeOH stock), 200 ppm streptomycin (100 mg/ml water, stock) and 50 µl/l Opus® Top (50 µl/ml water stock). Bacterial cultures were incubated at 26 °C.

Long-time conservation of bacteria was achieved in 20% glycerol at -80 °C.

**Table 1.** Origin of bacterial species and strains used to evaluate semiselective media

| Bacterial species | GSPB no.[a] | Designation or no. in other collections[b] | Origin[c] | Year of isolation | Isolated by[d] |
|---|---|---|---|---|---|
| *Clavibacter m.* subsp. *michiganensis* | 3199 | Amb-1 | Germany, R | 2006 | R. Ftayeh |
| *Clavibacter m.* subsp. *michiganensis* | 3200 | Ei-1 | Germany, NR | 2007 | R. Ftayeh |
| *Clavibacter m.* subsp. *michiganensis* | … | Ei-2 | Germany, NR | 2007 | R. Ftayeh |
| *Clavibacter m.* subsp. *michiganensis* | 3201 | Lu-1 | Germany, KL | 2006 | R. Ftayeh |
| *Clavibacter m.* subsp. *michiganensis* | 3202 | Mo-1 | Germany, R | 2006 | R. Ftayeh |
| *Clavibacter m.* subsp. *michiganensis* | … | Mo-2 | Germany, R | 2006 | R. Ftayeh |
| *Clavibacter m.* subsp. *michiganensis* | 3203 | Sc-2 | Germany, KL | 2006 | R. Ftayeh |
| *Clavibacter m.* subsp. *michiganensis* | 3204 | BO-RS | Germany, NR | 2006 | R. Ftayeh |
| *Clavibacter m.* subsp. *michiganensis* | 2972 | 78-s | Germany | 1979 | E. Griesbach |
| *Clavibacter m.* subsp. *michiganensis* | 3205 | AE-1 | Syria, L | 2007 | R. Ftayeh |
| *Clavibacter m.* subsp. *michiganensis* | 3206 | AH-1 | Syria, T | 2007 | R. Ftayeh |
| *Clavibacter m.* subsp. *michiganensis* | … | ES-1 | Syria, T | 2007 | R. Ftayeh |
| *Clavibacter m.* subsp. *michiganensis* | 3207 | HH-1 | Syria, L | 2007 | R. Ftayeh |
| *Clavibacter m.* subsp. *michiganensis* | … | La-1 | Syria, L | 2007 | R. Ftayeh |
| *Clavibacter m.* subsp. *michiganensis* | 3208 | OS-1 | Austria, STM | 2007 | E. Moltmann |
| *Clavibacter m.* subsp. *michiganensis* | … | OS-2 | Austria, STM | 2007 | E. Moltmann |
| *Clavibacter m.* subsp. *michiganensis* | … | OS-4 | Austria, STM | 2007 | E. Moltmann |
| *Clavibacter m.* subsp. *michiganensis* | 378 | 9/79 | Greece | 1979 | A. Mavridis |
| *Clavibacter m.* subsp. *michiganensis* | 382 | 24/78 | Greece | 1978 | A. Mavridis |
| *Clavibacter m.* subsp. *michiganensis* | 390 | 31/79 | Greece | 1979 | A. Mavridis |
| *Clavibacter m.* subsp. *michiganensis* | 392 | 45/78 | Greece | 1978 | A. Mavridis |
| *Clavibacter m.* subsp. *michiganensis* | … | Bulgarian 1 | Bulgaria | unknown | From Griesbach |
| *Clavibacter m.* subsp. *michiganensis* | 2973 | Cm8 | Bulgaria | unknown | From Griesbach |
| *Clavibacter m.* subsp. *michiganensis* | 2315 | KD/1-4 | Turkey | 1994 | Ö. Cinar |
| *Clavibacter m.* subsp. *michiganensis* | 2221 | NCPPB 1573 | Hungary | 1963 | Z. Klement |
| *Clavibacter m.* subsp. *michiganensis* | 2222 | NCPPB | Hungary | unknown | unknown |
| *Clavibacter m.* subsp. *michiganensis* | … | 399 | Unknown | unknown | From Griesbach |
| *Clavibacter m.* subsp. *michiganensis* | 3133 | NCPPB 3123 | USA | unknown | E. Echandi |
| *Clavibacter m.* subsp. *michiganensis* | … | 185 | USA | unknown | From Griesbach |
| *Clavibacter m.* subsp. *michiganensis* | … | Leningrad 3 | Russia | unknown | From Griesbach |
| *C. m.*subsp. *insidiosus* | 30 | NCPPB 1634 | UK | 1934 | From Lelliott |
| *C. m.* subsp. *nebraskensis* | 2223 | NCPPB 2581 | USA | 1971 | M. L. Schuster |
| *C. m.* subsp. *sepedonicus* | 1522 | NCPPB 2140, Cs 1 | USA | 1942 | L. T. Richardson |
| *C. m.* subsp. *sepedonicus* | 2823 | Solara 3 | Germany | 1998 | A. Mavridis |
| *C. m.* subsp. *tessellarius* | 2224 | ATCC 33566 | USA | 1982 | R.R. Carlson |
| *Curtobacterium f.* pv. *flaccumfaciens* | 2218 | NCPPB 559 | USA | 1958 | From Lelliott |
| *Bacillus subtilis* | 1769 | NCPPB 1246 | USA | 1956 | L.S. Bird |
| *Bacillus subtilis* | … | FZB 24 | Germany | unknown | unknown |
| *Pectobacterium c*. subsp. *carotovorum* | 436 | DSMZ 60442 | Germany | unknown | unknown |
| *Pantoea agglomerans* | 450 | NCPPB 651 | UK | 1985 | E. Billing |
| *Pseudomonas corrugata* | 2418 | PC 1 | Germany | 1995 | A. Mavridis |
| *P. fluorescens* | 1714 | G-1 | Germany | unknown | unknown |
| *P. syringae* pv.*syringae* | 1142 | R - 12 | Germany | 1967 | K. Rudolph |
| *P. syringae* pv. *tomato* | 1776 | 14-1 | Hungary | 1987 | S. Süle |
| *P. syringae* pv. *tomato* | 2317 | Nr.-1 | Turkey | 1994 | A. Mavridis |
| *P. syringae* pv. *tomato* | … | Syr-1 | Syria | 2007 | R. Ftayeh |
| *Ralstonia solanacearum* | 2607 | 180 a | Cameroon | 1996 | A. Mavridis |
| *Ralstonia solanacearum* | 2619 | Ps 24 | Brazil | 1995 | O. Martins |
| *Xanthomonas arboricola* pv. *juglandis* | 3148 | B- 102 | Germany | 2002 | W. Wohanka |
| *X. campestris* pv. *vesicatoria* | 2043 | S-08 | Hungary | 1964 | Z. Klement |
| 22 saprophytic bacteria[e] | … | S-1, S-2, ….S-23 | Germ. R, NR, KL | 2006- 2007 | R. Ftayeh |

[a] GSPB = Göttingen Collection (Sammlung) of Phytopathogenic Bacteria.

[b] NCPPB = National Collection of Plant Pathogenic Bacteria, UK; ATCC = American Type Culture Collection; DSMZ = German Collection of Microorganisms and Cell Cultures.

[c] R = Reichenau; NR = Niederrhein; KL = Knoblauchsland, Franken; L = Latakia; T = Tartous; STM = Steiermark.

[d] "From" indicates obtained from the person named.

[e] Saprophytes were isolated from tomato seed and tomato plants and differing in colour, morphology, Gram's reaction, or susceptibility to antibiotics, partially identified by fatty acid analysis as shown in Table 3

**Selection of the basic medium for *Cmm***

For selecting a basic medium with high potential plating efficiency of *Cmm*, compositions of nine semiselective media were prepared without addition of antibiotics, and the growth of *Cmm* was compared with growth on NGY medium. The original nine semiselective media were: D2 (Kado and Heskett, 1970); KBT (Dhanvantari, 1987); mCNS which was prepared as suggested by Gitaitis *et al*. 1991, based on CNS (Gross and Vidaver, 1979) and modified by omission of lithium chloride and Bravo 6F; D2ANX (Chun, 1982); SCM (Fatmi and Schaad, 1988); mSCM (Waters and Bolkan, 1992); CMM1 (Alvarez and Kaneshiro, 1999); the recently suggested medium for *Cmm* by the European Plant Protection Organization (OEPP/EPPO, 2005), named "EPPO" in our study; and MTNA (Jansing and Rudolph, 1998) which was developed for *Clavibacter michiganensis* subsp. *sepedonicus*. For evaluating the growth speed of *Cmm* on these media, bacterial suspensions were prepared in 0.01M $MgSO_4$, adjusted photometrically to ~$10^8$ cfu/ml (OD of 0.06 at 660 nm), and followed by serial dilution to 250-750 cfu/ml. Finally, 100 µl of each strain were surface streaked with an "L" shaped glass rod in triplicates per strain onto each of the above described basic media. Growth areas of *Cmm* strains were determined in $mm^2$ as average of three replicates on each medium at the 3rd and 5th day after plating.

**Growth area = cfu no. x $\pi$ $r^2$** (Figure 1).

**Screening of antibiotics**

Forty different antibiotics (Table 2) were initially screened for their inhibitory effect on two *Cmm* strains (GSPB 390 and 2973). The screening test was performed according to the technique of Bauer *et al*. (1966) by means of commercially available filter discs containing different concentrations of antibiotics (Oxoid Ltd, England). Bacterial suspensions of the *Cmm* strains tested were prepared from 24-hour-old NGY cultures in 0.01M $MgSO_4$. Bacterial concentrations were photometrically adjusted to approximately $10^8$ cfu/ml using a photometer (Spectronic 20, Bausch & Lomb), i. e. an optical density of 0.06 at 660 nm, and 150 µl of this bacterial suspension were streaked onto the surface of NGY medium with a Drigalski spatula. Within 10 to 20 min discs containing an antibiotic were placed on the agar with sterile forceps and gently pressed to ensure contact. The plates were kept for two hours at 4 °C to allow diffusion of antibiotics into the agar before incubating at 26 °C. After incubating at 26 °C for 24-48 h, inhibition's width around the discs was recorded in mm (Table 2).

**Susceptibility of accompanying bacteria towards antibiotics**

Antibiotics with no inhibitory effect on *Cmm* (Table 2) were further tested in several concentrations in NGY medium on their inhibitory effect against different accompanying bacteria. Susceptibility testing of accompanying bacteria was carried out to select antibiotics with potential selectivity. The *Cmm* strain GSPB 390 was also tested besides the accompanying bacteria, for determining the maximum concentration of each antibiotic which caused a strong inhibition of accompanying bacteria while maintaining good growth of *Cmm*. Highly concentrated suspensions of *Cmm* (GSPB 390) and accompanying bacterial species were prepared and streaked on NGY media with different concentrations of antibiotics, by dipping a sterile inoculating loop into each bacterial suspension and streaking on NGY media containing different concentrations of the following antibiotics: aztreaonam, metronidazole, mupirocin, nalidixic acid, polymyxin B sulfate, trimethoprim and fosfomycin. Agar plates were incubated at 25 °C for 24-48 h until evaluation (Table 3).

**Adjusting the optimum concentrations of inhibitors**

Antibiotics inhibiting a wide spectrum of accompanying bacteria, such as trimethoprim, polymyxin B sulfate and nalidixic acid, were furthermore tested in various combinations and concentrations with the new basic medium to adjust the optimum concentration of each antibiotic exerting high selectivity, while maintaining a good growth speed of two *Cmm* strains (GSPB 390 and 2073). For this purpose, field tomato seeds and plants that had been previously inoculated with the double mutant *Cmm* strain BO-RS (see above) and highly contaminated with saprophytes were homogenized in sterile water. Aliquots of the homogenates were streaked on the test plates. For comparison, the homogenates were also plated on NGY medium supplemented with rifampicin, streptomycin and Opus® Top to determine the actual number of *Cmm* cells occurring in the plant homogenates.

Furthermore, homogenates from healthy field plants (collected from different locations in Germany and Syria) were surface streaked in triplicates onto NGY agar and test compositions in order to estimate selectivity. Parallely, suspensions of two *Cmm* strains (GSPB 390 & GSPB 2973) differing in growth morphology and speed were also streaked, each in triplicates, onto agar plates with NGY or test compositions to estimate the growth area of *Cmm*. Only those compositions which allowed high selectivity concomitantly with large growth areas of *Cmm* were selected and modified repeatedly in further experiments.

Finally, the best compositions allowing high selectivity were tested with 30 *Cmm* strains (see

below).

**Determining the plating efficiency (recovery rate) of *Cmm* strains on semiselective media**

Cultures of 30 *Cmm* strains were grown for 24 h on NGY medium, and bacterial suspensions in 0.01M $MgSO_4$ containing 100-250 cfu were plated in triplicates on each medium for each strain. The recovery of *Cmm* was determined by counting the *Cmm* colonies of each variant. To avoid mistakes caused by the possible co-growth of several joining colonies, counting of colonies was started as soon as possible on each medium (for example on NGY after 48-72 h). Plating efficiency or recovery rate (Table 4) after 7, 10, 15 and 20 days was expressed in % recovered CFU of those detected on the NGY medium, i.e.:

**Plating efficiency of *Cmm* (%)** = (CFU on test medium/CFU on NGY medium) × 100.

**Evaluation of selectivity and detection sensitivity of semiselective media**

**Selectivity** means the suitability of selective media for supporting growth of target micro organisms or bacteria and preventing growth of nontarget microbes or bacteria.

**Detection sensitivity** means the lowest number of *Cmm* CFU occurring in plant homogenates which could be detected in the presence of high concentrations of nontarget bacteria (Table 7 & Figure 8).

Some media, such as mSCM, EPPO and mCNS, showed a rather higher toxicity than selectivity towards several *Cmm* strains, resulting in low detection sensitivity. Other media, such as D2, KBT, SCM, and CMM1, showed less selectivity and detection sensitivity as well, becuase *Cmm* growth was inhibited by saprophytic bacteria that rapidly occupied the agar background.

Thus, it was very important in our study to evaluate both the selectivity and detection sensitivity of the new media.

For initial evaluation of new medium compositions, field tomato plants were inoculated in 2007 and 2008 with very low concentrations of the double mutant *Cmm* strain BO-RS (30-50 cfu/_plant). After 30-70 days the field plants were only latently infected with *Cmm* and never showed disease symptoms. On the other hand, due to rainy weather conditions, the plants were highly contaminated by epiphytic or saprophytic microorganisms. Homogenates of plant stems were streaked on the test media, as well as on NGY agar supplemented with rifampicin, streptomycin and Opus® Top. In this way it was possible to evaluate detection sensitivity and selectivity of new medium-compositions.

Concomitantly, infected tomato plant samples which were collected in several locations in Syria, Germany and Austria or which had been sent to our laboratory in Göttingen between 2006 and 2008 were also evaluated by the medium-compositions being under development.

Finally, the selectivity of all tested semiselective media in comparison with the new media was evaluated using homogenates of healthy field tomato plants or seed lots which were highly contaminated with saprophytic bacteria and artificially infested ("spiked") with different strains of *Cmm*. The *Cmm* strains used were BO-RS, 382 and OS-2. Tomato stems or seeds were crushed in sterile mortars with sterile water, and serial dilutions were plated on NGY medium to estimate the density of saprophytic bacteria. Then a defined amount of each one of the above described *Cmm* strains was introduced separately into only one of the non-diluted or 1:10 diluted homogenates, and 100 µl aliquots were plated on each medium. Plates were incubated at 26 °C. As soon as bacteria began to grow, counting the colonies started for both, saprophytes and *Cmm.* Bacteria started to grow on each medium after different intervals (2 to 15 days).

To compare all media under the same conditions, the final colony number of saprophytes and *Cmm* was determined 10 dpi. *Cmm*-suspected colonies were purified and identified by re-streaking on new NGY agar plates or on rifampicin-, streptomycin-NGY agar, when the double mutant was applied.

The selectivity and detection sensitivity of each medium was evaluated as follows:
**Selectivity (%)** = [(Population of nontarget microbes on NGY - population of nontarget microbes on test medium) / population of nontarget microbes on NGY] × 100.

**Detection sensitivity (%)** = The CFU number of target bacteria (*Cmm*) detected from plant homogenate or seed extract × 100 / the total CFU number of target bacteria (*Cmm*) in the plant homogenate or seed extract.

# Results

### Selecting a new basic medium for *Cmm*

Three *Cmm* strains (GSPB 390, GSPB 2973 and Ei-2) with different growth morphology and growth speed were cultivated on the basic compositions of nine different semiselective media (without addition of antibiotics). After three and five days, all tested basic media showed significant differences in growth of *Cmm*. Compared with NGY agar, the growth of the three *Cmm* strains tested was very low or absolutely absent after three and five days on the basic media of D2, CMM1, SCM, mSCM and EPPO. In comparison to the reference NGY medium and to all the other tested basic media, the growth of the three *Cmm* strains was highest on the basic medium of MTNA after three and five days. On MTNA *Cmm* colonies appeared earlier and were larger in diameter (Figure 1). Therefore, the basic MTNA medium which had been developed for *Clavibacter michiganensis* subsp. *sepedonicus* (Jansing and Rudolph, 1998) was selected and adapted to *Cmm* by modifying the basic compounds and inhibitors.

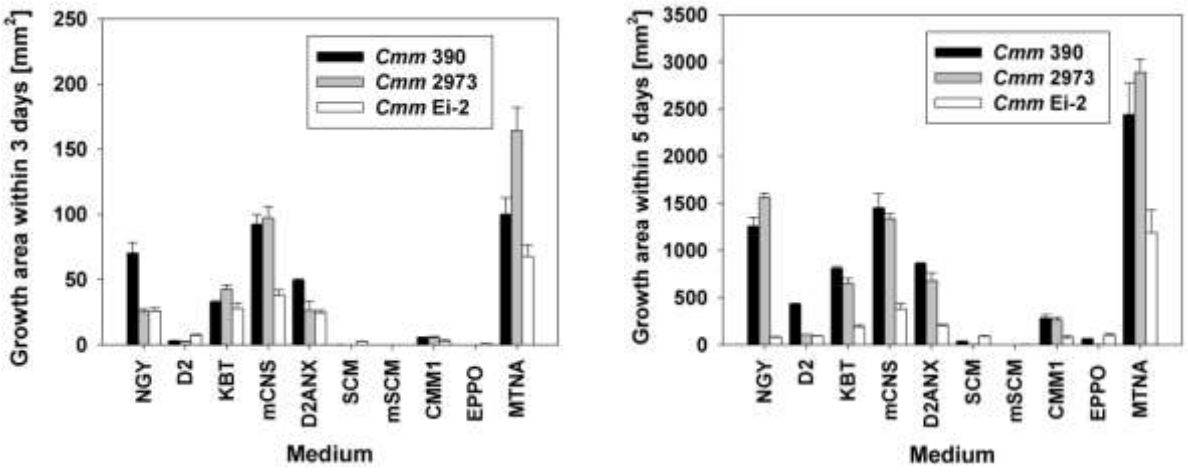


**Figure 1.** Growth areas in $mm^2$ of 3 *Cmm* strains (as the mean of three replicates for each strain) on NGY and on different semiselective media (without addition of antibiotics) at the $3^{rd}$ and $5^{th}$ day after plating. Growth area = number of CFU × $\pi$ $r^2$ (r: average radius of colonies in mm).

**Screening of different antibiotics**

Two *Cmm* strains with different growth speed and growth morphology on NGY medium were selected for screening 40 different antibiotics. The inhibitory effect of each antibiotic did not differ strongly against the two *Cmm* strains tested, but differed between antibiotics. Only co-trimoxazol which is a combination of trimethoprim and sulfamethoxazole inhibited one *Cmm* strain (GSPB 390) but did not inhibit the other strain (GSPB 2973). All the data are shown in Table 2.

**Table 2.** Reaction of two *Cmm* strains towards different antibiotics; results of inhibition zones for each antibiotic and strain were taken from two replicates on NGY medium

| **Compound,** | **Dosis on filter disc** | **Width of inhibition zone in mm** | |
|---|---|---|---|
| **Oxoid abbreviation** | **µg or IU[a]** | **GSPB 390** | **GSBP 2973** |
| Amikacin, AK | 30 µg | 11 | 11 |
| Amoxycillin/ Clavulanic acid, AMC | 30 µg | 24 | 27 |
| Ampicillin, AMP | 10 µg | 19 | 24 |
| Aztreonam, ATM | 30 µg | 0 | 0 |
| Bacitracin, B | 10 IU | 23 | 23 |
| Cefaclor, CEC | 30 µg | 19 | 17 |
| Cefepime, FEP | 30 µg | 23 | 25 |
| Cefotaxime sodium salt, CTX | 30 µg | 20 | 24 |
| Ceftazidime, CAZ | 10 µg | 10 | 18 |
| Cefuroxime, CXM | 30 µg | 21 | 19 |
| Cephazolin, KZ | 30 µg | 20 | 21 |
| Cepomdoxime, CPD | 10 µg | 16 | 15 |
| Ciprofloxacin, CIP | 5 µg | 16 | 17 |
| Co-Trimoxazol, SXT[b] | 25 µg | 8 | 0 |
| Erythromycin, E | 15 µg | 26 | 23 |
| Fosfomycin, FOS | 50 µg | 0 | 0 |
| Gentamycin, CN | 10 µg | 8 | 6 |
| Imipenem, IPM | 10 µg | 26 | 20 |
| Levofloxacin, LEV | 5 µg | 21 | 18 |
| Lincomycin, MY | 15 µg | 24 | 20 |
| Linezolid, LZD | 30 µg | 25 | 23 |
| Meropenem, MEM | 10 µg | 24 | 25 |
| Metronidazole, MTZ | 5 µg | 0 | 0 |
| Moxifloxacin, MXF | 5 µg | 21 | 21 |
| Mupirocin, MUP | 5 µg | 0 | 0 |
| Nalidixic acid, NA | 30 µg | 0 | 0 |
| Neomycin, N | 30 µg | 6 | 5 |
| Netilomicin, NET | 10 µg | 2 | 3 |
| Nitrofurantoin, F | 100 µg | 7 | 7 |
| Novobiocin, NV | 5 µg | 20 | 20 |
| Oxacillin, OX | 1 µg | 1 | 1 |
| Penicillin G, P | 10 IU | 16 | 21 |
| Piperacillin, PRL | 30 µg | 19 | 21 |
| Polymyxin B sulfate, PB | 300 IU | 0 | 0 |
| Rifampicin, RD | 2 µg | 25 | 24 |
| Tazobac, TZP[c] | 40 µg | 22 | 24 |
| Tetracycline, TE | 30 µg | 28 | 25 |
| Tobramycin, TOB | 10 µg | 4 | 4 |
| Trimethoprim, W | 5 µg | 0 | 0 |
| Vancomycin, VA | 30 µg | 16 | 16 |

[a] IU = International Units.

[b] Co-Trimoxazol (SXT) = Trimethoprim/ Sulfamethoxazole (1.25/ 23.75 µg).

[c] Tazobac (TZP) = Piperacillin/ Tazobactam (30/ 10 µg).

**Susceptibility of accompanying bacterial species and strains towards antibiotics**

Antibiotics which did not inhibit *Cmm,* such as aztreonam, fosfomycin, metronidazole, mupirocin, nalidixic acid, polymyxin B sulfate, sulfamethoxazole and trimethoprim were tested against accompanying bacteria in order to select the effective ones with a wide inhibiting spectrum. As shown on Table 3, nalidixic acid (10 and 20 mg/l) and trimethoprim (100-300mg/l) showed the strongest inhibition spectrum against the accompanying bacterial species tested, without inhibiting the growth of *Cmm*. A combination of 20 ppm nalidixic acid and 100 ppm trimethoprim seemed to inhibit all accompanying bacteria tested. Furthermore, polymyxin B sulfate was also tested separately and showed a broad inhibitory spectrum of accompanying bacteria (data not shown).

Therefore, nalidixic acid, trimethoprim and polymyxin B sulfate and the fungicide Opus® Top were tested furthermore in different compositions in the NGY medium and in different modifications of the selected basic medium of MTNA. Each composition was tested with two *Cmm* strains (GSPB 390 and 2973) for determining the growth speed of *Cmm.* Concomitantly, homogenates from naturally or artificially infected field tomato plants and seeds which were highly contaminated with diverse epiphytic microorganisms, were tested with these components in order to determine selectivity.

Compositions with low selectivity or low growth speed were excluded. Other compositions with high growth speed of *Cmm* and simultaneously high selectivity were further modified. In this way, every 10-15 days more than 15-20 different compositions were prepared and tested for growth speed and selectivity. After each experimental block the variants showing the highest potential for *Cmm* growth speed combined with a good selectivity were selected and modified again and again. In this way, the new selective media BCT and BCT-2 were finally developed.

**Recipes of the new selective media BCT and BCT-2**

Recipe of BCT for one liter: 2.5 g mannitol (Merck); 2.0 g yeast extract (Roth); 1.0 g $K_2HPO_4$ (AppliChem); 0.1 g $KH_2PO_4$ (Merck); 0.05 g NaCl (Merck); 0.1 g $MgSO_4 \times 7H_2O$ (Merck); 0.015 g $MnSO_4 \times H_2O$ (AppliChem); 0.015 g $FeSO_4 \times 7H_2O$ (Merck); 0.6 g $H_3BO_3$ (AppliChem), dissolved in 1 liter deionized $H_2O$. The resulting pH value should be between 7.0 and 7.1. Add 15 g/l agar agar (Roth).

After autoclaving at 121 °C for 15 min, cooling down to 50 °C and under stirring add the following: 20 mg/l nalidixic acid (AppliChem); 100 mg/l trimethoprim (Fluka); 20 mg/l polymyxin B sulfate (8,120 international units per milligram, from AppliChem); and 50 µl/l Opus® Top (commercially available from BSAF and containing 84.0 g/l expoxiconazol and 250.0 g/l fenpropimorph).

Antibiotics and Opus® Top must be added as stock solutions, freshly prepared and kept in sterile glasses at 4 °C. Stock solution of nalidixic acid (20 mg/ml 0.1N NaOH, filter-sterilized); trimethoprim (50 mg/ml Dimethyl sulfoxide, must be kept away from light); poymyxin B sulfate (10 mg/ml water, filter-sterilized); Opus® Top (50 µl/ml sterile water).

Recipe of **BCT-2** medium is similar to BCT: instead of 1.0 g $K_2HPO_4$ add 2.0 g/l to BCT-2 and instead of 0.1 g $KH_2PO_4$ add 0.5 g/l to BCT-2. The resulting pH value of BCT-2 should be between 7.15 and 7.2.

In most cases, both new selective media BCT & BCT-2 were filled into Petri dishes and stored for three days at room temperature. In some cases, when both media were used directly after preparation, we noticed some growth inhibition of several *Cmm* strains.

Therefore, we recommend using the new selective media BCT & BCT-2 at least three days after preparation.

**Table 3.** Growth of *Cmm* (GSPB 390), 5 phytopathogenic and 25 epiphytic or saprophytic bacterial strains isolated from tomato seeds and plants, on NGY media containing different concentrations of antibiotics

| Bacterial species or strain [a] | Gram react. [b] | Colony - colour / NGY | Bacterial growth on NGY medium amended with [c] | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Aztreonam | | Metronidazole | | | Sulfamethoxazole | | | | Mupirocin | | | | Nalidixic acid | | | | Trimethoprim | | | Fosfomycin | | |
| | | | 30 | 50 | 3 | 6 | 8 | 5 | 10 | 20 | 50 | 100 | 200 | 400 | 600 | 5 | 10 | 20 | 50 | 100 | 200 | 300 | 50 | 75 | 150 |
| *C . m. michiganensis* (GSPB 390) | G + | typical | + | + | + | + | ± | + | + | ± | - | + | + | + | + | + | + | + | ± | + | + | + | + | + | + |
| *Xanthomonas arboricola* pv. *juglandis* (GSPB 3148) | G – | typical | + | + | + | + | + | + | + | + | + | + | + | + | – | + | + | – | – | + | – | – | – | – | – |
| *X. c.* pv. *vesicatoria* (GSPB 2043) | G – | typical | + | + | + | + | + | + | + | + | + | + | + | – | – | + | + | – | – | + | – | – | – | – | – |
| *P. s.* pv. *syringae* (GSPB 1142) | G – | typical | – | – | + | + | – | + | + | + | + | – | – | – | – | – | – | – | – | + | – | – | – | – | – |
| *P. s.* pv. *tomato* (GSPB 2317) | G – | typical | – | – | + | + | + | + | + | + | + | + | + | + | + | – | – | – | – | + | – | – | – | – | – |
| *P. fluorescens* (GSPB 1714) | G – | typical | + | + | + | + | – | + | + | + | + | + | – | – | – | ± | – | – | – | + | – | – | – | – | – |
| *Pantoea agglomerans* (GSPB 450) | G – | typical | + | + | + | + | – | + | + | + | + | ± | – | – | – | ± | – | – | – | + | – | – | + | + | – |
| *Pectobacterium. c.* subsp. *carotovorum* (GSPB 436) | G – | typical | – | – | + | + | + | + | + | + | + | + | – | – | – | + | + | + | – | – | – | – | – | – | – |
| *Bacillus subtilis* (GSPB 1769) | G + | typical | + | + | + | + | + | + | + | + | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| S-1: *Pseudomonas putida* | G – | white - creamy | + | – | + | + | + | + | + | + | + | + | + | + | + | – | – | – | – | + | – | – | + | – | – |
| S-2: *Microbacterium lacticum* | G + | yellow - pink | + | – | + | + | + | + | + | + | + | + | + | + | + | + | + | + | – | – | – | – | + | + | + |
| S-3: not determined | G – | dark yellow | + | + | + | – | – | + | + | + | + | + | – | – | – | – | – | – | – | – | – | – | – | – | – |
| S-4: *Pantoea* sp. | G – | creamy yellowish | – | – | + | + | + | + | + | + | + | + | + | – | – | + | + | ± | – | – | – | – | + | + | + |
| S-5: *Pantoea* sp. | G – | white yellowish | – | – | + | + | + | + | + | + | + | ± | – | – | – | + | + | – | – | – | – | – | + | + | + |
| S-7: not determined | G + | creamy | + | + | + | + | + | + | + | + | + | – | – | – | – | + | + | + | – | – | – | – | + | + | + |
| S-8: *Bacillus cereus* | G + | yellow | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | – | – | – | + | + | + |
| S-9: not determined | G – | white - creamy | ± | ± | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | – | – | + | + | + |
| S-10: *Pseudomonas syringae* | G – | white - creamy | ± | – | + | + | + | + | + | + | + | – | – | – | – | ± | – | – | – | + | – | – | – | – | – |
| S-11: *Bacillus coagulans* | G + | light yellow - pink | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | – | ± | – | – | + | + | + |
| S-12: *Microbacterium* sp. | G + | Pink - yellowish | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | – | – | – | – | + | + | + |
| S-13: *Pantoea agglomerans* | G – | light yellow | + | + | + | + | + | + | + | + | + | + | + | + | + | – | – | – | – | + | – | – | + | + | + |
| S-14: *Pseudomonas putida* | G – | white creamy | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ± | – | – | + | ± | ± | + | + | + |
| S-15: *Pseudomonas putida* | G – | white creamy | + | + | + | + | + | + | + | + | + | + | + | + | + | + | – | – | – | + | ± | ± | + | + | + |
| S-16: not determined | G + | violet | – | – | nt | nt | nt | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | ± | – | – |
| S-17: not determined | G + | dark orange | + | ± | + | + | + | + | + | + | + | – | – | – | – | + | – | – | – | + | ± | – | – | – | – |
| S-18: not determined | G – | yellow | + | + | + | + | + | + | ± | – | – | + | + | + | + | – | – | – | – | – | – | – | + | – | – |
| S-19: *Rahnella aquatilis* | G – | white | – | – | + | + | + | + | + | + | + | + | – | – | – | – | – | – | – | + | – | – | – | – | – |
| S-20: not determined | G + | dark yellow | + | + | + | + | + | + | + | + | + | + | + | + | + | + | – | – | – | + | – | – | + | + | + |
| S-21: *Bacillus licheniformis* | G + | light yellow, creamy | + | + | + | + | + | + | + | + | + | – | + | + | + | + | + | + | – | – | – | – | + | + | + |
| S-22: not determined | G + | white | nt | nt | + | + | + | ± | ± | ± | ± | – | nt | nt | nt | – | – | – | – | – | – | – | nt | nt | nt |
| S-23: *Bacillus pumilus* | G + | yellow | + | + | + | + | + | + | + | + | + | + | – | – | – | + | + | ± | – | – | – | – | + | + | + |

[a] GSPB = Göttingen Collection (Sammlung) of Phytopathogenic Bacteria. [b] Gram reaction: G – = Gram negative and G + = Gram positive.

[c] Antibiotic concentrations are in milligrams per liter (mg/liter); + = growth; – = no growth; ± = slight growth; nt = not tested.

**Effect of boric acid**

Boric acid has also been used in other semiselective media because of its antimicrobial effect against saprophytic bacteria. We tested different concentrations of boric acid in the NGY medium (Figure 2) as well as in the new basic medium (600, 900, 1200, 1500 and 2000 ppm). By increasing amounts of boric acid in agar media, inhibition of saprophytes was stronger, but the growth of *Cmm* was retarded. This means that appearance of *Cmm*-colonies was delayed and colony-diameters were smaller resulting in less growth areas. The optimal concentration of boric acid was determined as 0.6 g/l causing high inhibition of accompanying bacteria by allowing good growth of *Cmm* (Figure 3).

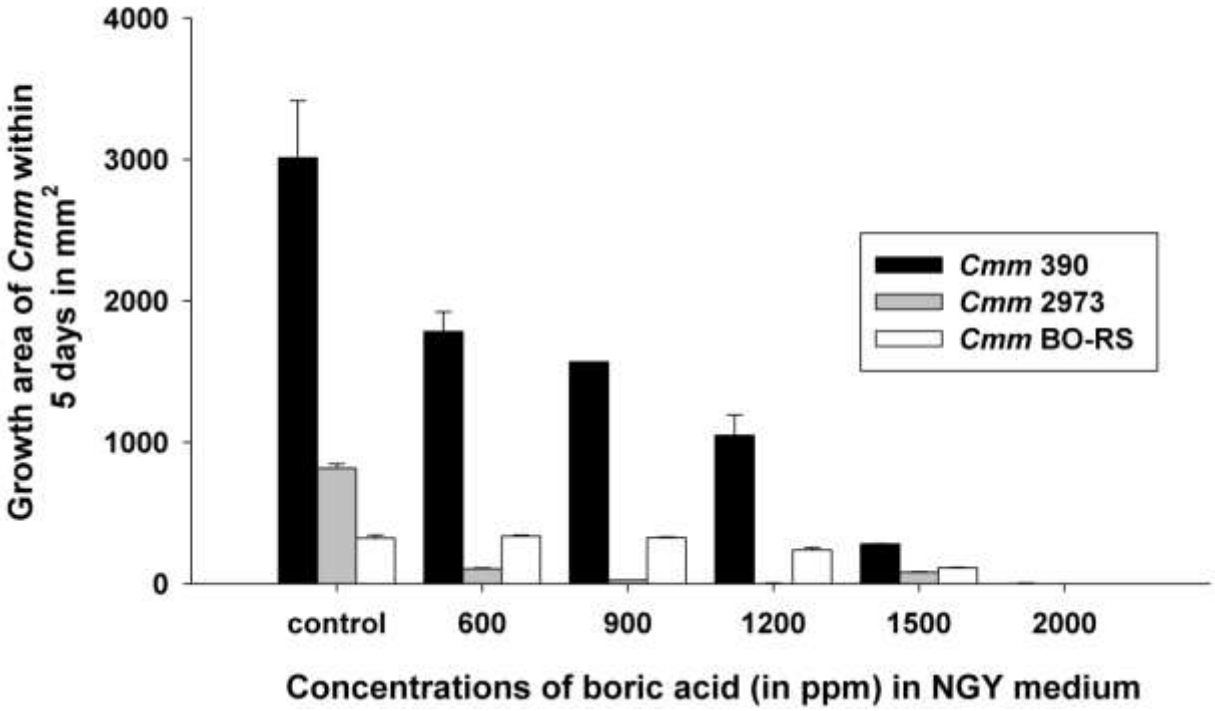


**Figure 2.** Effect of boric acid on growth areas of three *Cmm* strains (GSPB 390, GSPB 2973 and BO-RS = GSPB 3204) in the NGY medium.

However, when antibiotics were added to the medium the effect of boric acid was contrary. Thus, without addition of boric acid, the growth of *Cmm* on the new basic medium containing different compositions of antibiotics was never satisfactory, and the recovered colony forming units of *Cmm* were very low compared with NGY. When very low concentrations of

antibiotics were added, the selectivity of the medium was nearly lost completely. However, by addition of boric acid to some compositions, the recovery rate of *Cmm* was surprisingly high, even when increased amounts of antibiotics were added (Figure 4). Thus, by adding of boric acid together with high amounts of antibiotics a high selectivity of the medium for *Cmm* could be achieved.

For understanding the possible interactions between boric acid and different antibiotics and inhibitors we tested the basic medium with each inhibitor separately, with or without boric acid. Addition of 0.6 g/liter boric acid to compositions of the basic medium with either Opus® Top (100 µl/liter), or nalidixic acid (30 mg/liter), or trimethoprim (200 mg/liter), caused a slight reduction in recovery rate of *Cmm* compared with the same compositions without boric acid. Contrary results were obtained in case of polymyxin B sulfate. When the basic medium contained 30 mg/l polymyxin B sulfate and 0.6 g/l boric acid, the recovery of *Cmm* was normal and very high compared with the same composition without boric acid (Figure 5). The toxicity of polymyxin B sulfate to *Cmm* appeared to be reduced significantly when boric acid was added. Obviously, the reason for the very low recovery rate of *Cmm* without boric acid was due to the toxic acting of polymyxin B sulfate in the basic medium.

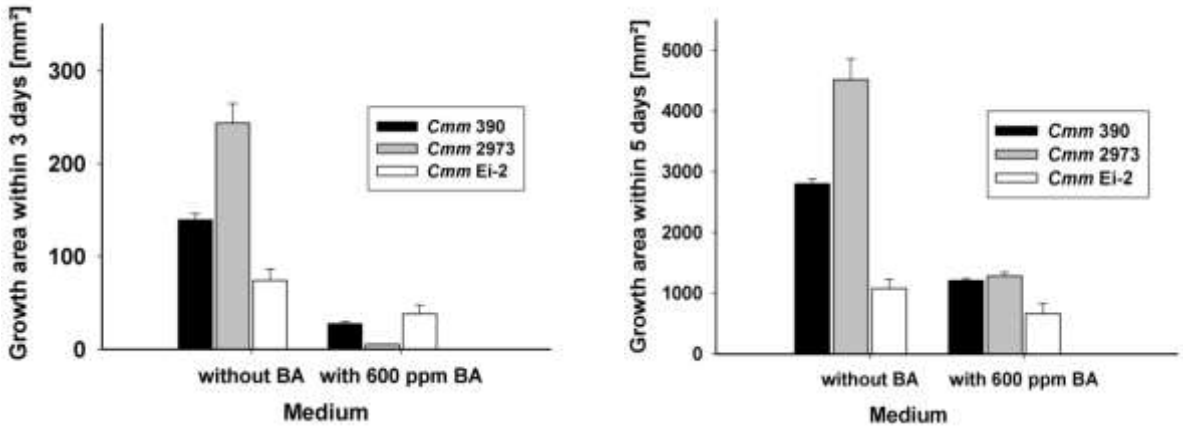


**Figure 3.** Effect of boric acid (BA) on the growth of three *Cmm* strains on the new basic medium BCT (**without antibiotics**), growth area of each strain represents the mean of three agar plates after three and five days.

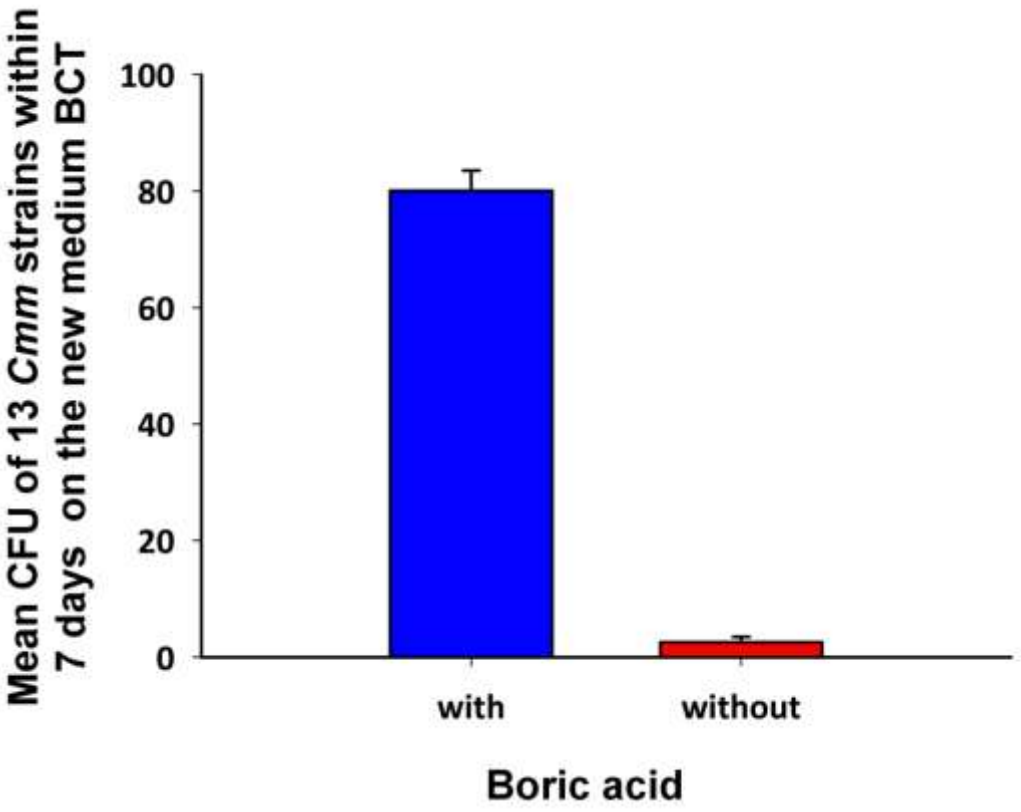


**Figure 4.** Mean number of CFU per agar plate recovered from pure cultures of 13 *Cmm* strains (each in three replicates) on the new medium (BCT) with and without boric acid (600 ppm), when ca. 90 cfu were streaked on each Petri dish.

