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# Eradication of *Clavibacter michiganensis* subsp. *michiganensis* by incorporating fresh crop debris into soil: Preliminary evaluations under controlled conditions

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ABSTRACT

A method for the eradication of *Clavibacter michiganensis* subsp. *michiganensis* was tested for its efficacy in three experiments carried out in the laboratory and greenhouse. In the first experiment, peat moss and sand mix in pots was amended with fresh tomato debris which was either artificially infected with the pathogen, or was not amended. Pots were enclosed in plastic bags or left open. Two temperatures (25 °C and 45 °C) were tested over a 6-week period. The pathogen was not detected in the amended soil after 4 weeks treatment at 45 °C, but was not eradicated after treatments in open pots at 25 °C.

In the second experiment, the survival of *C.m. michiganensis* in either artificially infested soil or in artificially infected tomato plants was studied to determine the behaviour of the pathogen under these conditions. Strains of saprophytic bacteria in the genera *Bacillus*, *Paenibacillus* and *Brevibacillus* were identified under the experimental conditions. *In vitro* antagonism between *Bacillus subtilis* and *C.m. michiganensis* was observed. Finally, the recovery of *C.m. michiganensis* introduced into disinfected substrate was determined. Survival of *C.m. michiganensis* in plates artificially inoculated with substrate was greatly reduced after a 4-week treatment at 25 °C, or after 1 week at 45 °C.

*C.m. michiganensis* remained pathogenic on plant tissue after 4 weeks of either thermal treatment.

It is important to take these results into account with regard the effect of different soil disinfection techniques or ecological alternatives such as biofumigation, solarization, and the addition of organic matter, as well as for integrated pest management systems.



## 1. Introduction

The production of several vegetable crops has depended on the use of methyl bromide soil fumigation to control a wide array of soil-borne pathogens, but this product was banned from 2005 in most developed nations. This situation has led to increased interest in the development of alternative strategies to control soil-borne pathogens (Lazzeri et al., 2004).

In recent years there has been emphasis on the importance of organic matter in cultivated soil to improve soil health for traditional farming and, particularly, organic farming. This has generated a growing interest in the use of composts, animal manure, organic industrial residues and green manure and cover crops. It is well known that different types of organic soil amendments can have different effects on soil-borne plant pathogens (Lazzeri et al., 2003).

Organic amendments have been applied to reduce plant pathogens, but the biology and chemistry of the suppressiveness of organic amendments is poorly understood. More research is required into biotic and abiotic factors affecting decomposition, as well as the release of plant chemicals and the potential of combining organic amendments with other pest management strategies (Zasada and Ferris, 2004).

The term "biofumigation" was originally applied to describe the suppression of soil-borne pests and diseases by glucosinolate-containing plants with biocidal properties released from either incorporated tissues or rotation crops (Matthiessen and Shackleton, 2005). Nonetheless, this concept has been expanded and is now applied to the beneficial effect of volatile compounds released into the soil by decomposing soil amendments (Bello, 1997; Bello et al., 2002; Stapleton, 2000).

The use of crop debris as biofumigant material has been demonstrated to give the same control of plant diseases as conventional pesticides (Bello et al., 2000). In Spain, many farmers keep their crop residues on the soil surface. Tonnes of vegetal debris are generated every year. Because the incorporation of these plant remains into the soil could be a solution for their negative environmental impact, infected plant residues are buried and used as organic amendments as well as biofumigant materials. The effectiveness of this practice for the control of plant pathogens is currently being proved.

The present study is part of a series of experiments to improve the efficacy of control of *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis et al. by plant debris soil amendments.

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A crucial factor in the management of diseases caused by soil-borne plant pathogens is reduction of their inoculum level below a critical threshold level before a susceptible crop is planted (Blok et al., 2000).

Bacterial canker of tomato (*Lycopersicum esculentum* Mill.), caused by *C.m. michiganensis*, is an important disease in many parts of the world. The disease causes the premature death of plants and reduces yields (Chang et al., 1992). The survival and detection of *C.m. michiganensis* in plants with infections and in soil is an important area of research into the role of this organism in the environment (Trevors and Finnen, 1990).

There have been several studies of the bacterial disease caused by *C.m. michiganensis*, survival of the bacterium in thermal treatments, and the behaviour of the pathogen in soil (Antoniou et al., 1995; Basu, 1970; Chang et al., 1991; Echandi, 1971; Fatmi and Schaad, 2002; Gleason et al., 1991; Moffet and Wood, 1984; Strider, 1967; Trevors and Finnen, 1990). The purpose of this research was to obtain more information about the *C.m. michiganensis* strain isolated from affected crops in Spanish fields. The results, under controlled conditions, provide additional information to be taken into account in the development and application of treatments for soil disinfection.