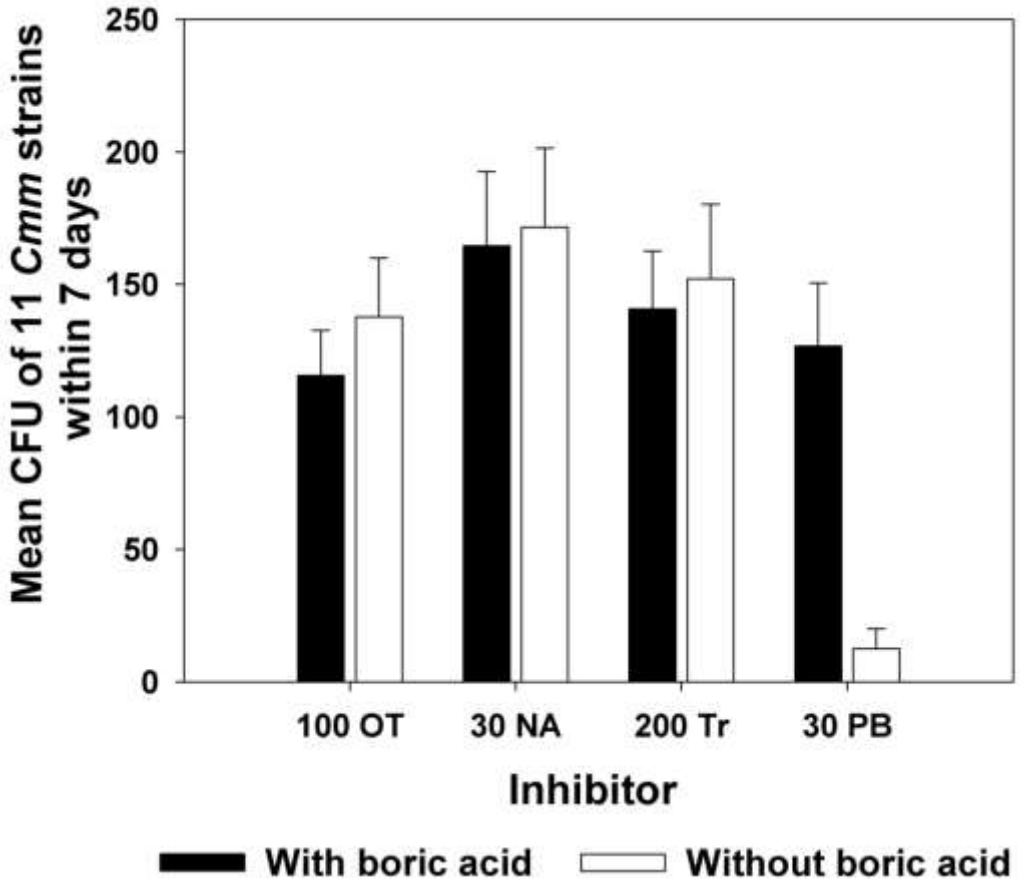


**Figure 5.** Interactive effects of boric acid (600 ppm) and different inhibitors in the basal BCT medium on the growth of *Cmm* (data represent the mean for 11 *Cmm* strains, each in three replicates), 100 OT = 100 µl/liter Opus® Top, 30 NA = 30 mg/liter nalidixic acid, 200 Tr = 200 mg/liter trimethoprim, 30 PB = 30 mg/liter polymyxin B sulfate containing 8120 IU/mg.

**Plating efficiency (recovery rate) of *Cmm* on the published and the new selective media**

Plating efficiency of 10 semiselective media was determined at 7, 10, 15 and 20 days, compared with NGY agar (Table 4). For a fast diagnosis it is most important, that the target bacteria grow within few days after plating. This was the case for the non-selective NGY medium, where *Cmm* colonies were visible within 2-3 days. Relative fast growth was also recorded on CMM1, KBT and D2 (4-5 days), followed by D2ANX and SCM (5-7 days). On the new medium BCT, most strains started to grow after 4-5 days, and finally 29 of the 30 strains tested grew within the first

**Table 4.** Plating efficiency (%) of 30 *Cmm* strains on different semiselective media compared with the standard NGY medium within 7/ 10/ 15/ 20 days after plating

| Strain [a] | Plating efficiency [d] (%) within 7/ 10/ 15/ 20 days respectively on | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | D2 | KBT | mCNS | D2ANX | SCM | mSCM | CMM1 | EPPO [c] | BCT | BCT-2 |
| Amb-1 | 117/ 117/ 117/ 117 | 120/ 120/ 120/ 120 | 0/ 0/ 0/ 0 | 61/ 61/ 61/ 61 | 125/ 125/ 125/ 125 | 0/ 0/ 86/ 113 | 109/ 109/ 109/ 109 | 0/ 0/ 0/ 0 | 67/ 90/ 90/ 90 | 76/ 76/ 90/ 90 |
| Ei-1 | 121/ 121/ 121/ 121 | 123/ 123/ 123/ 123 | 0/ 0/ 1/ 1 | 104/ 104/ 104/ 104 | 119/ 119/ 119/ 119 | 0/ 0/ 101/ 103 | 106/ 106/ 106/ 106 | 0/ 0/ 19/ 43 | 87/ 109/ 109/ 109 | 119/ 119/ 119/ 119 |
| Ei-2 | 110/ 110/ 110/ 110 | 95/ 95/ 95/ 95 | 0/ 0/ 0/ 0 | 13/ 13/ 13/ 13 | 94/ 94/ 94/ 94 | 0/ 84/ 84/ 96 | 89/ 89/ 89/ 89 | 0/ 0/ 0/ 0 | 73/ 73/ 73/ 73 | 98/ 98/ 98/ 98 |
| Lu-1 | 127/ 127/ 127/ 127 | 92/ 92/ 92/ 92 | 0/ 0/ 0/ 0 | 98/ 98/ 98/ 98 | 94/ 94/ 94/ 94 | 0/ 69/ 69/ 98 | 105/ 105/ 105/ 105 | 0/ 0/ 0/ 0 | 102/ 102/ 102/ 102 | 114/ 114/ 114/ 114 |
| Mo-1 | 112/ 112/ 112/ 112 | 89/ 89/ 89/ 89 | 47/ 47/ 51/ 51 | 91/ 91/ 91/ 91 | 105/ 105/ 105/ 105 | 0/ 0 /113/ 113 | 93/ 93/ 93/ 93 | 0/ 0/ 104/ 104 | 96/ 96/ 96/ 96 | 97/ 97/ 97/ 97 |
| Mo-2 | 103/ 103/ 103/ 103 | 103/ 103/ 103/ 103 | 0/ 0/ 3/ 6 | 90/ 90/ 90/ 90 | 104/ 104/ 104/ 104 | 0/ 51/ 85/ 102 | 111/ 111/ 111/ 111 | 0/ 0/ 0/ 2 | 95/ 95/ 95/ 95 | 87/ 87/ 87/ 87 |
| Sc-1 | 112/ 112/ 112/ 112 | 106/ 106/ 106/ 106 | 0/ 0/ 3/ 6 | 90/ 90/ 90/ 90 | 107/ 107/ 107/ 107 | 0/ 35/ 86/ 95 | 108/ 108/ 108/ 108 | 0/ 0/ 0/ 0 | 112/ 112/ 112/ 112 | 110/ 110/ 110/ 110 |
| BO-RS | 91/ 91/ 91/ 91 | 68/ 68/ 68/ 68 | 0/ 0/ 0/ 0 | 36/ 36/ 36/ 36 | 25/ 35/ 35/ 35 | 0/ 0/ 0/ 0 | 67/ 67/ 67/ 67 | 0/ 0/ 0/ 0 | 76/ 76/ 76/ 76 | 71/ 71/ 71/ 71 |
| GSPB [b] 2972 | 84/ 84/ 84/ 84 | 61/ 61/ 61/ 61 | 0/ 0/ 0/ 0 | 107/ 107/ 107/ 107 | 80/ 80/ 80/ 80 | 0/ 0/ 0/ 0 | 97/ 97/ 97/ 97 | 0/ 0/ 0/ 57 | 92/ 92/ 92/ 92 | 81/ 81/ 88/ 88 |
| AE-1 | 112/ 112/ 112/ 112 | 87/ 87/ 87/ 87 | 0/ 0/ 0/ 0 | 56/ 56/ 56/ 56 | 102/ 102/ 102/ 102 | 0/ 10/ 24/ 38 | 94/ 94/ 94/ 94 | 0/ 12/ 12/ 21 | 106/ 106/ 106/ 106 | 94/ 94/ 94/ 94 |
| AH-1 | 117/ 117/ 117/ 117 | 114/ 114/ 114/ 114 | 0/ 0/ 0/ 0 | 89/ 89/ 89/ 89 | 107/ 107/ 107/ 107 | 0/ 44/ 77/ 89 | 118/ 118/ 118/ 118 | 0/ 111/ 111/ 122 | 100/ 100/ 100/ 100 | 68/ 68/ 68/ 68 |
| ES-1 | 98/ 98/ 98/ 98 | 90/ 90/ 90/ 90 | 0/ 0/ 0/ 0 | 95/ 95/ 95/ 95 | 100/ 100/ 100/ 100 | 0/ 0/ 99/ 99 | 90/ 90/ 90/ 90 | 0/ 0/ 92/ 92 | 93/ 93/ 93/ 93 | 79/ 79/ 79/ 79 |
| HH-1 | 109/ 109/ 109/ 109 | 99/ 99/ 99/ 99 | 0/ 0/ 4/ 5 | 75/ 75/ 75/ 75 | 104/ 104/ 104/ 104 | 0/ 76/ 77/ 79 | 86/ 86/ 86/ 86 | 0/ 0/ 0/ 0 | 45/ 45/ 45/ 45 | 107/ 107/ 107/ 107 |
| La-1 | 96/ 96/ 96/ 96 | 64/ 64/ 64/ 64 | 0/ 0/ 0/ 0 | 43/ 43/ 43/ 43 | 89/ 89/ 89/ 89 | 71/ 79/ 87/ 88 | 89/ 89/ 89/ 89 | 0/ 87/ 87/ 91 | 87/ 87/ 87/ 87 | 73/ 73/ 73/ 73 |
| OS-1 | 93/ 93/ 93/ 93 | 74/ 74/ 74/ 74 | 0/ 0/ 4/ 4 | 93/ 93/ 93/ 93 | 101/ 101/ 101/ 101 | 0/ 0/ 99/ 99 | 94/ 94/ 94/ 94 | 0/ 0/ 79/ 84 | 96/ 96/ 96/ 96 | 93/ 93/ 93/ 93 |
| OS-2 | 91/ 91/ 91/ 91 | 84/ 84/ 84/ 84 | 0/ 0/ 0/ 0 | 81/ 81/ 81/ 81 | 129/ 129/ 129/ 129 | 0/ 0/ 103/ 105 | 91/ 91/ 91/ 91 | 0/ 0/ 65/ 73 | 91/ 91/ 91/ 91 | 93/ 93/ 93/ 93 |
| OS-4 | 91/ 91/ 91/ 91 | 87/ 87/ 87/ 87 | 0/ 0/ 0/ 0 | 47/ 47/ 47/ 47 | 120/ 120/ 120/ 120 | 0/ 89/ 89/ 101 | 91/ 91/ 91/ 91 | 0/ 0/ 0/ 10 | 109/ 109/ 109/ 109 | 83/ 83/ 83/ 83 |
| GSPB 378 | 88/ 88/ 88/ 88 | 88/ 88/ 88/ 88 | 0/ 0/ 0/ 0 | 43/ 43/ 43/ 43 | 93/ 93/ 93/ 93 | 0/ 76/ 98/ 98 | 75/ 75/ 75/ 75 | 0/ 79/ 87/ 88 | 66/ 66/ 66/ 66 | 81/ 81/ 81/ 81 |
| GSPB 382 | 83/ 83/ 83/ 83 | 55/ 55/ 55/ 55 | 0/ 0/ 0/ 0 | 0/ 0/ 0/ 0 | 65/ 65/ 65/ 65 | 48/ 67/ 67/ 67 | 49/ 49/ 49/ 49 | 0/ 65/ 65/ 66 | 45/ 45/ 45/ 45 | 20/ 42/ 46/ 46 |
| GSPB 390 | 111/ 111/ 111/ 111 | 102/ 102/ 102/ 102 | 0/ 0/ 0/ 0 | 85/ 85/ 85/ 85 | 88/ 88/ 88/ 88 | 0/ 76/ 76/ 93 | 103/ 103/ 103/ 103 | 0/ 113/ 113/ 116 | 108/ 108/ 108/ 108 | 111/ 111/ 111/ 111 |
| GSPB 392 | 106/ 106/ 106/ 106 | 91/ 91/ 91/ 91 | 0/ 0/ 0/ 1 | 72/ 72/ 72/ 72 | 92/ 92/ 92/ 92 | 0/ 0/ 115/ 115 | 96/ 96/ 96/ 96 | 0/ 0/ 113/ 113 | 102/ 102/ 102/ 102 | 107/ 107/ 107/ 107 |
| Bulgarian 1 | 97/ 97/ 97/ 97 | 104/ 104/ 104/ 104 | 0/ 0/ 0/ 0 | 79/ 79/ 79/ 79 | 103/ 103/ 103/ 103 | 0/ 85/ 102/ 102 | 87/ 87/ 87/ 87 | 0/ 0/ 96/ 96 | 0/ 23/ 58/ 64 | 0/ 0/ 0/ 0 |
| GSPB 2973 | 94/ 94/ 94/ 94 | 99/ 99/ 99/ 99 | 0/ 7/ 9/ 9 | 94/ 94/ 94/ 94 | 82/ 82/ 82/ 82 | 0/ 0/ 0/ 46 | 100/ 100/ 100/ 100 | 0/ 0/ 55/ 73 | 84/ 84/ 84/ 84 | 85/ 85/ 85/ 85 |
| GSPB 2315 | 128/ 128/ 128/ 128 | 78/ 78/ 78/ 78 | 0/ 0/ 0/ 4 | 79/ 79/ 79/ 79 | 91/ 91/ 91/ 91 | 0/ 14/ 14/ 101 | 106/ 106/ 106/ 106 | 0/ 0/ 0/ 38 | 102/ 102/ 102/ 102 | 82/ 82/ 82/ 82 |
| GSPB 2221 | 103/ 103/ 103/ 103 | 79/ 79/ 79/ 79 | 7/ 7/ 13/ 13 | 86/ 86/ 86/ 86 | 99/ 99/ 99/ 99 | 0/ 0/ 95/ 97 | 98/ 98/ 98/ 98 | 0/ 0/ 95/ 97 | 91/ 91/ 91/ 91 | 90/ 90/ 90/ 90 |
| GSPB 2222 | 105/ 105/ 105/ 105 | 61/ 61/ 61/ 61 | 0/ 0/ 0/ 0 | 66/ 66/ 66/ 66 | 90/ 90/ 90/ 90 | 0/ 0/ 0/ 81 | 95/ 95/ 95/ 95 | 0/ 0/ 0/ 22 | 103/ 103/ 103/ 103 | 74/ 74/ 74/ 74 |
| 399 | 102/ 102/ 102/ 102 | 47/ 47/ 47/ 47 | 0/ 0/ 2/ 7 | 103/ 103/ 103/ 103 | 75/ 75/ 75/ 75 | 0/ 0/ 87/ 87 | 100/ 100/ 100/ 100 | 0/ 0/ 99/ 101 | 97/ 97/ 97/ 97 | 87/ 87/ 90/ 90 |
| GSPB 3133 | 103/ 103/ 103/ 103 | 88/ 88/ 88/ 88 | 0/ 0/ 0/ 0 | 23/ 23/ 23/ 23 | 87/ 87/ 87/ 87 | 0/ 59/ 59/ 66 | 92/ 92/ 92/ 92 | 0/ 0/ 34/ 47 | 85/ 85/ 85/ 85 | 94/ 94/ 94/ 94 |
| 185 | 98/ 98/ 98/ 98 | 81/ 81/ 81/ 81 | 0/ 0/ 0/ 0 | 29/ 29/ 29/ 29 | 98/ 98/ 98/ 98 | 86/ 98/ 98/ 98 | 80/ 80/ 80/ 80 | 0/ 114/ 115/ 115 | 100/ 100/ 100/ 100 | 89/ 89/ 89/ 89 |
| Leningrad 3 | 107/ 107/ 107/ 107 | 61/ 61/ 61/ 61 | 0/ 0/ 42/ 43 | 81/ 81/ 81/ 81 | 110/ 110/ 110/ 110 | 0/ 55/ 80/ 81 | 98/ 98/ 98/ 98 | 0/ 0/ 83/ 85 | 84/ 85/ 85/ 85 | 106/ 106/ 106/ 106 |

[a] 100 to 250 CFU were plated in triplicates onto each medium.

[b] GSPB = Göttingen Collection (Sammlung) of Phytopathogenic Bacteria.

[c] EPPO = This medium was suggested in 2005 by the European Plant Protection Organization, therefore we named it EPPO.

[d] Plating efficiency % = (CFU of *Cmm* on test medium / CFU of *Cmm* on NGY) × 100. Each value was derived from triplicates.

seven days. Only one *Cmm* strain grew between 7 and 10 days. On the BCT-2 medium, due to a higher buffering capacity of this medium, the growth of each *Cmm* strain was delayed one day compared with BCT, and just one strain did not grow at all on BCT-2.

In contrast, on mSCM some strains started to grow between 7 and 10 days, other strains between 10 and 15 days, or between 15 and 20 days, and few strains did not grow at all. On the EPPO medium, only 7 from 30 strains and other 12 from 30 strains grew after 7 and 10 days, respectively, and 5 strains did not grow after 20 days (Table 4).

Compared with the NGY medium, most tested semiselective media showed significant differences in recovery rates (Figure 6 and Table 4). Seven and ten days after plating, the maximum mean number was recorded on D2, followed by SCM, CMM1, BCT, BCT-2, KBT and D2ANX respectively. On most of the media (D2, CMM1, BCT, BCT-2, KBT and D2ANX) maximum bacterial growth was recorded within the first seven days (Figure 6). The media which have been used or recommended recently most often, are mSCM, D2ANX, SCM, CMM1 and EPPO. However, after 7 and 10 days, the mean recovery rate on medium mSCM reached only 6% and 29% respectively and was 0% and 17% on the EPPO medium after 7 and 10 days, respectively. On both of these media, the bacteria reached maximum growth between 10 and 20 days. Thus, the recovery rates were about 70% and 84% after 15 and 20 days, respectively, on the mSCM medium, and on the EPPO medium only 53% and 62% were reached after 15 and 20 days, respectively. Very little growth was recorded on the mCNS medium, even after 10 and 20 days only about 2% and 5% cfu, respectively, were recovered. In contrast, the new media BCT and BCT-2 allowed recovery rates of about 89% and 88%, respectively within seven days (Table 4).

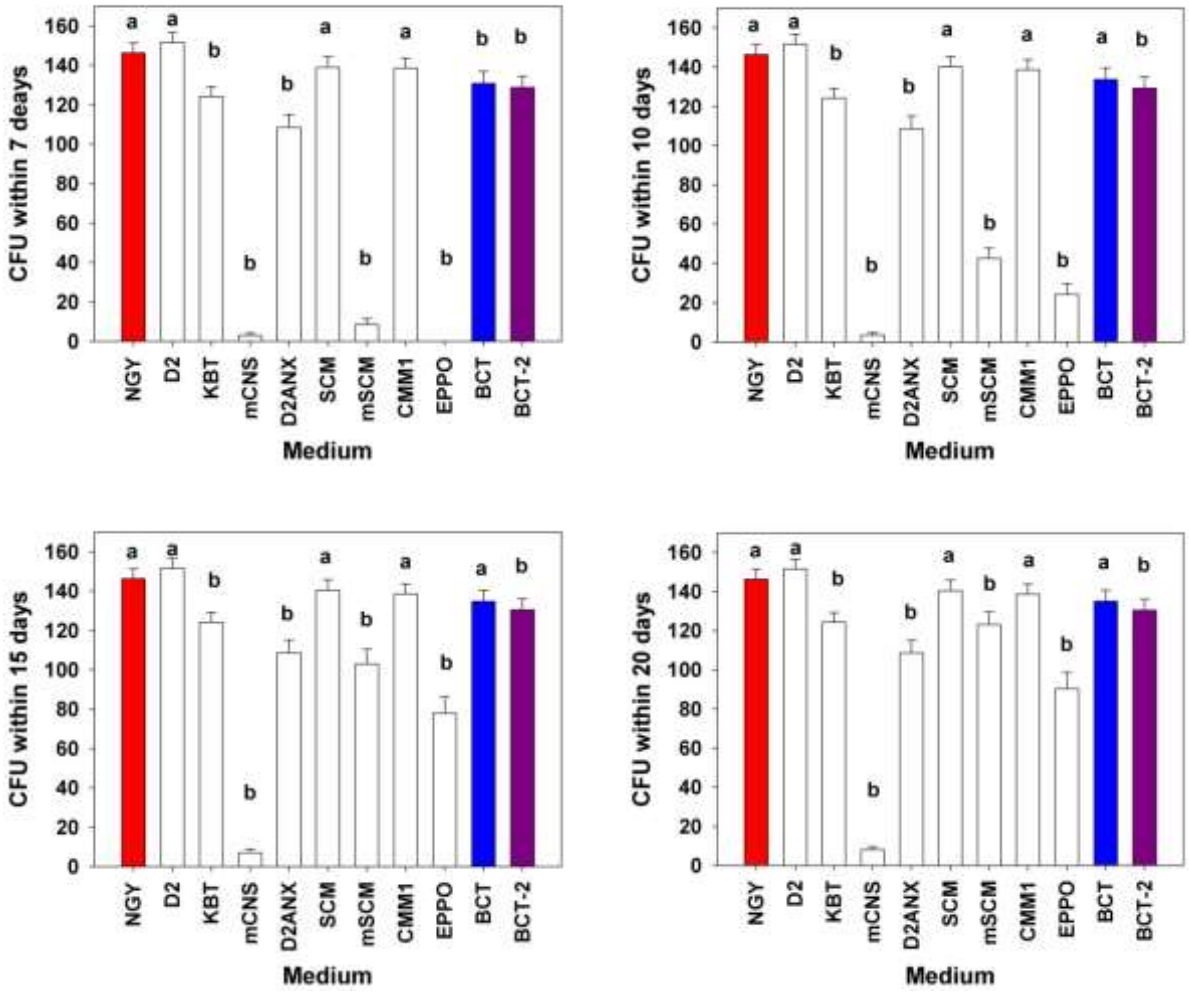


**Figure 6.** Recovery (CFU) of 30 *Cmm* strains on the standard NGY medium and different semiselective media. The starting inoculum contained 100-250 CFU per strain and plate. Results represent the mean of 30 *Cmm* strains, each in three replicates. Letters a and b above each column indicate the significance compared to the NGY medium, a means no significant difference in the mean number of colony forming units compared with those on the NGY medium. Statistical analysis was performed by Fisher's LSD test. $P \leq 0.05$; n = 990.

Further tests with 13 selected *Cmm* strains of different origin (Table 5) revealed sizes of *Cmm*- colonies differing strongly between five semiselective media and the new medium BCT after 7 days. Three media (mCNS, mSCM and EPPO) showed no or very little growth, whereas the other three media (D2ANX, SCM and BCT) allowed large colony sizes (Table 5).

**Table 5.** Colony diameter (mm) of 13 *Cmm* strains on 6 selective media 7 days after plating

| Strain | Diameter of colonies in mm 7 days after plating | | | | | |
|---|---|---|---|---|---|---|
| | **mCNS** | **D2ANX** | **SCM** | **mSCM** | **EPPO** | **BCT** |
| Amb-1 | 0.0 | 4.0 | 2.0-4.0 | 0.0 | 0.0 | 2.0-4.0 |
| Ei-1 | 0.0 | nd. | 2.0-4.0 | ≤ 0.2 | 0.0 | 3.0-4.0 |
| Mo-2 | 0.0 | nd. | 3.0-5.0 | 0.0 | 0.0 | 3.0 |
| GSPB 2972 | 0.0 | 3.0 | 1.5-2.5 | 0.0 | 0.0 | 1.0-2.2 |
| La-1 | 0.0 | 2.5 | 2.0-5.0 | 0.0 | 0.0 | 3.0-6.0 |
| OS-4 | 0.0 | 3.0 | 3.0 | ≤ 0.2 | 0.0 | 3.0-5.0 |
| GSPB 382 | 0.0 | 0.0 | 0.5-4.0 | 0.0 | 0.0 | 1.0-2.0 |
| GSPB 392 | 0.0 | nd. | 1.0-3.0 | 0.0 | 0.0 | 2.0-4.0 |
| GSPB 2221 | 0.0 | 3.0 | 1.0 | ≤ 0.2 | 0.0 | 2.0 |
| GSPB 2222 | 0.0 | nd. | 2.0-5.0 | ≤ 0.5-1.0 | 0.0 | 3.0-5.0 |
| 399 | 0.0 | 3.0 | 0.5-1.0 | ≤ 0.5 | 0.0 | 1.0-2.0 |
| GSPB 3133 | 0.0 | 4.0 | 2.0 | 0.0 | 0.0 | 2.0-5.0 |
| Leningrad 3 | 0.0 | 2.3 | 2.0 | 0.0 | 0.0 | 2.0 |

nd. = not determined

**Selectivity of the new media BCT and BCT-2**

Accompanying bacterial species and pathovars tested (Table 6), such as *Bacillus subtilis*, *Pantoea agglomerans*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Pseudomonas corrugata*, *P. fluorescens*, *P. syringae* pv. *syringae*, *P. s.* pv. *tomato*, *Ralstonia solanacearum*, *Xanthomonas arboricola* pv. *juglandis*, *Xanthomonas campestris* pv. *vesicatoria* and 22 different saprophytic bacterial isolates from tomato plants grew very well on NGY or KB medium but were **unable to grow** on both of the new media BCT and BCT-2.

In contrast, exclusively all the 30 *Cmm* strains tested grew on the new medium BCT as well as on NGY, whereas 29 strains out of 30 grew on the new medium BCT-2 (Table 6). Also, when homogenates from field tomato plants and seeds containing very high levels of unknown saprophytic bacteria were tested, the new medium BCT showed a very high selectivity, since more than 98% of accompanying bacterial cells contained in homogenates were inhibited on the new medium (Table 7 & Fig. 7).

**Table 6.** Growth of 30 *Clavibacter michiganensis* subsp. *michiganensis* strains and different accompanying bacterial species on the new selective media BCT and BCT-2 compared with NGY agar and King's medium B (KB)

| **Bacterial species or pathovar**[a] | **Growth on**[b] | | |
|---|---|---|---|
| | **KB/ NGY** | **BCT** | **BCT-2** |
| *Clavibacter michiganensis* subsp. *michiganensis* (30 strains) | + | + (30) | + (29) |
| *Bacillus subtilis* (FZB 24 & GSPB 1769) | + | – | – |
| *Pectobacterium carotovorum* subsp. *carotovorum* (GSPB 436) | + | – | – |
| *Pantoea agglomerans* (GSPB 450) | + | – | – |
| *Pseudomonas corrugata* (GSPB 2418) | + | – | – |
| *Pseudomonas fluorescens* (GSPB 1714) | + | – | – |
| *Pseudomonas syringae* pv. *syringae* (GSPB 1142) | + | – | – |
| *Pseudomonas syringae* pv. *tomato* (GSPB 1776, GSPB 2317 & Syr 1) | + | – | – |
| *Ralstonia solanacearum* (GSPB 2607 & GSPB 2619) | + | – | – |
| *Xanthomonas arboricola* pv. *juglandis* (GSPB 3184) | + | – | – |
| *Xanthomonas campestris* pv. *vesicatoria* (GSPB 2043) | + | – | – |
| Saprophytic bacteria (22 different isolates) | + | – | – |

[a] Saprophytic bacteria were isolated from tomato seed and tomato plants and differing in colour, morphology, Gram's reaction, or susceptibility to antibiotics, partially identified by fatty acid analysis as shown in Table 3.
[b] + = growth; – = no growth.

**Table 7.** Inhibition of saprophytic bacteria (in %) and detection sensitivity of *Cmm*-cells (in %) from tomato seed and plant homogenates on different semiselective media

| Medium | Inhibition of saprophytes & detection sensitivity of *Cmm* (%)* | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | | B | | C | | D | | E | | F | | average % | |
| | inhib. | det. | inhib. | det. | inhib. | det. | inhib. | det. | inhib. | det. | inhib. | det. | **inhib.** | **det.** |
| **D2** | 70.0 | 0.0 | 91.2 | 0.0 | 93.0 | 0.0 | 93.9 | 0.0 | 90.8 | 0.0 | 84.9 | 0.0 | **87.3** | **0.0** |
| **KBT** | 84.9 | 0.0 | 81.0 | 0.0 | 80.9 | 0.0 | 98.6 | 0.0 | 72.5 | 0.0 | 74.8 | 0.0 | **82.1** | **0.0** |
| **mCNS** | 95.1 | 0.0 | 98.6 | 0.0 | 98.4 | 0.0 | 100 | 0.0 | 99.2 | 0.0 | 98.3 | 0.0 | **98.3** | **0.0** |
| **EPPO** | 95.6 | 0.0 | 98.3 | 0.0 | 97.8 | 0.0 | 100 | 0.0 | 94.7 | 0.0 | 97.9 | 0.0 | **97.4** | **0.0** |
| **CMM1** | 88.0 | 0.0 | 87.8 | 0.0 | 98.6 | 0.0 | 98.1 | 0.0 | 98.8 | 0.0 | 77.4 | 0.0 | **91.5** | **0.0** |
| **D2ANX** | 82.0 | 0.0 | 89.1 | 0.0 | 99.0 | 0.0 | 86.9 | 0.0 | 91.6 | 0.0 | 91.5 | 0.0 | **90.0** | **0.0** |
| **SCM** | 79.9 | 0.0 | 99.3 | 0.0 | 95.1 | 0.0 | 95.8 | 0.0 | 99.6 | 0.0 | 94.1 | 0.0 | **94.0** | **0.0** |
| **mSCM** | 88.4 | 0.0 | 95.5 | 0.0 | 95.8 | 0.0 | 97.6 | 0.0 | 99.7 | 0.0 | 95.2 | 0.0 | **95.4** | **0.0** |
| **BCT** | 97.8 | 67.3 | 98.0 | 39.7 | 98.7 | 100 | 99.4 | 66.7 | 98.6 | 100 | 98.2 | 25.0 | **98.5** | **66.4** |
| **BCT-2** | 99.8 | 63.6 | 98.0 | 50.0 | 99.3 | 98.4 | 99.6 | 0.0 | 98.1 | 0.0 | 100 | 0.0 | **99.1** | **35.3** |

*) **A, B, C, D, E and F: different seed or plant homogenates which were naturally contaminated with saprophytic bacteria (S) and spiked with defined cell-numbers of *Cmm*,** **A**: field seed homogenate (11,500 S + 110 *Cmm* BO-RS/ agar plate); **B**: field plant homogenate (18,000 S + 58 *Cmm* 382/ agar plate); **C**: homogenate from greenhouse plants (15,000 S + 250 *Cmm* BO-RS/ agar plate); **D**: field seed homogenate (1,150 + 21 *Cmm* BO-RS/ agar plate); **E**: homogenate of field plants (1,200 S + 3 *Cmm* OS-2/ agar plate);
**F**: homogenate of field plants (12,750 S + 8 *Cmm* 382/ agar plate).

**Detection sensitivity of the new media for latent infection by *Cmm***

Healthy field tomato plants and seeds naturally contaminated with high levels of saprophytic bacteria were homogenized in sterile water. Afterwards, defined **very small amounts** of *Cmm*-suspensions were added ("spiked") to the homogenates. Aliquots (100 µl) of the spiked homogenates were plated on different semiselective media. As shown in Table 7, Fig. 7 and Fig. 8, these very small amounts of *Cmm*-cells were only detected on the newly developed media BCT and BCT-2, whereas on all the other 8 media *Cmm*-cells could not be detected at all. These results were confirmed by repeated re-streaking of suspected colonies on NGY agar plates. On the other hand, more than 98% of saprophytic bacteria were inhibited on the new medium BCT, so that between 25% and 100% of the existing *Cmm*-cells added or contained in plant and seed homogenates were detected on BCT. The BCT-2 medium showed an even higher selectivity than BCT, however, the growth of *Cmm* was delayed compared with BCT. Therefore, detection of the *Cmm*-cells added was visibly impossible in some cases, so that the detection sensitivity of *Cmm* on BCT-2 was lower than on BCT (Table 7 and Fig. 8).

Thus, our experiments showed very clearly, that all other 8 semiselective media tested revealed false negative results, because low levels of *Cmm*-cells were not detectable when the impact of saprophytes was very high (Table 7, Fig. 7). In other experiments, when the differences in population densities of saprophytic bacteria and *Cmm* were lower, by increasing the amounts of *Cmm* in plant homogenates, detection of *Cmm* was possible on some of the other media. However, distinguishing between *Cmm* and saprophytes was often difficult, and in order to prove the presence of *Cmm* on the other media, dilution re-streaking of bacterial cultures on NGY medium would have been necessary. Therefore we do not have quantitative data for such experiments.

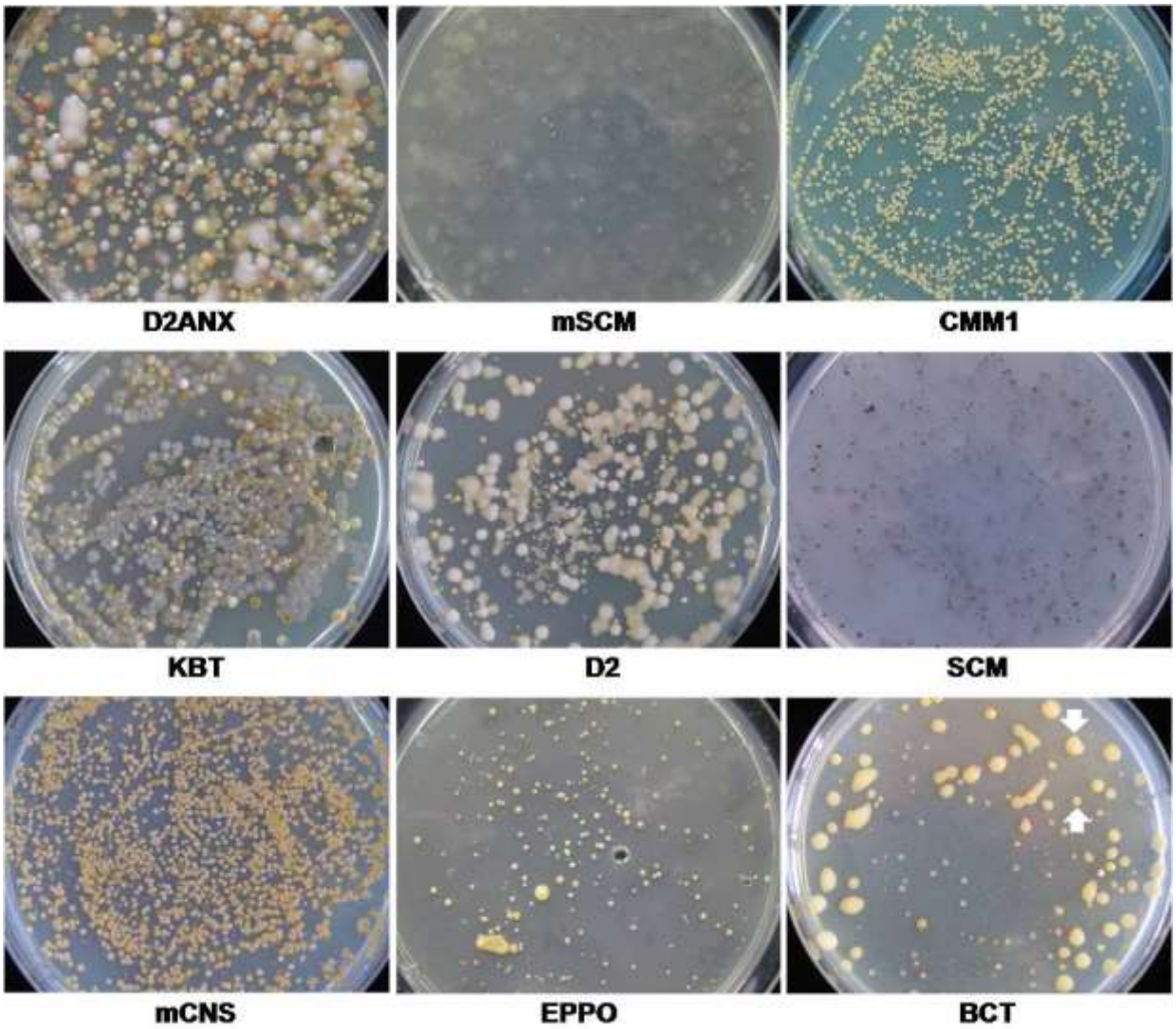


**Figure 7.** Detection of *Cmm* in asymptomatic plant samples on different media. When plant samples were only slightly infected with *Cmm* and highly contaminated with saprophytic bacteria, detection of *Cmm* was only possible on the new medium BCT. On BCT, *Cmm* colonies were easily recognized (creamy to yellow in colour, convex, shining and had increased size with time), whereas colonies of saprophytes were depressed (small, faint, and mostly white in colour).

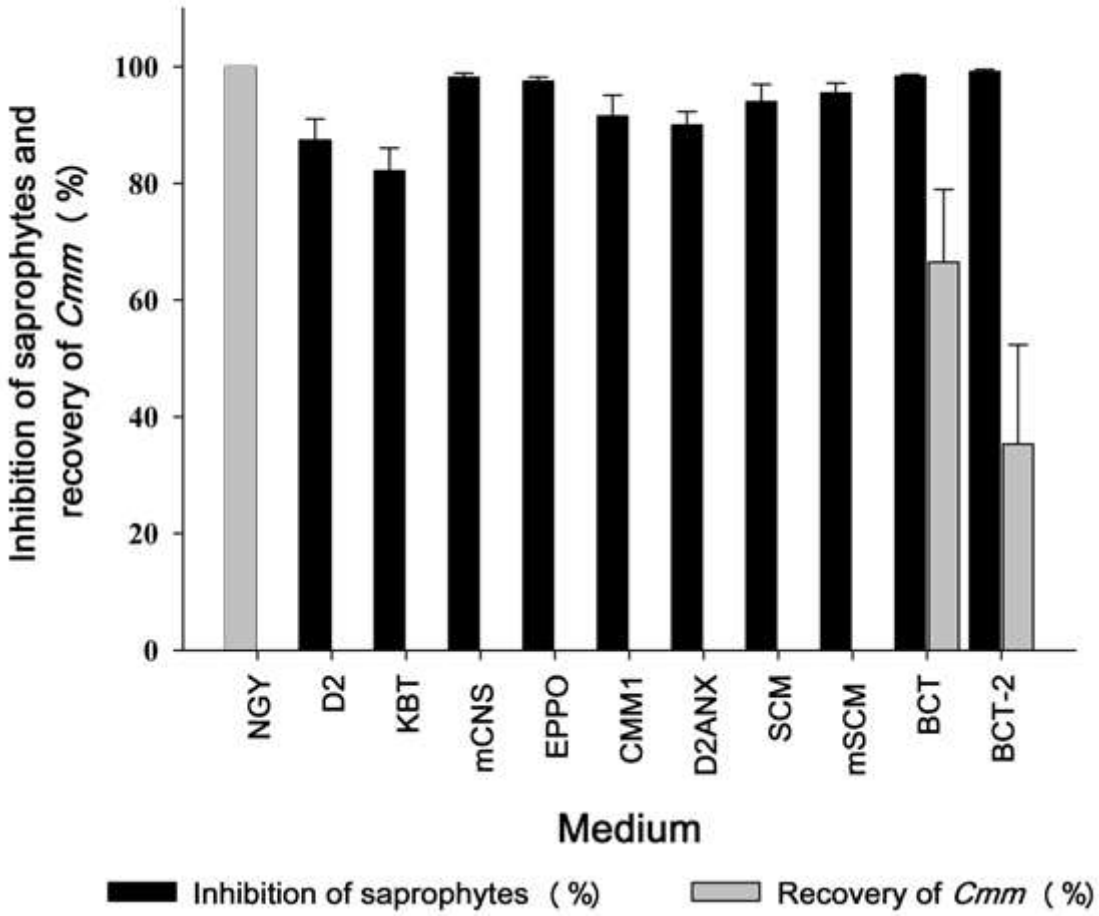


**Figure 8.** Inhibition of naturally occurring saprophytic bacteria and recovery rate of *Cmm* by semiselective media compared with the new selective media (BCT and BCT-2) from tomato plant and seed homogenates spiked with *Cmm*. Inhibition of saprophytes and recovery rate of *Cmm* was determined in % of growth on NGY. The figure shows that, only on BCT and BCT-2 *Cmm* was detected, but not on the earlier published semiselective media under these conditions. The homogenates contained 1,150-18,000 cfu of saprophytes but only 3-250 cfu of *Cmm* per Petri plate. Results are the mean of 6 experiments with the following relations of **saprophytes / *Cmm***: 11,500 / 110; 18,000 / 58; 1,150 / 22; 1,200 / 3; 12,750 / 8 and 15,000 / 250.

**Selectivity for other pathovars/species of coryneform bacteria**

In additional experiments, we tested the new selective medium BCT for detection of different pathovars of coryneform phytopathogenic bacteria (Table 8, Figs. 10 and 11). The results revealed that only those bacteria which are very closely related to *Cmm* according to Davis *et al.* (1984) (i.e., the *C. michiganensis* subspecies *tessellarius, insidiosus* and *nebraskensis)* grew exclusively on the new selective medium BCT wiith a similar appearance as *Cmm* (Fig. 9).

The appearance of these 3 other subspecies on BCT can be described as follows:

- *C. m.* ssp. *nebraskensis* (GSPB 2223): colonies similar to *Cmm*, yellow, convex, brilliant and slimy colonies, about 2.0-3.0 mm in diameter after 7 days.
- *C. m.* ssp. *tessellarius* (GSPB 2224): light pink colour, convex, shining and slimy colonies, diameter of colonies between 1.5-2.5 mm after 7 days (Fig. 10).
- *C. m.* ssp. *insidiosus* (GSPB 30): convex, shining and pink colonies with violet internal flecks, diameter of colonies about 1.0-1.8 mm after 7 days (Fig. 11).

**Table 8.** Growth of other coryneform phytopathogenic bacterial species on NGY and BCT and colony diameter after 7 days

| Bacterial species and GSPB no. | Growth on | | Colony diameter in mm on | | Recovery (%) on BCT |
|---|---|---|---|---|---|
| | NGY | BCT | NGY | BCT | |
| *C. m.* subsp. *insidiosus* 30 | + | + | 2.0 - 5.0 | 1.0 - 1.8 | 68.0 |
| *C. m.* subsp. *nebraskensis* 2223 | + | + | 2.0 - 5.0 | 2.0 - 3.0 | 99.0 |
| *C. m.* subsp. *tessellarius* 2224 | + | + | 2.0 - 5.0 | 1.5 - 2.5 | 98.0 |
| *C. m.* subsp. *sepedonicus* 1522 | + | – | 0.5 | 0.0 | 0.0 |
| *C. m.* subsp. *sepedonicus* 2823 | + | – | 0.2 | 0.0 | 0.0 |
| *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* 2218 | + | – | 2.0 - 4.0 | 0.0 | 0.0 |

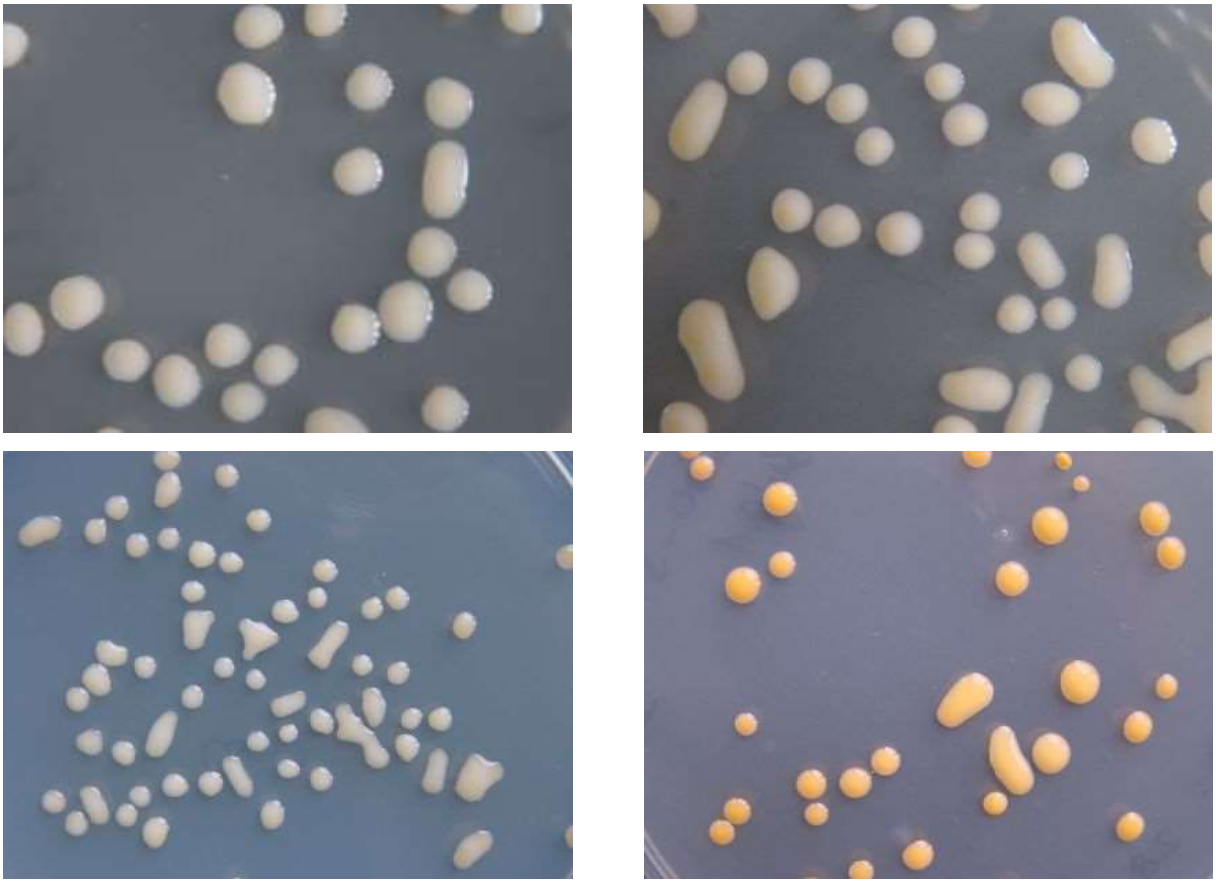

**Figure 9.** Growth of *Cmm* on the new medium BCT, colonies are brilliant, convex, and colour of *Cmm* strains ranges from white yellowish (white creamy) to yellow

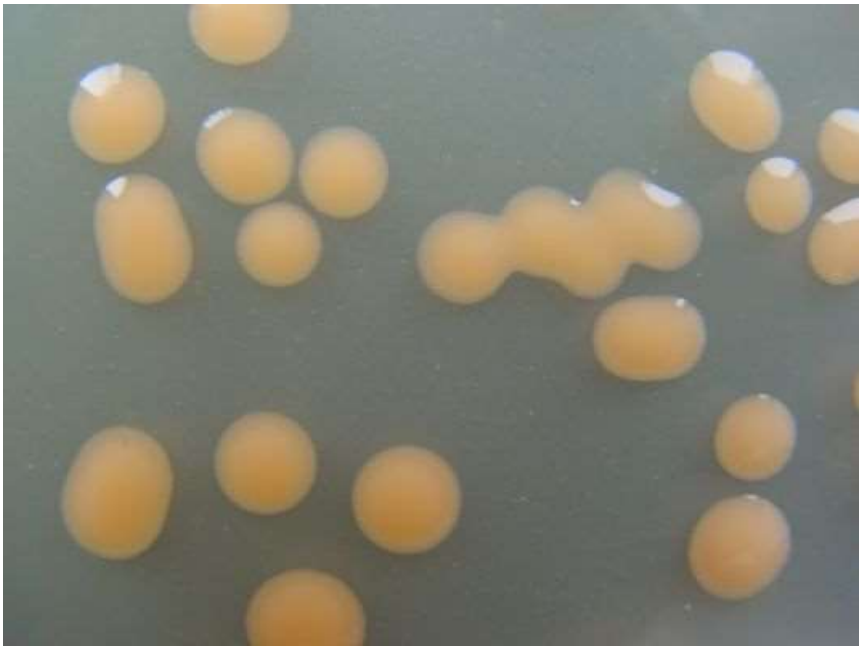

**Figure 10.** *C. m.* subsp. *tessellarius* GSPB 2224 on the new medium BCT.

**Figure 11.** *C. m.* subsp. *insidiosus* GSPB 30 on the new medium BCT.

**Modifications of the new media BCT and BCT-2**

The following modifications (Table 9) did not improve the new media (BCT & BCT-2):

- Replacement of Opus® Top by cycloheximide (BCT-2-C),
- Replacement of Opus® Top by nystatin (BCT-2-N),
- Replacement of Opus® Top by cycloheximide and higher concentrations of polymyxin B sulfate and nalidixic acid (BCT-M & BCT-2-M),
- Omission of polymyxin B sulfate (BCT-3),
- Omission of polymyxin B sulfate and higher concentration of trimethoprim (BCT-4),
- Omission of nalidixic acid and higher concentration of trimethoprim (BCT-5).

**Table 9.** Inhibition of saprophytic bacteria from naturally contaminated field tomato seeds and plants on the new media BCT and BCT-2 and several modifications of them

| Medium | Description | Inhibition (%)* | |
|---|---|---|---|
| | | Range | Mean |
| **BCT** | (As described on page 30) | 96.62 - 99.96 | 98.41 |
| **BCT-2** | (As described on page 30) | 97.97 - 99.98 | 99.02 |
| **BCT-M** | BCT with additional 5 mg/liter of each polymyxin and nalidixic acid, and Opus® Top was replaced by 100 mg/liter cycloheximide | 94.69 - 99.52 | 96.3 |
| **BCT-2-C** | BCT-2 but Opus Top was replaced by cycloheximide (100 mg/liter) | 95.6 - 99.41 | 96.86 |
| **BCT-2-N** | BCT-2 but Opus® Top was replaced by nystatin (10mg/liter) | 93.53 - 99.37 | 97.21 |
| **BCT-2-M** | BCT-2-C with additional 5 mg/liter of each polymyxin and nalidixic acid | 96.53 - 99.73 | 98.03 |
| **BCT-3** | BCT but without polymyxin B sulfate | 87.51 - 92.37 | 89.13 |
| **BCT-4** | BCT but without polymyxin and with additional 100 mg/liter Trimethoprim | 88.45 - 96.8 | 93.47 |
| **BCT-5** | BCT but without nalidixic acid and with additional 100 mg/liter trimethoprim | 97.16 - 97.47 | 96.93 |

*) **Inhibition of saprophytic bacteria indicates selectivity (%)** = [(Population of nontarget microbes on NGY - population of nontarget microbes on test medium) / population of nontarget microbes on NGY] × 100.

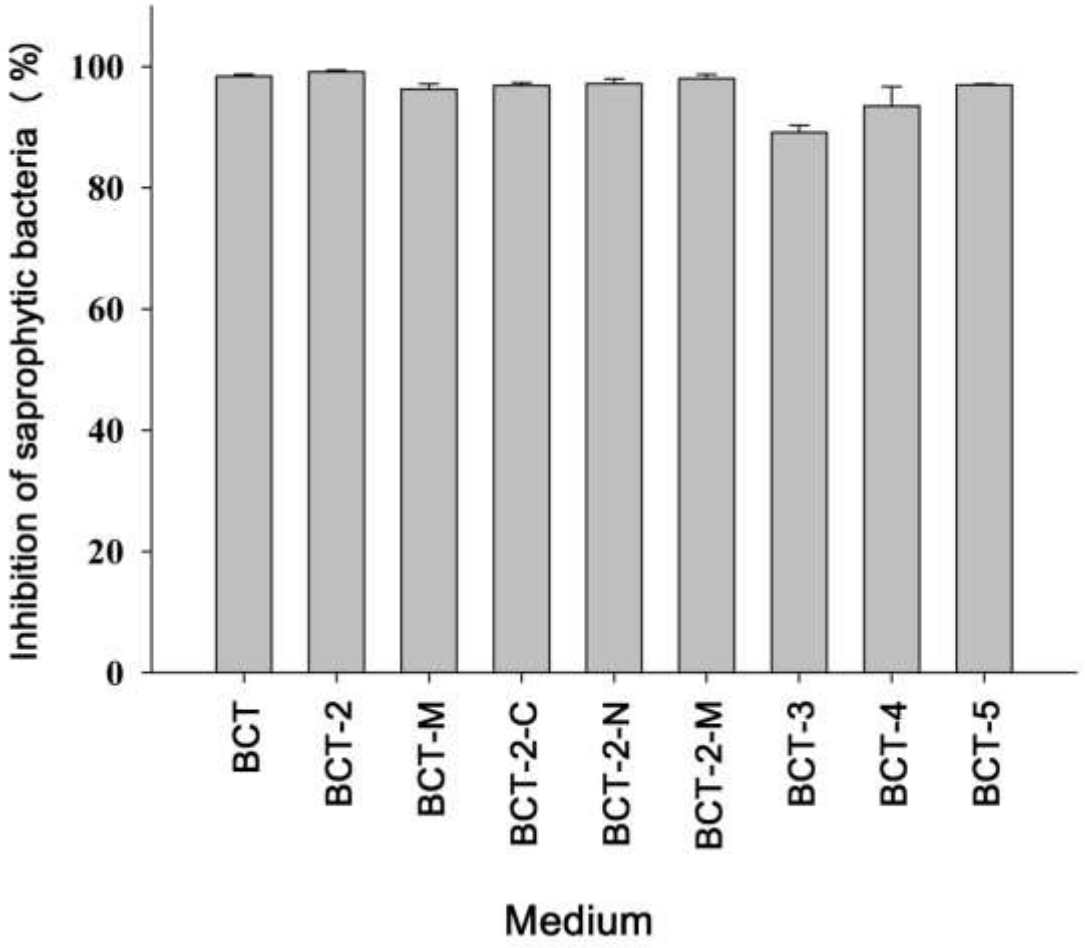


**Figure12.** Inhibition of saprophytic bacteria from naturally contaminated field tomato seeds and plants by several modifications of the new selective media **BCT and BCT-2**; **BCT-M** = BCT but with additional 5 mg nalidixic acid and 5 mg polymyxin B, Opus® Top was replaced by 100 mg/l cycloheximide; **BCT-2-C** = BCT-2 but with 100 mg/l cycloheximide instead of Opus® Top; **BCT-2-N** = BCT-2 but with 10 mg/l nystatin instead of Opus® Top; **BCT-2-M** = BCT-2-C with additional 5 mg nalidixic acid and 5 mg polymyxin B; **BCT-3** = BCT but without polymyxin B; **BCT-4** = BCT but without polymyxin B and with additional 100 mg trimethoprim.; **BCT-5** = BCT without nalidixic acid but with additional 100 mg Trimethoprim.

**Effect of the buffering system and other fungicides**

When the new medium BCT was modified to BCT-2 by increasing the buffering system, the resulting pH value was increased from 7 - 7.05 in BCT to 7.2 in BCT-2 resulting in increased selectivity from ~ 98% to ~ 99%, but the appearance of *Cmm* strains was delayed one day for each strain, except for strain Bulgarian-1 which was unable to recover on BCT-2.

Since Opus® Top has a certain general antimicrobial activity; addition of this fungicide improved the selectivity. By replacing this fungicide with either cycloheximide or nystatin which are contained in earlier used semiselective mediea, growth speed and recovery rates of *Cmm* increased (Fig. 13 & Table 10), but the **selectivity was reduced** (Fig. 12 & Table 9). According to our observations it is therefore not recommended to replace Opus® Top by other fungicides. If Opus® Top is not available it might be replaced by 100 mg/l cycloheximide (dissolved in MeOH, 100 mg/ml stock) or 10 mg/l nystatin (dissolved in MeOH, 10 mg/ml stock), However, in this case the whole buffering system should be adjusted as in BCT-2 (BCT-2-C & BCT-2-N), or the amounts of antibiotics should be higher than in BCT (as in BCT-M).

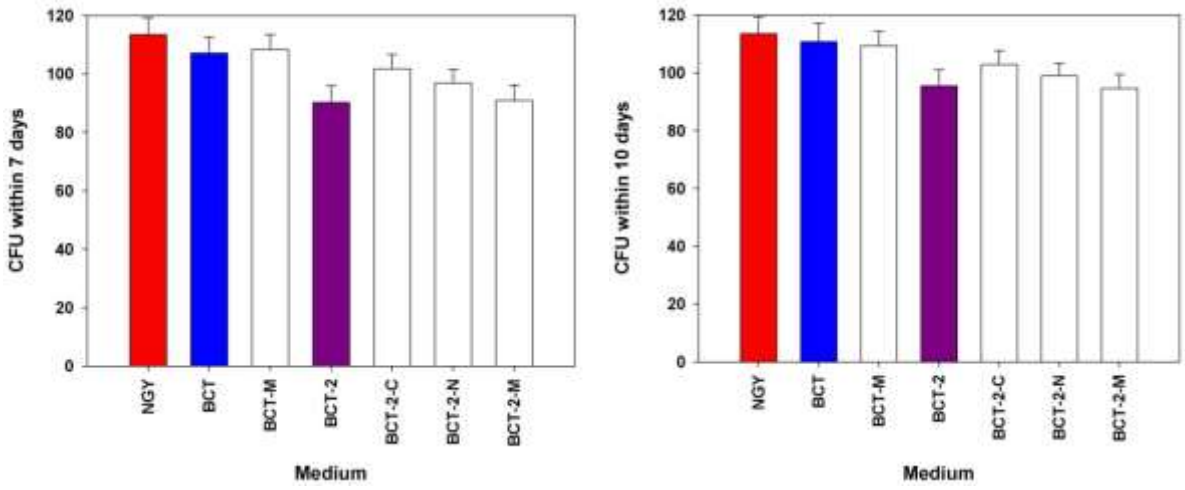


**Figure 13.** Recovery rates of 30 *Cmm* strains on NGY, the new selective media BCT and BCT-2, and several modifications of them. Results represent the mean of colony forming units recovered on each agar plate from 30 *Cmm* strains and three replicates for each strain.
**NGY:** nutrient broth-glucose-yeast extract agar;
**BCT-M:** like BCT, but with additional 5 mg/l each of nalidixic acid and polymyxin B, and Opus® Top was replaced by 100 mg/l cycloheximide;
**BCT-2-C:** like BCT-2 but Opus® Top was replaced by 100 mg/l cycloheximide;
**BCT-2-N:** like BCT-2 but Opus® Top was replaced by 10 mg/l nystatin;
**BCT-2-M**: Like BCT-2 but with additional 5 mg/l each of nalidixic acid and polymyxin B, and Opus® Top was replaced by 100 mg/l cycloheximide.

**Table 10.** Plating efficiency (%) of 30 *Cmm* strains on the new medium and different modifications after 7/ 10/ 14/ 20 days respectively, each value represents the mean of three replicates

| ***Cmm* strain** | **Semiselective media*** | | | | | |
|---|---|---|---|---|---|---|
| | **BCT** | **BCT-M** | **BCT-2** | **BCT-2-C** | **BCT-2-N** | **BCT-2-M** |
| Amb-1 | 117/ 117/ 117/ 117 | 58/ 103/ 103/ 103 | 61/ 94/ 94/ 95 | 77/ 93/ 93/ 93 | 109/ 123/ 123/ 123 | 1/ 30/ 46/ 46 |
| Ei-1 | 93/ 93/ 93/ 93 | 88/ 88/ 88/ 88 | 93/ 93/ 93/ 93 | 88/ 88/ 88/ 88 | 98/ 98/ 98/ 98 | 98/ 99/ 99/ 99 |
| Ei-2 | 107/ 107/ 107/ 107 | 103/ 103/ 103/ 103 | 105/ 105/ 105/ 105 | 107/ 107/ 107/ 107 | 93/ 93/ 93/ 94 | 84/ 84/ 84/ 84 |
| Lu-1 | 90/ 90/ 90/ 90 | 97/ 97/ 97/ 97 | 80/ 80/ 80/ 80 | 127/ 127/ 127/ 127 | 109/ 109/ 109/ 109 | 93/ 93/ 93/ 93 |
| MO-1 | 75/ 75/ 75/ 75 | 95/ 95/ 95/ 95 | 84/ 84/ 84/ 84 | 91/ 91/ 91/ 91 | 89/ 89/ 89/ 89 | 91/ 91/ 91/ 91 |
| MO-2 | 89/ 89/ 89/ 89 | 107/ 107/ 107/ 107 | 101/ 101/ 101/ 101 | 97/ 97/ 97/ 97 | 90/ 90/ 90/ 90 | 92/ 92/ 92/ 92 |
| Sc-2 | 110/ 110/ 110/ 110 | 109/ 109/ 109/ 109 | 106/ 106/ 106/ 106 | 112/ 112/ 112/ 113 | 107/ 109/ 109/ 109 | 123/ 123/ 123/ 123 |
| BO-RS | 92/ 93/ 93/ 93 | 130/ 130/ 130/ 130 | 82/ 83/ 83/ 83 | 107/ 107/ 107/ 107 | 102/ 105/ 105/ 105 | 92/ 94/ 94/ 94 |
| GSPB 2972 | 101/ 101/ 101/ 101 | 108/ 108/ 108/ 108 | 78/ 78/ 78/ 80 | 105/ 105/ 105/ 105 | 95/ 95/ 95/ 95 | 99/ 99/ 99/ 99 |
| AE-1 | 86/ 86/ 86/ 86 | 78/ 78/ 78/ 78 | 61/ 61/ 61/ 61 | 88/ 88/ 88/ 88 | 87/ 87/ 87/ 87 | 77/ 77/ 77/ 77 |
| AH-1 | 78/ 78/ 78/ 78 | 93/ 93/ 93/ 93 | 84/ 84/ 84/ 84 | 85/ 85/ 85/ 86 | 96/ 96/ 96/ 97 | 95/ 95/ 95/ 95 |
| ES-1 | 90/ 90/ 90/ 90 | 118/ 118/ 118/ 118 | 78/ 79/ 79/ 79 | 105/ 105/ 105/ 105 | 103/ 104/ 104/ 104 | 98/ 98/ 98/ 98 |
| HH-1 | 101/ 101/ 101/ 101 | 83/ 83/ 83/ 83 | 95/ 95/ 95/ 95 | 86/ 86/ 86/ 86 | 69/ 70/ 70/ 70 | 92/ 92/ 92/ 92 |
| La-1 | 71/ 71/ 71/ 71 | 80/ 80/ 80/ 80 | 62/ 62/ 62/ 62 | 72/ 72/ 72/ 73 | 74/ 74/ 74/ 74 | 81/ 81/ 81/ 81 |
| OS-1 | 95/ 95/ 95/ 95 | 86/ 86/ 86/ 86 | 87/ 87/ 87/ 87 | 91/ 91/ 91/ 91 | 84/ 84/ 84/ 85 | 98/ 98/ 98/ 98 |
| OS-2 | 65/ 65/ 65/ 65 | 75/ 75/ 75/ 75 | 68/ 68/ 68/ 68 | 79/ 79/ 79/ 79 | 79/ 79/ 79/ 79 | 71/ 71/ 71/ 71 |
| OS-4 | 96/ 96/ 96/ 96 | 95/ 95/ 95/ 95 | 77/ 77/ 77/ 77 | 90/ 90/ 90/ 90 | 84/ 84/ 84/ 84 | 85/ 85/ 85/ 85 |
| GSPB 378 | 112/ 112/ 112/ 112 | 100/ 100/ 100/ 100 | 75/ 75/ 75/ 75 | 97/ 97/ 97/ 97 | 93/ 93/ 93/ 93 | 74/ 85/ 85/ 85 |
| GSPB 382 | 87/ 98/ 98/ 98 | 125/ 125/ 125/ 125 | 35/ 85/ 85/ 90 | 126/ 126/ 126/ 126 | 122/ 122/ 122/ 123 | 90/ 90/ 90/ 90 |
| GSPB 390 | 84/ 84/ 84/ 84 | 106/ 106/ 106/ 106 | 84/ 84/ 84/ 84 | 98/ 98/ 98/ 98 | 94/ 94/ 94/ 94 | 87/ 87/ 87/ 87 |
| GSPB 392 | 99/ 99/ 99/ 99 | 87/ 87/ 87/ 87 | 93/ 93/ 93/ 94 | 120/ 120/ 120/ 120 | 93/ 93/ 93/ 93 | 105/ 105/ 105/ 105 |
| Bulgarian 1 | 94/ 112/ 112/ 112 | 102/ 104/ 104/ 104 | 0/ 0/ 0 / 0 | 69/ 81/ 81/ 81 | 59/ 71/ 73/ 73 | 0/ 59/ 69/ 71 |
| GSPB 2973 | 78/ 84/ 84/ 84 | 84/ 84/ 84/ 84 | 22/ 80/ 80/ 80 | 86/ 86/ 86/ 86 | 91/ 91/ 91/ 91 | 99/ 101/ 101/ 101 |
| GSPB 2315 | 100/ 100/ 100/ 100 | 96/ 96/ 96/ 98 | 108/ 108/ 108/ 108 | 108/ 108/ 108/ 109 | 120/ 120/ 120/ 120 | 104/ 104/ 104/ 104 |
| GSPB 2221 | 106/ 106/ 106/ 106 | 96/ 96/ 96/ 96 | 96/ 96/ 96/ 96 | 109/ 109/ 109/ 109 | 100/ 100/ 100/ 100 | 98/ 98/ 98/ 98 |
| GSPB 2222 | 79/ 79/ 79/ 79 | 83/ 83/ 83/ 83 | 62/ 62/ 62/ 62 | 91/ 91/ 91/ 92 | 77/ 77/ 77/ 77 | 72/ 72/ 72/ 72 |
| 399 | 84/ 84/ 84/ 84 | 80/ 82/ 83/ 83 | 87/87/ 90/ 90 | 2/ 6/ 12/ 12 | 4/ 15/ 29/ 29 | 0/ 2/ 2/ 2 |
| GSPG 3133 | 88/ 88/ 88/ 88 | 101/ 101/ 101/ 101 | 113/ 113/ 113/ 135 | 115/ 115/ 115/ 115 | 94/ 94/ 94/ 98 | 99/ 99/ 99/ 99 |
| 185 | 109/ 109/ 109/ 109 | 101/ 101/ 101/ 101 | 83/ 84/ 84/ 84 | 106/ 106/ 106/ 107 | 97/ 97/97/ 97 | 97/ 98/ 98/ 98 |
| Leningrad 3 | 108/ 108/ 108/ 109 | 97/ 97/ 97/ 97 | 111/ 111/ 111/ 111 | 111/ 111/ 11/ 111 | 100/ 100/ 100/ 101 | 90/ 90/ 90/ 90 |

*) see footnotes from Figure 13

**Effect of the pH value on *Cmm* growth**

The pH value inside tomato fruits is normally very low (between 4 and 3 or less). We found out that *Cmm* can survive inside infected fruits for many months, even when the fruits were harvested and stored at 4 °C. However, on several agar media, growth of *Cmm* was decreased by lower pH values (Figure 14) and *Cmm* colonies were smaller in diameter. We also found that during fermentation of tomato seeds for 72 or 96 h inside the pulp of tomato fruits, *Cmm*-cells did not survive in the pulp of tomato fruits, whereas populations of some other saprophytic bacteria increased in fermented pulp with a low pH-value. Therefore, it appeared senseless to further improve the new selective medium by lowering its pH value. When the pH-value was lower than 5.0, the agar media were semi-solid and not suited for streaking of plant and seed extracts as well as for determining bacterial growth.

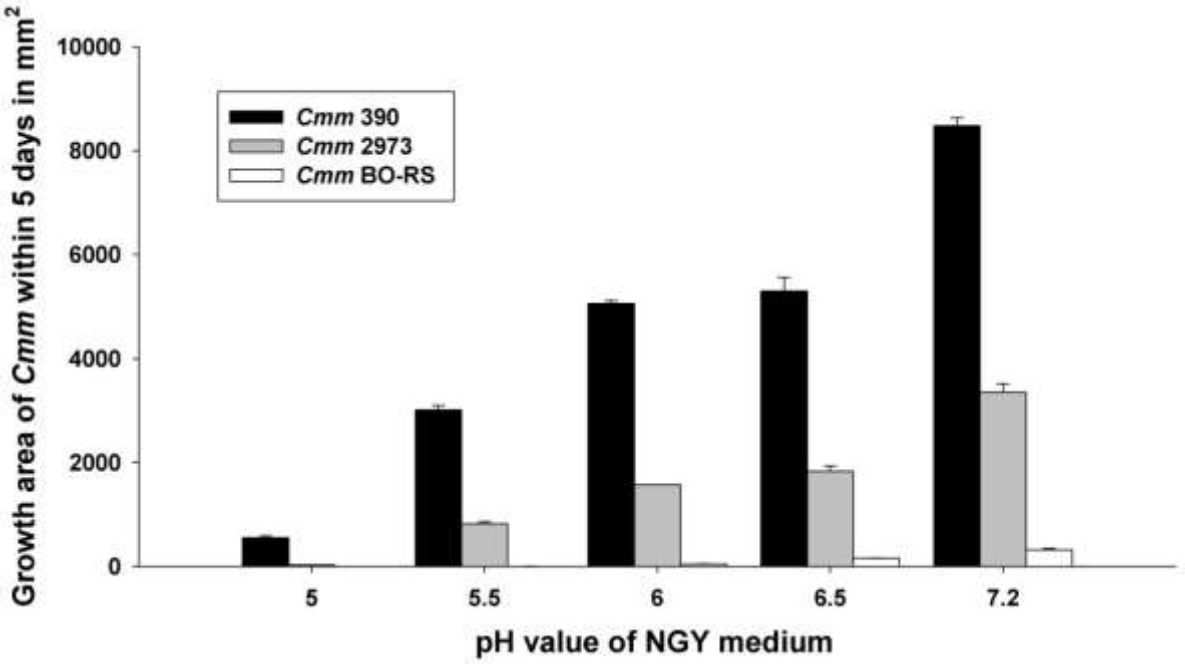


**Figure 14.** Effect of different pH values on growth of three *Cmm* strains on the NGY medium.

**Effect of fruit juice**

Several experiments were conducted trying to develop a semiselective medium on the basis of host plant extracts, such as tomato juice. We found that on media prepared only of agar and juice from tomato fruits (100 ml/l), without further additions, *Cmm* could grow very well. In other compositions of agar and tomato juice, with or without mannit, with yeast extract and antibiotics, we noticed that the delayed growth of *Cmm* by antibiotics was reduced by addition of tomato juice and that *Cmm* colonies appeared faster when juice was added to these compositions. Nevertheless, we stopped completing these experiments by further modifications, because tomato juice is not always available as a standardized product, can vary considerably depending on the source of the tomatoes and the procedure to produce the juice, and tomato juice might also be a very good nutrient source for other accompanying bacteria.

**Selection of a fungicide**

During our investigations it was often noticed that while testing other semiselective media for *Cmm* that contained cycloheximide or nystatin with homogenates from field plants allowed fungal growth. Therefore, 29 fungicides were tested for inhibition of these fungi (Table 11). Results showed that Opus® Top was very effective against the two most often appearing fungi and did not inhibit *Cmm*.

In addition, Opus® Top showed antimicrobial effects against some accompanying saprophytic bacteria. However, Opus® Top may act toxically against *Cmm* when it is added to other semiselective media (for example when we replaced cycloheximide by Opus® Top in mSCM medium) due to some unknown interactions between Opus® Top and basal components of such media. Thus, *Cmm* was completely inhibited, when cycloheximide was replaced by Opus® Top in case of mSCM medium. However, we did not record toxical interactions when Opus® Top was added to NGY medium or to the new selective media BCT and BCT-2.

In further experiments, Opus® Top was tested in compositions with several antibiotics. The optimal concentration of Opus® Top was 50 µl/L in the new media BCT and BCT-2. We added Opus® Top as a diluted stock solution (50 µl/ml sterile water stock) to facilitate the addition of the exact amount of Opus® Top, because it is a thick product and cannot be filter-sterilized.

**Table 11.** Fungicides tested against the two most prevalent appearing fungi

| Commercial name | Active ingredient | content g/l | Inhibition of [*] | |
|---|---|---|---|---|
| | | | Fungus 1 | Fungus 2 |
| Acrobat plus | Dimethomorph/ Mancozeb | 90 / 600 | + | – |
| Afugan | Pyrazophos | 293 | + | – |
| Amistar | Azoxystrobin | 250 | + | – |
| Bayfidan | Triadimenol | 250 | + | + |
| Calixin | Tridemorph | 750 | + | + |
| Capitan | Flusilazol | 250 | + | + |
| Cercobin- M | Thiophanate | 70% | + | + |
| Corbel | Fenpropimorph | 750 | + | + |
| Daconil 2787 extra | Chlortalonil | 500 | + | + |
| Derosal | Carbendazim | 360 | + | + |
| Desmel | Propiconazol | 250 | – | + |
| Euparen WG | Dichlofluanid | 50% | + | + |
| Folicur | Tebuconazole | 250 | + | + |
| Fortress | Quinoxyfen | 500 | + | – |
| Harvesan | Flusilazol / Carbendazim | 250 / 125 | + | + |
| Juwel Top | Epoxiconazol/ Fenpropimorph/ Kresoximmethyl | 125 / 150/ 125 | + | + |
| Maneb80 Spritzpulver | Maneb | 80% | + | + |
| Milgo | Ethirimol | 280 | + | – |
| Opus Top | Epoxiconazol / Fenpropimorph | 84 / 250 | + | + |
| Previcur N | Proparmocarb hyd. | 722 | + | – |
| Ronilan fl. | Vinclozolin | 500 | + | – |
| Saprol Neu | Triforin | 190 | – | – |
| Shirlan | Fluazinam | 500 | – | – |
| Simbo | Propiconazol / Fenpropimorph | 125 / 300 | + | + |
| Sportak alpha | Prochloraz / Carbendazim | 300 / 80 | + | + |
| Sportak delta | Prochloraz / Cyproconazol | 360 / 48 | + | + |
| Sumisclex WG | Procymidone | 50% | + | + |
| Taspa | Propiconazol / Difenoconazol | 250 / 250 | + | + |
| Verisan | Iprodion | 260 | + | + |

[*)] + = Inhibitory effect; – = no inhibitory effect

## Discussion

In recent years, substantial economic losses in commercial tomato cultures caused by *Clavibacter michiganensis* subsp. *michiganensis*, the incitant of bacterial canker and wilt, increased worldwide (Gleason *et al.*; 1993). High yielding resistant tomato cultivars are not available (Boelema, 1980; Coaker *et al.*, 2004; Poysa, 1993; van Steekelenburg, 1985), and during tomato cultivation the disease cannot be controlled by effective chemicals. Therefore, hygienic measures are most important for controlling bacterial wilt.

Depending on weather conditions or the micro-climate in greenhouses, plant residues in the soil may be a source of the primary inoculum (Kleitman *et al.*, 2008). In most cases, however, transmission by soil appears to be of minor importance (Ftayeh, 2004; Ftayeh *et al.*, 2004). Thus, recent outbreaks of the disease in greenhouses happened even in tomato cultures on sterilized artificial substrate (Ftayeh and Maeritz, person. observations). Therefore, a high effort to detect the pathogen in seeds and transplants is required (ISHI, 2008; Olivier *et al.*, 2009; Werner *et al.*, 2002). In most cases, the primary infections appeared to originate from infected seeds or transplants. For instance, in one greenhouse in Germany with tomato hydro culture the disease was observed in 2006 at first three months after transplanting. In the beginning only 5 tomato plants showed the disease within a total of 25,000 plants (infection ratio 0.02%). Because infected and neighbouring plants were eradicated and hygienic rules were strictly observed disease incidence could be kept less than 10% until the end of the season. In another greenhouse with hydro cultivation, the disease started by 5 primary infected plants within 13,000 (infection ratio of 0.038%) at the beginning of April. Because hygienic measures were not realized there, 80% of all plants were absolutely wilted in the mid of October, and all other plants showed strong wilt symptoms. Although the necessity of hygienic measures in tomato cultures under glass should not be neglected, there exists an urgent demand for absolutely pathogen-free tomato seeds and tomato plantlets.

Thus, plant protection inspectors confirmed that disease incidence in hydro-cultures with artificial substrates was often more destructive than in traditional soil-cultivations, although hydro-cultures are organized very professionally. Infected plants monitored during 2007 in some locations with no previously disease occurrence were obtained from tomato seeds and planting material which had been previously tested according to the recommended protocol by EPPO (OEPP/EPPO, 2005), indicating that the detection protocol was not reliable (Weber and Fuchs, personal commun.; IPPC, 2007).

Therefore, a high effort in this research project dealt with developing a new selective medium which allows a very sensitive detection of infected seeds or plantlets. At first, all the semiselective media recommended for *Cmm* to our knowledge were scrutinized thoroughly (although some of them are not used anymore today). Our investigations revealed that all these media proved to be not satisfactory for a sensitive detection of *Cmm* in infected tomato plants and seeds.

Some semiselective media such as mSCM, mCNS and the recent one suggested by EPPO, are not suited for sufficient growth of many *Cmm* strains. These media are rather toxic than selective, and the growth of many *Cmm* strains is time consuming. The media appear to be selective in the beginning, but after about days 7 days of incubation, non-target bacteria start to grow even before the growth of target *Cmm*-bacteria starts. Thus, measuring the selectivity of these media was performed 10 days after plating, since *Cmm* strains needed at least this time to be identified, if they grew at all.

Except for the SCM medium, all semiselective media developed for *Cmm* were not studied thoroughly on selectivity and plating efficiency. In fact, some semiselective media which were suggested for *Cmm*, such as the EPPO medium, mCNS and mSCM were more or less toxic to many *Cmm* strains. Other media, such as SCM, CMM1, KBT, D2 and D2ANX showed a high plating efficiency but a low selectivity. The media mSCM, SCM and D2ANX are the most cited and applied ones for detection of *Cmm*, whereas KBT, D2, mCNS are very seldom used or not used at all today.

Our results concerning the plating efficiency of the medium SCM are similar to those of Fatmi and Schaad (1998). These authors found that the inhibition of accompanying bacteria on SCM was more than 98%, and we obtained similar results with some plant samples (Table 7), but with other plant samples inhibition of accompanying bacteria was only 79.9%. We noted a similar effect for all semiselective media tested, because different seed or plant samples may also harbour a different diversity of accompanying bacteria.

Many semiselective media, such as mSCM, mCNS, D2ANX, KBT and EPPO were not evaluated by the original authors on plating efficiency for *Cmm*, but other authors confirmed our results in this respect. Thus, Hadas *et al.* (2005) obtained similar results, since some of their *Cmm*-strains tested were not able to grow on D2ANX, CNS, or mSCM, and other *Cmm*-strains grew with very low plating efficiency. Recently, Koenraadt *et al.* (2009) reported that antagonistic bacteria occurring in tomato seed extracts seriously hampered the recovery of

*Cmm* on the semiselective media D2ANX, CMM1, SCM and mSCM.

In summary, some of the previous developed semiselective media for *Cmm* allowed high plating efficiency, such as the media D2, KBT, SCM, CMM1 and D2ANX, but these media showed a very low selectivity level, so that detection and growth of *Cmm* was inhibited by the overgrowth of accompanying bacteria. In case of high inhibition of the accompanying bacteria on semiselective media, such as mSCM, EPPO, and mCNS, this feature was due to a general toxicity, so that also many *Cmm*-strains could not grow on these media. Thus, a sensitive detection with a very low threshold (Hadas *et al.*, 2005) was impossible with any of these earlier developed semiselective media.

Therefore, we always tested newly designed diverse semiselective media in plant homogenates containing high concentrations of saprophytic accompanying bacteria but only very few cells of *Cmm.* By following this strategy we finally could develop media with high inhibition of accompanying bacteria but simultaneously allowing high growth speed and plating efficiency of *Cmm*.
Developing a selective medium for *Cmm* was difficult because in nearly all cases some of the diverse accompanying bacteria existing with tomato seeds and plants showed higher tolerance towards inhibitors or antibiotics than *Cmm.* On the other side, also *Cmm*-strains differed considerably in sensitivity to inhibitors. Further difficulties arose because *Cmm*-strains differed in growth ability on nutrient media.

Development of absolute synthetic selective media for *Cmm* was impossible because of its partial fastidious nature. Thus, complete omission of yeast extract was impossible. Unlike previous semiselective media for *Cmm,* the new medium BCT contains D(-)-mannit which is more selective than glucose or sucrose. Mannose is also selective but it does not support growth of *Cmm*. These results are similar to those of De la Cruz (1990) and Jansing and Rudolph (1998) in case of *C. michiganensis* subsp. *sepedonicus*. Similarly to the MTNA medium (Jansing and Rudolph, 1998), our new selective media contain the antibiotic **trimethoprim**, as was reported by Ftayeh *et al.* (2008a). Later on, Koenraadt *et al.* (2009) confirmed that trimethoprim was well suited in semiselective media for detection of *Cmm*.

We excluded **sodium azide** because we found it to be toxic to *Cmm* when combined with other inhibitors. **Lithium chloride** delayed growth of *Cmm,* which was also reported for *C. m.* ssp. *nebraskensis* by Smidt and Vidaver (1986). Amendment of the new medium with **potassium tellurite** which was used in the SCM medium was not preferable, because it

caused a gray to black colour of *Cmm* colonies as well as colonies of saprophytic bacteria making it impossible to distinguish between *Cmm* and accompanying bacteria.

Kaup *et al*. (2005) identified the enzyme tomatinase in *Cmm* (NCPPB 382) which deglycosylates α-tomatine to tomatidine. Therefore we tried to develop a semiselective medium containing **α-tomatine** which has antimicrobial activity and could only be utilized by *Cmm.* However, this approach was not successful, because tomatine failed to be filter-sterilized and was contaminating the medium. Possibly, we did not follow this strategy thoroughly enough and it should be further investigated in future studies.

The new medium BCT (with pH value of 7.00 - 7.05) was modified to **BCT-2** which is more basic and has a pH value of 7.2. BCT-2 showed a higher selectivity than BCT, but one *Cmm* strain (Bulgarian-1) did not grow at all, and all *Cmm* strains grew with one day delay compared to BCT. We recommend use of BCT because it possesses acceptable selectivity, offers high plating efficiency and fast growth of *Cmm* and also allows high detection sensitivity. The medium BCT-2 was modified further by replacing the fungicide Opus® Top with cycloheximide or nystatin. Suggested different compositions of BCT-2 with cycloheximide or nystatin could be useful when the contamination with other saprophytic bacteria is low. Therefore, one of these other compositions could be used parallely with the BCT medium. BCT, BCT-2 and the different new modifications might be useful for application or development of new semiselective media suited for other subspecies of *Clavibacter michiganensis*. Although Opus® Top has antimicrobial effect, it should not be added to other basal media, without testing its possible toxical effect against different *Cmm* strains due to some interactions with media's components. Thus, Opus® Top prevented growth of some *Cmm* strains when it was used instead of cycloheximide in mSCM medium.