## 2. Material and methods

### 2.1. Culture media and growth of the bacterium

A highly pathogenic *C.m. michiganensis* strain (GenBank accession no. EU857429), was grown on a selective D2 medium (Kado and Heskett, 1970) for 48 h at 30 °C (Trevors and Finnen, 1990). Direct sequencing revealed that this strain has nucleotide sequences identities of 98% to strain NCPPB 382 (GenBank accession no. AM7111867.1). Cultures were maintained in the dark in an incubator, and were subcultured once a week. Infiltrations of the intracellular spaces of tobacco plant leaves with suspensions of bacteria ($10^8$ colony forming units (cfu) $ml^{-1}$) were performed once a month to determine the phytopathogenicity of the pure cultures based on the development of internervial necrosis (Gitaitis, 1990; Klement et al., 1963).

The virulence of this strain in tomato was assessed in greenhouse tests by stem-inoculating tomato plants (cv. Money-Maker) at the 4-leaf stage with 30 μl of a bacterial suspension containing approximately $10^8$ cfu $ml^{-1}$ (Antoniou et al., 1995). To standardize inoculum density, a $BaSO_4$ turbidity standard was used, this being the equivalent to a 0.5 McFarland standard (Andrews, 2004). Plants were maintained under controlled conditions at 18–30 °C and symptoms were assessed 15 days after inoculation, as described below.

### 2.2. Infiltration of tomato plants: Controlled infection

Healthy tomato plants (cv. Money-Maker) were infiltrated with a pathogenic bacterial solution, as previously described. Once all the plants were inoculated and symptoms of bacterial canker were observed (15 days after inoculation) the presence of the bacterium was confirmed using a Double Antibody Sandwich- Enzyme Linked Immunosorbent Assay (DAS-ELISA) with the Loewe commercial antiserum No. 07063 (Loewe Biochemica GmbH, Sauerlach, Germany).

### 2.3. Use of scanning electron microscope (SEM) to view bacteria in infected tomato plants

In order to prove successful infection after the first inoculation of tomato plant wounds, ultrastructural interactions between bacteria and host tissues at infection sites were observed by SEM (Jeol JSM-5410 Scanning Microscope) at the "Servicio de Microscopia de la Universidad Politécnica de Valencia" (Hagen et al., 1968; Huang, 1986). The presence of the pathogen inside the plant was observed.

### 2.4. Treatments in pots: The addition of tomato debris as an organic amendment

Plant debris was added to pots (15 cm diameter, 15 cm deep) containing 500 g of growing medium, consisting of a mixture of peat moss and sand (4:1 v/v) previously disinfected for 1 h at 121 °C. Infected tomato plants (as previously described) were cut and stem pieces (2–4 cm) were obtained. These highly infected stem pieces were mixed with the growing medium in three doses (5 g, 10 g and 15 g, per 500 g of growing medium with four replications). Negative controls were prepared with the same doses using either healthy tomato plants or only growing medium (dose 0). The dose was chosen according to the biomass of tomato plants, and 5 g, 10 g and 15 g in each 500 g of substrate were related with 25, 50 and 100 tonnes of plant material $ha^{-1}$, respectively. Six groups were prepared to coincide with the weeks of treatment. Half of the pots of each group (12 with infected material, 12 with healthy material, and four without plant material) were enclosed in hermetically sealed plastic bags (B; thermal PE, 300 g thick, transparent, 25 × 40 cm) to avoid the release of volatile substances produced during the decomposition of the plant material. The remaining pots were left open (NB; no bag). The temperatures used to study the survival of the pathogen were 25 and 45 °C (Basu, 1970). These temperatures were selected because 25 °C is easily reached in Spanish fields. Biofumigation could be combined with other techniques, such as solarization, during which soil temperatures may reach 45 °C under field conditions. Although the use of a single temperature throughout the treatment does not simulate solarization under field or greenhouse conditions, the effect of two temperatures on the survival of *C.m. michiganensis* can be studied.

All the pots (56 in each group or "week of treatment") were placed into climatic chambers at the experimental temperatures. When pots were removed from the chamber, healthy tomato seedlings (2 weeks old, cv. Money-Maker) were planted in them (one seedling per pot). The pots were then placed in a greenhouse, controlled at 18–30 °C. Disease was evaluated 40 days after planting and all the plants (with and without symptoms) were analysed using the DAS-ELISA technique, as previously described. When the analyses gave negative results for all the plants, it was considered that the pathogen had not infected the plants and that *C.m. michiganensis* levels had been reduced below the detection limit of the specific detection method used. However, low levels of the pathogen in question might still be present in the soil (Noble and Roberts, 2004).

### 2.5. Statistical analyses

Disease incidence, expressed as the percentage of plants with a positive result using DAS-ELISA, was analysed by ANOVA factorial analysis using the Statgraphics Plus software 4.1. (Manugistics Inc., Rockville, MD, USA). Data comparing controls containing healthy plants and pots containing infected debris were analysed by dose, weeks of treatment and pot treatment as fixed factors. *F* statistics ($P < 0.05$) using the *t*-test (LSD) were used to compare means. Regression analyses were performed with the EXCEL program (Microsoft Office, 2003).

### 2.6. Introduction of C.m. michiganensis to artificially infested growing media and its recovery

The pathogenic strain was isolated from diseased tomato plants and grown for 48 h at 30 °C in D2 medium, as previously described.