On the new media BCT and BCT-2, colour of *Cmm* colonies ranges from white creamy to yellow, brilliant and slimy. *Cmm*-colonies are easily distinguished from saprophytes once they have increased size by time, while saprophytic bacteria remain smaller, well inhibited and are mostly white in colour (Figure 7). In contrast, on other semiselective media *Cmm* colonies could not always be distinguished from contaminants, since certain *Cmm* strains do not always show the typical morphology as has been described on those media, so that some contaminants seem to be similar to *Cmm*.

Because the new media BCT and BCT-2 proved to be superior in selectivity, sensitivity and reliability for detecting *Cmm* in seeds and plant material compared to all published semiselective media, the new media BCT & BCT-2 were appropriately denoted as **selective media.**

To our knowledge, no other researcher has reported similar experiments in terms of detecting *Cmm* in plant homogenates or seed extracts occurring in low concentrations in the presence of high concentrations (up to thousand-fold more) of saprophytic bacteria (Table 7 & Figure 8).

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# Chapter 2

## Establishment of a Bio-PCR assay for a sensitive detection of Clavibacter michiganensis subsp. michiganensis in seed and plant material


## Summary

A Bio-PCR protocol for a highly sensitive detection of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), the causal agent of bacterial canker of tomato (*Solanum lycopersicum*), was developed. The protocol is based on the enrichment of viable cells of the target bacterium by plating seed or plant extracts on the newly developed selective medium BCT. Grown up cells are directly used as template for PCR detection.

However, the PCR primer systems for *Cmm* published by: Dreier *et al.* (1995); Pastrik and Rainey (1999); Sousa-Santos *et al.* (1995); and Kleitman *et al.* (2008) proved to be not satisfactory in our study, because several *Cmm*-strains were not amplified (false negative) in some cases, or cross-reactions (false positive results) appeared in other cases with several associated bacterial species that may exist with tomato plants and seeds, such as *Pectobacterium carotovorum* subsp. *carotovorum; Pseudomonas fluorescens, Pseudomonas syringae* pv. *syringae, P. syringae* pv. *tomato, Xanthomonas campestris* pv. *vesicatoria,* and diverse species of saprophytic bacteria.

Therefore, two new primer sets "**B-rev-CM/B-fw-PCM**" and "**L-fw-CM/L-rev-PCM**" were deduced and converted from TaqMan PCR into classical PCR protocols. The new primer pairs showed to be specific for detecting *Cmm* and amplified all the 76 *Cmm* strains tested without any exception. These *Cmm*-strains had been obtained from different origins and included virulent, hypovirulent and avirulent isolates.

This new Bio-PCR protocol allowed a sensitive detection of very small *Cmm* cell numbers in plant homogenates (12 cfu or less/agar plate), although the population of saprophytic bacteria was very high ($2 \times 10^6$ - $2 \times 10^7$ cfu/agar plate). Furthermore, a reliable detection was possible within short time (4-5 days). In contrast, Bio-PCR-detection of these small numbers of *Cmm* cells occurring together with very high numbers of saprophytic bacteria was impossible on most of the earlier published semiselective media or required much longer time (10 days or

more).

Only viable cells of *Cmm* that exist in plant and seed extracts are detected. There is no need for DNA extraction. The effect of PCR inhibitors present in seeds and plant extracts is avoided and further complementary tests such as pathogenicity or biochemical tests to determine the identity of the pathogen are limited or not required.

Because the new Bio-PCR protocol improves the detection reliability and sensitivity and also reduces the time for *Cmm*-detection significantly, the protocol appears to be very useful for seed health certifications and for testing asymptomatic tomato plants for latent infection by *Cmm*.

# Introduction

The phytopathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is classified as an A2 quarantine organism according to the European Plant Protection Organisation (EPPO). *Cmm* causes bacterial canker of tomato, which is considered as one of the most serious bacterial diseases of tomato plants worldwide. As few as 0.01 - 0.05% contaminated seeds or transplants can cause an epidemic under suitable conditions (Chang *et al.*, 1991). Therefore, very strict international quarantine regulations have been issued for controlling the trade of commercial seeds that can be infested with such quarantine organisms.

Molecular-based methods such as PCR or Real-Time PCR are widely used today for detection of plant pathogens and have greatly improved detection of bacteria in environmental samples. Classical and Real-Time PCR protocols are available for many different bacteria (Alvarez, 2004; Schaad *et al*., 2001; Schaad *et al.*, 2003). These protocols can be very specific, but the **sensitivity** of PCR is not high enough when compared to agar plating assays. Several factors may limit the detection by PCR, especially inhibitors often present in plant samples (Nabizadeh-Ardekani, 1999; Prosen *et al*., 1993; Rossen *et al.*, 1992; Schaad *et al.*, 1999), or a relatively low sensitivity due to extremely small sample size requirements (Schaad *et al.*, 2007; Weller *et al.*, 2000a and 2000b).

The sensitivity of PCR technique can be improved 10 to 100 fold by combination with plating assays on selective media (Bio-PCR). In this way, viable cells of the target bacterium are enriched on nutrient agar media and detected in extremely low levels in seeds and other propagative materials (Ito *et al.*, 1998; Schaad *et al.*, 1995; Schaad & Frederick, 2002; Schaad *et al.*, 2007).

Bio-PCR considerably increases sensitivity by detecting very small bacterial cell numbers that exist in asymptomatic plant tissues, also in the presence of numerous other microorganisms (Schaad *et al.*, 2007; Schaad *et al.*, 1999). Additional advantages of Bio-PCR assays are: minimizing the effect of plant inhibitors, DNA extraction is not required, and the need for further complementary tests such as pathogenicity or biochemical tests to determine the identity of the pathogen is limited.

PCR and Real-time PCR protocols are widely applied and available for most plant bacterial pathogens, but until recently Bio-PCR assays were applied only for very few phytopathogenic bacterial species, such as *Clavibacter michiganensis* subsp. *sepedonicus* (Schaad *et al.*, 1999),

*Pseudomonas syringae* pv. *phaseolicola* (Schaad *et al.*, 2007), *Ralstonia solanacearum* (Ito *et al.*, 1998; Weller *et al.*, 2000a and 2000b), *Agrobacterium tumefaciens* (Weller and Stead, 2002), *Acidovorax avenae* subsp. *citrulli* (Randhawa *et al.*, 2001), *Xanthomonas albilineans* (Wang *et al.*, 1999).

For detection of *Clavibacter michiganensis* subsp. *michiganensis* by Bio-PCR, Burokiené published a paper in 2006. However, these results cannot be utilized in practice for detection of *Cmm*, because Burokiené used a combination of non-selective media and PCR for detecting *Cmm* in very young plants that were artificially inoculated with high *Cmm* concentrations ($10^8$ cfu/ml). These plants were probably only slightly contaminated with accompanying microorganisms.

Although the international quarantine regulations for seed trade restrict the occurrence of *Cmm* in tomato seeds and transplants for import and export to zero tolerance for the EU, Canada, the USA (Bach *et al.*, 2003) and many other countries, increased outbreaks of bacterial canker of tomato were recently recorded in several European countries, including Austria, Belgium, Czech Republic, France, Netherlands, Serbia, Slovakia, Slovenia and Spain, and in some more locations world-wide (CABI/EPPO, 2009). Obviously, the current applied detection protocols for *Cmm* used for issuing seed heath certificates were not effective to prevent further distribution of the pathogen into new areas. Therefore, questions arose about the reliability of the diagnostic protocols that have been described for *Cmm.* Thus, the urgent need for highly sensitive detection methods of *Cmm* in order to prevent any further distribution of the pathogen *via* infested seeds and young plants became obvious. As pointed out by Louws *et al*. (1999), most sampling protocols cannot detect an infestation or infection at a threshold of one seed in 10,000, but such a threshold still represents an economic risk.

The objective of our investigations was to significantly improve the detection protocols for *Cmm* which have been recommended by EPPO (OEPP/EPPO, 2005). The aim was to develop a new protocol for detecting *Cmm* in seed lots and asymptomatic plant tissues. This protocol should be applicable in a routine laboratory test for a highly sensitive detection of *Cmm* and for issuing reliable seed health certificates.

# Materials and Methods

## Bacterial cultures and growth conditions

Hundred bacterial isolates of different species and origins as listed in Table 1 were tested, the including 76 *Cmm* strains and 24 strains of other bacterial species. Most of the *Cmm* strains were self-isolated from different locations in Germany or Syria and identified microscopically by cell shape, size and mobility as well as by biochemical tests, PCR and pathogenicity tests. Additional *Cmm* strains were obtained from other bacterial collections.

Pseudomonads were grown on King's medium B (King *et al.*, 1954) and incubated at 26 °C for 24 h, whereas all other bacterial strains were grown on NGY medium and incubated for 24-72 h at 26 °C. The NGY medium (Mavridis, personal commun.) contains: 0.8% nutrient broth (Roth, Karlsruhe, Germany), 1% glucose (AppliChem, Darmstadt, Germany) and 0.3% yeast extract (Roth). Bacteria were stored on NGY or KB plates at 4 °C for short periods and at -80 °C in 20% glycerol for long time storage.

For DNA isolation a typical single colony of each bacterial strain was suspended in test-tubes filled with 12 ml of Rhodes liquid medium (Rhodes, 1959). Test-tubes were incubated overnight in a circular shaker at 20-26 °C and 1-1.5 ml bacterial suspensions were sedimented by centrifugation. Sedimented cells were used for DNA isolation directly or stored at -20 °C until the extraction date.

## DNA extraction

DNA of both Gram-positive as well as Gram-negative bacteria was extracted from *in-vitro*-grown pure bacterial cultures with the MasterPure™ Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). Extracted DNA was resuspended in TE buffer and stored at -20 °C.

Concentrations of DNA were assessed after standard gel electrophoresis (1.2% w/v of agarose dissolved in 0.5% TBE-Puffer, pre-stained with 0.3 µg/ml ethidium bromide, 3V/cm, 120 min) in comparison with different concentrations of Lambda DNA (Fermentas, St. Leon-Rot, Germany). For routine PCR, working DNA-dilutions of 4 ng/ µl were prepared and stored at -20 °C.

**Table 1.** Source of bacterial species and isolates used in this study

| No. | Bacterial species/ pathovar | GSPB[a] | Designation/ Collect. no.[b] | Origin[c] | Isolation date | Host | Isolated by[d] |
|---|---|---|---|---|---|---|---|
| 1 | *Clavibacter m.* subsp. *michiganensis* | 3199 | Amb-1 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 2 | *Clavibacter m.* subsp. *michiganensis* | … | Bo-1 | Germany, NR | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 3 | *Clavibacter m.* subsp. *michiganensis* | … | Bo-2 | Germany, NR | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 4 | *Clavibacter m.* subsp. *michiganensis* | … | Bo-3 | Germany, NR | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 5 | *Clavibacter m.* subsp. *michiganensis* | … | Bo-4 | Germany, NR | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 6 | *Clavibacter m.* subsp. *michiganensis* | … | Deg-1 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 7 | *Clavibacter m.* subsp. *michiganensis* | … | Deg-2 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 8 | *Clavibacter m.* subsp. *michiganensis* | … | Deg-3 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 9 | *Clavibacter m.* subsp. *michiganensis* | … | Deg-4 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 10 | *Clavibacter m.* subsp. *michiganensis* | … | Deg-5 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 11 | *Clavibacter m.* subsp. *michiganensis* | 3200 | Ei-1 | Germany, NR | 2007 | *Solanum lycopersicum* | R. Ftayeh |
| 12 | *Clavibacter m.* subsp. *michiganensis* | … | Ei-2 | Germany, NR | 2007 | *Solanum lycopersicum* | R. Ftayeh |
| 13 | *Clavibacter m.* subsp. *michiganensis* | … | Ei-3 | Germany, NR | 2007 | *Solanum lycopersicum* | R. Ftayeh |
| 14 | *Clavibacter m.* subsp. *michiganensis* | … | GL-1 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 15 | *Clavibacter m.* subsp. *michiganensis* | … | GL-2 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 16 | *Clavibacter m.* subsp. *michiganensis* | … | JBL-1 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 17 | *Clavibacter m.* subsp. *michiganensis* | … | KBL-1 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 18 | *Clavibacter m.* subsp. *michiganensis* | … | Kn-4 | Germany, KL | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 19 | *Clavibacter m.* subsp. *michiganensis* | 3201 | Lu-1 | Germany, KL | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 20 | *Clavibacter m.* subsp. *michiganensis* | … | Me-1 | Germany, KL | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 21 | *Clavibacter m.* subsp. *michiganensis* | 3202 | Mo-1 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 22 | *Clavibacter m.* subsp. *michiganensis* | … | Mo-2 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 23 | *Clavibacter m.* subsp. *michiganensis* | … | Mo-3 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 24 | *Clavibacter m.* subsp. *michiganensis* | … | Mo-4 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 25 | *Clavibacter m.* subsp. *michiganensis* | … | Mo-5 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 26 | *Clavibacter m.* subsp. *michiganensis* | … | Mo-6 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 27 | *Clavibacter m.* subsp. *michiganensis* | … | Mo-7 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 28 | *Clavibacter m.* subsp. *michiganensis* | … | Mo-8 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 29 | *Clavibacter m.* subsp. *michiganensis* | … | Ok-1 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 30 | *Clavibacter m.* subsp. *michiganensis* | … | Ru-1 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 31 | *Clavibacter m.* subsp. *michiganensis* | … | Ru-2 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 32 | *Clavibacter m.* subsp. *michiganensis* | … | Ru-3 | Germany, R | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 33 | *Clavibacter m.* subsp. *michiganensis* | … | Sc-1 | Germany, KL | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 34 | *Clavibacter m.* subsp. *michiganensis* | 3203 | Sc-2 | Germany, KL | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 35 | *Clavibacter m.* subsp. *michiganensis* | … | Bo-R | Germany, NR | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 36 | *Clavibacter m.* subsp. *michiganensis* | 3204 | Bo-RS | Germany, NR | 2006 | *Solanum lycopersicum* | R. Ftayeh |
| 37 | *Clavibacter m.* subsp. *michiganensis* | 3135 | *CMM* 2 | Germany, R | 2001 | *Solanum lycopersicum* | R. Ftayeh |
| 38 | *Clavibacter m.* subsp. *michiganensis* | 3136 | *CMM* 4 | Germany, R | 2001 | *Solanum lycopersicum* | R. Ftayeh |
| 39 | *Clavibacter m.* subsp. *michiganensis* | 3137 | *CMM* 6 | Germany, R | 2001 | *Solanum lycopersicum* | R. Ftayeh |
| 40 | *Clavibacter m.* subsp. *michiganensis* | 3138 | *CMM* 8 | Germany, R | 2001 | *Solanum lycopersicum* | R. Ftayeh |
| 41 | *Clavibacter m.* subsp. *michiganensis* | 3139 | *CMM* 10 | Germany, R | 2001 | *Solanum lycopersicum* | R. Ftayeh |
| 42 | *Clavibacter m.* subsp. *michiganensis* | 2972 | 78-s | Germany | 1979 | *Solanum lycopersicum* | E. Griesbach |
| 43 | *Clavibacter m.* subsp. *michiganensis* | 3205 | AE-1 | Syria, L | 2007 | *Solanum lycopersicum* | R. Ftayeh |
| 44 | *Clavibacter m.* subsp. *michiganensis* | … | AE-2 | Syria, L | 2007 | *Solanum lycopersicum* | R. Ftayeh |
| 45 | *Clavibacter m.* subsp. *michiganensis* | … | AE-3 | Syria, L | 2007 | *Solanum lycopersicum* | R. Ftayeh |
| 46 | *Clavibacter m.* subsp. *michiganensis* | 3206 | AH-1 | Syria, T | 2007 | *Solanum lycopersicum* | R. Ftayeh |
| 47 | *Clavibacter m.* subsp. *michiganensis* | … | AH-2 | Syria, T | 2007 | *Solanum lycopersicum* | R. Ftayeh |
| 48 | *Clavibacter m.* subsp. *michiganensis* | … | ES-1 | Syria, T | 2007 | *Solanum lycopersicum* | R. Ftayeh |
| 49 | *Clavibacter m.* subsp. *michiganensis* | 3207 | HH-1 | Syria, L | 2007 | *Solanum lycopersicum* | R. Ftayeh |
| 50 | *Clavibacter m.* subsp. *michiganensis* | … | HH-2 | Syria, L | 2007 | *Solanum lycopersicum* | R. Ftayeh |
| 51 | *Clavibacter m.* subsp. *michiganensis* | … | La-1 | Syria, L | 2007 | *Solanum lycopersicum* | R. Ftayeh |
| 52 | *Clavibacter m.* subsp. *michiganensis* | 3208 | OS-1 | Austria, STM | 2007 | *Solanum lycopersicum* | E. Moltmann |

**Table 1.** Source of bacterial species and isolates used in this study

| No. | Bacterial species/ pathovar | GSPB[a] | Designation/ Collect. no.[b] | Origin[c] | Isolation date | Host | Isolated by[d] |
|---|---|---|---|---|---|---|---|
| 53 | *Clavibacter m.* subsp. *michiganensis* | … | OS-2 | Austria, STM | 2007 | *Solanum lycopersicum* | E. Moltmann |
| 54 | *Clavibacter m.* subsp. *michiganensis* | … | OS-3 | Austria, STM | 2007 | *Solanum lycopersicum* | E. Moltmann |
| 55 | *Clavibacter m.* subsp. *michiganensis* | … | OS-4 | Austria, STM | 2007 | *Solanum lycopersicum* | E. Moltmann |
| 56 | *Clavibacter m.* subsp. *michiganensis* | 378 | 9/ 79 | Greece | 1979 | *Solanum lycopersicum* | A. Mavridis |
| 57 | *Clavibacter m.* subsp. *michiganensis* | 382 | 24/ 78 | Greece | 1978 | *Solanum lycopersicum* | A. Mavridis |
| 58 | *Clavibacter m.* subsp. *michiganensis* | 390 | 31/ 79 | Greece | 1979 | *Solanum lycopersicum* | A. Mavridis |
| 59 | *Clavibacter m.* subsp. *michiganensis* | 392 | 45/ 78 | Greece | 1978 | *Solanum lycopersicum* | A. Mavridis |
| 60 | *Clavibacter m.* subsp. *michiganensis* | 3132 | 34-Mut. | Greece | 1979 | *Solanum lycopersicum* | A. Mavridis |
| 61 | *Clavibacter m.* subsp. *michiganensis* | … | Bulgarian 1 | Bulgaria | unknown | *Solanum lycopersicum* | From E. Griesbach |
| 62 | *Clavibacter m.* subsp. *michiganensis* | 2973 | … | Bulgaria | unknown | *Solanum lycopersicum* | From E. Griesbach |
| 63 | *Clavibacter m.* subsp. *michiganensis* | 2315 | KD/ 1-4 | Turkey | 1994 | *Solanum lycopersicum* | Ö. Cinar |
| 64 | *Clavibacter m.* subsp. *michiganensis* | 2221 | NCPPB 1573 | Hungary | 1963 | *Solanum lycopersicum* | Z. Klement |
| 65 | *Clavibacter m.* subsp. *michiganensis* | 2222 | … | unknown | unknown | *Solanum lycopersicum* | unknown |
| 66 | *Clavibacter m.* subsp. *michiganensis* | … | *Cmm* 399 | unknown | unknown | *Solanum lycopersicum* | From E. Griesbach |
| 67 | *Clavibacter m.* subsp. *michiganensis* | 3133 | NCPPB 3123 | USA | unknown | *Solanum lycopersicum* | E. Echandi |
| 68 | *Clavibacter m.* subsp. *michiganensis* | 3134 | … | USA | unknown | *Solanum lycopersicum* | E. Echandi |
| 69 | *Clavibacter m.* subsp. *michiganensis* | … | *Cmm* 185 | USA | unknown | *Solanum lycopersicum* | From E. Griesbach |
| 70 | *Clavibacter m.* subsp. *michiganensis* | … | Leningrad 3 | Russia | unknown | *Solanum lycopersicum* | From E. Griesbach |
| 71 | *Clavibacter m.* subsp. *michiganensis* | … | 80 A-4 | Austria | 2010 | *Solanum lycopersicum* | A. Mavridis |
| 72 | *Clavibacter m.* subsp. *michiganensis* | … | 93 B-1 | Austria | 2010 | *Solanum lycopersicum* | A. Mavridis |
| 73 | *Clavibacter m.* subsp. *michiganensis* | … | 92 B-7 | Austria | 2010 | *Solanum lycopersicum* | A. Mavridis |
| 74 | *Clavibacter m.* subsp. *michiganensis* | … | 81 A-4 | Austria | 2010 | *Solanum lycopersicum* | A. Mavridis |
| 75 | *Clavibacter m.* subsp. *michiganensis* | … | 83 A-1 | Austria | 2010 | *Solanum lycopersicum* | A. Mavridis |
| 76 | *Clavibacter m.* subsp. *michiganensis* | … | 86 A-2 | Austria | 2010 | *Solanum lycopersicum* | A. Mavridis |
| 77 | *Rathayibacter iranicus* | 2220 | NCPPB 2253 | Iran | 1966 | wheat | F. Eskandari |
| 78 | *C. m.* subsp. *insidiosus* | 30 | NCPPB 1634 | UK. | 1964 | *Medicago sativa* | R. A. Lelliott |
| 79 | *C. m.* subsp. *insidiosus* | 2225 | NCPPB 1109 | USA | 1955 | *Medicago sativa* | W. H. Burkholder |
| 80 | *C. m.* subsp. *nebraskensis* | 2223 | NCPPB 2581 | USA | 1971 | *Zea mays* | M. L. Schuster |
| 81 | *C. m.* subsp. *sepedonicus* | 1522 | NCPPB 2140 | USA | 1942 | *Solanum tuberosum* | L. T. Richardidson |
| 82 | *C. m.* subsp. *sepedonicus* | 2823 | Solara 3 | Germany | 1998 | *Solanum tuberosum* | A. Mavridis |
| 83 | *C. m.* subsp. *tessellarius* | 2224 | ATCC 33566 | USA | 1982 | *Triticum aestivum* | R.R. Carlson |
| 84 | *Rathayibacter tritici* | 2749 | Isolat 2 | Pakistan | 1997 | wheat | A. Mavridis |
| 85 | *Rathayibacter tritici* | 2753 | Isolat 6 | Pakistan | 1997 | wheat | A. Mavridis |
| 86 | *Bacillus* sp. | … | unknown | unknown | unknown | unknown | A. Mavridis |
| 87 | *Pectobacterium c.* sp. *carotovorum* | 436 | DSMZ 60442 | Germany | … | *Solanum tuberosum* | unknown |
| 88 | *Pseudomonas fluorescens* | 1714 | G-1 | unknown | unknown | unknown | Microb. Göttingen. |
| 89 | *P. syringae* pv. *syringae* | 1142 | R-12 | Germany | 1967 | *Phaseolus vulgaris* | K. Rudolph |
| 90 | *P. syringae* pv. *tomato* | 1776 | 14-1. | Hungary | 1987 | *Solanum lycopersicum* | S. Süle |
| 91 | *P. syringae* pv. *tomato* | 2317 | Nr.-1 | Turkey | 1994 | *Solanum lycopersicum* | A. Mavridis |
| 92 | *P. syringae* pv. *tomato* | 3209 | Syr-1 | Syria, T | 2007 | *Solanum lycopersicum* | R. Ftayeh |
| 93 | *Xanthomonas arboricola* pv. *juglandis* | 3148 | B-102 | Germany | 2002 | *Juglans regia* | W. Wohanka |
| 94 | *X.campestris* pv. *vesicatoria* | 2043 | S- 08 | Hungary | 1964 | *Solanum lycopersicum* | Z. Klement |
| 95 | *Pantoea agglomerans* | 450 | NCPPB 651 | UK. | 1958 | *Pyrus communis* | E. Billing |
| 96 | *Bacillus subtilis* | 1769 | NCPPB 1246 | USA | 1956 | *Gossypium* sp. | L.S. Bird |
| 97 | *Bacillus subtilis* | … | FZB 24 | unknown | unknown | unknown | unknown |
| 98 | *Ralstonia solanacearum* | 2607 | 180 a | Cameron | 1996 | *Solanum lycopersicum* | A. Mavridis |
| 99 | *R. solanacearum* | 2619 | Ps 24 | Brazil | 1995 | *Solanum lycopersicum* | O. Martins |
| 100 | *Pseudomonas corrugata* | 2418 | Pc1 | Germany | 1995 | *Solanum lycopersicum* | A. Mavridis |

**a)** **GSPB** = Göttingen Collection of Phytopathogenic Bacteria (Göttinger Sammlung Phytopathogener Bakterien).

**b)** **NCPPB** = National Collection of Plant Pathogenic Bacteria; **ATCC** = American Type Culture Collection; **DSMZ** = German Collection of Microorganisms and Cell Cultures.

**c)** **R** = Reichenau. **NR** = Niederrhein. **KL** = Knoblauchsland, Franken. **L** = Latakia. **T** = Tartous. **STM** = Steiermark.

**d)** "From" indicates obtained from the person named.

**Primer Design**

Two new primer pairs were derived from PCR assays published by Bach *et al.* (2003) and Luo *et al.* (2008).

**Designing new primers based on the publication of Bach *et al.* (2003)**

The original primers of Bach *et al.* (2003) suggested for the application of TaqMan PCR technique for different subspecies of *Clavibacter michiganensis*, were designed by targeting intergenic spacer sequences of the rRNA operon (ITS) that are contained in all subspecies of *Clavibacter michiganensis* and extracted from the Genomatix DiAlign program (http://genomatix.gsf.de/cgi-bin/dialign/dialign.pl). In case of *Cmm* the intergenic spacer sequence was U09379 and U09380. Specificity of PCR was reached by Bach *et al*. (2003) *via* the TaqMan probe.

We used the described reverse primer (B-rev-CM) for our protocol and deduced a primer from the TaqMan probe (B-fw-PCM). The combination of both primers will result in an amplicon size of 139 bp.

B*-rev-CM: GGA.GAC.AGA.ATT.GAC.CAA.TGA.T
B-fw-PCM**: C.CGT.CGT.CCT.GTT.GTG.GAT.G

**Designing new primers based on the publication of Luo *et al.* (2008)**

The subspecies-specific primers and probe sequences suggested for *Cmm* by Luo *et al.* (2008) were designed according to the ITS sequences of U09379 and U09380 that were obtained from the NVBI database and aligned with DNAMAN software (Li & De Boer, 1995a and b). The Luo protocol is a TaqMan protocol. We converted this protocol to a standard PCR procedure by using the sequence of the TaqMan probe for primer design. Targeting sufficient specificity, the new primer sequences derived from Luo *et al.* (2008) were designed by elongating the forward primer "Spm4f" of Luo *et al.* (2008) with three nucleotide bases [GGT] into L-fw-CM, and the reverse primer sequence, L-rev-PCM, was as reverse sequence of the specific probe used by Luo *et al.* (2008). Combining this primer set results in an amplicon of 181bp.

L*-fw-CM***: TCA.GGC.GTC.TGT.TCT.GGC.GGT
L-rev-PCM**: GAA.ACC.AGA.CAC.ACC.CAG.AAG.G

*) First letter indicates the initial source of the primer sequence, B = Bach *et al.* (2003); L = Lou *et al.* (2008).
**) Indicates deduced primers from the probe sequence of the corresponding work.
***) Orignial Luo primer elongated by 3 additional nucleotides.

**Amplification conditions**

**- The new primers "B-rev-CM/B-fw-PCM" and "L-fw-CM/L-rev-PCM"; and the primer set tomA-F/tomA-R (Kleitman *et al.*, 2008)**

Optimization of annealing temperatures and PCR reaction buffers for all primers was accomplished using a T-Gradient Thermoblock (Biometra, Göttingen, Germany).

Further amplifications were carried out using a PTC-100 Thermo Cycler (MJ Research, INC., Watertown, USA) for both primer sets (B-rev-CM/B-Fw-PCM and tomA-F/tomA-R) or using a Biometra T-Gradient Thermoblock (L-fw-CM/L-rev-PCM).

For B-rev-CM/B-fw-PCM; L-fw-CM/L-rev-PCM; and tomA-F/tomA-R, PCR was performed in a total volume of 25 µL containing 0.5 µL of template DNA (equal to 2 ng), 1µM of each forward and reverse primer, 0.7mM $MgCl_2$, 0.2mM dNTPs (Fermentas, St. Leon-Rot, Germany), 1 x reaction buffer and 1 U of Dream Taq DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany) as in Table 2.

Initial denaturation at 95 °C for 4 min, 37 cycles of amplification at 95 °C for 1 min, at 61 °C (B-rev-CM/B-fw-PCM and tomA-F/tomA-R) or at 66 °C (L-fw-CM/L-rev-PCM) for 1 min and 72 °C for 10 sec. The final elongation step was accomplished at 72 °C for 10 min (Table 3).

**- The primers CMM-5/CMM-6 (Dreier *et al.*, 1995); CM3/CM4 (Sousa-Santos *et al.*, 1997); and PSA-4/PSA-R (Pastrik and Rainey, 1999)**

Amplifications were carried out using a PTC-100 Thermo Cycler (MJ Research, Inc) for CM3/CM4 and PSA-4/PSA-R and a Biometra T-Gradient Thermoblock for CMM5/CMM6.

PCR was performed for these primers in a total volume of 25 µl containing 1 µl of template DNA (equal to 4 ng), 1µM of each forward and revers primer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 1 x reaction buffer and 1 U of Dream Taq DNA polymerase (the same reaction mixture of the new primers but with 1.5mM $MgCl_2$ instead of 0.7mM $MgCl_2$ (Table 2)). The PCR program for each primer is shown in Table 3.

PCR products and DNA markers (GenRuler$^{TM}$ 100 bp DNA Ladder or GenRuler$^{TM}$ 100 bp Plus DNA Ladder) were separated on 1.5% agarose gels. Gels were pre-stained after cooling at 55 °C with 0.3 µg/ml ethidium bromide.

**Table 2.** PCR Reaction mixture for different primer pairs

| | Primer | | | | | |
|---|---|---|---|---|---|---|
| **Component** | **CMM-5/CMM-6** | **PSA-4/PSA-R** | **CM3/CM4** | **tomA-F/tomA-R** | **L-fw-CM/L-rev-PCM** | **B-rev-CM/B-fw-PCM** |
| PCR reaction buffer | 1 x | 1 x | 1 x | 1 x | 1 x | 1 x |
| $MgCl_2$ | 1.5 x | 1.5 x | 1.5 x | 0.7 x | 0.7 x | 0.7 x |
| dNTP (each) | 0.2mM | 0.2mM | 0.2mM | 0.2mM | 0.2mM | 0.2mM |
| each of forward / reverse primer | 1µM | 1µM | 1µM | 1µM | 1µM | 1µM |
| Template DNA | 4 ng | 4 ng | 4 ng | 2 ng | 2 ng | 2 ng |
| Dream Taq DNA polymerase | 1 U | 1 U | 1 U | 1 U | 1 U | 1 U |
| End volume | 25 µl | 25 µl | 25 µl | 25 µl | 25 µl | 25 µl |

**Table 3.** PCR program for different primer pairs

| | Primer | | | | | |
|---|---|---|---|---|---|---|
| **Step** | **CMM-5/CMM-6** | **PSA-4/PSA-R** | **CM3/CM4** | **tomA-F/tomA-R** | **L-fw-CM/L-rev-PCM** | **B-rev-CM/B-fw-PCM** |
| Initial denaturation | 95°C, 4 min | 95°C, 4 min | 94°C, 4 min | 95°C, 4 min | 95°C, 4 min | 95°C, 4 min |
| Denaturation | 95°C, 1 min | 95°C, 1 min | 94°C, 1 min | 95°C, 1 min | 95°C, 1 min | 95°C, 1 min |
| Annealing | 56°C, 1 min | 63°C, 1 min | 60°C, 1 min | 61°C, 1 min | 66°C, 1 min | 61°C, 1 min |
| Elongation | 72°C, 1 min | 72°C, 1 min | 72°C, 1 min | 72°C, 10 sec. | 72°C, 10 sec. | 72°C, 10 sec. |
| Cycles | 35 × | 35 × | 35 × | 37 × | 37 × | 37 × |
| Final elongation | 72°C, 10 min | 72°C, 10 min | 72°C, 10 min | 72°C, 10 min | 72°C, 10 min | 72°C, 10 min |

**Direct PCR**

Direct PCR assay with pure bacterial suspension of *Cmm* was performed using the primer pair B-rev-CM/B-fw-PCM, without DNA extraction, to determine the minimal concentration of bacterial cells needed for a visible amplification of the diagnostic amplicon.

Initial *Cmm* suspension of 2.5 x $10^8$ cfu/ml was prepared in sterile $H_2O$. Dilutions of 1:10 were prepared serially down to 2.5 x $10^3$ cfu/ml. From each bacterial dilution, 2.5 µl were added to a final volume of 25 µl for each PCR reaction tube, so that approximately 6; 62; 620; 6,200; 62,000; and 620,000 cfu of *Cmm* were contained in each PCR reaction.

**Inhibitor tests**

Inhibitors from plants are often a problem for detecting phytopathogenic bacteria by classical PCR (Schaad *et al.*, 2007; Schaad *et al.*, 1995; Schaad *et al.*, 1999; Weller *et al.*, 2000a) as well as for detecting fungi (Zhonghuo and Michaillides, 2006). The occurrence of such inhibitors was reported by Nabizadeh-Ardekani (1999) in tomato plant homogenates.

For testing whether and which parts of tomato plants contain substances that inhibit the amplification of PCR, different parts of healthy tomato plants including leaves, stems, seeds and fruit pulp were tested. Leaves and stem parts were obtained either from 50-day-old young plants or from 6-month-old plants. Samples of 0.5 g from fresh leaves or stem parts from young or adult plants, as well as 0.5 g of adult fruit pulp or 25 dried tomato seeds (which were commercially extracted 3 years earlier and kept at 4 °C) were crushed separately in 2 ml of TE buffer in sterile mortars. For supplementing the homogenates with defined amounts of *Cmm,* a bacterial suspension of 0.06 optical density at 660 nm (~$10^8$ cfu/ml) was diluted 1:100, so that a working concentration of ~$10^6$ cfu/ml was obtained. Plant homogenates were diluted so that each PCR reaction tube with a final volume of 25 µl contained either 1:1; 1:10; 1:100; 1:1,000 or 1:10,000 diluted plant extracts (stem, leaves, seeds or fruit pulp) and 2.5 µl *Cmm* bacterial suspension of ~$10^6$ cfu/ml (equal to 2,500 cfu of *Cmm* in each PCR reaction tube).

For inhibitor tests, direct cell PCR (without DNA extraction) was carried out with the newly derived primers from the Bach and Luo protocols **(B-rev-CM/B-fw-PCM) & (L-fw-CM/L-rev-PCM)**. Bacterial cells were lysed by an initial denaturation step at 95 °C for 4 min. PCR amplification products were visualised on 1.5% agarose gel, pre-stained with ethidium

bromide (0.3 µg/ml).

**Bio- PCR**

Bio-PCR-experiments were accomplished by plating of plant extracts artificially infested with *Cmm* on the new selective medium BCT (this thesis chapter 1) and two additional semiselective media, i.e. the medium recommended recently by EPPO (OEPP/EPPO, 2005), and the mSCM medium (Waters & Bolkan, 1992), the internationally most often used medium for seed health certifications and for detection of *Cmm*. PCR with bacterial up-growth was carried out using our two new primer sets (B-rev-CM/B-fw-PCM & L-fw-CM/L-rev-PCM) and the primer pair tomA-F/tomA-R proposed by Kleitman *et al*. (2008).

From healthy field tomato plants (5 plants) 1 cm stem slices were crushed together in 10 ml sterile water without primary surface disinfection to ensure high contamination with epiphytic microorganisms. Population density of saprophytic bacteria was determined by plating of serial dilutions on NGY medium supplemented with a fungicide (50 µg/liter Opus® Top). *Cmm* strain 185 (origin USA) was used because this strain was growing faster than the other 29 *Cmm* strains tested on the semiselective media mSCM and EPPO. This strain was selected to give these two media an additional chance for detection of *Cmm*, since several other *Cmm* strains did not grow at all on these two media (Chapter 1, Table 4). Two concentrations of plant homogenates were used for the Bio-PCR test, undiluted **(A)** and 1:10 diluted **(B).** Defined amounts of *Cmm*-cells were added to A and B, so that the final concentrations of saprophytes in A and B were 200,000,000 and 20,000,000 cfu/ml, respectively. In contrast, only 120 cfu/ml of *Cmm* were contained in samples A and B.

From these mixtures of plant homogenates and *Cmm* bacterial cells 0.1 ml aliquots of each variant were plated in three replicates on the media mSCM, EPPO and the new medium BCT for each time of evaluation. As positive controls, 0.1 ml of pure *Cmm* suspension containing 12 cfu of *Cmm* were plated onto three replicates of each selective medium, to compare growth of pure *Cmm* cultures without saprophytes on these agar media.

Bacterial up-growth was washed from three replicates of each tested medium with 4 ml sterile water after 4, 7, 10 and 13 days of incubation at 26 °C. 1.0-1.5 ml of pooled samples (wash-aliquots) were put in 2 ml E-cups. Samples were incubated in a water bath at 97-98 °C for 15 min and cooled on ice. Samples were either used directly for Bio-PCR or stored at -20 °C for later use. An aliquot of 2.5 µl of each variant was added for amplification to 22.5 µl of the

PCR reaction-mixture of each one of the three primers applied, *i.e*. the two new primers (B-rev-CM/B-fw-PCM) & (L-fw-CM/L-rev-PCM) and the primer tomA-F/tomA-R. The PCR reaction mixture and PCR program are shown in Tables 2 and 3.

Amplification by PCR was visualized by gel electrophoresis (3V/cm for 120 min) by loading 6 µl of PCR products on 1.5% agarose gel, pre-stained with 0.3 µg/ml ethidium bromide.

# Results

## Specificity

Initial PCR tests were carried out with DNA isolated from different bacterial species with the primers developed by Dreier *et al.* (1995); Pastrik & Rainey (1999) and Sousa-Santos *et al.* (1997) for *Cmm.* These primers may still be used in many laboratories. The results shown in Figs. 1-6, and summarized in Table 4 revealed strong positive results for several *Cmm* strains, but also weak positive results for many other phytopathogenic and non-phytopathogenic species, such as *Bacillus subtilis, Pectobacterium carotovorum* subsp. *carotovorum, Pseudomonas syringae pv.* syringae, *P. fluorecens, P. syringae pv. tomato* and *Xanthomonas campestris pv. vesicatoria* etc., *i.e.* **false positive results.** Even DNA extracted from the host plant (*Solanum lycopersicum*) lead to a weak positive reaction. We noticed that generally increasing the template DNA from accompanying bacteria often resulted in amplification, while even small DNA amounts of *Cmm* (1-2 ng/ reaction) were enough for a strong amplification. Since tomato seeds and plant samples could be strongly colonized with other pathogenic and non pathogenic bacteria, the use of the old primers may often reveal false positive results, even when plant and seed samples are *Cmm*-free. Therefore, we excluded these primer sets from our further experiments, and instead we searched for other primers that were more specific.

Two new PCR protocols were established based on TaqMan protocols published by Bach *et al.* (2003) and Luo *et al.* (2008). Besides the two newly derived primer sets we also tested one primer pair that was suggested recently by Kleitman *et al.* (2008). The new primers possessed a much better specificity, since the other tested bacterial species were never amplified (Figs. 7, 8 and 9). Therefore, we used only the new pimers for our further studies.

DNA of the *Cmm* strain GSPB 3133 (NCPPB 3123) that was reported by Griesbach *et al.* (2000a and 2000b) and by Dreier *et al.* (1995) to be avirulent, was not amplified by Dreier *et al*. (1995). This strain was also not amplified in our tests using the primer of Dreier *et al.* (Figs**.** 1 and 2). However, this strain was amplified in other repetitions when more DNA template was applied (≥ 4.0-8.0 ng/ reaction). The *Cmm* strain 399 obtained from K. Richter (the bacterial collection of E. Griesbach) is also considered as avirulent (according to Griesbach) and was amplified by Dreier *et al.* (1995). However, this strain was also amplified in our study with the primer of Dreier *et al.* (Figs. 1 and 2), but not amplified by the primers

tomA-R/tomA-F of Kleitman *et al.* (Fig. 7). Both of those avirulent *Cmm* strains (GSPB 3133 & 399) were amplified with our new primer pairs (Figs. 8 and 9) and with the primers of Pastrik and Rainey (Figs. 3 and 4) & Sousa-Santos *et al*. (Figs. 5 and 6).

Furthermore, 6 newly isolated and weakly virulent (hypovirulent) *Cmm* strains from Austria (80 A-4,; 93 B-1; 92 B-7; 81 A-4; 83 A-1 and 86 A-2) were not amplified by the primer pair CMM-5/CMM-6 of Dreier *et al*. (1995), whereas the primer pair tomA-R/tomA-F of Kleitman *et al.* (2008) amplified only 4 of the 6 weakly virulent *Cmm* strains, but two strains (80 A-4 and 81 A-4) were not amplified. However, with our newly adapted primer pairs all the six hypovirulent strains were amplified.

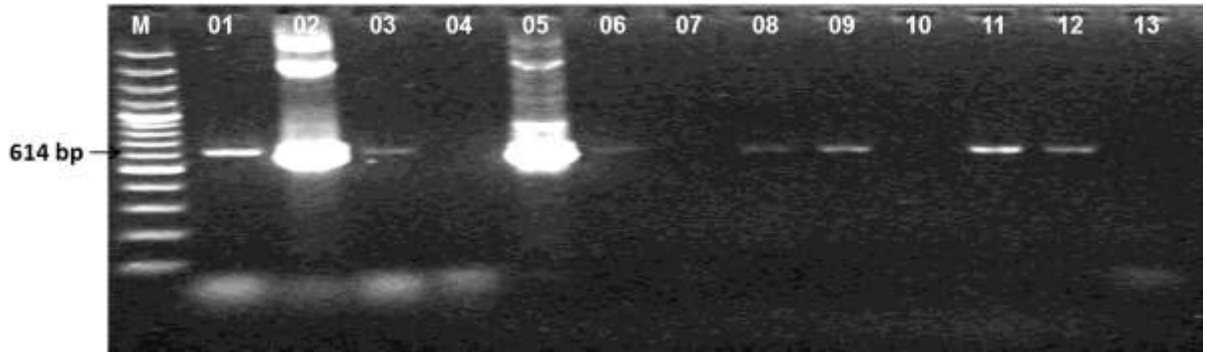


**Fig. 1.** Amplification of different bacterial species with the primers CMM-5/CMM-6 (Dreier *et al*., 1995)

**M**: GeneRuler™ 100bp Plus DNA ladder (Fermentas), **1-4**: *C. m*. subsp. *michiganensis* (GSPB 378, GSPB 382, *Cmm* 399 and GSPB 3133)**, 5**: *C. m*. subsp. *nebraskensis* (GSPB 2223), **6**: *Rathayibacter tritici* (GSPB 2749), **7**: *Bacillus* sp., **8**: *Pectobacterium carotovorum* subsp. *carotovorum* (GSPB 436), **9**: *Pseudomonas fluorescens* (GSPB 1714), **10**: *Pseudomonas syringae* pv. *tomato* (GSPB 2317), **11**: *Xanthomonas arboricola* pv. *juglandis* (GSPB 3148), **12**: *X***.** *campestris* pv. *vesicatoria* (GSPB 2043), 13: negative control (water).

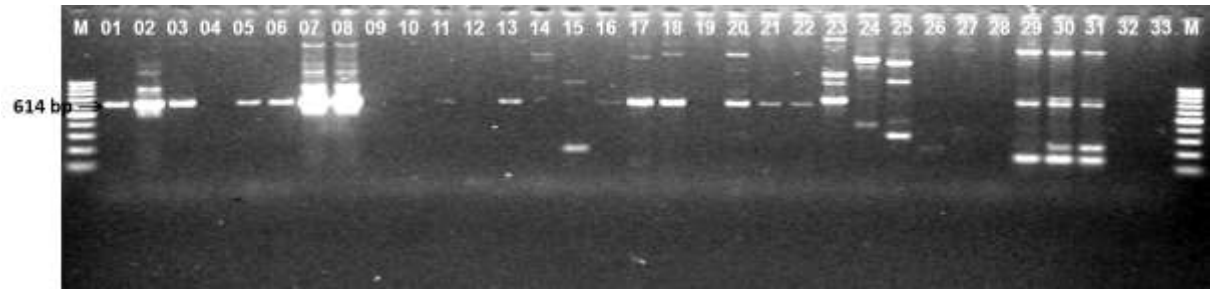


**Fig. 2.** Amplification of different bacterial species with the primers CMM-5/CMM-6 (Dreier *et al.*, 1995)

**M**: GeneRuler™ 100 bp DNA ladder, 1: *Cmm* 378; **2**: *Cmm* 382; **3**: *Cmm* 399; **4**: *Cmm* 3133; **5**: *Rathayibacter iranicus* 2220; **6**: *C. m. insidiouses* 30; **7**: *C. m. insidiouses* 2225; **8**: *C. m. nebraskensis* 2223; **9**: *C. m. sepedonicus* 1522; **10**: *C. m. sepedonicus* 2823; **11**: *C. m. tessellarius* 2224; **12**: *Rathayibacter tritici* 2749; **13**: *R. tritici* 2753; **14**: *Bacillus* sp.; **15**: *Pectobacterium carotovorum* subsp. *carotovorum* 436; **16**: *Pseudomonas fluorecens* 1714; **17**: *P. s. syringae* 1142; **18**: *P. s. tomato* 1176; **19**: *P. s. tomato* 2317; **20**: *P. s. tomato* (Syr.1); **21**: *Xanthomonas arboricola* pv. *juglandis* 3148; **22**: *X. c. vesicatoria* 2043; **23**: *Pantoea agglomerans* 450; **24**: *Bacillus ubtilis* 1769; **25**: *B. subtilis* (FZB 24); **26**: *Ralstonia solanacearum* 2607; **27**: *Ralstonia solanacearum* 2619; **28**: *P. corrugate* 2418; **29**; **30** and **31**: DNA from tomato seeds, leaves of young plant and leaves from adult plant respectively. **32** and **33**: negative control (water).

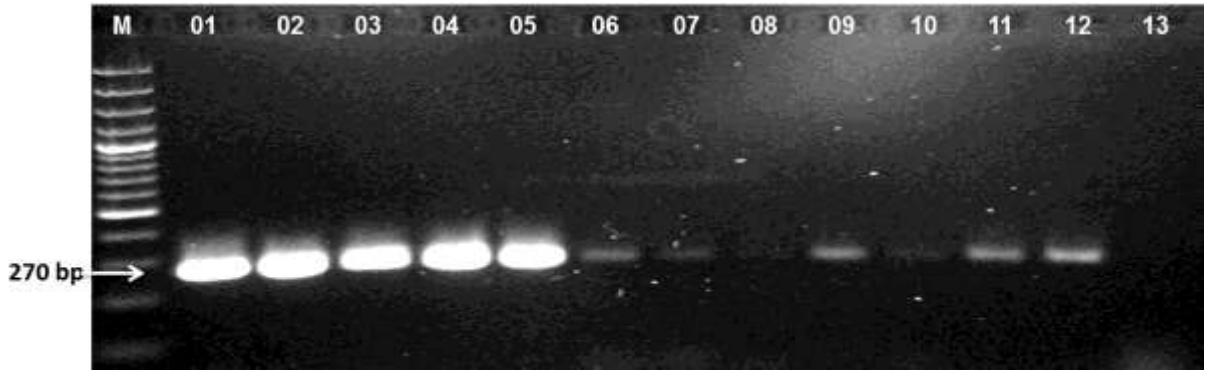


**Fig. 3.** Amplification of different bacterial species with the primers PSA-4/ PSA-R (Pastrik and Rainey, 1999)

**M**: GeneRuler™ 100bp Plus DNA ladder (Fermentas), **1-4**: *C. m*. subsp. *michiganensis* (GSPB 378, GSPB 382, *Cmm* 399 and GSPB 3133)**, 5**: *C. m*. subsp. *nebraskensis* (GSPB 2223), **6**: *Rathayibacter tritici* (GSPB 2749), **7**: *Bacillus* sp., **8**: *Pectobacterium carotovorum* subsp. *carotovorum* (GSPB 436), **9**: *Pseudomonas fluorescens* (GSPB 1714), **10**: *Pseudomonas syringae* pv. *tomato* (GSPB 2317), **11**: *Xanthomonas arboricola* pv. *juglandis* (GSPB 3148), **12**: ***X. campestris*** pv. *vesicatoria* (GSPB 2043), **13**: negative control (water).

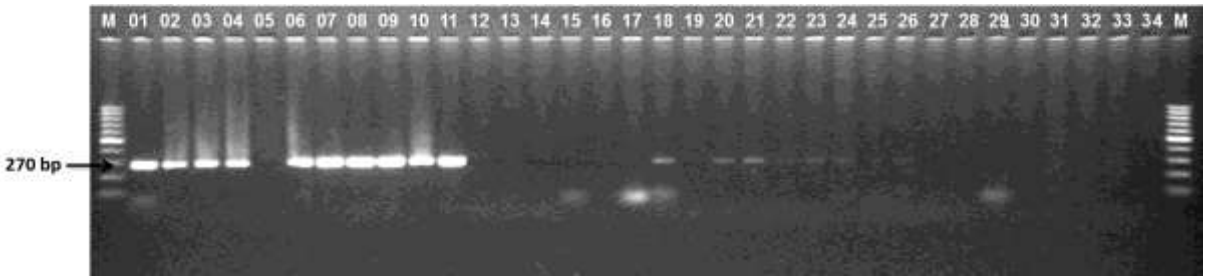


**Fig. 4.** Amplification of different bacterial species with the primers PSA-4/ PSA-R of Pastrik and Rainey (1999)

**M**: GeneRuler™ 100 bp DNA ladder, 1: *Cmm* 378; **2**: *Cmm* 382; **3**: *Cmm* 399; **4**: *Cmm* 3133; **5**: *Rathayibacter iranicus* 2220; **6**: *C. m. insidiosus* 30; **7**: *C. m. insidiosus* 2225; **8**: *C. m. nebraskensis* 2223; **9**: *C. m. sepedonicus* 1522; **10**: *C. m. sepedonicus* 2823; **11**: *C. m. tessellarius* 2224; **12**: *Rathayibacter tritici* 2749; **13**: *R. tritici* 2753; **14**: *Bacillus* sp.; **15**: *Pectobacterium carotovorum* subsp. *carotovorum* 436; **16**: *Pseudomonas fluorescens* 1714; **17**: *P. s. syringae* 1142; **18**: *P. s. tomato* 1176; **19**: *P. s. tomato* 2317; **20**: *P. s. tomato* (Syr.1); **21**: *Xanthomonas arboricola* pv. *juglandis* 3148; **22**: *X. c. vesicatoria* 2043; **23**: *Pantoea agglomerans* 450; **24**: *Bacillus subtilis* 1769; **25**: *B. subtilis* (FZB 24); **26**: *Ralstonia solanacearum* 2607; **27**: *Ralstonia solanacearum* 2619; **28**: *P. corrugata* 2418; **29**; **30** and **31**: DNA from tomato seeds, from leaves of young plant and from leaves of adult plant respectively. **32** and **33**: negative control (water).

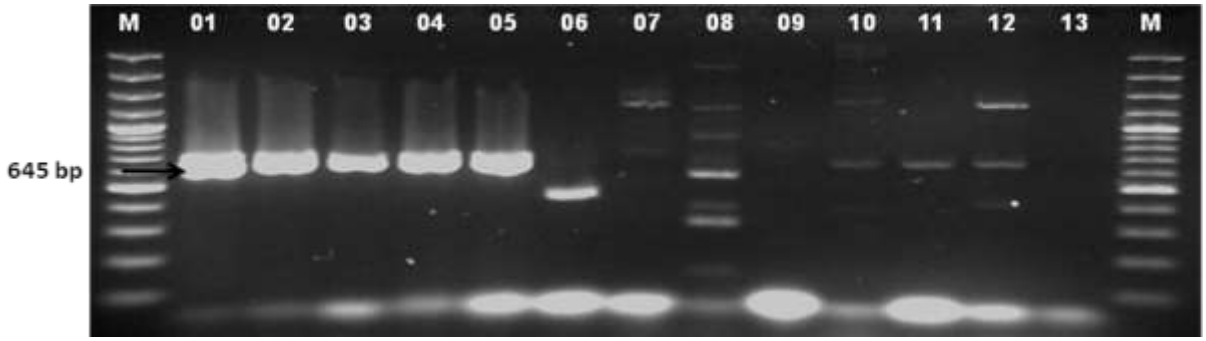


**Fig. 5.** Amplification of different bacterial species with the primers CM3/CM4 (Sousa-Santos *et al*., 1997)

**M**: GeneRuler™ 100bp Plus DNA ladder (Fermentas); **1-4**: *C. m*. subsp. *michiganensis* (GSPB 378, GSPB 382, *Cmm* 399 and GSPB 3133)**, 5**: *C. m*. subsp. *nebraskensis* (GSPB 2223), **6**: *Rathayibacter tritici* (GSPB 2749), **7**: *Bacillus* sp., **8**: *Pectobacterium carotovorum* subsp. *carotovorum* (GSPB 436), **9**: *Pseudomonas fluorescens* (GSPB 1714), **10**: *Pseudomonas syringae* pv. *tomato* (GSPB 2317), **11**: *Xanthomonas arboricola* pv. *juglandis* (GSPB 3148), **12**: *X***.** *campestris* pv. *vesicatoria* (GSPB 2043), **13**: negative control (water).

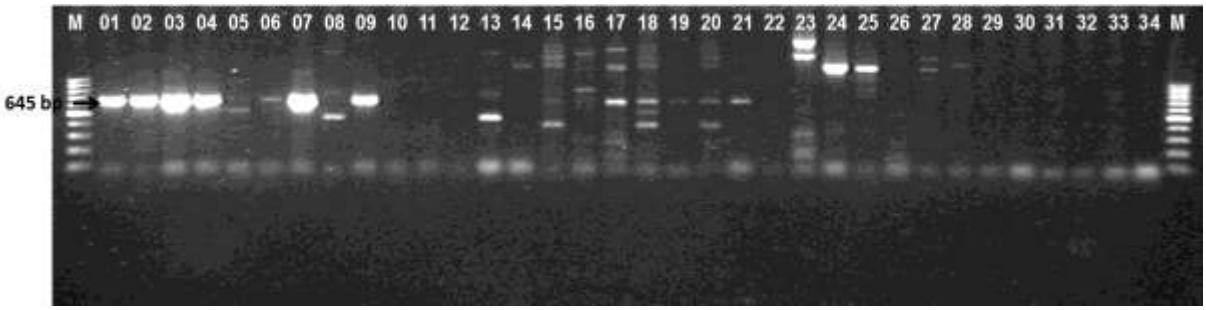


**Fig. 6.** Amplification of different bacterial species with the primers CM3/CM4 (Sousa-Santos *et al*., 1997)

**M**: GeneRuler™ 100 bp DNA ladder, 1: *Cmm* 378; **2**: *Cmm* 382; **3**: *Cmm* 399; **4**: *Cmm* 3133; **5**: *Rathayibacter iranicus* 2220; **6**: *C. m. insidiosus* 30; **7**: *C. m. insidiosus* 2225; **8**: *C. m. nebraskensis* 2223; **9**: *C. m. sepedonicus* 1522; **10**: *C. m. sepedonicus* 2823; **11**: *C. m. tessellarius* 2224; **12**: *Rathayibacter tritici* 2749; **13**: *R. tritici* 2753; **14**: *Bacillus* sp.; **15**: *Pectobacterium carotovorum* subsp. *carotovorum* 436; **16**: *Pseudomonas fluorescens* 1714; **17**: *P. s. syringae* 1142; **18**: *P. s. tomato* 1176; **19**: *P. s. tomato* 2317; **20**: *P. s. tomato* (Syr.1); **21**: *Xanthomonas arboricola* pv. *juglandis* 3148; **22**: *X. c. vesicatoria* 2043; **23**: *Pantoea agglomerans* 450; **24**: *B. subtilis* 1769; **25**: *B. subtilis* (FZB 24); **26**: *Ralstonia solanacearum* 2607; **27**: *Ralstonia solanacearum* 2619; **28**: *P. corrugata* 2418; **29**; **30** and **31**: DNA from tomato seeds, leaves of young plant and leaves from adult plant respectively; **32** and **33**: negative control (water).

**Table 4.** Results of amplifications of DNA extracted from different bacteria or from host plant with the previously described primers for *C. m.* subsp. *michiganensis*

| DNA of | GSPB or other collection no. | Amplification result with primers* | | |
|---|---|---|---|---|
| | | CMM5/CMM6 | PSA-F/PSA-R | CM3/CM4 |
| *Clavibacter m.* subsp. *michiganensis* | GSPB 378 | a | a | a |
| *Clavibacter m.* subsp. *michiganensis* | GSPB 382 | a, b | a | a |
| *Clavibacter m.* subsp. *michiganensis* | *Cmm* 399, avirulent | a | a | a |
| *Clavibacter m.* subsp. *michiganensis* | GSPB 3133/NCPPB 3133, avirulent | a (±) | a | a |
| *Rathayibacter iranicus* | GSPB 2220/ NCPPB 2253 | a | – | a, b |
| *C. m.* subsp. *insidiosus* | GSPB 30/ NCPPB 1634 | a | a | (a) |
| *C. m.* subsp. *insidiosus* | GSPB 2225/ NCPPB 1109 | a, c | a | a |
| *C. m.* subsp. *nebraskensis* | GSPB 2223 / NCPPB 2581 | a, c | a | c |
| *C. m.* subsp. *sepedonicus* | GSPB 1522/ NCPPB 2140 | – | a | a |
| *C. m.* subsp. *sepedonicus* | GSPB 2823 | – | a | – |
| *C. m.* subsp. *tessellarius* | GSPB 2224/ ATCC 33566 | a | a | – |
| *Rathayibacter tritici* | GSPB 2749 | – | – | – |
| *Rathayibacter tritici* | GSPB 2753 | a | – | c |
| *Bacillus* sp. | ... | c | – | b |
| *Pectobacterium carotovorum* subsp. *carotovorum* | GSPB 436 / DSMZ 60442 | c | – | a, c |
| *P. fluorescens* | GSPB 1714 | a | – | a, c |
| *P. syringae* pv. *syringae* | GSPB 1142 | a, b | – | a, c |
| *P. syringae* pv. *tomato* | GSPB 1776 | a, b | a | a, c |
| *P. syringae* pv. *tomato* | GSPB 2317 | – | – | a |
| *P. syringae* pv. *tomato* | GSPB 3209 | a, c | a | a, c |
| *Xanthomonas arboricola* pv. *juglandis* | GSPB 3148 | a | a | a |
| *Xanthomonas campestris* pv. *vesicatoria* | GSPB 2043 | a | a | – |
| *Pantoea agglomerans* | GSPB 450 / NCPPB 651 | a, c | a | c |
| *Bacillus subtilis* | GSPB 1769/ NCPPB 1246 | a, c | a | b |
| *Bacillus subtilis* (FZB 24) | ... | a, c | – | c |
| *Ralstonia solanacearum* | GSPB 2607 | b | a | – |
| *R. solanacearum* | GSPB 2619 | – | – | c |
| *Pseudomonas corrugata* | GSPB 2418 | – | – | b |
| Tomato seeds cv. Marmande | ... | c | – | – |
| Tomato leaves (cv. Lyconorma, young plant) | ... | c | – | – |
| Tomato leaves (cv. Lyconorma, adult plant) | ... | c | – | – |

*) –: for no amplification; ±: variable negative and positive amplification; **a**: amplification of the diagnostic fragment; **b**: the amplification of one other fragment and **c**: amplification of two or more other fragments.

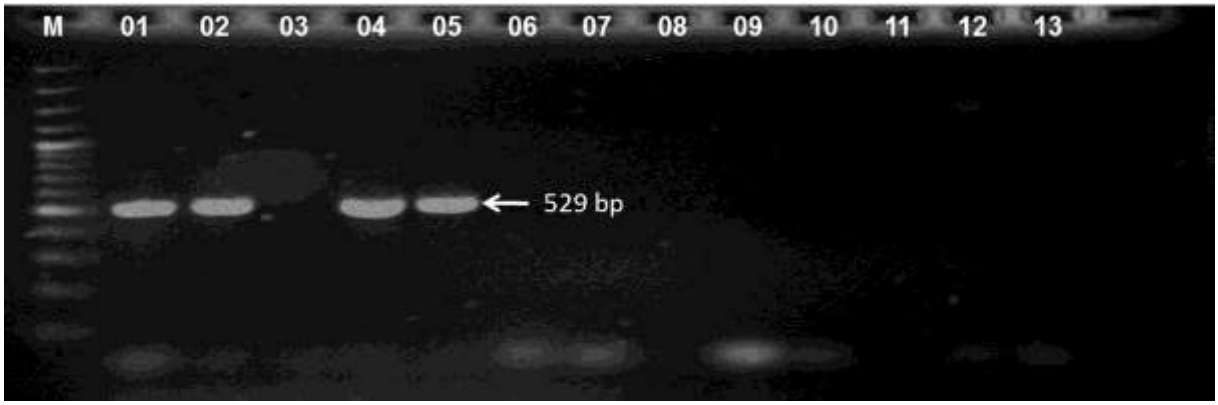


**Fig. 7.** Amplification of different bacterial species with tomA-F/tomA-R (Kleitman *et a*l., 2008)


**Fig. 8.** Amplification of different bacterial species with B-rev-CM/B-fw-PCM

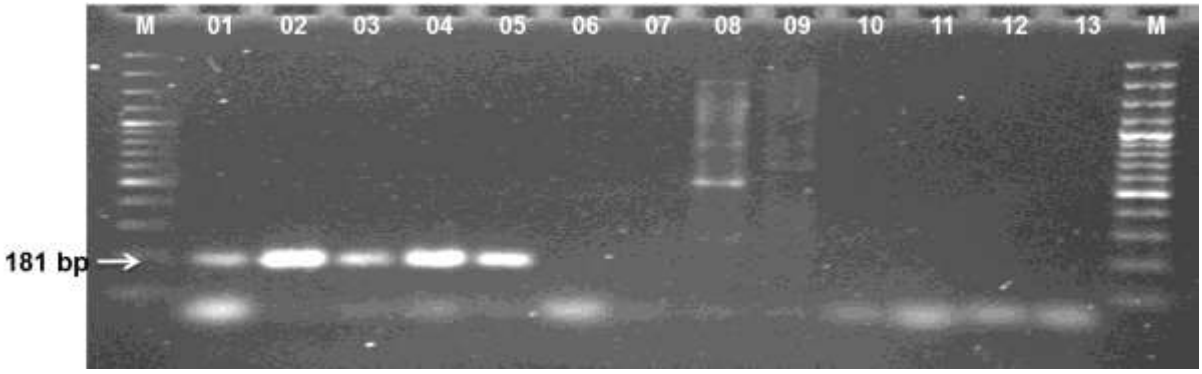


**Fig. 9.** Amplification of different bacterial species with L-fw-CM/L-rev-PCM

**Fig 7, 8 and 9. M**: GeneRuler™ 100bp Plus DNA ladder (Fermentas), **1-4**: *C. m.* subsp. *michiganensis* (GSPB 378, GSPB 382, *Cmm* 399 and GSPB 3133), **5**: *C. m.* subsp. *nebraskensis* (GSPB 2223), **6**: *Rathayibacter tritici* (GSPB 2749), **7**: Bacillus subtilis, **8**: *Pectobacterium carotovorum* subsp. *carotovorum* (GSPB 436), **9**: *Pseudomonas fluorescens* (GSPB 1714), **10**: *Pseudomonas syringae* pv. *tomato* (GSPB 2317), **11**: *Xanthomonas arboricola* pv. *juglandis* (GSPB 3148), **12**: *X.* **.** *campestris* pv. *vesicatoria* (GSPB 2043), **13**: negative control (water).

**Efficiency of the new primers for amplification of different *Cmm* strains**

Tests with the newly derived primers (B-rev-CM/B-fw-PCM; L-fw-CM/L-rev-PCM) with DNA extracted from 76 *Cmm* strains listed in Table 1, resulted in the expected diagnostic amplicons of 131 bp and 181 bp for each primer, respectively, with all of the 76 *Cmm* strains (including virulent and hypovirulent *Cmm* strains).

**Direct PCR**

When pure suspensions of *Cmm* cells were amplified directly (without DNA extraction) with the primer B-rev-CM/B-fw-PCM, amplification was detectable using 620 cfu/ each PCR reaction-tube in a final volume of 25 µl (Fig. 10). By a final amount of only 62 or 6 cells of *Cmm* in each PCR reaction tube, no amplification was detectable.

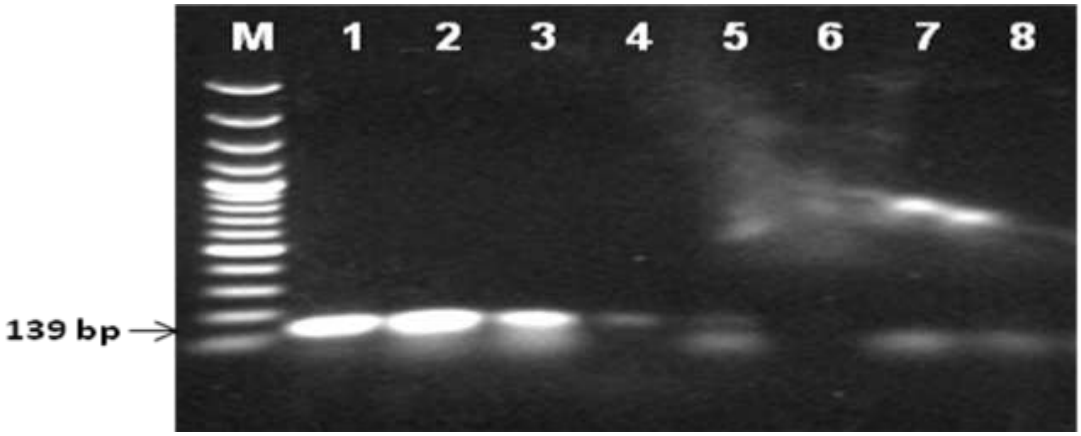


**Fig. 10.** Direct PCR using the primers B-rev-CM/B-fw-PCM with different amounts of *Cmm* cells in each reaction.

**M:** GeneRuler™ 100bp Plus DNA ladder (Fermentas), **1**: 620,000; **2**: 62,000; **3**: 6,200; **4 & 5**: 620; **6**: 62; **7**: 6 and **8**: 0 cfu of *Cmm* in each PCR reaction tube, respectively.

**Inhibitor tests**

When seed-, leaf-, stem- and fruit-homogenates were artificially infested with *Cmm* and amplified with PCR, the two new primers (B-rev-CM/B-fw-PCM) or (L-fw-CM/L-rev-PCM) showed similar results but the primer set B-rev-CM/B-fw-PCM was more efficient for detecting *Cmm* and the diagnostic amplicon were clearer. Otherwise there were no differences in inhibitor effects in young or in adult plants.

The effect of inhibitors in plant homogenates was clearly observed. Thus, amplifications by PCR were not detectable in undiluted homogenates of plant tissues, although the concentration of *Cmm* cells was very high (2,500 cfu/each PCR reaction tube). First amplifications were recorded when stem homogenates were diluted 1:10, then in 1:100 diluted fruit pulp, followed by 1:1,000 diluted leaf homogenates and finally in 1:10,000 diluted seed homogenates (Figs. 11 and 12).

According to these experiments it was concluded that inhibitors occure in similar concentrations in adult and young tomato plants. The inhibitors showed the highest concentration in tomato seeds, followed by leaves, then in fruit pulp, and inhibitors were very weak in stems (Figs. 11 and 12).

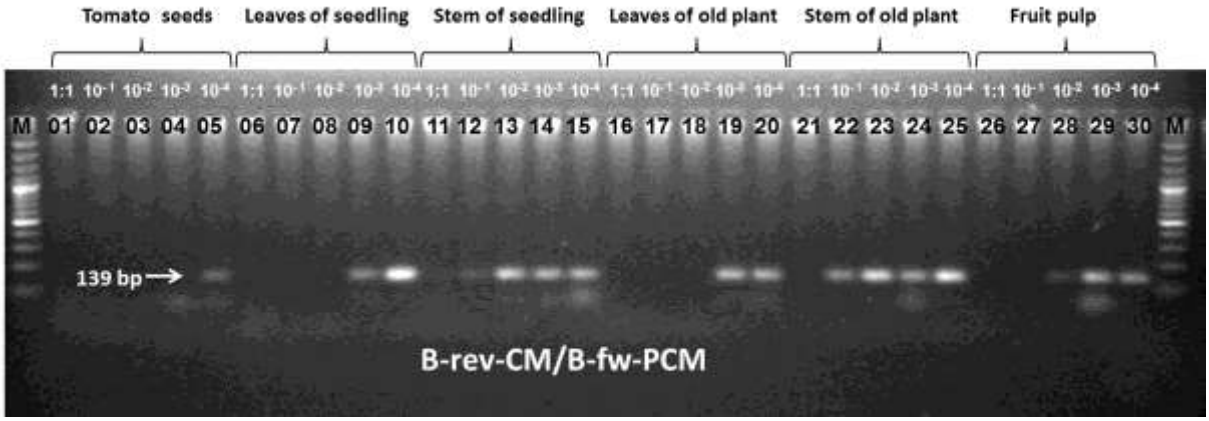


**Fig. 11.** The amplification of 2,500 cfu of *Cmm* contained in plant homogenates (extracts) diluted 1:1, 1:10, 1:100, 1:1,000 and 1:10,000 and obtained from different parts of tomato plants: from seeds (1-5), from leaves of young plants (6-10), from stem of young plant (11-15), from leaves of adult plants (16-20), from stem of adult plant (22-25) and from fruit pulp (26-30) with the primer set B-rev-CM/B-fw-PCM (the diagnostic amplicon is 139 bp). M: GeneRuler™ 100bp Plus

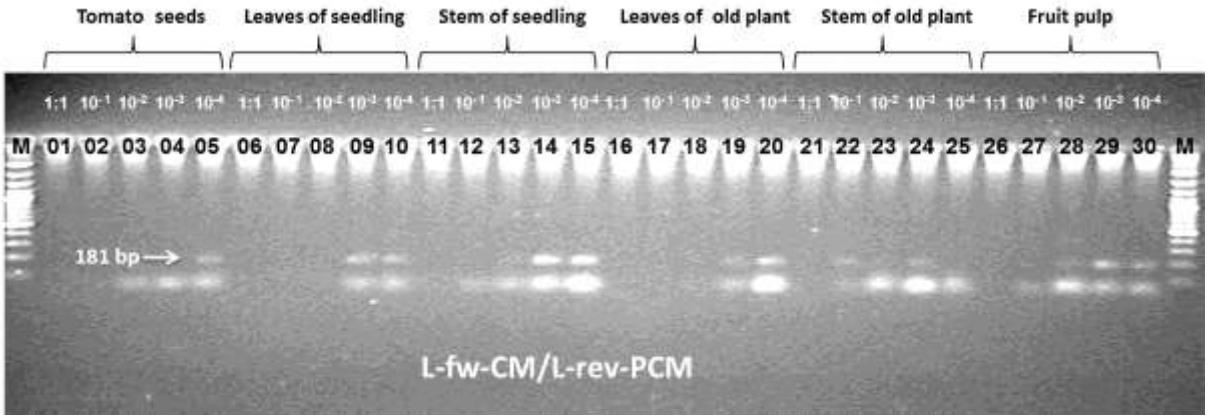


**Fig. 12.** The amplification of 2,500 cfu of *Cmm* contained in plant homogenates (extracts) diluted 1:1, 1:10, 1:100, 1:1,000 and 1:10,000 and obtained from different parts of tomato plants: from seeds (1-5), from leaves of young plants (6-10), from stem of young plant (11-15), from leaves of adult plant (16-20), from stem of adult plant (22-25) and from fruit pulp (26-30) with the primer set L-fw-CM/L-rev-PCM (the diagnostic amplicon is 181 bp). M: GeneRuler™ 100bp Plus DNA ladder.

**Bio-PCR**

Samples obtained from three agar plates of each medium (the new medium BCT, mSCM and EPPO) of each concentration (A & B) and for each time of evaluation (4, 7, 10 and 13 days after incubation at 26 °C) revealed the first detection results **four days** after incubation for both concentrations A (Fig. 13) and B (Fig. 14) only with washate-aliquots taken from all replicates of the new mediuma BCT nd with all primers. These results proved very clearly that only the new medium allowed detection of the very small amounts of *Cmm* on each agar plate (12 cfu), although the amount of saprophytic bacteria on each agar plate was very high, i. e. 20,000,000 and 2,000,000 cfu for A and B, respectively.

Results after **7 days** did not change, the amplification was only positive on all replicates of the new medium with all primers used, but not for the other media (Figs. 15 & 16). Also on control plates, when the same amount of *Cmm*-cells was plated on each agar medium, but without saprophytes, the amplification was only possible with washates from the new medium BCT, indicating that the growth of *Cmm* did not start on both of the other selective media mSCM and EPPO, even when competing saprophytic microorganisms were not present (Fig. 17). When NGY Petri dishes which had been previously streaked with concentration B were washed after 7 days, we did not obtain amplifications, indicating that recovery of *Cmm* was inhibited by other saprophytes on the non-selective agar medium (Fig. 18). The diagnostic bands shown on Fig. 18 were obtained only from DNA of *Cmm* as positive control for amplifications with each primer.

After **10 days**, amplification from washate samples of the EPPO plates started on A with primers B-rev-CM/B-fw-PCM and L-fw-CM/L-rev-PCM (Fig. 19) and on B with all primers, whereas the washate samples from the mSCM medium did not show any amplification (Fig. 20). The washate-aliquots of the positive control only showed strong amplifications when obtained from the new medium BCT and slight amplifications when obtained from the EPPO medium (Fig. 21), indicating that the mSCM medium did not allow growth of low concentrated *Cmm*.

Results did not change after **13 days** (Figs. 22 & 23). These results clearly indicate, that 12 cfu of *Cmm* were not detectable on the widely used medium mSCM, and that this low number of *Cmm* cells was under the sensitivity threshold of this medium.

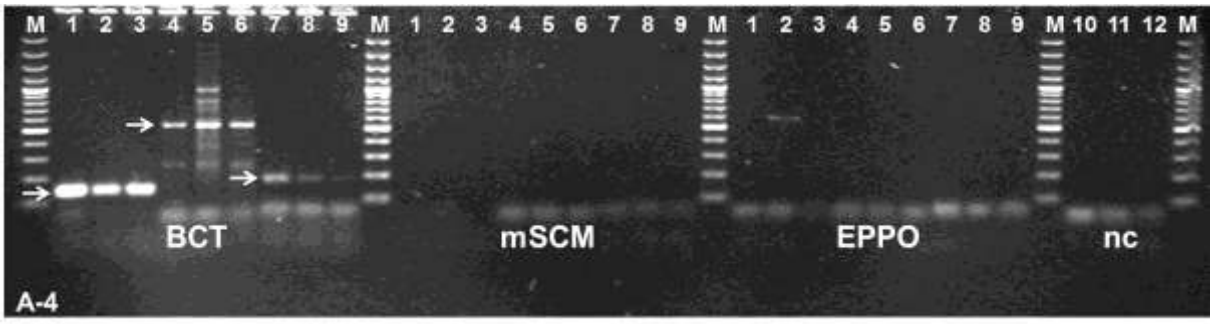


**Fig. 13**

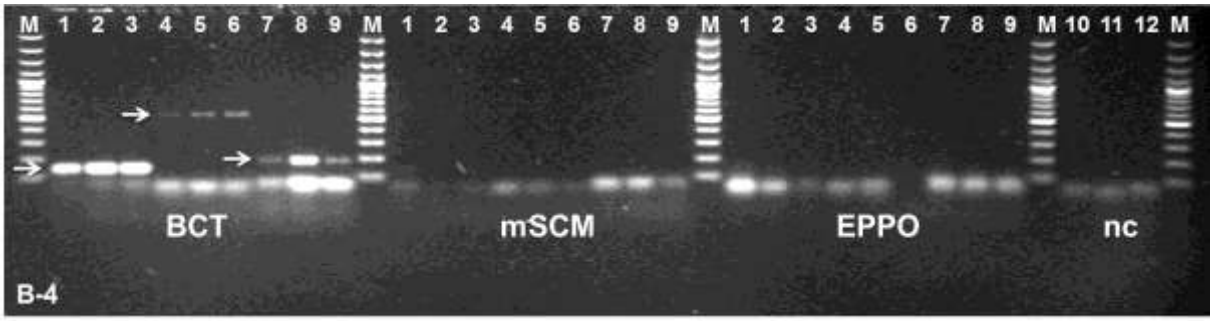


**Fig. 14**

**Fig. 13 & 14.** Amplification of washate-aliquots obtained from 3 agar plates of each medium: BCT (the new medium), mSCM and EPPO, from concentration A (Fig. 13) and B (Fig. 14), 4 days after plating with the primers B-rev-CM/B-fw-PCM (1-3, 131 bp), with the primers tomA-F/tom-A-R (4-6, 529 bp) and with the primers L-fw-CM/L-rev-PCM (7-9, 181 bp), 10, 11 and 12: negative control (water) for each of the mentioned primers, respectively, M: GeneRuler™ 100bp Plus DNA ladder. Agar plates A were streaked with 100 µl of plant homogenates containing 12 cfu of *Cmm* and 20,000,000 cfu saprophytic bacteria. Agar plates B were streaked with 100 µl plant homogenate containing 12 cfu of *Cmm* and 2,000,000 cfu saprophytic bacteria.

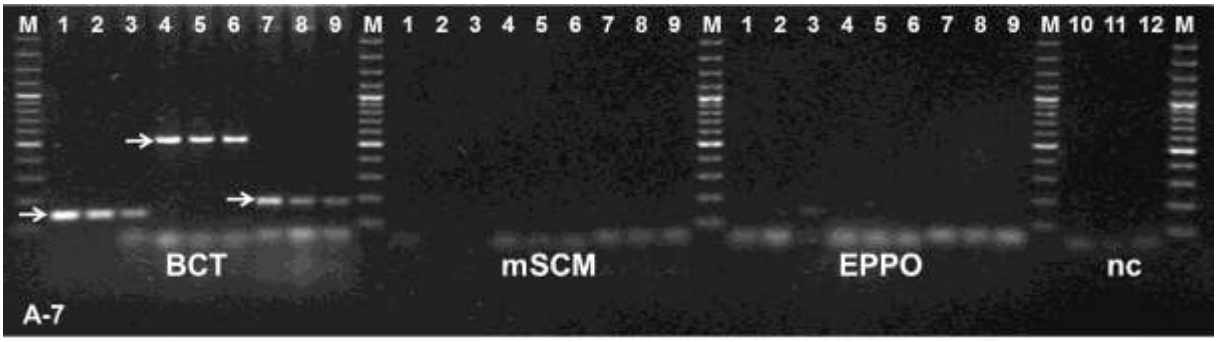


**Fig. 15**

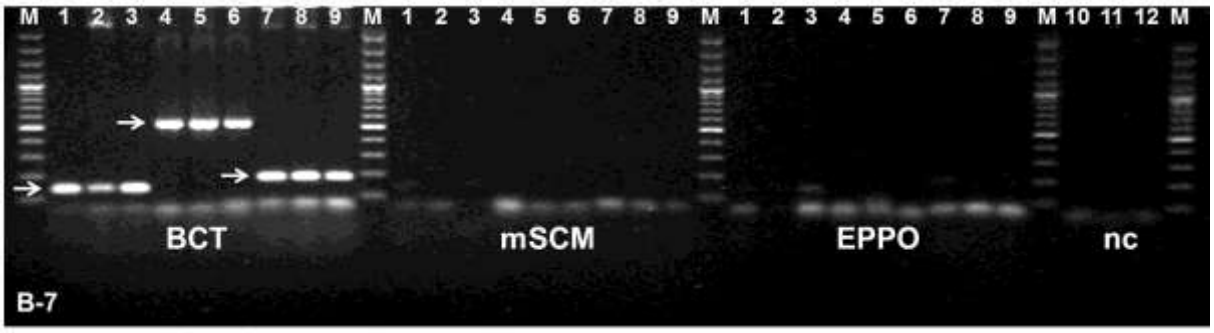


**Fig. 16**

**Fig. 15 & 16.** Amplification of washates obtained from 3 agar plates of the new medium BCT, mSCM and EPPO, from concentration A (Fig. 15) and B (Fig. 16), 7 days after plating with the primers B-rev-CM/B-fw-PCM (1-3, 131 bp), with the primers tomA-F/tomA-R (4-6, 529 bp) and with the primers L-fw-CM/L-rev-PCM (7-9, 181 bp), 10, 11 and 12: negative control (water) for each of the mentioned primers, respectively, M: GeneRuler™ 100bp Plus DNA ladder .

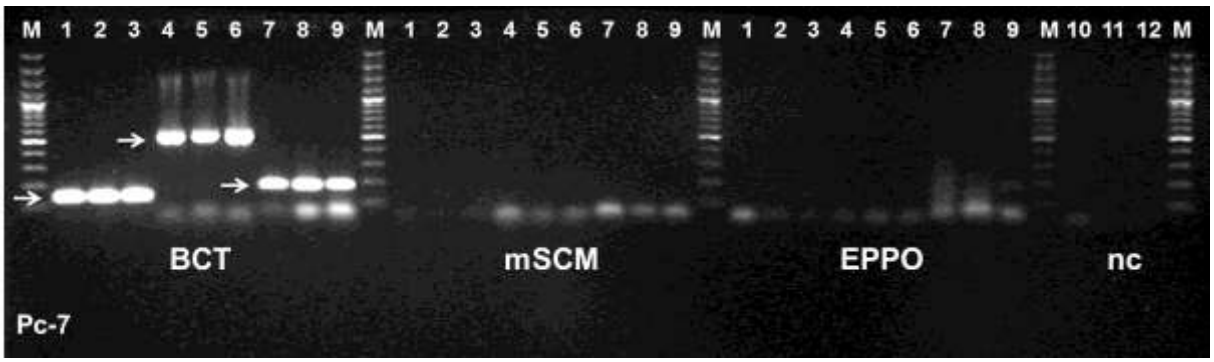


**Fig. 17.** Amplification of pooled samples of the positive controls obtained from three replicates of each medium, 7 days after plating, when only 12 cfu of *Cmm* were plated onto each agar plate without saprophytes with the primers B-rev-CM/B-fw-PCM (1-3, 131 bp), with the primers tomA-F/tomA-R (4-6, 529 bp) and with the primers L-fw-CM/L-rev-PCM (7-9, 181 bp), 10, 11 and 12: negative control (water) for each of the mentioned primers, respectively. M: GeneRuler™ 100bp Plus DNA ladder.

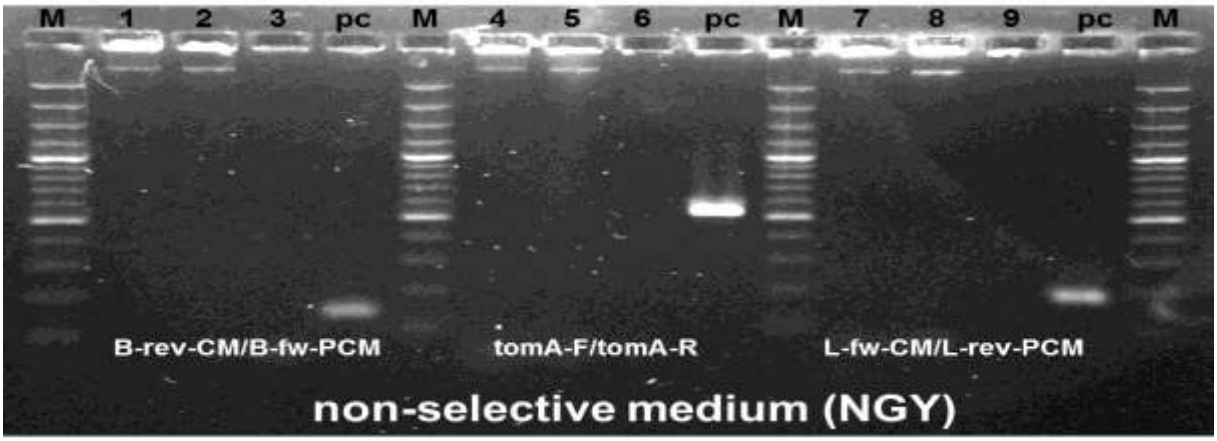


**Fig. 18.** Amplification of pooled samples obtained from three replicates of non-selective medium (NGY), 7 days after plating, with the primer B-rev-CM/B-fw-PCM (1-3, 131 bp), with the primers tomA-F/tomA-R (4-6, 529 bp) and with the primers L-fw-CM/L-rev-PCM (7-9, 181 bp), pc: positive control for each primer with DNA of *Cmm*. M: GeneRuler™ 100bp Plus DNA ladder.

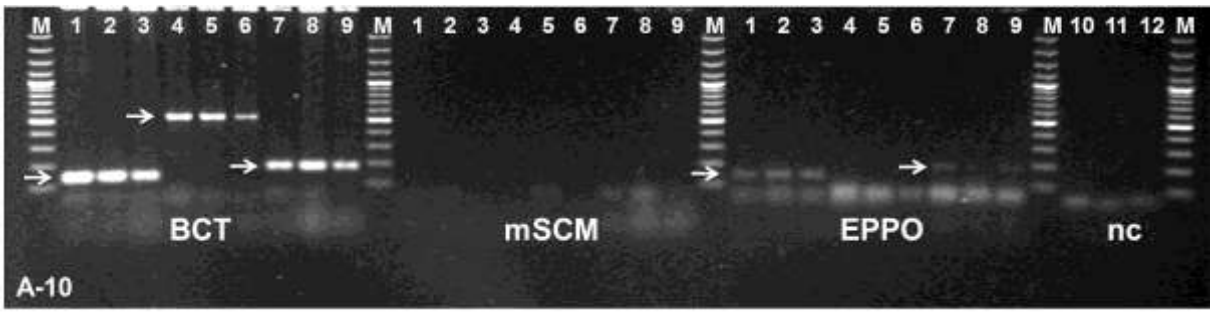


**Fig. 19**

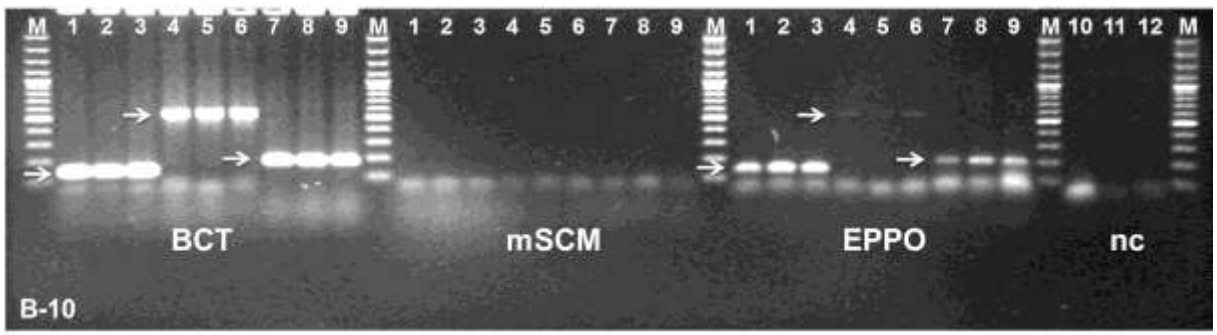


**Fig. 20**

**Fig. 19 & 20.** Amplification of washate aliquots obtained from 3 agar plates of each medium BCT, mSCM and EPPO, from concentration A (Fig. 19) and concentration B (Fig. 20), 10 days after plating with the primers B-rev-CM/B-fw-PCM (1-3, 131 bp), with the primers tomA-F/tomA-R (4-6, 529 bp) and with the primers L-fw-CM/L-rev-PCM (7-9, 181 bp), 10, 11 and 12: negative control (water) for each of the mentioned primers, respectively, M: GeneRuler™ 100bp Plus DNA ladder .

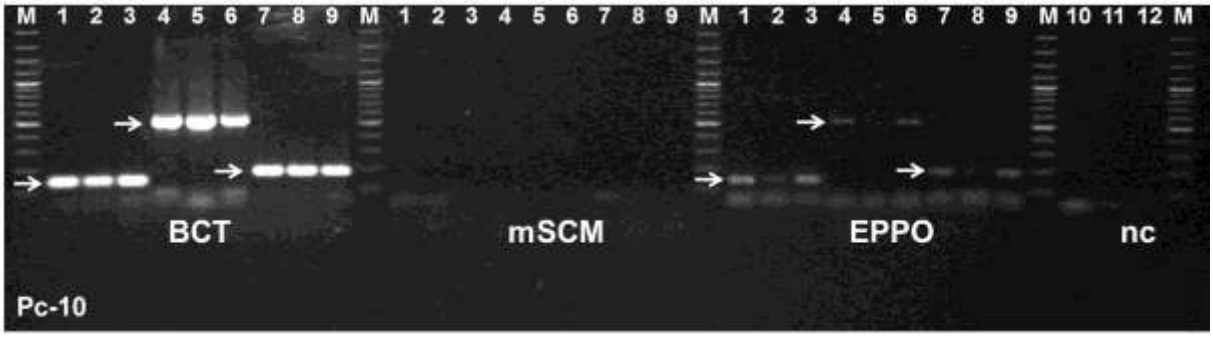


**Fig 21.** Amplification of pooled samples of the positive controls obtained from three replicates of each medium, 10 days after plating, when only 12 cfu of *Cmm* were plated onto each agar plate without saprophytes with the primers B-rev-CM/B-fw-PCM (1-3, 131 bp), with the primers tomA-F/tomA-R (4-6, 529 bp) and with the primers L-fw-CM/L-rev-PCM (7-9, 181 bp), 10, 11 and 12: negative control (water) for each of the mentioned primers, respectively. M: GeneRuler™ 100bp Plus DNA ladder.

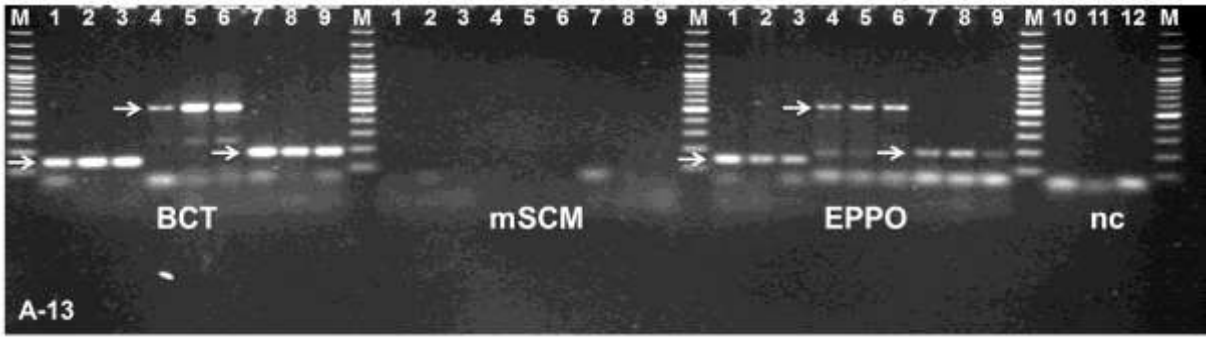


**Fig 22**

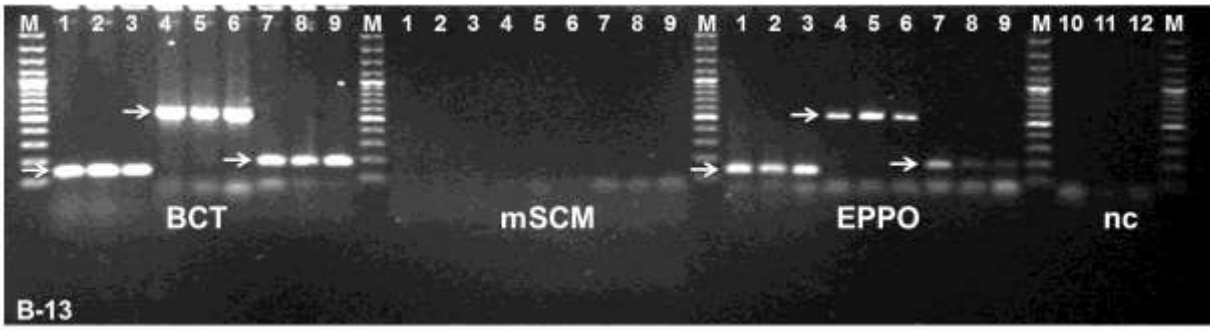


**Fig. 23**

**Fig. 22 & 23.** Amplification of washate aliquots obtained from 3 agar plates of each medium BCT, mSCM and EPPO, from each concentration A (Fig. 22) and B (Fig. 23), 13 days after plating with the primers B-rev-CM/B-fw-PCM (1-3, 131 bp), with the primers tomA-F/tomA-R (4-6, 529 bp) and with the primers L-fw-CM/L-rev-PCM (7-9, 181 bp), 10, 11 and 12: negative control (water) for each of the mentioned primers, respectively, M: GeneRuler™ 100bp Plus DNA ladder.

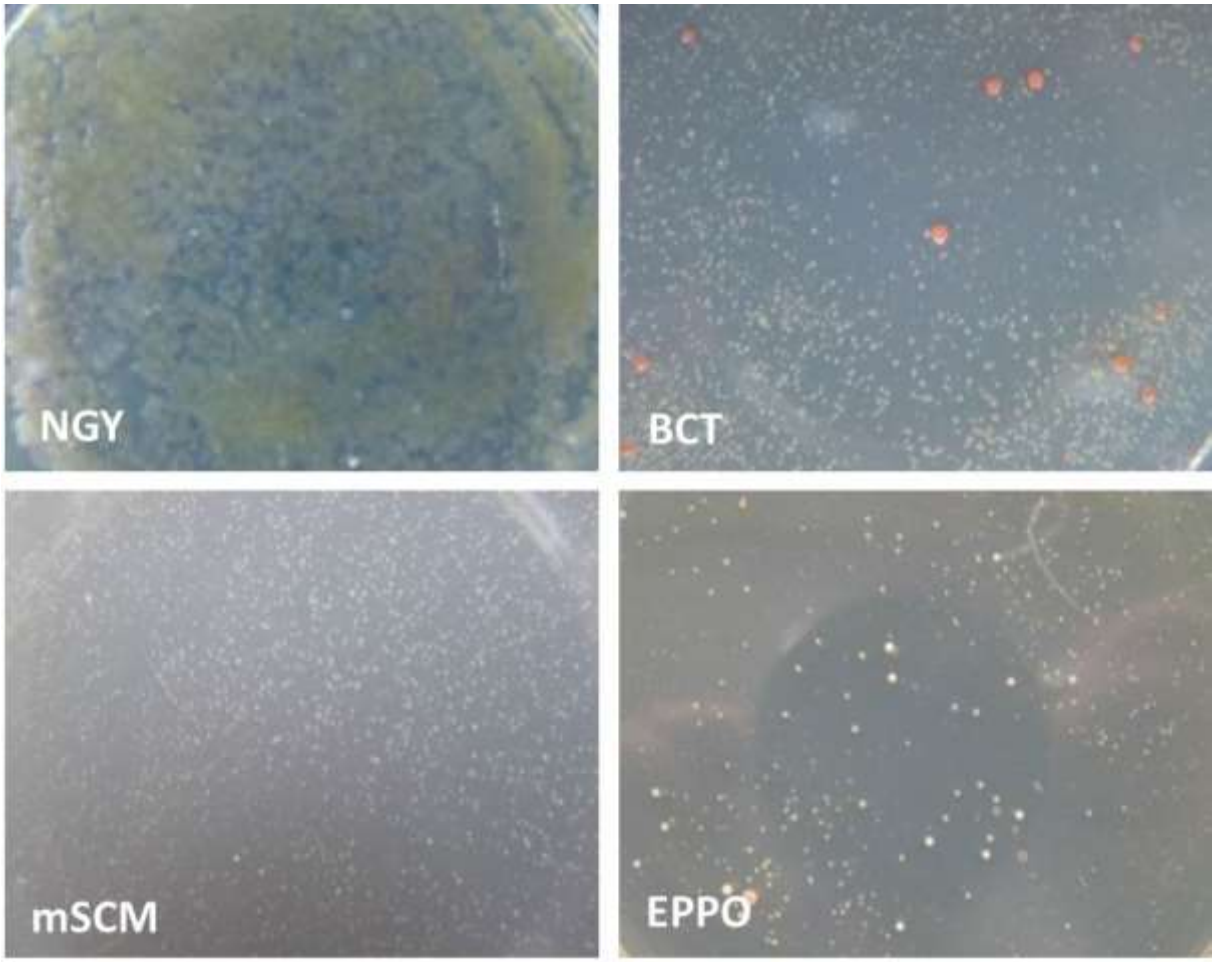


**Fig. 24.** Agar plates of NGY medium, the new semiselective medium BCT, mSCM medium and EPPO medium, when "inoculated" with 20,000,000 cfu of saprophytic bacteria and only 12 cfu of *Clavibacter michiganensis* subsp. *michiganensis*.
A visible detection of *Cmm* on these agar plates was impossible. Only with Bio-PCR it was possible to detect *Cmm* on the new medium BCT within 4 days and on the EPPO medium within 10 and 13 days.

## Discussion

Several PCR primer systems for detection of *C. m.* subsp. *michiganensis* (*Cmm*) have been described in the literature. Our aim was to carefully check the value of all published primer pairs in terms of specificity and effectiveness. Finally, the best suited sets were applied in combination with the newly developed selective medium (BCT) in comparison with other semiselective media for sensitivity of detecting *Cmm* in plant or seed material which was highly contaminated by saprophytic or other accompanying bacteria.

In the first experiments, three primer sets described by Dreier *et al.* (1995), Pastrik & Rainey (1999) and Sousa-Santos *et al.* (1997) were tested. Whereas Dreier *et al.* derived the primers from the *pat*1 gene of *Cmm*, Pastrik & Rainey used intergenic spacer sequences of the rRNA cistron to develop *Cmm* specific primers. The origin of the primers CM3 and CM4 of Sousa-Santos *et al.* is a shot gun cloned *Cmm* DNA-Fragment of a total DNA preparation. To our knowledge, these three primer sets were never scrutinized for specificity against a broad range of bacterial species and strains from diverse origin and relatedness to *Cmm*. Therefore, we compared these primer sets in a specificity check with several other bacterial species many of which were probably never checked before in this regard.

It turned out that all three primer sets showed cross-reactions with several accompanying bacterial species that may occur on tomato plants and seeds, such as *Pectobacterium carotovorum* subsp. *carotovorum*, *Pseudomonas fluorescens*, *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *tomato*, *Xanthomonas campestris* pv. *vesicatoria*, *Bacillus* sp. and various accompanying non-pathogenic bacteria of different species.

Sousa-Santos *et al.* (1997) reported no amplification of the diagnostic fragment of 645 bp when the primer set CM3/CM4 was tested with one strain each of: *Clavibacter michiganensis* subsp. *sepedonicus*, *C. m.* subsp. *insidiosus*, *P. syringae* pv. *syringae* and *Ralstonia solanacearum*. In contrast, we found amplifications of the diagnostic fragment of 645 bp with *C. m. sepedonicus* (GSPB 1522), but no amplification with the *C. m. sepedonicus* strain GSPB 2823. Also, both *C. m. insidiosus* strains (GSPB 30 and GSPB 2225) were positive as well as *P. syringae* pv. *syringae* (GSPB 1142). No amplicon was detected for two strains of *R. solanacearum* (GSPB 2607 and GSPB 2619).

Pastrik and Rainey (1999) reported that there were no cross-reactions with other bacterial genera using the primers PSA-4 and PSA-R. We obtained the same results with strains of

*Ralstonia solanacearum* and *Pectobacterium carotovorum* spp. *carotovorum*. However, by testing different strains of other genera we recorded some amplifications of the diagnostic band (270 bp) and even stronger amplifications when other subspecies of *Clavibacter michiganensis* were tested (Figs. 3 & 4).

Since these three primer sets proved to be not specific, we excluded them from further experiments, although all or some of them are still being recommended by EPPO (OEPP/EPPO, 2005). Therefore, we carried out further tests with a primer set described by Kleitman *et al.* (2008) derived from the tomatinase gene *tom*A. In addition, TaqMan based PCR protocols, as described by Bach *et al.* (2003) and Luo *et al.* (2008) were included in our studies. These protocols rely on specific TaqMan probes, which were either deduced from intergenic sequences or internal transcribed spacer regions of the rRNA operon, respectively. We translated these protocols to a conventional PCR using one of the described primers together with a specific primer, which was deduced from the sequence of the TaqMan probe.

These **newly adapted PCR systems** proved to be **superior** when compared with the three afore-mentioned primer sets (see Figs. 7, 8 and 9). Thus, our results clearly revealed that all possibly accompanying bacterial species tested, such as *Bacillus subtilis*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Pseudomonas fluorescens*, *P. syringae* pv. *tomato* and *X. campestris* pv. *vesicatoria*, were not amplified by the newly adapted primer sets. On the other hand, all the 76 different *Cmm* strains tested were amplified without any exception by both new primer sets, i.e. B-rev-CM/B-fw-PCM as well as L-fw-CM/L-rev-PCM.

With the primers tomA-R/tomA-F developed by Kleitman *et. al.* (2008) all virulent *Cmm* strains tested were amplified, except a variable amplification of the **avirulent strain** 399. Similar results for the avirulent *Cmm* strain NCPPB 3123 (GSPB 3133) were reported by Dreier *et al.* (1995) with the primer set CMM-5/CMM-6. However, 6 newly obtained hypovirulent *Cmm* strains from Austria (80 A-4,; 93 B-1; 92 B-7; 81 A-4; 83 A-1 and 86 A-2) were not amplified by the primer pair CMM-5/CMM-6 of Dreier *et al*. (1995), whereas the primer pair tomA-R/tomA-F of Kleitman *et al.* (2008) amplified only 4 of the 6 weakly virulent *Cmm* strains. Nevertheless, all 6 strains induced typical, but very late disease symptoms on the tomato cv. Lyconorma (first symptoms were recorded after 3 months). Accordinly, our newly adapted primer sets B-rev-CM/B-fw-PCM and L-fw-CM/L-rev-PCM showed a reliable amplification of all the new and hypovirulent 6 Austrian *Cmm* isolates, as well as of strain NCPPB 3123. Since these strains were capable to induce typical disease symptoms on tomato plants in our pathogenicity tests, it is necessary that the occurrence of

such strains can be detected by a reliable PCR protocol. Also, Kaneshiro and Alvarez (2001) and Alvarez *et al.* (2005a and 2005b) pointed out that hypovirulent and avirulent *Cmm*-strains potentially play a role in disease development. Especially since these strains can occure rather frequently, up to 50% of all *Cmm* strains on tomato seeds, the hypovirulent or avirulent *Cmm* strains should not be neglected in diagnosis.

For **direct PCR**, a significant **inhibitory effect** of tomato tissues was found, which is consistent to results reported by Nabizadeh-Ardekani (1999) in tomato, but was also proved by Prosen *et al.* (1993) in beans; by Rossen *et al.* (1992) in food and by Schaad *et al.* (1999) in potato tubers. Inhibitors showed the highest concentration in seed-homogenates, followed by leaves, fruit pulp and stems. Nabizadeh-Ardekani (1999) reported the presence of inhibitors also in tissues of tomato plants that inhibited detection of *Pseudomonas syringae* pv. *tomato* with direct PCR in non-diluted or slightly diluted plant samples, so that first amplifications were accomplished in 1:100 diluted plant extracts. But it was not clear, whether the final concentrations of plant extracts in the PCR reaction mixture were 1:100 or 1:500 diluted. According to our results, homogenates of crushed seeds had to be diluted 1:10,000, leaf homogenates 1:1,000, fruit pulp 1:100 and stem homogenates 1:10-diluted in order to enable amplifications by direct PCR. In the laboratory, seeds should not be crushed but soaked in buffer overnight. Then, the buffer should be centrifuged and the supernatant discarded, whereby the effect of inhibitors could be considerably diminished or omitted in the sedimented bacterial template, compared to homogenates of crushed seeds. On the other hand, Hadas *et al.* (2005) found that grinding of seeds was significantly better for detection of *Cmm* in seed lots than other methods based only on seed-soaking. Obviously, *Cmm* cells attached to or existing in tomato seeds are only partly released by seed-soaking. However, hybrid tomato seed is very expensive, so that a non-destructive soaking method has been developed (Biggerstaff *et al.*, 2000).

By direct PCR with pure cell-suspensions of *Cmm* (without DNA extraction) using the primer **B-rev-CM/B-fw-PCM,** visible amplification was started by a minimum of 620 cfu each in the PCR reaction-tube with a final volume of 25 µl (Figure 10). Assuming that 620 cfu are needed for a visible amplification are contained in ~6.0 µl of initial bacterial suspension, means that the minimal bacterial concentration required for a visible amplification is equal to about $10^5$ cfu/ml by direct PCR. Due to the possible effect of inhibitors in plant tissue we recommend dilution of homogenates from stems, fruits or leaves to be 1:10, 1:100, or 1:1,000. This means that the minimal *Cmm* concentration should be $10^6$, $10^7$ and $10^8$ cfu/ml in

homogenates of stems, fruits and leaves, respectively, in order to be detected by direct PCR. Thus, the sensitivity of direct PCR-based detection is very low, since inhibitors play an important role. The inhibition of PCR by plant samples was one of the factors which led to the development of Bio-PCR by Schaad *et al.* (1999).

The **classical PCR** for detecting *Cmm* according to the method suggested by Dreier *et al.* (1995) is based on isolation of bacterial DNA from plant tissues and amplification of the extracted DNA by the primer set CMM-5/CMM-6. In this case the effect of inhibitors will be limited, but the detection sensitivity will also be reduced, because very low *Cmm* populations in plant tissues do not allow sufficient DNA yield that can be detected by classical PCR. According to Dreier *et al.* (1995), a specific amplification of the target DNA was observed in extracts prepared from infected plants with strain NCPPB 382 which induced symptoms and effectively colonized the host plant (1-3 x $10^9$ cfu per g of plant material), and the pathogen was also detected in extracts of 50 seeds containing 1 x $10^3$ bacteria. In fact, the sensitivity of detecting *Cmm* in plant or seed extracts according to Dreier *et al.* (1995) is not satisfactory, since **tolerance to the pathogen is zero** and seed or plant samples could be latently infected with *Cmm* and very highly contaminated with other saprophytic bacteria.

Therefore, the most **challenging situation** in the field of diagnosis of bacterial diseases, is to detect the pathogens in asymptomatic samples from latently infected or contaminated plants that are very highly contaminated with other microorganisms but contain very small numbers of the pathogen. Also in case of *Cmm*, it has been observed again and again that very few infected seeds can cause serious losses during the vegetation period (Chang *et al*. 1991). Such very low numbers of *Cmm* colonies are very difficult to be recognized when the impact of saprophytic bacteria is very high, as shown by Figure 24. Obviously, the **detection protocols** for *Cmm* recommended by EPPO (OEPP/EPPO, 2005) and the International Seed Health Initiative (ISHI, 2008) were not sufficient to prevent further distribution of the pathogen into new areas. Thus, new questions arose regarding the reliability of diagnostic protocols published for *Cmm* and the urgent need for highly sensitive detection methods (Olivier *et al*., 2009).

Even with our newly adapted PCR systems it is impossible to detect very few *Cmm* cells by direct PCR of tomato tissue. Instead, the bacteria have to be cultivated on an agar nutrient medium which allows good growth of *Cmm* but inhibits nearly every other bacterial species occurring under these conditions. However, such a medium was not available, when we started this research project. When *Cmm* concentrations were very low in plant extracts and

accompanied by high concentrations of saprophytic bacteria, *Cmm* could not be visually detected or recognized on any of the published semiselective media for *Cmm*. Either the starting *Cmm*-colonies were overgrown by saprophytic bacteria on media with lower selectivity or the *Cmm*-colonies were partially inhibited on the media with increased selectivity.

Thus, the detection of *Cmm* according to the described protocols was not sensitive enough and false negative results could not be excluded. On all the previously published semi-selective media for *Cmm*, it was difficult to distinguish between *Cmm* colonies and other bacterial species when the contamination by accompanying bacteria was high or when *Cmm* populations were very low.

Therefore, the main weakness in the suggested protocols for detecting *Cmm* by EPPO (OEPP/EPPO, 2005) and ISHI (2008) is due to the semiselective media still being recommended and used. The lack of a good, semiselective and highly sensitive medium for *Cmm* is probably the main reason why Bio-PCR for detecting *Cmm* is not widely accepted until now. According to our knowledge, very few laboratories started to apply Bio-PCR assays for detecting *Cmm* in seeds years ago, using the earlier semiselective media and the earlier PCR protocols. However, until recently a standard Bio-PCR protocol for *Cmm* has not been worked out, although many laboratories may apply now a Taq-Man PCR according to Bach *et al.* (2003) or Luo *et al.* (2007). Our experiments proved the importance of a reliable semiselective or selective medium, since Bio-PCR with other media was not satisfactory, resulting in false negative or much delayed results (10 days or more).

Therefore, we spent more than 3 years for designing a new selective medium for *Cmm*. Fortunately, this great effort was finally successful (chapter 1), and we were able to develop a **new Bio-PCR protocol** by combining the new selective medium with PCR based on the new primer system. Due to their better performance, the three new primer sets were tested in a Bio-PCR assay. In this case, the *tom*A primers were inferior to the other two primer sets that showed better efficiencies of direct *Cmm* detection in plant extracts that were highly contaminated with saprophytes. The best suited primer set was the **B-rev-CM/B-fw-PCM** combination, resulting in very clear fragments.

However, when plant and seed samples were very highly contaminated with saprophytes and only weakly infected with *Cmm*, as was the case in our Bio-PCR experiments with $2 \times 10^8$ or $2 \times 10^7$ cfu/ml saprophytes in plant extracts A or B, respectively, and only 120 cfu/ml *Cmm* in

variants A or B, it was impossible to recognize *Cmm* colonies, even though our new medium was superior in selectivity, detection sensitivity and plating efficiency to all other semi-selective media, and although *Cmm* strains grow very fast on this medium (within 4 and 7 days). The only way for detection very few numbers of *Cmm* cells under these conditions was to apply Bio-PCR (Figure 24). In other plant extracts with higher numbers of *Cmm* or with less numbers of saprophytic bacterial cells, *Cmm* colonies can be visually recognized very easily on the new medium compared to the published semiselective media. There may be no need for Bio-PCR if time is not so important (additional one to three days are needed to recognize *Cmm* colonies easily).

Hadas *et al*. (2005) found out that detection of one infected tomato seed in 10,000 was only possible by Bio-PCR, and only one sample out of five infected replicates was positive by Bio-PCR. But Hadas *et al.* (2005) used the semiselective media CNS (Gross and Vidaver, 1979), mSCM (Waters and Bolkan, 1992) and D2ANX (Chun, 1982) for their Bio-PCR assay. The publication of Hadas *et al.* (2005) and the Bio-PCR assay described by Burokiené (2006) are the only papers on applying Bio-PCR for detecting *Cmm* to our knowledge. The Bio-PCR protocol of Burokiené is not applicable in practice, because this author tested artificially highly infected young plants by plating of plant extracts on a non-selective medium (yeast glucose mineral agar, YGMA) prior to PCR. Thus, detection of *Cmm* was probably only possible in these experiments, because a high *Cmm* inoculum of $10^8$ cfu/ml was used for artificial inoculation of young tomato plants that were not highly contaminated with saprophytes. Such infections are normally detectable without highly sensitive detection methods and without Bio-PCR. Therefore, the Bio-PCR method described by Burokiené is not of any practical impact for detecting *Cmm* in latently infected plant samples that are highly contaminated with saprophytic bacteria. In contrast, by our Bio-PCR assay, as few as 120 cfu/ml of *Cmm* were detectable in the presence of 200,000,000 or 20,000,000 cfu/ml of saprophytic bacteria in all replicates and at all times of evaluations. Theoretically, only one or two bacterial cells of the pathogen/ml could be detected by Bio-PCR, since this bacterial cell can recover on a good selective and sensitive medium, building a colony that contains millions of bacterial cells that are easily detectable.

For a rapid detection of *Cmm*, the agricultural advisers often use "**Immune Strips**" of the company Linaris (Linaris Biologische Produkte GmbH, Hotelstrasse 11, D-97877 Wertheim-Bettingen). This test method is proposed for a rapid diagnosis of *Cmm*-infections of tomato plants, even before the first symptoms appear. However, in our experiments this test

procedure proved to be not reliable, and first positive signals were recorded with a minimum *Cmm*-population of $10^5$ cfu/ml plant extract. Infection levels of $10^4$, $10^3$, $10^2$ and 10 cfu/ml were not detected. In comparison, detection of *Cmm* by the new Bio-PCR assay, or only by plating plant extracts on the new selective media is much more sensitive.

Visual tests of all other semiselective media developed for *Cmm*, such as D2ANX (Chun, 1982), CMM1 (Alvarez and Kaneshiro, 1999), mCNS (Gitaitis *et al.*, 1991), SCM (Fatmi and Schaad, 1988), D2 (Kado and Heskett, 1970), KBT (Dhanvantari, 1987) on their efficiency for detection of *Cmm* in latently infected seed and plant materials often revealed false negative results. Such false negative results occurred because *Cmm*-colonies were overgrown and inhibited by saprophytes. Therefore, the use of these published media is not suited for a sensitive Bio-PCR protocol.

Obviously, the biological amplification (by Bio-PCR), based on application of the new selective medium in combination with PCR using the new primers improved the detection sensitivity considerably. Thus, very few bacterial cells contained in plant extracts in the presence of high populations of numerous other microorganisms allowed detection of very few non-recognizable *Cmm* colonies within 4 days (Figure 24).

In very short **summary**, our new Bio-PCR protocol reduced the time needed for detection of *Cmm*, eliminated the effect of inhibitors present in plant tissues and significantly improved the sensitivity of classical PCR, allowing detection of very small numbers of the target bacterium, also in presence of high populations of numerous saprophytic bacteria in plant extracts which prevented recognizing of *Cmm* colonies on the new selective medium. This new Bio-PCR protocol is faster than earlier Bio-PCR protocols using other semiselective media. False negative results were never obtained and all the tested 76 *Cmm* strains of different origin were amplified without exception.

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# Chapter 3

## Efficacy of different disinfection methods for eradication of *Clavibacter michiganensis* subsp. *michiganensis* from tomato seed


## Summary

Various chemical, physical and fermentative treatments were tested on their efficacy for eradicating the phytopathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), the causal agent of bacterial canker of tomato, from systemically infected tomato seed. The pathogen was absolutely eradicated from 100% infected seed by several chemical and physical treatments without significant reduction in seed germination capacity.

Initial experiments were carried out with healthy tomato seed for adjusting and selecting the most effective concentrations of chemicals, soaking time and treating temperatures without reduction of seed germination capacity.

Selected treatments were applied in further experiments parallely on systemically infected tomato seed produced in greenhouse trials and on healthy seed. All treatments were evaluated on their efficacy in eradicating *Cmm* from seed, in reducing populations of saprophytic bacteria accompanied with tomato seed, and in their impact on seed germination capacity.

Seed infection was determined by the plating assay technique of seed homogenates on agar media from 200-300 seeds of each treatment. Germination capacity was determined for three 100-seed replicates of each treatment in blotter (filter paper) and for other three 100-seed replicates in soil at greenhouse conditions.

Complete eradication (100%) of *Cmm* from seeds without a significant reduchtion of germination rates was obtained by **soaking 100% systemically** and very heavily infected seeds at room temperatures in a solution of:

- 5% MENNO-Florades$^{TM}$ for 120 min,
- 3% HCl for 60 min,
- 3% HCOOH for 60 min,
- 5% HCOOH for 30 min,
- 5% $CH_3COOH$ for 120 min,
- in warm water at 52 °C for 60 min,
- in warm water at 54 °C for 30 min.

Also, **fermentative treatments** for 96 h of slightly infected seeds, as well as **acid seed extraction** with 0.1M, 0.6M or 1.0M HCl for one hour, reduced seed infection from 40% or 48% to zero with each treatment, respectively, without significant reduction of seed germination capacity.

All the other treatments reduced seed infection with *Cmm* from 100% to levels between 0.3% and 3.0%.

All treatments were also evaluated on their efficacy against accompanying saprophytic bacteria existing in or on tomato seeds. All treatments, except fermentative treatments, reduced the population of saprophytic bacteria but did not eradicate them absolutely from seeds. Saprophytic bacteria were much more resistant to all the disinfection treatments by chemicals, high temperatures or fermentations, compared with *Cmm*.

When seed germination capacity was determined 8 months after treatment by chemicals, no reduction in germination rates was recorded.

In addition, **hot air treatments** of dry and wet seeds at 63-64 °C for 15, 48 and 96 h were investigated. However, these treatments were not successful.

Thus, several treatments proved to be effective in eradicating *Cmm* from tomato seed without significant reduction of seed germination capacity, and these treatments can be recommended to the seed industry for avoiding disease transmission *via* infested seeds into new areas.

## Introduction

*Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), Smith 1910 (Davis *et al.*, 1984) the causal agent of bacterial canker of tomato (*Solanum lycopersicum*) is considered an $A_2$ quarantine organism by the European Plant Protection Organization (Council Directive 2000/29/EC; OEPP/EPPO, 1982) and was transmitted worldwide by infested or infected seeds within the last century into nearly all main tomato production areas. The disease can be very destructive. The incidence of the disease has increased recently in several European countries and many other locations worldwide (CABI/EPPO, 2009).

Disease control in the field or in greenhouses is very difficult, once the disease appears, because bactericides for control are not available. Disease resistance is known (Coaker *et al.*, 2004; Poysa, 1993; van Steekelenburg, 1985) but has not been incorporated into commercial tomato cultivars.

Therefore, hygienic measures play an important role in disease control. Infected and neighboring plants must be destroyed as soon as possible, and very strict hygienic measures have to be applied after disease appearance (Strider, 1969). The pathogen survives in plant debris (Farley, 1971; Fatmi and Schaad, 2002; Ftayeh *et al.*, 2004; Gleason *et al.*, 1991; Moffett and Wood, 1984; Strider, 1967; Strider, 1969). When residues from infected plants are not decomposed between the vegetation periods, or the bacteria are not killed by high temperatures in dry soil in warm climates, the disease may be transmitted by the soil (Ftayeh, 2004; Ftayeh *et al.*, 2004). However, disease occurrence was observed even when all possible hygienic measures were applied and when tomato plants were grown in artificial substrates, indicating that infected seeds play a very important role in disease-transmission.

Obviously, tomato seeds are the most important inoculum source (Bryan, 1930; Kruger, 1959; Moffett *et al.*, 1983; Shoemaker and Echandi, 1976; Strider, 1969; Thyr, 1969; Tsiantos, 1987), and even symptomless young tomato plants can contain high populations of *Cmm* (Werner *et al.*, 2002). Seed transmission has been reported to be less than 1% (Grogan and Kendrick, 1953). However, only 0.01 to 0.05% infested seeds can cause an epidemic in suitable conditions (Chang *et al.*, 1991). Tomato seeds can be surface infested with the pathogen (Bryan 1930; Shoemaker and Echandi, 1976) or internally infected (Bryan 1930; Moffett *et al.*, 1983).

Although seed infection or infestation is not well understood, the use of pathogen-free seed,

whether obtained naturally or by treating seeds with chemical eradicants, could eliminate a potential source of inocula (Fatmi *et al.*, 1991) and is considered to be the best strategy for controlling the disease.

Many scientists have worked on treatments of tomato seed for eradication of *Cmm*, e.g. Ark (1944); Blood (1933 & 1937); Dhanvantari (1989); Dhanvantari and Brown (1993); Fatmi *et al.* (1991); Thyr *et al.* (1973). However, most of the treatments investigated by previous researchers were either not effective to eradicate the pathogen absolutely from seeds or were effective but simultaneously severely reducing seed germination capacity.

The aim of this chapter was to test the efficacy of different treatments for eradicating *Cmm* from systemically infected seeds, in order to select effective ones that could be applied in practice by the seed industry, by nurseries or even by individual growers to eliminate the pathogen from tomato cultures.

# Materials and Methods

### Antibiotic resistant *Cmm*-mutant

From a highly virulent strain of *Cmm* (BO) we selected the spontaneous mutant BO-RS with resistance against two antibiotics (100 ppm rifampicin and 600 ppm streptomycin). With this antibiotic resistant mutant it was much easier to determine bacterial concentrations in seeds, by plating aliquots of seed homogenates on NGY agar, supplemented with both antibiotics. The NGY medium contained: 0.8% nutrient broth (Roth, Karlsruhe, Germany), 1% glucose (AppliChem, Darmstadt, Germany) and 0.3% yeast extract (Roth). The mutant was obtained by cultivating *Cmm* strain BO in 100 ml of Rhodes liquid medium (Rhodes, 1959) for 12 h at 25 °C and 110 rpm, using a HT Multitron 2 incubator (Basel, Switzerland) or ETI incubator from Clim-O-Shake (Adolf Kühner AG, Basel, Switzerland) and then introducing 5 ppm of rifampicin into the bacterial culture. After 24 h, one ml from this culture was introduced into a new Erlenmeyer flask with 100 ml Rhodes liquid medium, and after 12 h a higher concentration of rifampicin was added so that a final concentration of 15 ppm resulted. After visible growth of the bacterial culture, one ml of this culture was introduced into a new Erlenmeyer flask, and the process was repeated again with increasing concentrations of rifampicin of 25, 40, 60, 80 and 100 ppm. Mutation and culture purity were tested on NGY agar plates with or without rifampicin. In order to obtain the second mutation against streptomycin, the same procedure was followed with increasing streptomycin concentrations of 25, 50, 100, 200, 300, 400, 500 and 600 ppm, on the rifampicin-resistant *Cmm* strain. Thus, we obtained the *Cmm*-strain BO-RS which proved to be virulent in pathogenicity tests.

### Bacterial inocula and testing of different inoculation methods with *Cmm*

Inocula were prepared by transferring a loopful of a 24-h-old bacterial culture into 0.01M $MgSO_4$ and adjusting the optical density (OD) to 0.06 at 660 nm ($\approx 10^8$ cfu/ml) using a photometer (Spectronic 20, Bausch & Lomb, USA). The needed bacterial concentrations were prepared by 1:10- serial dilutions.

Different inoculation methods were tested on efficiency for production of *Cmm*-infected seeds (**Table 1**), in order to obtain tomato seeds with a very high infection level with *Cmm* for our experiments.

The following inoculation methods were tested (summarized in table 1):

- Inoculating the axil of the 3rd or 4th leaf of two-month-old plants with 50 µl of a bacterial suspension of $10^4$ cfu/ml (A1), $10^6$ cfu/ml (A2), or with $10^8$ cfu/ml (A3).
- Spraying a bacterial suspension of $10^6$ cfu/ml (B1) or $10^8$ cfu/ml (B2) onto the flowers.
- Spraying a bacterial suspension of $10^6$ cfu/ml (C1) or $10^8$ cfu/ml (C2) onto very small fruits (directly after pollination).
- Inoculating the bases of fruit peduncles (with small two-week-old fruits) with 50 µl of bacterial suspensions of $10^4$ cfu/ml (D1), $10^6$ cfu/ml (D2) or with $10^8$ cfu/ml (D3).
- Injection into small fruits using a syringe with 50 µl of bacterial suspension of $10^2$ cfu/ml (E1) or $10^4$ cfu/ml (E2).
- Spraying small fruits using a force-pump with bacterial suspensions of $10^4$ cfu/ml (F1) or $10^6$ cfu/ml (F2).
- Artificial inoculation of tomato seeds under vacuum (G): Tomato seeds were soaked in a bacterial suspension of $10^8$ cfu/ml for 30 min, then evacuated at -0.95 bar for 15 min followed by release of the vacuum, so that the bacterial suspension was infiltrated into the seeds.

Tomato seeds obtained after these different inoculation methods were extracted with water, dried, labelled and stored at 4 °C until evaluation of infection with *Cmm*. Twenty-five single seeds from each trial were crushed (each single seed separately) in 0.5 ml sterile water and 100 µl each of three serial dilutions ($10^0$, $10^{-1}$ and $10^{-2}$) were plated onto NGY plates, incubated at 26 °C and finally grown *Cmm* colonies were counted to determine the efficiency of each inoculation method for seed infection.

**Table 1.** Designation of bacterial suspensions of different inoculation methods tested for production of infected seeds

| Inoculation method | Designation of bacterial suspension |
|---|---|
| Inoculation of leaf axil | **A1**: $10^4$; **A2**: $10^6$; **A3**: $10^8$ |
| Spraying of flowers | **B1**: $10^6$; **B2**: $10^8$ |
| Spraying of small fruits | **C1**: $10^6$; **C2**: $10^8$ |
| Inoculating the basis of fruit peduncles | **D1**: $10^4$; **D2**: $10^6$; **D3**: $10^8$ |
| Injecting small fruits with syringe | **E1**: $10^2$; **E2**: $10^4$ |
| Spraying small fruits with pressure pump | **F1**: $10^4$; **F2**: $10^6$ |
| Infection under air pressure after vacuum release | **G:** $10^8$ (at -0.95 bar for 15 min) |

**Screening of different chemical and physical seed treatment methods**

For adjusting acid concentrations, temperatures and soaking time for each treatment, several treatment methods using different chemicals or warm water were carried out with healthy tomato seeds of the cultivar Marmande. Marmande cultivar was obtained from International Seed Processing GmbH, Quedlinburg, Germany. Pre-treatments were evaluated on their effect on seed germination capacity on wet filter paper at room temperature.

Treatments with no or with low impact on seed germination capacity were selected and applied later on *Cmm*-infected tomato seeds. From these treatments, only those with strongest concentrations, longest soaking time or highest temperatures with very low or without significant reductions of seed germination capacity were selected and later applied on systemically infected seeds to achieve an absolute eradication of *Cmm* from seeds without significant reduction in seed germination capacity.

**Seed lots, seed infection and storage conditions**

For evaluating the efficacy of each treatment, two different seed cultivars were used. Healthy seeds of the cultivar Marmande were used for evaluating the impact of different treatments on seed germination capacity. For evaluating the impact of each treatment on infection with *Cmm*, systemically infected seeds of the cultivar Lyconorma were used. Seed infection was achieved in greenhouse trials by inoculating the bases of very small fruit peduncles. Thirty µl of bacterial suspensions of $10^4$ cfu/ml (strain BO-RS) were placed on the axil of fruit or flower peduncles (during flowering or one to three days directly after pollination of the first flowers), and the axils of fruit/ flower peduncles were stabbed by a needle through the suspension drop.

In some experiments this inoculation was supported by a spray application of *Cmm*-suspension (ca. $10^5$ cfu/ ml) onto the flowers.

Seeds of the tomato cultivar Lyconorma used for chemical and physical treatments were highly infected with *Cmm* with an infection rate of 100%, and the bacterial concentration ranged from 4 x $10^2$ to 1 x $10^5$ cfu/ seed.

For the fermentation treatments or seed extraction with hydrochloric acid, freshly extracted seeds of cultilvar Lyconorma with an infection level of 40% or 48%, respectively, were used. The Lyconorma seeds used for fermentation or extraction with hydrochloric acid were colonized with low populations of *Cmm* (5-200 cfu/infected seed).

In order to remove residual chemicals after treatments, acid-extracted seeds were immediately rinsed and soaked for 10-15 min in sterile water. Fermented seeds were washed with sterile water. Finally, seeds were dried on a laminar flow bench and stored:

- at 4 °C for evaluation of infection by *Cmm*,
- or at room temperature for evaluation of germination capacity.

**Evaluation of seed germination capacity**

Evaluation of seed germination capacity was carried out with healthy seeds of the cultivar Marmande and started within two and 10 weeks after treatments. For each treatment 300 seeds of cultivar Marmande were tested in three replicates (each with 100 seeds) on germination capacity on wet filter paper at room temperature (**Figure 1**) and 300 other seeds from different replicates of each treatment were evaluated in three replicates (each100 seeds) on germination capacity in soil in multi-pot trays at greenhouse conditions (**Figure 2**). The soil mixture containing 1/3 sand, 1/3 compost and 1/3 loamy field soil was autoclaved at 121 °C for 15 min. Seed germination capacity was determined after 2-3 weeks on filter paper and after 3-4 weeks in soil. Greenhouse temperatures ranged between 17 and 30 °C and the relative humidity ranged between 15 and 70%. Room temperatures ranged between 14 and 25 °C.

For both, soil and blotter germination trials, only seedlings with fully expanded cotyledons and first true leaves were considered as germinated. Seedlings that did not develop true leaves or that were damaged were determined as not germinated.

**Figure 1.** Seed germination tests at room conditions in blotter (on wet filter paper).

**Figure 2.** Seed germination tests in soil at greenhouse conditions.

### Evaluation of seed infection

For each treatment 200 or 300 Lyconorma seeds from different replicates were evaluated for infection with *Cmm* within one and three weeks after each treatment. Evaluation of tomato seeds for germination capacity was carried out within one to two months after treatments.

For evaluating the residual infection with *Cmm* after each treatment, 200 or 300 seeds of each treatment were crushed (every single, 3, 5 or 10 seeds together) in sterile water and 100 µl of the homogenates were plated onto NGY medium supplemented with 25-50 ppm rifampicin

and 200 ppm streptomycin for evaluating the infection with *Cmm*. Another 100 µl, each from the non-diluted and 1:10 diluted homogenates, were plated onto NGY medium without antibiotics to determine the survival rates of saprophytes. **Initially 100 single seeds** of each treatment were ground separately in 0.5 ml sterile water and further on, every 3, 5 or 10 seeds - (according to the residual infection with *Cmm*) - were crushed together in one ml sterile water and 100-200 µl of seed homogenates were plated on the agar media described above to determine the infection with *Cmm* or contamination with saprophytes.

**Seed treatments**

Infected (100%) 'Lyconorma' seeds as well as healthy 'Marmande' seeds were treated with chemical solutions, or physically with hot water or hot air. Seed infection was determined with 'Lyconorma' seeds, whereas germination capability was determined with 'Marmande' seeds. In other experiments, freshly extracted Lyconorma seeds, 48% or 40% infected with *Cmm*, were either extracted with hydrochloric acid for 1 h, or were fermented with the pulp juice for 72 or 96 h, respectively.

**Chemical and hot water treatments**

Chemical and hot water treatments were applied on 100% infected 'cv. Lyconorma' seeds and on healthy 'Marmande' seeds in parallel (Table 3). These chemical treatments were carried out using the disinfectant MENNO Florades$^{TM}$; benzoic acid ($C_6H_5COOH$); hydrochloric acid (HCl); formic acid (HCOOH); lactic acid ($CH_3CHOHCOOH$); sodium hypochlorite (NaOCl); or with acetic acid ($CH_3COOH$).

Solutions of chemicals were freshly prepared and their concentrations were adjusted with bidest water. Concentrations of sodium hypochlorite (NaOCl) were adjusted depending on chlorine concentrations (1% or 2% NaOCl means final chlorine concentrations of 1% and 2%).

As shown in **Table 3**, different soaking times and temperatures were tested for the chemical treatments.

Chemical treatments were carried out in small Erlenmeyer flasks (50 ml) with a relation of 10 ml of each solution for each gram of seeds, and during seed treatment flasks were covered to inhibit acid evaporation. In parallel, non-infected seeds of cultivar Marmande were subjected to the same treatments for determining the possible reduction of seed germination capacity.

Hot water treatments or chemical treatments at high temperatures, such as treatment with MENNO Florades$^{TM}$ or sodium hypochlorite, were performed in Erlenmeyer flasks that were kept in a digital water bath (Memmert, Germany). Soaking of the seeds was started when the exact temperatures were reached inside the flasks for avoiding possible differences in temperatures between water bath and the content of the flasks.

Seeds treated with chemicals were washed immediately with sterile water several times, soaked in sterile water for 15 min and washed again after soaking to remove residual acid from the seeds. Seeds treated with hot water were also washed with sterile water for normalizing seed temperatures.

**Seed fermentation**

Infected 'Lyconorma' seeds were removed from the ripe fruits together with the surrounding gelatinous pulp. After mixing the pulp on a magnetic stirrer a portion was taken from the juice as non-fermented control. The other part was fermented at 20 °C for 72 or 96 h. Immediate analysis of the non-fermented juice revealed that about 40% of the seeds were colonized by a low level of *Cmm* (5 to 200 cfu per infected seed). After fermentation, seeds were washed several times with sterile water, dried on a laminar flow bench and evaluated for *Cmm*-infection and germination capacity.

**Seed extraction with hydrochloric acid**

In separately performed experiments, infected (48%) 'Lyconorma' seeds were extracted together with the surrounding gelatinous pulp. This pulp was mixed well using a magnetic stirrer and was divided into four portions, one for control, and three portions for the treatments with HCl. The volume of the three portions was adjusted with water and supplemented with defined volumes of hydrochloric acid so that final concentrations of 0.1, 0.6 and 1.0M HCl were obtained inside the pulp that was stirred by a magnetic stirrer during the time of treatment (60 min). Then seeds were filtered in a strainer, washed three times with sterile water, soaked in sterile water for 15 min, washed again for releasing residual acids and dried. Finally, seeds were evaluated on germination capacity and infection with *Cmm.*

**Seed treatments with hot air**

Hot air treatments of healthy "Marmande" and 100% infected "Lyconorma" seeds were accomplished in a digital incubator (Memmert, Germany) at 63-64 °C for 15, 48 or 96 h. The treatments were carried out with dry and wet seeds. For wetting 1-2 ml water were mixed with 1 g seed before beginning the treatment. Seeds were placed in plastic petri dishes to avoid direct contact with the incubator. After finishing the treatment, Lyconorma seeds were evaluated for infection with *Cmm* and Marmande seeds were evaluated for germination capacity.

**Statistical analysis**

Results of the different treatments were evaluated for **germination capacity** in soil or in blotter separately and analyses were done with ONEWAY ANOVA, using SPSS Statistics 17 (Version 17.0.0), comparing germination capacity after each treatment with the control (untreated) for each soil or blotter trials separately.

Statistical analyses of the treatments' effects on **infection with *Cmm*** were not done because the infections were significantly reduced from 100% to values between 3% and zero by all chemical and physical treatments as listed in Table 3, or from 40% to values between 2% and zero by seed fermentation compared with non-fermented seeds (Table 4) and from 48% to zero when seeds were extracted with hydrochloric acid (Table 5).

# Results

### Screening of different inoculation methods with *Cmm*

Seed infection with *Cmm* was evaluated for each inoculation method. Inoculation methods differed strongly in efficacy of seed infection ranging between zero and 100% (**Figure 3**).

The highest infection rates were obtained by:
- artificial inoculation with air pressure after evacuation of soaked seeds (G: 100%),
- spraying small fruits without pressure (C1: 84% and C2: 92%),
- injection of small fruits with a bacterial suspension (E1: 84% and E2: 68%).

Spraying flowers with $10^8$ cfu/ml (B2) also caused a high infection rate (56%), but all these inoculation methods have several disadvantages that are listed in **Table 2**. Therefore, these methods were not used to produce systemically infected tomato seeds that were needed for the seed disinfection experiments.

Inoculating the basis of fruit peduncles with a bacterial suspension of $10^4$ cfu/ml (D1) caused an infection of 68%. This method was modified into (H) by inoculating very small flower peduncles while in pollination or directly after pollination, supported with spray aplications of *Cmm*-suspension ($10^5$ cfu/ml) onto the flowers, so that all the seeds were infected (100%) with a very high level of the *Cmm* population. This method was used for production of infected seeds, because it was easier to be carried out than the other methods and closer to natural infections when plants are injured or cut and the pathogen can infect the plant tissue systemically.

Induction of **birds' eye spots** was only possible when very small fruits were sprayed with a bacterial suspension of $10^8$ cfu/ml, but not with $10^6$ cfu/ml and not with any other inoculation method (**Figure 4**). Naturally, birds' eye spots seem to occur due to external infections when dew drops containing high bacterial populations fall in early morning from infected plants onto the small fruits, and the bacteria infect the fruits through stomata (**Figure 5 A and B**). This may be the explanation why birds' eye spots often appeared only on one side of naturally infected fruits, as shown in **Figure 5 (A** and **B**).

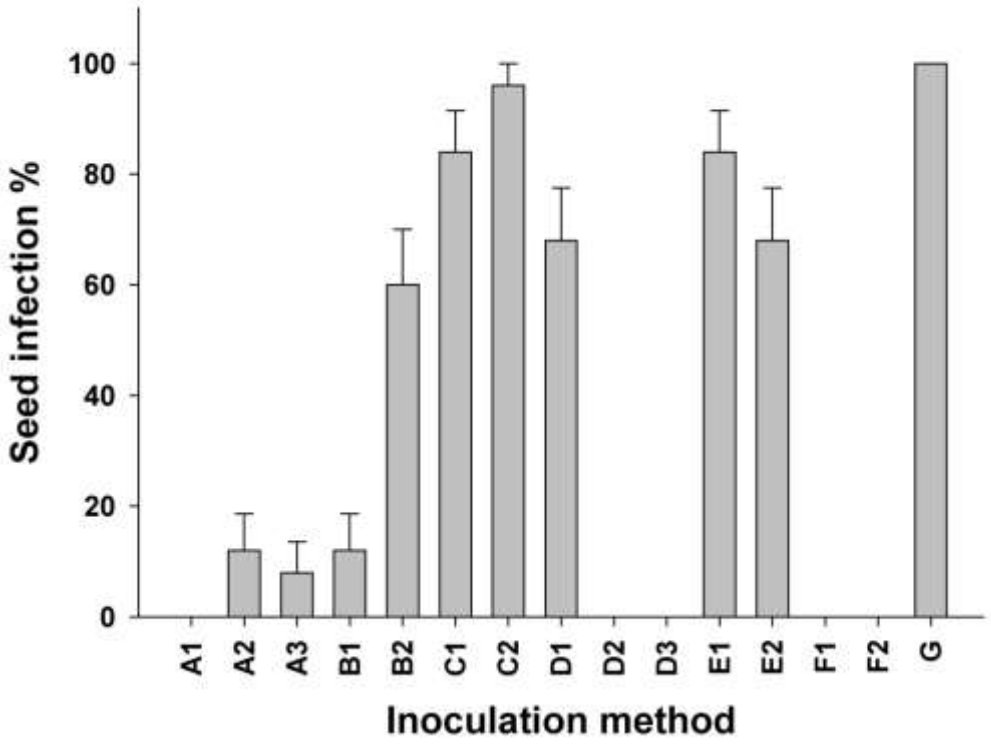


**Figure 3.** Seed infection rate with *Cmm* resulting from different inoculation methods. Inoculation methods are explained in Table 1.

**Figure 4.** Formation of bird's eye spots on a young fruit that was sprayed in a younger stage with a *Cmm*-suspension of $10^8$ cfu/ml.


**Figure 5.** Bird's eye spots concentrated on one side of tomato fruits collected from naturally infected open field **(A)**, whereas no spots appeared on the other sides of the fruits (**B**).

**Table 2.** Advantages and disadvantages of different inoculation methods

| Inoculation method | Advantages | Disadvantages |
|---|---|---|
| Inoculating leaf axils | Easy to do | Fruits that are far from the inoculation site do not become infected, especially by low inocula |
| Spraying the flowers | By $10^8$ cfu/ml seed infection was 56% | Difficult to do and time consuming, new growing flowers must be sprayed 1-2 times weekly |
| Spray onto small fruits (diameter ≤ 1.0 cm) | By $10^8$ cfu/ml bird's eye spots developed and seed infection was 92% | Time consuming, newly developing fruits must be sprayed once a week |
| Inoculating the basis of fruit peduncles | Easy, seed infection by suspensions of $10^4$ cfu/ml was 68%, but by higher concentrated suspensions, seed infection was zero. | Inoculation points in field trials were infected with *Phytophthora infestans*, but the method was good in greenhouse trials |
| Injection of small fruits | High infection rates | Fruits remained small producing seeds very seldom or very few seeds |
| Spraying small fruits with pressure pump | | Difficult to do, although fruits were infested, we never obtained infected seeds |
| Vacuum inoculation | Very fast, easy to do in the lab, time saving and no need to inoculate plants, seeds infection was 100% | Far away from natural infection ways, seeds might be only externally infested |

**Effect of seed treatments towards *Cmm* bacteria**

Dry tomato seeds of the cultivar 'Lyconorma' infected systemically to 100% with *Cmm* were treated chemically (with acids) or physically (with hot water or hot air). The *Cmm*-population ranged between $4 \times 10^2$ and $1 \times 10^5$ cfu/ seed. The infection rate of other 'Lyconorma' freshly extracted seeds used for fermentative treatments or for extraction with HCl was 40% or 48%, respectively, with *Cmm*-populations between 5 and 200 cfu/ infested seed. Except of hot air treatment of dry seeds, all the treatments tested reduced *Cmm*-infection to levels between zero and 3.0%.

**Successful eradication of *Cmm* from infested seeds by chemical or hot water treatments**

All the treatments listed in **Table 3** differed in efficacy against *Cmm*. **A complete eradication** (100%) of *Cmm* from seeds, without any significant reduction in seed germination capacity, was achieved by the following treatments:

- 5% MENNO-Florades™ for 120 min at room temperature
- 3% HCl for 60 min at room temperature
- 3% HCOOH for 60 min at room temperature
- 5% HCOOH for 30 min at room temperature
- 5% $CH_3COOH$ for 120 min at room temperature
- warm water at 52 °C for 60 min
- warm water at 54 °C for 30 min

All the other treatments reduced *Cmm*-infection to 0.3-3.0%. The highest residual infection rate (3%) was recorded for treatments with 1 or 2% NaOCl at 40 °C for 60 min (Table 3).

**Table 3.** Effect of tomato seed treatments with chemical disinfectants or hot water on infection with *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), on seed germination rate and on population densities of *Cmm* and seed-associated saprophytic bacteria

| Treatment[w] | Seed infection with *Cmm* (%)[x] ± std. dev. | Seed germination (%)[y] ± std dev. | | CFU[z] / 100 seed | |
|---|---|---|---|---|---|
| | | in soil | in blotter | *Cmm* ± std. dev. | Saprophytes |
| Untreated seeds (control) | 100.0 ± 0.0 | 88.3[a] ± 1.2 | 79.0[d] ± 13.2 | (2.8 ± 0.9) × $10^6$ | 3.9 × $10^6$ |
| 5% MF, 60 min, rt | 0.5 ± 0.7 | 82.3[a] ± 7.4 | 83.0[d] ± 7.0 | (2.6 ± 3.6) × $10^3$ | 7.8 × $10^4$ |
| 5% MF, 120 min, rt | 0.0 ± 0.0 | 80.3[a] ± 6.7 | 73.0[d] ± 16.5 | 0.0 ± 0.0 | 4.9 × $10^4$ |
| 15% MF, 60 min, rt | 0.7 ± 0.6 | 83.3[a] ± 11.0 | 79.0[d] ± 4.0 | (7.3 ± 13.0) × $10^2$ | 1.1 × $10^4$ |
| 0.45% $C_6H_5COOH$, 60 min, rt | 0.5 ± 0.7 | 84.0[a] ± 5.3 | 74.7[d] ± 13.5 | 70.0 ± 99.0 | 1.1 × $10^5$ |
| 0.45% $C_6H_5COOH$, 120 min, rt | 0.5 ± 0.7 | 85.3[a] ± 7.6 | 80.7[d] ± 5.5 | 40.0 ± 56.6 | 9.4 × $10^4$ |
| 3% HCl, 30 min, rt | 0.3 ± 0.5 | 90.3[a] ± 0.6 | 76.3[d] ± 15.0 | 18.0 ± 35.0 | 1.1 × $10^5$ |
| 3% HCl, 60 min, rt | 0.0 ± 0.0 | 88.3[a] ± 2.9 | 78.0[d] ± 7.0 | 0.0 ± 0.0 | 4.8 × $10^3$ |
| 3% HCOOH, 60 min, rt | 0.0 ± 0.0 | 84.0[a] ± 3.0 | 75.0[d] ± 5.2 | 0.0 ± 0.0 | 1.8 × $10^4$ |
| 5% HCOOH, 15 min, rt | 0.5 ± 0.7 | 85.0[a] ± 10.8 | 71.0[d] ± 8.9 | 50.0 ± 70.7 | 1.7 × $10^4$ |
| 5% HCOOH, 30 min, rt | 0.0 ± 0.0 | 82.7[a] ± 3.2 | 80.0[d] ± 3.5 | 0.0 ± 0.0 | 2.6 × $10^4$ |
| 5% HCOOH, 60 min, rt | 0.0 ± 0.0 | 62.3[b] ± 6.4 | 51.7[e] ± 17.1 | 0.0 ± 0.0 | 1.2 × $10^4$ |
| 5% lactic acid, 60 min, rt | 1.0 ± 1.4 | 84.3[a] ± 6.0 | 81.0[d] ± 9.54 | (2.0 ± 2.8) × $10^2$ | 3.5 × $10^4$ |
| 1% NaOCl, 60 min, 40°C | 3.0 ± 1.7 | 86.3[a] ± 3.2 | 82.7[d] ± 2.1 | (3.4 ± 4.1) × $10^2$ | 1.8 × $10^4$ |
| 2% NaOCl, 60 min, 40°C | 3.0 ± 1.4 | 82.3[a] ± 14.2 | 80.3[d] ± 7.2 | (5.1 ± 2.7) × $10^3$ | 4.5 × $10^3$ |
| 5% $CH_3COOH$, 60 min, rt | 0.3 ± 0.6 | 90.0[a] ± 1.7 | 83.3[d] ± 5.0 | (4.9 ± 8.5) × $10^2$ | 9.3 × $10^4$ |
| 5% $CH_3COOH$, 120 min, rt | 0.0 ± 0.0 | 82.7[a] ± 6.8 | 81.7[d] ± 11.9 | 0.0 ± 0.0 | 6.1 × $10^4$ |
| $H_2O$, 60 min, 52°C | 0.0 ± 0.0 | 86.0[a] ± 9.2 | 80.0[d] ± 1.7 | 0.0 ± 0.0 | 5.4 × $10^4$ |
| $H_2O$, 30 min, 54°C | 0.0 ± 0.0 | 81.3[a] ± 6.0 | 67.7[d] ± 11.9 | 0.0 ± 0.0 | 3.3 × $10^4$ |
| $H_2O$, 60 min, 54°C | 0.0 ± 0.0 | 71.7[b] ± 9.9 | 59.0[e] ± 17.6 | 0.0 ± 0.0 | 3.2 × $10^4$ |

[w] rt = room temperature.

[x] Data represent the mean of seed infection taken from 200 or 300 seeds (cv. Lyconorma) from different replicates for each treatment; std. dev. = standard deviation.

[y] Data represent the mean of germinated seeds (cv. Marmande) from three 100-seed replicates in soil under greenhouse conditions and other three 100-seed replicates in the blotter test at room temperature; values followed by the same letters (a or d) do not differ significantly ($P \leq 0.05$) when compared to the control, according to Fisher's least significant differences (LSD) test.

[z] Data represent the mean number of colony forming units (CFU) of *Cmm* or seed-associated saprophytes in 100 seeds; data for *Cmm* populations were derived from two or three replicates each with 100 seeds and from only 100 seeds for saprophytic bacteria.

**Effect of seed fermentation**

Fermenting seeds for 96 h at 20 °C reduced infection from 40% to zero, whereas fermenting seeds for 72 h at 20 °C reduced infection from 40% to 2% (Table 4).

The populations of saprophytic bacteria in seeds increased 100-fold after seed fermentation for 72 h, i. e. from $4.4 \times 10^6$ cfu to $1.0 \times 10^8$ cfu/ 100 seeds (**Table 4**). However, the diversity of saprophytes decreased after fermentation for 72 h compared with non-fermented seeds, because those saprophytes that were resistant to low pH values survived and increased in population.

**Table 4.** Effect of seed fermentative treatments (cv. Lyconorma) on infection rate with *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), on germination rate, and on population densities of *Cmm* and seed-associated saprophytes

| Treatment | Infection (%) with *Cmm*[w] ± std. dev. | Germination (%)[w] ± std. dev.[c] | | CFU per 100 seed | |
|---|---|---|---|---|---|
| | | in soil | on filter paper | *Cmm*[w] ± std. dev. | saprophytes[y] |
| Control (non-fermented) | 40.0 ± 7.71 | 93.3[*] ± 0.58 | 62.0[*] ± 35.09 | 1425 ± 318.2 | $4.4 \times 10^6$ |
| Fermentation, 72 h, 20°C | 2.0 ± 2.83 | 85.3[*] ± 5.13 | 66.67[*] ± 1.15 | 180.0 ± 254.56 | $1.1 \times 10^8$ |
| Fermentation, 96 h, 20°C | 0.0 ± 0.0 | 90.0[*] ± 1.73 | 78.8[*] ± 5.66 | 0.0 ± 0.0 | not available |

[w)] data represent the mean from three replicates, each wilth 100 seeds; std. dev. = standared deviation.
[y)] values of saprophytic bacteria were taken from 100 seed only.
[*)] indicates no significant differences when compared to the control, according to LSD test ($P \leq 0.05$).

**Seed extraction with hydrochloric acid**

The tomato seeds used in these experiments were slightly infected with *Cmm* (48%). By seed extraction with 0.1, 0.6 or 1.0M hydrochloric acid, seed infection was reduced to zero without any significant reduction in seed germination capacity compared with the control seeds that were extracted with water only (Table 5).

**Table 5.** Effect of seed extractions with hydrochloric acid on the infection rate with *Cmm* and on seed germination

| Seed-extraction, duration, temperature | Germination capacity in soil ± std. dev. | infection % with *Cmm* | cfu of *Cmm*/ 100 seeds ± std. dev. |
|---|---|---|---|
| water (control) | 95.59[a] ± 1.88 | 48 | 5095 ± 3296 |
| 0.1M HCl, 60 min, rt | 97.06[a] ± 1.13 | 0.0 | 0.0 ± 0.0 |
| 0.6M HCl, 60 min, rt | 95.59[a] ± 2.47 | 0.0 | 0.0 ± 0.0 |
| 1.0M HCl, 60 min, rt | 97.55[a] ± 1.88 | 0.0 | 0.0 ± 0.0 |

Same letters following values of seed germination capacity mean no significant differences.

**Effect of hot air treatments**

Treating dry infected seeds with hot air at 63-64 °C for 15, 48 or 96 h did not reduce germination capacity or infections with *Cmm*. In contrast, treating wetted seeds with hot air caused a reduction in seed infection with *Cmm*, but also in seed germination capacity, to zero (**Table 6**). Thus, hot air treatments under the conditions of these experiments cannot be used for eradicating *Cmm* from seeds. It is possible that treating wetted seeds at lower temperatures than 63 °C might reduce or eliminate infection with *Cmm* without reduction in seed germination capacity, but we did not test such lower temperatures.

**Table 6.** Effect of hot air treatment of tomato seed at 63-64 °C on infection with *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) and on the seed germination rate (wet blotter test)

| Treatment | Seed infection (%)[w] of | | Seed germination (%)[x] ± std. dev. | |
|---|---|---|---|---|
| | dry seeds | wetted seeds | dry seeds | wetted seeds |
| untreated | 100.0 | 100.0 | 88.0 ± 5.66 | 88.0 ± 5.66 |
| 15 h | 100.0 | 0.0 | 83.0± 2.12 | 0.0 ± 0.0 |
| 48 h | 100.0 | 0.0 | 80.5 ± 3.54 | 0.0 ± 0.0 |
| 96 h | 90.0 | 0.0 | 74.0 ± 2.83 | 0.0 ± 0.0 |

In all experiments, seeds were either dry or wetted with 1-2 ml water/g seeds directly before starting the treatment.

[w)] Data represent the mean of 100 seeds (cv. Lyconorma), systemically infected with *Cmm*.

[x)] Data represent the mean of three replicates of 100 seeds each (cv. Marmande).

**Effect of seed treatments on saprophytic bacteria**

In general, accompanying saprophytic bacteria that exist on seeds were more tolerant to all chemical, physical and fermentaive treatments tested than *Cmm*. However, *Cmm* bacteria were eradicated from seed by several treatments, whereas saprophytic bacteria were not eradicated completely by any of the treatments tested. By chemical and physical treatments, both the diversity and population density of saprohhytic bacteria were reduced but not compeletly eradicated, whereas by fermentative treatments the diversity of saprophytes was reduced, but some species increased in population during fermentation.

The populations of saprophytic bacteria decreased by chemical and hot water treatments between 10 and 1000-fold after treatments, from $3.9 \times 10^6$ cfu/ 100 seeds (control) to levels

between 4.5 × $10^3$ and 1.1 × $10^5$ (**Table 3**). In contrast, the populations of some saprophytic bacteria occurring on the seeds increased 100-fold after seed fermentation for 72 h, i.e. from 4.4 × $10^6$ cfu to 1.0 × $10^8$ cfu/100 seeds (**Table 4**). Similarly, the population of accompanying saprophytic bacteria increased in the pulp juice from 1.8 x $10^6$ cfu/ml to 7.8 x $10^6$, 1.1 x $10^7$, and 4.4 x $10^8$ cfu/ml pulp juice after fermentation for 24, 48 and 72 h, respectively (**Table 7**).

The approximate pH value of pulp juice was 4 or less, and this value decreased to about 3 during fermentation. *Cmm*-cells contained in seeds or inside the fruits can tolerate these low pH value as long as fruits are not injured or decayed. After seed extraction *Cmm*-cells cannot tolerate these low pH values inside the pulp juice during fermentation. Therefore, the population of *Cmm* decreased significantly during fermentaion, due to the low pH values inside the fermented fruit pulp.

When fermentation was started, the diversity of accompanying bacteria in pulp juice or on seeds was higher, and their population densities were lower. Thus, at the beginning of seed fermentation, saprophytes varied stronger in colony color and in shape on NGY medium, but lateron the diversity of saprophytic bacteria decreased on the NGY medium, although the population of recovering saprophytes that could tolerate the low pH value increased (**Table 7)**.

**Table 7.** Effect of fermentation on the populations of *Cmm* and accompanying bacteria and on pH value in pulp juice

| **Duration of fermentation in h** | **cfu/ml pulp juice*** | | **pH value of pulp juice*** |
|---|---|---|---|
| | ***Cmm*** | **Saprophytes** | |
| **0 (control)** | 1.4 x $10^8$ | 1.8 x $10^6$ | 4.05 |
| **24** | 1.5 x $10^6$ | 7.8 x $10^6$ | 4.0 |
| **48** | 0.0 | 1.1 x $10^7$ | 3.65 |
| **72** | 0.0 | 4.4 x $10^8$ | 3.3 |

*) Each value represents the mean of 4 replicates.

**Effect of treatments on seed germination capacity**

Seed germination capacity after treatments with chemicals, with hot water, or with hot air was determined using healthy tomato seeds of cultivar Marmande. Seed germination of fermented seeds or seeds extracted with hydrochloric acid and their control was determined with freshly extracted Lyconorma seeds. Seed germination rate was assessed for each chemical, hot water and fermentative treatment in soil and on blotter (filter paper) separately and compared with germination of untreated (control) seeds (**Table 3 and 4**). Germination of HCl-extracted seeds

was determined only in soil and germination of hot air treated seeds was determined only on blotter. Except for seed treatments with 5% HCOOH for 60 min at room temperature and with warm water treatment at 54 °C for 60 min (**Table 3**), or treatment with hot air of wetted seeds (**Table 6**), there were no significant differences in germination rates between treated and untreated seeds in all the experiments. These results were anticipated, because numerous pre-treatments were initially done with each disinfectant on healthy seeds to adjust and select concentrations, soaking time and temperatures with potential absolute eradication of *Cmm*, but without reduction in seed germination capacity.

However, it should not be disregarded that most of the **average values** of the seed germination capacity of treated seeds were lower than those of untreated seeds (**Table 3**). Fermentative treatments did not cause a significant reduction in germination rates when compared with the control in both soil and blotter trials. However, germination rates of fermented seeds and their control were significantly lower in blotter test compared to germination rate in soil, because of fungal attack that was recorded in blotter tests **(Table 4)**.

**Seed germination capacity at two and eight months after treatments**

In one of the treatment trials, seeds were evaluated for germination capacity in soil at greenhouse conditions nearly two months after the treatments. Another seed-portion of the same variants was stored at room temperature and evaluated 6 months later (about 8 months after treatments) on germination capacity under the same conditions to find out whether germination rates of treated seeds decreased during storage. Surprisingly, seed germination capacity increased when tested 8 months after treatments in most experiments (**Table 8**) comparing germination within 2 months after treatments. In general, no negative effect was recorded on seed germination capacity by storage at room temperature. The mean germination rates of all variants after two and eight months are summarized in **Figure 6**. The increase in seed germination capacity is probably due to the degradation of the residual chemicals in seeds during storage, since we did not normalize the pH values of seeds after treatments by rinsing seeds in 0.1M phosphate buffer (pH 7.0). It is known that seeds must be washed very well after acid-treatments and soaked in 0.1M phosphate buffer (pH 7.0), because high residual acid concentrations in seeds reduce the germination capacity when the storage temperatures are high. In our experiments the storage temperatures ranged between 15-23 °C, and the seeds were washed well after treatment, but were not soaked in phosphate buffer to normalize the seed-pH-values.

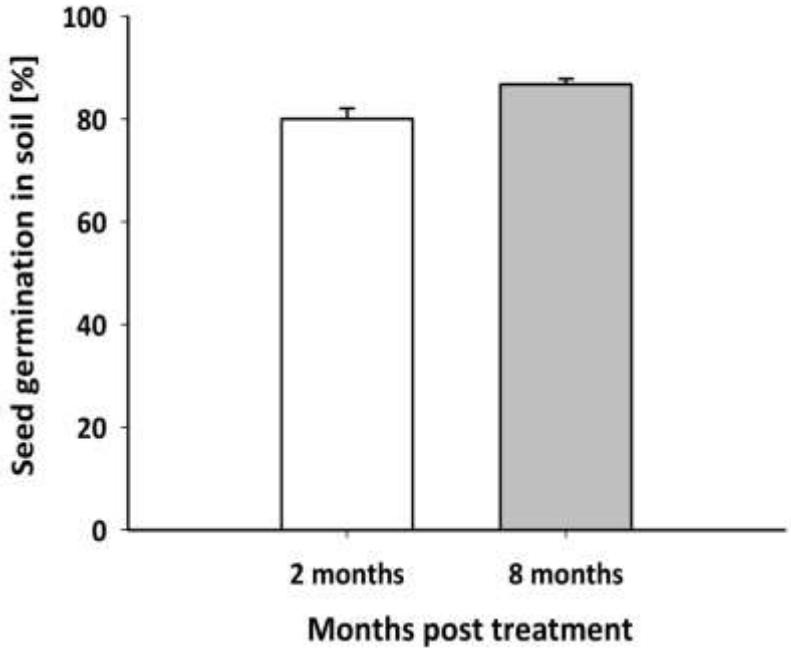


**Figure 6.** Mean of seed germination capacity from all treatments listed in Table 8 at two months and 8 months post treatments

**Table 8.** Effect of storage on germination capacity of treated seeds

| Treatment | Seed germination capacity in soil (%)* | |
|---|---|---|
| | after 50 days | after 8 months |
| Untreated (control) | 89 | 89 |
| 5%MF, 60 min, rt | 74 | 85 |
| 5% MF, 120 min, rt | 77 | 88 |
| 15% MF, 60 min, rt | 92 | 87 |
| 0.45% $C_6H_5COOH$, 60 min, rt | 78 | 88 |
| 0.45% $C_6H_5COOH$, 120 min, rt | 77 | 87 |
| 3% HCl, 30 min, rt | 91 | 90 |
| 3% HCl, 60 min, rt | 85 | 90 |
| 3% HCOOH, 60 min, rt | 81 | 84 |
| 5% HCOOH, 15 min, rt | 73 | 88 |
| 5% HCOOH, 30 min, rt | 79 | 84 |
| 5% HCOOH, 60 min, rt | 55 | 67 |
| 5% lactic acid, 60 min, rt | 78 | 85 |
| 1% NaOCl, 60 min, 40°C | 85 | 90 |
| 2% NaOCl, 60 min, 40°C | 66 | 91 |
| 5% $CH_3COOH$, 60 min, rt | 92 | 89 |
| 5% $CH_3COOH$, 120 min, rt | 75 | 85 |
| $H_2O$, 60 min, 52°C | 76 | 94 |
| $H_2O$, 30 min, 54°C | 87 | 82 |
| $H_2O$, 60 min, 54°C | 65 | 83 |
| control of fermentation (non-fermented) | 94 | 92 |
| Fermentation, 72 h, 20°C | 81 | 86 |
| Fermentation, 96 h, 20°C | 91 | 91 |

*) Each value was obtained from 100 seeds.

## Discussion

Different **inoculation methods** with *Cmm* were tested to select the best suited one for production of highly and systemically infected seeds for carrying out investigations on different seed treatments. The best method was to inoculate very small (young) peduncles of fruits or flowers three days after or during pollination of the first lower flowers with a bacterial suspension of $10^4$ cfu/ml. Thus, 100% infected seeds were obtained when the fruit/ flower peduncles were very young and also when the inoculation method was supported with a spray application of *Cmm*-suspension ($10^5$ cfu/ ml) onto the flowers.

The typical symptom of **birds' eye spots** could be produced only when very small fruits were sprayed with a bacterial suspension of $10^8$ cfu/ml but not with $10^6$ cfu/ml and not with any other inoculation method. These results were similar to those of Medina-Mora *et al.* (2001).

Naturally, birds' eye spots seem to appear when dew drops containing many bacterial cells fall in early morning from infected plants onto small fruits, so that the bacteria can infect the fruits through open stomata. This may be the explanation why birds' eye spots often appeared only on one side of naturally infected fruits (**Figure 5**).

By using the **double mutant *Cmm* strain** BO-RS (GSPB 3204) that was mutated against 100 ppm rifampicin and 600 ppm streptomycin for seed infection, it was very easy to determine *Cmm* population densities in infected seeds by plating seed homogenates on NGY medium supplemented with 25-50 ppm rifampicin and 200 ppm streptomycin. The combination of these two antibiotics effectively excluded most of the accompanying saprophytic bacteria in our experiments. We did not carry out any bio-assay trials *in planta* for determining seed-infection with *Cmm* after treatments, because this method proved to be not reliable in other studies, since the bacterial cells can mask themselves inside infected plants and the incubation time could be more than 5-6 months. Using dilution plating of seed homogenates on the above mentioned medium was much easier, faster and more accurate in order to determine seed-infections with *Cmm* when compared with bio-assay trials and allowed a quantative determination of *Cmm* populations in seeds. The new selective medium BCT (Chapter 1) was not yet developed when this study was carried out. Therefore, we used the NGY medium amended with rifampicin and streptomycin.

We excluded any grow-out assays (i.e. planting of seeds in soil and determination of *Cmm* infection according to symptom appearance of canker disease) for evaluating the efficacy of

treatments. These assays proved not to be reliable in our study, since the incubation period of *Cmm in planta* can last 5 or 6 months before latent infections with *Cmm* can be visually detected, and because this method does not allow any quantitative determination of bacterial populations.

Fatmi *et al.* (1991) mentioned that soaking seeds in **warm water at 56 °C** for 30 min was effective in eradicating the pathogen without affecting seed germination. In contrast, we found in our pre-experiments that **seed germination** after such treatments was **reduced to 1%.** This contradiction could be due to difficulties in adjusting the correct temperature. In our experiments treated seeds were soaked in Erlenmeyer flasks within a water bath. Soaking the seeds was started once the adjusted temperature was reached inside the flask, since we realized that temperature inside the flasks could be 1-2 °C lower than in the surrounding water-bath.

Treatments with 1% or 2% **sodium hypochlorite** at 40 °C were not effective enough to eradicate *Cmm* from seeds. These results are similar to those from other researchers, such as Fatmi *et al.* (1991) and Dhanvantari and Brown (1993). Fatmi *et al.* (1991) reported that a treatment with sodium hypochlorite was disinfecting the seed surface but not eradicating the internal seed infection. This finding corresponds to our results. However, an effetcive seed treatment must destroy the bacteria on as well as beneath the seed coat (Bryan, 1930; Patino, 1964).

The active substance of MENNO Florades$^{TM}$ is 9% benzoic acid. In our experiments we applied both, benzoic acid (0.45%) as well as MENNO Florades$^{TM}$. In fact 5% of MENNO Florades$^{TM}$ is equivalent to 0.45% benzoic acid, and the treatments with both solutions for 60 or 120 min at room temperature resulted in nearly similar results.

**Seed extraction with acid** has been recommended by the Council Directive of Eulropean Communities (Council Directive 2000/29/EC, 2000). In addition, it was also recommended by the European Plant Protection Organization (EPPO) to obtain tomato seed by acid extraction (OEPP/EPPO, 1990). However, a uniformly standardized extraction method has never been developed, which was internationally accepted (Council Directive 2000/29/EC; Petter, 2009, personal commun.). It is unknown whether this method is applied exactly in the same way by different seed companies or other laboratories. Thus, different acids, different concentrations and different treating durations may be applied. For instance, some seed companies use 0.6% HCl for 1-2 h, 3% tartaric acid ($C_4H_6O_6$) for 1 h, wet seeds + 0.8% solution of acetic acid for

24 h below 21 °C, or 25 ml of HCl/ 10 liter of tomato pulp for 30 min, etc.. All these seed extraction methods by acids may not be very effective in eradicating *Cmm*, since each company uses another different extraction method. This uncertainty may also explain why in recent years latently infected tomato seeds were sold in several European and neighbouring countries.

Some reports of the European Plant Protection Organization (CABI/ EPPO 90/ 399003) were referring to the acid extraction methods of Thyr *et al.* (1973) and Dhanvantari (1989), although these methods were not absolutely effective against *Cmm.* However, later on Dhanvantari and Brown (1993) referred to improved seed treatments based on earlier methods that were mentioned in the previous paper (Dhanvantari, 1989). Therefore, we tested the effect of three processing methods of seed extraction with hydrochloric acid on infected tomato seed (seed infection about 48%), which were slightly colonized with low populations of *Cmm*. Seed extraction with 0.1M HCl, 0.6M HCl and 1.0M HCl for 60 min for each treatment eliminated the infection with *Cmm* to zero, without any significant reduction in seed germination capacity. Probably, these extraction methods should be repeated with seeds that are higher infected with *Cmm*, to determine the efficacy in eliminating *Cmm* from 100% infected and heavily colonized seeds. Such treatments combined with seed extraction are easy to carry out and save time and labour, and a standardized acid extraction method could be used worldwide by the seed industry. At present, there exist no recommendations for a standardized method to extract seeds from tomato fruits by acid.

Our seed treatments with chemicals, hot air and hot water were accomplished between December 2007 and April of 2008 and seed germination capacity trials were carried out using tomato seeds of the cultivar “Marmande” that was extracted before 2006 and obtained from the seed company International Seed Processing GmbH, Quedlinburg, Germany. It is unknown whether these seeds had been also extracted with acids before. The germination capacity of these so-called untreated (control) “Marmande” seeds, as obtained from the seed company, was about 88.33%.

Internationally, the accepted **seed germination capacity** of commercial tomato seeds must be at least 85% according to the International Seed Federation (ISF, 2009). Most of our treatments were eradicating *Cmm* absolutely from seeds without any significant reduction in seed germination capacity. Furthermore, many of our treatments reduced *Cmm* infection to zero, and the seed germination capacity was maintained above the internationally accepted level of 85%, although we treated commercially available tomato seeds (Marmande), the

germination capacity of which was only 88% before the treatments.

Our results concerning the seed germination capacity were anticipated, because numerous pre-treatments were initially done with each disinfectant on healthy seeds to adjust and select concentrations, soaking time and temperatures with potential absolute eradication of *Cmm*, but without reducing the seed germination capacity. However, it should not be neglected that most of the **average values** of the seed germination capacity of treated seeds were lower than those of untreated seeds (**Table 3**).

The seed extraction processing with hydrochloric acid (0.1, 0.6 or 1.0M HCl) for one hour was effective in reducing the *Cmm*-population to zero. However, the seeds that were extracted with HCl were latently infected and infested with *Cmm* (infection ratio 48%), and the seed colonization with *Cmm* was rather low (about $5.1 \times 10^3$ cfu/ 100 seeds). Nevertheless, we concluded that **soaking dry seeds** in acid solutions (as it was done in our main experiments, Table 3) is much more effective in eradicating *Cmm* than **seed extraction by acids** of **wet and freshly extracted seeds,** because dry seeds absorb more acid solutions that allow killing and eradicating the bacteria under the seed coat much more than treating wet seeds during seed extraction. Thus, these two aspects must be tested again carefully on very strongly infected seeds. In this way it should be possible to develop a standard method that could really eradicate the bacteria from seeds. This strategy is now possible and easy, because we established a suitable inoculation method for production of highly infected seeds with *Cmm* as well as a highly sensitive Bio-PCR protocol (chapter 2 of this thesis) for detection of *Cmm* that is based on a combination of new specific primers (chapter 2) and the new sensitive and selective BCT medium developed for *Cmm* (chapter 1).

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# Chapter 4

## Occurrence of *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker of tomato, in Syria

This paper was accepted for publication in *Phytopathologia Mediterranea* (2010) **49:**172-178, and a related first report had been previously published in Plant Disease (2008) **92:** 649.

## Summary

Several surveys were carried out to evaluate the occurrence of bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) in the North-West provinces of Syria (Latakia and Tartous). The surveys revealed typical disease symptoms in greenhouses where the tomato cvs. Dima, Huda and Astona were grown, such as dark brown to black lesions on the leaf margins, wilting of whole plants, stunting, and vascular discoloration. The disease incidence in such greenhouses was 15% in the spring of 2007, and up to 70% by the end of July. Ten isolates obtained from diseased plants at different locations in these two provinces were identified as *Clavibacter michiganensis* subsp. *michiganensis* using classical microbiological tests as well as PCR. This is the first detailed proof of the occurrence of bacterial canker of tomato in Syria.

## Introduction

*Clavibacter michiganensis* subsp. *michiganensis* (Smith, 1910) Davis *et al*., 1984 (*Cmm*) causes one of the most injurious bacterial diseases of tomato (*Solanum lycopersicum* L.). It is listed as an A2 quarantine pathogen by EPPO and now occurs in many tomato-growing areas worldwide, including the EPPO region (EPPO/CABI, 1998) and many neighbouring countries. In Syria; too; *Cmm* is a quarantine organism and imported tomato seeds must be free of this pathogen. So far the occurrence of the disease in Syria has not been comprehensively studied, apart from one abstract (Ftayeh *et al.*, 2008). The bacterium causes yield losses of up to 60% (Griesbach *et al*., 2000) and it has several alternative host plants, such as *Capsicum annuum, Solanum melongena*, *S. nigrum* and *S. triflorum* (Strider, 1969). The pathogen survives in seeds, on greenhouse structures, in plant debris (Strider, 1969; Fatmi & Schaad, 2002), and to a certain extent in soil (Ftayeh *et al*., 2004).

Contaminated seeds and young plants are the principal means for long-distance transmission of the pathogen (Strider, 1969). A minute number of contaminated seeds (1-5 in 10,000) can cause an epidemic in field-grown tomatoes (Chang *et al*., 1991; Gitaitis *et al.*, 1991). Even symptomless tomato seedlings may harbour high populations of *Cmm* (Werner *et al*., 2002) and infect other tomato plants later. Since there are as yet no effective bactericides, or high-yielding *Cmm*-resistant tomato cultivars available (Boelema, 1980), strict hygienic measures are currently the only way to control the disease. Most important is the use of pathogen-free tomato seeds.

The aim of this study was to survey bacterial canker of tomato in the Syrian provinces Latakia and Tartous along the Mediterranean Sea, where almost all Syrian greenhouse tomatoes are grown, destined for the Syrian market in winter and for export.

**Table 1.** Areas and yield of tomatoes in Syria and in the Syrian provinces Latakia and Tartous in 2007 (Anonymous, 2007)

| Tomato Production | Open field | | | Greenhouse | | |
|---|---|---|---|---|---|---|
| | Syria | Latakia province | Tartous province | Syria | Latakia province | Tartous province |
| Area (ha) | 15235 | 677 | 404 | 3759 | 418 | 3287 |
| Yield (ton) | 731251 | 13440 | 6371 | 501204 | 55740 | 438300 |

# Materials and Methods

## Surveys and sample collection

Between March and mid-April of 2007, and again at the end of July 2007, a number of surveys were carried out in greenhouses (plastic tunnels 2.5-3.0 m in height, and about 450 $m^2$) in Latakia & Tartous along the Mediterranean Sea in North-West Syria (Figure 1), where 82,340 greenhouses were cultivated with tomatoes in 2007 (Table 1). One hundred and fifty greenhouses with a total acreage of 6.75 ha were surveyed. Most of the greenhouses were randomly selected, but a few were chosen because local agricultural advisers observed wilt symptoms in them. Disease incidence caused by *Cmm* in these greenhouses was estimated by dividing the number of plants with wilt symptoms by the total number of plants in the greenhouse. From each greenhouse with disease occurrence, stem samples of wilted tomato plants were taken and stored under cool conditions until isolating the causal organism.

**Figure 1.** Intensive greenhouse tomato cultivation in the coastal Mediterranean provinces of Syria.

## Isolation and identification

Bacterial isolates were obtained and purified in the laboratory of the Plant Protection Directorate in Damascus, Syria, and all further laboratory tests were conducted at the Division of Plant Pathology and Crop Protection, University of Göttingen, Germany. Stem samples from

diseased plants were surface-disinfected with 70% ethanol and homogenized in a sterilized mortar in sterile water. Serial dilutions (1:10) until $10^{-5}$ of the homogenate were made in 0.01M $MgSO_4$, and 0.1 ml from each dilution was plated on NGY agar containing 0.8% nutrient broth, 1% glucose, 0.3% yeast extract (Mavridis, University of Göttingen, Germany, personal communication), as well as on the new selective medium for *Cmm* (chapter 1, this thesis). The Petri dishes were incubated at 26°C and evaluated after 3 or 5 days on NGY or the new selective medium, respectively.

Putative colonies of *Cmm* were purified by sub-culturing and repeated re-streaking on Petri dishes containing **NGY**. Isolates were initially identified on the basis of colony characteristics and cell morphology (colour, shape, motility and size), Gram's reaction with 3% KOH (Gregersen, 1978), and a hypersensitive reaction on the leaves of four-o'clock plants (*Mirabilis jalapa*) (Gitaitis, 1990) using bacterial suspensions of $10^8$ cfu/mL prepared photometrically (Spectronic 20, Bausch & Lomb Inc., Rochetser, NY, USA). Final identification of the isolates was confirmed by both PCR and pathogenicity tests. At all identification steps, two reference *Cmm* strains (2973 and 390) obtained from the Göttinger Sammlung phytopathogener Bakterien (GSPB), were used as positive controls. As negative controls, plants were inoculated with sterile 0.01M $MgSO_4$.

**Pathogenicity**

Pathogenicity of the isolates was tested by mechanically inoculating 6-week-old tomato plants (cv. Lyconorma). Each isolate and strain was inoculated into three tomato seedlings. The inoculum was prepared by suspending a loopful of a 24-h-old bacterial culture grown on **NGY** in sterile 0.01M $MgSO_4$, and the suspension was adjusted to an optical density of 0.06 at 660 nm (Spectronic 20, Bausch & Lomb Inc., Rochetser, NY, USA) corresponding to about $10^8$ cfu/mL. A 35 µL drop was placed in the axil of the second or third true leaf (Mavridis *et al.*, 1990). Inoculation was performed by pricking the stem through the drop with a sterile needle. For negative control, the tomato seedlings were inoculated with sterile 0.01M $MgSO_4$. The plants were kept at room temperature (18°C) for 12 h and later in a glasshouse at 25/20°C (day/night) with a relative humidity between 50 and 90%. Plants were checked regularly for symptom development.

Symptoms were recorded within 10 to 15 days after inoculation. To fulfil Koch´s postulates, the pathogen was re-isolated and re-identified from the inoculated plants showing disease symptoms.

**PCR identification**

DNA of all *Cmm* strains was isolated from *in-vitro*-grown pure bacterial strains with the MasterPure Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). Concentrations of DNA were assessed after standard gel electrophoresis (1.2% w/v of agarose dissolved in 0.5% TBE-Puffer, stained with 0.5 µg/mL ethidium bromide, 3 V/cm, 120 min) in comparison with different concentrations of Lambda DNA (MBI Fermentas, St. Leon-Rot, Germany).

The polymerase chain reaction (PCR) was carried out using the specific primer set PSA-4 and PSA-R proposed by Pastrik and Rainey (1999). Amplification was performed in a total volume of 25 µL. The reaction mix contained 1x reaction buffer (10mM Tris-HCl of pH 8.8 at 25°C, 50mM KCl, 0.8% Nonidet P40) and was supplemented with 1.5mM $MgCl_2$, 0.2mM dNTPs, 1µM of each primer, 1 U Taq DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany) and 1 ng of template DNA. The PCR profile consisted of an initial denaturation step at 95°C for 4 min, followed by 35 amplification cycles at 95°C for 1 min, 63°C for 1 min and 72°C for 1 min. The final elongation step was done at 72°C for 10 min. Amplification was performed using a PTC 100 thermo cycler (MJ Research, Watertown, MD, USA). PCR products and the GeneRuler™ 100 bp DNA ladder (MBI Fermentas, St. Leon-Rot, Germany) were separated on 1.5% agarose gel. Gels were stained in 0.5 µg/ml ethidium bromide solution for 10 min.

# Results

## Disease incidence

Typical symptoms of bacterial canker were observed in 10 of the 150 greenhouses. Symptoms such as stunting, dark brown-to-black lesions on the leaf margins (Figure 2 A), and vascular discoloration followed by wilting (Figure 2 B) were seen on the tomato cvs. Dima, Huda and Astona. Disease incidence in these greenhouses was estimated at up to 15% by the middle of April 2007. By the end of July, disease incidence had increased to a maximum of 70% in two of these greenhouses, to 30-40% in 6 greenhouses, and was still 15% in the remaining two greenhouses. Obviously, disease incidence varied depending on how actively farmers destroyed infected and adjacent plants and followed the recommended hygienic measures. In 2008 and 2009 no surveys were conducted. Wilt symptoms were seen by agricultural advisers (M. Eshbani) in some greenhouses, but laboratory tests for isolation of the causal pathogen were not done.


**Figure 2.** Symptoms seen in greenhouses: **A**, discoloration of leaf margins and **B**, wilting of whole plants.

## Isolate identification

Ten bacterial isolates, subsequently identified as *Clavibacter michiganensis* subsp. *michiganensis*, were obtained from various greenhouses at different locations in both provinces: from Ayn Erraheb and Bostan Eljamee in Latakia, and from Banyas, Hryson and Alkhrab Alshamali in Tartous. Three days after streaking these strains onto NGY and 5 days after streaking them on the new selective medium, typical *Cmm* colonies appeared when

incubated at 26°C. Colonies were 2 to 3 mm, light yellow, brilliant, convex and slimy, round or with irregular margins. *Cmm* colonies on NGY and on the new medium were very similar. However, the new medium strongly suppressed saprophytic bacteria.

Microscopically, the bacterial cells were coryneform in shape and non-motile. All the isolates were Gram-positive and induced hypersensitive reactions on four-o'clock plants (*Mirabilis jalapa*) within 24 h after inoculation.

**Pathogenicity**

All the isolates and the reference strains induced the typical symptoms of bacterial canker on mechanically inoculated young tomato plants in 10 to 15 days. These symptoms included unilateral wilt of leaflets (Figure 3 A) and cankers on the stems (Figure 3 B) followed finally by wilting of entire plants. Control plants inoculated with sterile 0.01M $MgSO_4$ solution did not show any symptoms. In order to fulfill Koch's postulates, re-isolation and re-identification of the pathogen was performed from these artificially inoculated plants.

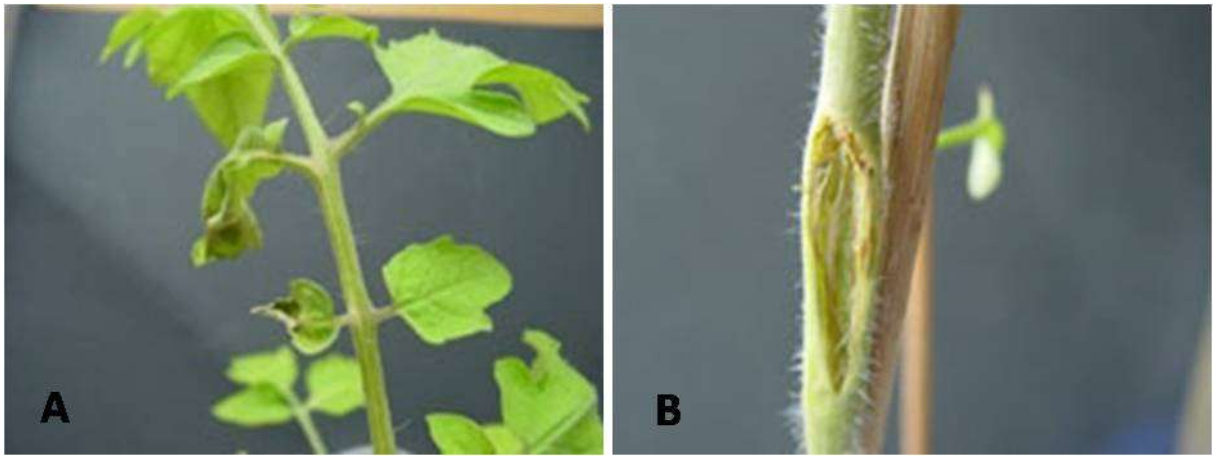


**Figure 3.** Symptoms seen after inoculation: **A**, unilateral wilt of leaflets and **B**, canker on tomato stem.

**PCR identification**

Amplifications using the primer pair PSA-4 and PSA-R produced the expected amplicons of 270 bp with both the two reference strains and all the 10 Syrian isolates (Figure 4), as described by Pastrik and Rainey (1999).

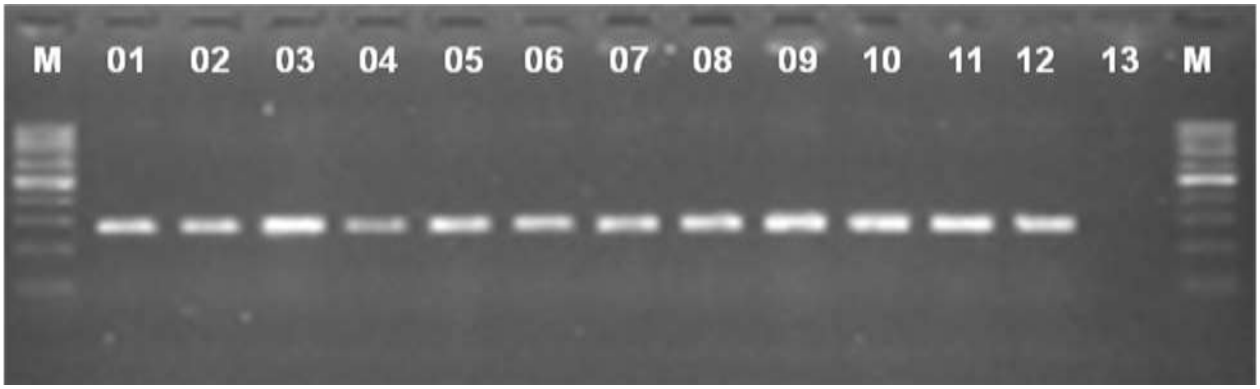


**Figure 4.** Gel electrophoresis of amplicons after PCR. **M,** GeneRuler™ 100bp DNA ladder; **01-10,** Syrian *Cmm* isolates; **11 & 12,** positive controls (*Cmm* GSPB 2973 & 390); **13**, negative control (water).

## Discussion

Bacterial canker of tomato has not been reported before in Syria (Ftayeh *et al*., 2008). Similar symptoms such as stunting or wilting of tomato plants and discoloration of the vascular system may have been seen in the past but they were not further investigated in Syria, probably because they were mistaken for Fusarium wilt (M. Eshbani, personal communication). In addition, the exchange of information between Syria and the EPPO was not very intensive in the past. This is therefore the first detailed report and confirmation of bacterial canker occurring on tomatoes in Syria.

Although the total yield of greenhouse grown tomatoes in Syria is lower than that of field tomatoes (Table 1), greenhouse tomatoes are economically very important because they are harvested in winter and represent the only source of fresh tomatoes in winter for the market in Syria, and they are also exported. The price of fresh tomatoes is much higher in winter than in summer. Consequently this study focused on greenhouse tomatoes.

The economic losses caused by *Cmm* in this part of the country can only roughly be estimated. In the surveys, 150 greenhouses in Latakia and Tartous out of 82,340 existing greenhouses (Anonymous 2007), or only 0.18% of the total, were carefully inspected for *Cmm*. Ten infected greenhouses out of 150 signifies an infection rate of 6.6%. However, since some of the greenhouses examined were not selected at random but on the basis of information provided by agricultural advisers, it is assumed that overall only 2% of all greenhouses were infected with *Cmm*; or 1,647 greenhouses. In this part of Syria the average yield of tomatoes per greenhouse is 6 t (Table 1), so that a loss of 20% due to *Cmm* would amount to 1.2 t per greenhouse, or 2000 t for all infected greenhouses. Assuming a wholesale selling-price of 0.50 € per kg for the farmer and a retail market price of 1.00 € per kg, this would signify that *Cmm* caused an economic loss of 1 million € to farmers and a loss of 2 million € on the market.

Discussions with Syrian farmers and agricultural advisers revealed that bacterial canker had not been noticed in this part of the country before. The typical symptoms were certainly not detected in the year before the present survey was initiated in any of the greenhouses later found to be infected in the survey. This suggests that the pathogen may have been introduced recently by infected or contaminated seeds, although the seed from which the diseased tomato plants were grown had been certified as healthy. The survey also revealed that the disease did not turn into an epidemic. Instead, disease incidences occurred in diverse locations in both

provinces Latakia and Tartous, obviously scattered all over this region. These findings also suggest that bacterial canker when it occurred derived from a very few and only slightly infested tomato seeds which remained undetected in the tomato seed lots that are regularly imported from overseas. It is therefore strongly recommended that in future all lots of tomato seeds and young plants should be carefully inspected for latent infection or contamination by *Cmm* before permitting them to enter the country.

After the survey was completed, some recommendations were given to Syrian farmers to help them manage bacterial canker and avoid further infections. The recommendations were: to destroy all infected and adjacent plants together with their root system, to disinfect all cutting tools with 70% ethanol, not to exchange or move tools between greenhouses, and to make all workers aware of the symptoms of bacterial canker. It is vital to eradicate all plants with their main root systems at the end of the vegetation period. When severe outbreaks of bacterial canker occur, the soil should be damped or solarized if possible. And in any case, it is strongly recommended to use certified healthy seeds every year.

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# General Discussion

The newly developed selective medium BCT (a selective medium for bacterial canker of tomato) proved to be superior to all other previously known semiselective media for *Cmm* in selectivity, in detection sensitivity and in allowing a fast growth of a very wide range of *Cmm* strains without exceptions. In addition, the new selective medium appears to be very promising for a sensitive detection of other subspecies of *Clavibacter michiganensis,* too.

Also the **newly adapted PCR primers** were significantly superior in specificity to the published ones tested and allowed amplifications of all 76 tested *Cmm* strains without exception. Even some so-called avirulent *Cmm* strains were amplified.

A Bio-PCR protocol for a highly sensitive detection of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), the causal agent of bacterial canker of tomato (*Solanum lycopersicum / Lycopersicon esculentum*) was developed. The protocol is based on the enrichment of viable cells of the target bacterium by plating seed extracts or plant homogenates on the **newly developed selective medium**. Bacterial upgrowth was directly used as template for PCR detection using new sets of *Cmm*-specific primers. This Bio-PCR assay allows a sensitive detection of very few *Cmm* cells in plant homogenates and seed extracts, even when populations of saprophytic bacteria are very high. Furthermore, the protocol allows very fast and early detection of *Cmm* (within 4 days). In contrast, false negative or significantly delayed results were obtained using earlier recommended semiselective media. The new Bio-PCR protocol improves reliability and sensitivity, and also reduces the time for *Cmm*-detection significantly, and further additional tests for identifying Cmm are no longer necessary. The protocol could be useful for issuing seed-health certifications and for testing asymptomatic tomato plants on latent infection by *Cmm*.

Using this **new Bio-PCR protocol** for testing of tomato seed lots may allow detection of **only one infected seed within 10,000 seeds**. This high sensitivity in detecting infected seeds was not possible before, and this may be the reason of further distribution of the pathogen *via* infested seeds in recent years, although the seed had been tested and certified as pathogen free in the past.

Other advantages of the new Bio-PCR for *Cmm* are*:*

- the assay needs less time for detection (detection is possible within 4 days),
- the effect of PCR inhibitors that exist in plant and seed extracts is eliminated,

- also the impact of saprophytic bacteria that are found in plant extracts is minimized,
- additional tests for identifying the target bacterium are not necessary,
- no need for DNA extraction,
- even non-recognizable small *Cmm* colonies in the bacterial upgrowth which cannot be distinguished due to the potentially high recovering number of saprophytes are deteced.

Compared with all previous reports, **seed treatment methods achieved** by this study allow a radical and absolute eradication of *Cmm* from seed lots without any significant impact on seed germination. The treatment by **soaking dry tomato seeds in chemical solutions has the potential to be more effective** than the acid seed extraction recommended by EPPO. The EPPO method relies on treating fresh seeds with acid during seed extraction from the tomato pulp, however without a defined standard acid extraction method. Soaking dry seeds in acids allows **absorption of acids by soaked dry seeds** and finally allows eradicating the internal population of the pathogen that may exist under the hard seed coat. Therefore, soaking dry seeds can be carried out within very short time (30-120 minutes), but an additional seed drying process is required after the treatments. This method allows external seed surface disinfection and internal eradicating of the bacterial population. The internal *Cmm*-population may be able to survive during seed storage for many years and can be a potential danger later on, when these seeds are placed in a seed bed for germination.

Our **surveys on the occurrence** of bacterial canker of tomato in the Mediterranean Syrian provinces and discussions with Syrian plant protection inspectors and growers, as well as our observations on the occurrence of *Cmm* in many new locations in Germany and Austria where this disease was unknown before, indicated that seed transmission was responsible for introducing the pathogen into these locations, although tested and healthy certified seeds were used in these locations. Our assumption was confirmed when we found out that the protocols applied for detecting *Cmm* in seeds, cannot detect low *Cmm* populations in seeds and may, therefore, often reveal false negative results.

The European directions for issues of Seed-Health Certifications require that seeds must be obtained from plants that did not develop any disease symptoms and that either seeds were extracted by acids or were tested according to an internationally approved testing method. Our comments on such regulations are:

1) Our investigations revealed that healthy appearing plants may nevertheless harbour viable *Cmm* populations in low concentration. Thus, we proved that the incubation period could last five or six months, depending on the inoculum dose, the plant age during

infection and the weather temperatures. We observed that even tomato plants grown from highly infected seeds did not develop symptoms during the first five months. These results were confimed by field observations when the new plants showed disease symptoms at an age of approximately five months (three months after transplanting two month-old plantlets).

2) Since no standardized and effective acid seed extraction method is recommended, seed companies process their seed lots differently. Therefore, this weak rule cannot be an alternative for a scrutinized seed testing protocol in order to certify seeds as pathogen free.
3) The recommended seed testing protocols that are based on plating assays of seed extracts on the old semiselective media often revealed false negative results.

Since such weaknesses of seed health testing are internationally known (Olivier, 2009), alternative solutions are very urgently required. For the time being it is suggested to require fulfilling of **all criteria** to certify seeds as pathogen free, this means:
- seeds must be obtained from plants without any disease symptoms,
- seed extraction by acids is obligatory,
- seeds must be tested for *Cmm* infestation as thoroughly as possible.

We hope that this thesis can help to understand the present weaknesses of tomato seed health tests regarding *Cmm* and that it will be possible in the future to provide pathogen free seed to the grower which can be obtained by effective seed treatments as well as by highly sensitive and reliable detection methods.

# General Summary

The main objective of these investigations was to improve the diagnostic methods for *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), since the previous detection methods often failed to detect infections by *Cmm* in seed lots and asymptomatic plant samples. An improvement of *Cmm*-detection was achieved by developing two new sensitive and selective media for *Cmm*, deducing and designing two specific primer sets and finally by establishment of a novel Bio-PCR assay for a sensitive detection of the pathogen. Another objective of the study was to investigate different seed treatment methods for eradicating *Cmm* from infected tomato seed.

**1)** All the previously published semiselective media for *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) tested, *i. e.* SCM, mSCM, D2ANX, CMM-1, KBT, D2 and the semiselective medium recently recommended by EPPO (in 2005) proved to be not satisfactory for a sensitive detection of *Cmm* in infected tomato plants and seeds. Therefore **new selective agar media** (**BCT & BCT-2**) for **B**acterial **C**anker of **T**omato were developed in three steps: 1) Selection of a basic medium allowing good growth of *Cmm* but excluding or slowing down several other bacterial species; 2) screening a wide range of antibiotics and other inhibitors for selective inhibition of often accompanying bacterial or fungal species; 3) optimizing the composition of inhibitors and nutrient components.

Initial tests for selection of antibiotics which did not inhibit *Cmm* were conducted with 32 strains of accompanying pathogenic and non-pathogenic bacterial species isolated from tomato seeds and plants that were obtained from different locations. For these experiments, tomato plants were cultivated in the field and artificially inoculated with very low concentrations of a rifampicin and streptomycin resistant strain of *Cmm.* These tomato plants did not develop disease symptoms but were latently infected with the pathogen. On the other hand, homogenates from leaves, stems, or tomato fruits from these plants were heavily contaminated with various microorganisms (bacteria and fungi). The exact concentration of *Cmm* cells contained in the homogenates was determined by dilution plating on NGY agar medium amended with rifampicin, streptomycin and a fungicide. Parallely, dilution plating assays from the same homogenates were conducted on many newly designed compositions for a potential semiselective medium. The best suited new media were then tested for isolation of *Cmm* from naturally infected plants obtained from different locations in Germany, Syria and Austria, in order to enlarge the diversity of naturally occurring microorganisms on or in

tomato plants.

Compared to the published semiselective media for *Cmm* (see above), the new media (BCT and BCT-2) proved to be well suited for sufficient and fast growth of a wide range of *Cmm* strains. On the other hand, the new media inhibited growth of naturally occurring microorganisms to an extent of 98 to 100%, and the main recovery rate of 30 different *Cmm* strains (tested as pure cultures) reached 89 and 88% on BCT and BCT-2, respectively, within 7 days.

By testing seed and stem homogenates of field-grown tomato plants which were latently infected with *Cmm* (between 30 and 1,100 cfu/ml) and highly contaminated with various saprophytic bacteria (between 11,500 and 180,000 cfu/ml), the average recovery rates of *Cmm* were 66.4% and 35.3% on BCT and BCT-2, respectively, whereas all the tested published semiselective media revealed false negative results under these conditions.

On the new media BCT and BCT-2, *Cmm* colonies were creamy to yellow, shining, slimy, convex and easily distinguishable from saprophytes, once they increased in size by time, while the colonies of saprophytic bacteria were suppressed and remained smaller, were strongly inhibited and mostly white in colour. In contrast, on the published semiselective media *Cmm* colonies were often interfered by saprophytic bacteria, so that distinction from contaminants was difficult. Several *Cmm* strains tested did not show the typical morphology or did not grow at all on some of the published semiselective media.

Summarizing, the new selective media are superior in selectivity, sensitivity and reliability for detecting *Cmm* in seeds and plant material compared with all published semiselective media for *Cmm.* The new media are recommended for *Cmm* isolation and detection in latently infected tomato plants as well as in infested tomato seed by a routine seed testing procedure.

**2)** The **PCR primer systems** for detection of *Cmm* published by Dreier *et al.* (1995); Pastrik and Rainey (1999); Sousa-Santos *et al.* (1995); and Kleitman *et al.* (2008) proved to be not satisfactory in our study, because several *Cmm*-strains were not amplified (false negative) or cross-reactions (false positive results) appeared with several associated bacterial species that may exist with tomato plants and seeds, such as *Pectobacterium carotovorum* subsp. *carotovorum*, *Pseudomonas fluorescens*, *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *tomato, Xanthomonas campestris* pv. *vesicatoria* and *Bacillus* spp..

Therefore, the **new primer sets** "**B-rev-CM/B-fw-PCM**" and "**L-fw-CM/L-rev-PCM**" were deduced and designed in our experiments from TaqMan-based PCR protocols, as described by Bach *et al.* (2003) and Luo *et al.* (2008), respectively. These TaqMan based PCR protocols rely on specific TaqMan probes, which were either deduced from intergenic sequences or internal transcribed spacer regions of the rRNA operon, respectively. We translated these protocols to a conventional PCR using one of the described primers together with a specific primer, which was deduced from the sequence of the TaqMan probe.

These PCR systems proved to be **more specific** compared with the above mentioned primer sets and amplified all the 76 different *Cmm* strains tested containing virulent, hypo-virulent and avirulent strains without any exception.

The new primer sets were finally applied in combination with the newly developed selective medium (Bio-PCR). The Bio- PCR protocol is based on the enrichment of viable cells of the target bacterium by plating seed or plant extracts on the newly developed selective medium BCT. Grown-up cells are directly used as template for PCR detection. This Bio-PCR assay allowed a sensitive detection of very few *Cmm* cells in seed and plant extracts (12 cfu or less/agar plate) within 4 days, although the population of saprophytic bacteria was very high ($2 \times 10^6$ - $2 \times 10^7$ cfu/agar plate). In contrast, Bio-PCR-detection of these few *Cmm* cells in the presence of very high numbers of saprophytic bacteria was impossible on the earlier published semiselective media tested, or required much longer time (10 days). Very few viable *Cmm*-cells that existed in plant or seed extracts were detected. There is no need for DNA extraction. The effect of PCR-inhibitors present in seeds and plant extracts is avoided, and further complementary tests such as pathogenicity or biochemical tests to determine the identity of the pathogen are limited or not required.

Because this new Bio-PCR protocol improves reliability and sensitivity and also reduces the time required for *Cmm* detection significantly, this protocol appears very useful for seed health certifications and for testing asymptomatic tomato plants latently infected by *Cmm*.

**3)** The efficacy of different **seed-treatment methods** in eradicating *Cmm* from systemically infected seeds was investigated, because no standardized seed extraction method was suggested by EPPO and because some published tomato seed treatments were either not effective enough or effective but severely affecting seed germination. Selected treatments were applied on systemically infected tomato seed produced in greenhouse trials. All treatments were evaluated on their efficacy for eradicating *Cmm* from seeds, on their efficacy

in reducing populations of saprophytic bacteria accompanied with tomato seed as well as on their impact on seed germination. Seed infection with *Cmm* was determined by plating seed homogenates on agar media. A total number of 200 or 300 seeds of each treatment were evaluated in this way. Germination capacity of control and treated seeds was determined for three 100-seed replicates of each treatment in blotter (filter paper) and for other three 100-seed replicates in soil at greenhouse conditions.

Best treatments of 100% systemically infected and very heavily colonized seeds with *Cmm* were recorded when seed infection was reduced to zero without any significant reduction in seed germination, by soaking seeds at room temperature in a solution of: 5% MENNO-Florades$^{TM}$ for 120 min, 3% HCl for 60 min, 3% HCOOH for 60 min, 5% HCOOH for 30 min, or 5% $CH_3COOH$ for 120 min; as well as by soaking seeds in warm water at 52°C for 60 min, or at 54°C for 30 min. All these treatments eradicated *Cmm* from seeds without any significant reduction in seed germination capacity compared with untreated seeds.

Other treatments with other concentrations or soaking time of the above mentioned chemicals or using other chemicals, such as 0.45% benzoic acid; 5% lactic acid at room temperature as well as using 1 or 2% NaOCl at 40°C, caused a reduction in seed infections with *Cmm* from 100% to levels between only 0.3% and 3.0%.

**4)** During these investigations and by extensive **field surveys** it was possible to reveal the situation of canker disease caused by *Cmm* in many locations in **Germany** and also to report disease occurrence for the first time in the Mediterranean Syrian provinces where the largest proportion of greenhouse tomatoes in **Syria** is grown**.** Thus, 50 strains of *Cmm* were isolated from different locations and identified by classical microbiological as well as by PCR tests.

**5)** The results obtained by these investigations, regarding the development of new selective nutrient agar media and development of a new very specific Bio-PCR protocol, as well as the suggested seed treatments may be very helpful to the seed industry in improving the production of healthy tomato seed which is the key for disease control.

# Related Publications

## Refereed journals

- **Ftayeh RM**, von Tiedemann A, Koopmann B, Abu-Ghorrah M and Rudolph K, 2010. Occurrence of *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker of tomato, in Syria. Phytopathologia Mediterranea **49**, 172-178.

- **Ftayeh R**, von Tiedemann A, Koopmann B, Abu-Ghorrah M and Rudolph K, 2008. First record of *Clavibacter michiganensis* subsp. *michiganensis* causing canker of tomato plants in Syria. Plant Disease **92**, 649.

- **Ftayeh RM**, von Tiedemann A and Rudolph KWE. A new selective medium for isolation of *Clavibacter michiganensis* subsp. *michiganensis* from tomato plants and seed. Accepted by Phytopathology.

## Presentations at national and international conferences

- Ftayeh R, von Tiedemann A, Koopmann B and Rudolph K, 2010. Reliability and sensitivity of diagnostic methods for detection of *Clavibacter michiganensis* subsp. *michiganensis* in seeds and plant material. *J. Plant Disease and Protection* **117**, 40. Oral presentation on the 30th workshop "Phytobacteriology" of the German Phytopathological Society (DPG) in Dossenheim, 2009.

- Ftayeh R, von Tiedemann A und Rudolph K, 2008. Untersuchungen zum Vorkommen und Nachweis von *Clavibacter michiganensis* ssp. *michiganensis* an Tomatenkulturen. *Nachrichtenbl. Deut. Pflanzenschutzd*. **60**, 91. Oral presentaion on the 28th workshop "Phytobacteriology" of the German Phytopathological Society (DPG) in Quedlinburg, 2007.

- Ftayeh R, von Tiedemann A und Rudolph K, 2008. Entwicklung eines semi-selektiven Mediums für *Clavibacter michiganensis* ssp. *michiganensis*. (Development of a new semi-selective medium for *Clavibacter michiganensis* ssp. *michiganensis*). *Mitt. Julius-Kühn-Institut* **417**, 145. Oral presentation on the 56th German Plant Protection Conference in Kiel, 2008.

- Ftayeh R, von Tiedemann A und Rudolph K, 2008. Versuche zur Abtötung des bakteriellen Schaderregers, *Clavibacter michiganensis* ssp. *michiganensis*, im Tomatensaatgut. (Investigations on eradication of the phytopathogenic bacterium, *Clavibacter michiganensis* ssp. *michiganensis*, in tomato seeds). *Mitt. Julius-Kühn-Institut* **417**, 165. Oral presentation on the 56th German Plant Protection Conference in Kiel, 2008.

## Conferences and workshops attended

| | |
|---|---|
| Sept. 01 - Sept. 04, 2009: | Participation in the 30th meeting of the working group Phytobacteriology in Dossenheim, one presentation. *J. of Plant Disease and Protection* **117**, 40. |
| Sept. 22 - Sept. 25, 2008: | Participation in the 56th German Plant Protection Conference in Kiel, two presentations. *Mitt. Julius Kühn-Institut* **417**, 145 and 165. |
| Sept. 14 - Sept. 15, 2007: | Participation in the 28th meeting of the working group Phytobacterioloy in Quedlinburg, one presentation. *Nachrichtenbl. Deut. Pflanzenschutzd*. **60**, 91. |

# Abbreviations

| | |
|---|---|
| **°C**: | degree Celsius |
| **µg**: | microgram ($10^{-6}$ g) |
| **µl**: | microliter ($10^{-6}$ l) |
| **BCT/ BCT-2**: | new selective media for **B**acterial **C**anker of **T**omato |
| **bidest.**: | *bidestillata* = double-distilled |
| **bp**: | base pair |
| **cfu**: | colony forming units |
| **cfu no.**: | number of colony forming units |
| **cm**: | centimetre ($10^{-2}$ m) |
| ***Cmm***: | *Clavibacter michiganensis* subsp. *michiganensis* |
| **cv.**: | cultivar |
| **DNA**: | deoxyribonucleic acid |
| **dNTP**: | deoxynucleotide triphosphates |
| **EDTA**: | ethylenediaminetetraacetate |
| **e.g.**: | *exempli graciā* (for example) |
| ***et al.***: | *et alii*, (and others) |
| **etc.**: | *et cetera* = and so on |
| **EV**: | end volume |
| **FAME**: | fatty acid methyl esters |
| **Fig.**: | figure |
| **g**: | gram |
| **h**: | hours |
| **ha**: | hectare (10,000 $m^2$) |
| **i.e.**: | *id est* (that is; in other words) |
| **IU**: | international unit |
| **l**: | litre |
| **m**: | metre |
| **M**: | molar |
| **MF**: | MENNO Florades™ |
| **mg**: | milligram ($10^{-3}$ g) |
| **min**: | minute |
| **MIS**: | Microbial Identification System (Hewlett-Packard HP5898A) |

**ml**: millilitre ($10^{-3}$ l)

**mm**: millimetre ($10^{-3}$ m)

**mM**: millimolar ($10^{-3}$ molar)

**µM**: micromolar ($10^{-6}$ molar)

**$mm^2$**: square millimetre

**ng:** nanogram ($10^{-9}$ g)

**nm**: nanometre ($10^{-9}$)

**OD**: optical density

**p:** pico ($10^{-12}$)

**PCR**: polymerase chain reaction

**pmol**: picomole ($10^{-12}$)

**ppm**: parts per million ($10^{-6}$)

**pv.**: pathovar

**r**: radius

**rt**: room temperature

**sec**: second

**std. dev.**: standard deviation

**subsp.**: subspecies

**TBE-buffer**: tris-borate-EDTA-buffer

**Tris**: tris-(hydroxymethyl)-aminomethan

**U**: unite

**V**: Volt

**w/v**: weight/volume

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# Eidesstattliche Erklärung

Hiermit erkläre ich eidesstattlich, dass diese Dissertation selbständig und ohne unerlaubte Hilfe angefertigt wurde.

**Göttingen, im Januar 2010**

# Curriculum Vitae

## Personal data


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| Name: | Radwan M. Ftayeh |
| Date & Place of Birth: | 1967 in Deir Al-Bakht, Daraa, Syria |
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## Educational background:

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| 1973 - 1979: | Primary school in Damascus |
| 1979 - 1985: | Secondary school in Damascus |
| 1985 - 1990: | Student at the Faculty of Agricultural Sciences, University of Damascus, Syria. |
| Nov. 01, 95 - Feb. 28, 1997: | Advanced professional training in the Federal Republic of Germany, supported by the German Foundation for International Development/ Centre for Food, Rural Development and the Environment (DSE)/ ZEL). |
| July 17, 2004: | Master of agricultural sciences (MSc. agr.), at the Institute of Plant Pathology and Plant Protection, University of Göttingen, Germany. |
| Since June 01, 2006: | PhD student at Department of Crop Sciences, Division of Plant Pathology and Crop Protection, University of Göttingen, Germany. |

## Professional experience:

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| May 26, 1991: | Permanent position at the Ministry of Agriculture and Agrarian Reform (MAAR), Directorate of Plant Protection, Damascus, Syria. |
| March 01, 1997 - Feb. 28, 2000: | Participation in a German/ Syrian cooperation project, supported by the German Agency for Technical Cooperation GTZ (Deutsche Gesellschaft für technische Zusammenarbeit) in the field of plant quarantine, Damascus, Syria. |
| Since June, 2006: | Research Assistant at the Department of Crop Sciences, Division of Plant Pathology and Crop Protection, Georg-August-University of Göttingen, Germany. |