

Clavibacter michiganensis subsp. *michiganensis* strains virulence and genetic diversity. a first study in Argentina

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Abstract Tomato bacterial wilt and canker, caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is considered one of the most important bacterial diseases of tomato worldwide. During the last two decades, severe outbreaks have occurred in greenhouses in the horticultural belt of Buenos Aires-La Plata, Argentina. Cmm strains collected in this area over a period of 14 years (2000–2013) were characterized for genetic diversity by rep-PCR genomic fingerprinting and level of virulence in order to have a better understanding of the source of inoculum and virulence variability. Analyses of BOX-, ERIC- and REP-PCR fingerprints revealed that the strains were genetically diverse; the same three fingerprint types were obtained in all three cases. No relationship could be established between rep-PCR

clustering and the year, location or greenhouse origin of isolates, which suggests different sources of inoculum. However, in a few cases, bacteria with identical fingerprint types were isolated from the same greenhouse in different years. Despite strains differing in virulence, particularly within BOX-PCR groups, putative virulence genes located in plasmids (*celA*, *pat-1*) or in a pathogenicity island in the chromosome (*tomA*, *chpC*, *chpG* and *ppaA*) were detected in all strains. Our results suggest that new strains introduced every year via seed importation might be coexisting with others persisting locally. This study highlights the importance of preventive measures to manage tomato bacterial wilt and canker.

Keywords Epidemiology · Rep-PCR · Virulence genes · Tomato bacterial wilt and canker

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Introduction

Tomato bacterial wilt and canker is caused by the Gram positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). The disease, characterized by sporadic occurrence with important economic losses, is considered one of the most important bacterial diseases of tomato (*Solanum lycopersicum* L.) worldwide (de León et al. 2011; Sen et al. 2015). It has been reported in the five continents (EFSA Panel on Plant Health, 2014). Moreover, Cmm is a quarantine organism under the plant health legislation of Europe, Africa, the Caribbean and Asia and is present in the United States of

America, Canada, South America and countries in the Pacific region (EPPO 2015; 2013).

In Argentina, the horticultural belt of Buenos Aires-La Plata is a main producing zone of fresh market tomato. There, tomato is usually grown in two periods per year (spring and autumn) in the same greenhouse. Growers buy seedlings of different varieties to plant nurseries, which produce them from seeds imported from various countries. Bacterial canker was detected for the first time in this area in 1957 (Fernandez Valiela 1975). Since the early 2000's, severe outbreaks have been observed, affecting as much as 92% of the plants by the end of the crop season (Vega and Romero 2016).

Bacterial canker severity and yield losses may vary according to environmental conditions and genetic plant susceptibility (de León et al. 2011; Gitaitis et al. 1991; Gleason et al. 1993). Warm temperatures, between 25 °C and 28 °C, and high humidity are optimal for symptoms appearance (Chang et al. 1992; Sharabani et al. 2014). Young, succulent and well fertilized plants allow a more rapid disease development (Chang et al. 1992). Also, there are differences in virulence among Cmm strains (Ialacci et al. 2015; Strider and Lucas 1970), which could be related to the presence of virulence genes either in the chromosome or plasmids. Several virulence genes have been studied in Cmm isolates (Croce et al. 2016; Kleitman et al. 2008; Tancos et al. 2015) since the whole genome sequence of Cmm strain NCPPB382 was published (Gartemann et al. 2008). These genes are present either in the two known plasmids carried by this strain (i.e. *celA* encoding an endo- β -1,4-glucanase and *pat-1* encoding for a serine protease), or in a pathogenicity island in the chromosome, where pathogenicity genes encoding for several serine proteases (*chpC*, *chpG* and *ppaA*) as well as a tomatinase (*tomA*) were described. When some of these genes are missing, the bacterium is either less virulent or loses completely its pathogenicity (Kleitman et al. 2008; Meletzus et al. 1993).

When Cmm invades the xylem vessels, symptoms start with the unilateral wilting of leaflets or whole leaves. Sometimes stems show brown streaks which may finally split open as cankers (Gleason et al. 1993). The vascular tissue of infected stems appears brown colored, especially near the nodes (EPPO 2013). The proportion of stem discoloration and the progress of wilting leaves were successfully used as a measure of disease development (Yogev et al. 2009).

Bacterial canker epidemics can be initiated by the introduction of infected seeds or seedlings, or by the

persistence of the pathogen in infested plant debris in a farm (Gleason et al. 1993; Vega and Romero 2016). Several molecular-based methods have been used to study Cmm strain diversity, persistence and dissemination. Among them, restriction fragment length polymorphism (RFLP) or multilocus sequence typing (MLST) analysis of housekeeping genes, amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and repetitive sequence-based PCR (rep-PCR) have the ability to discriminate *Clavibacter michiganensis* subspecies and allow the differentiation between Cmm strains (Croce et al. 2016; de León et al. 2009; Ialacci et al. 2015; Louws et al. 1998; Milijašević-Marčić et al. 2012; Waleron et al. 2011). Of all, rep-PCR genomic fingerprinting has proven to be very convenient since it is easy to apply, rapid and useful in assessing Cmm variability. Genotyping of Cmm isolates by this method allowed epidemiological studies in Iran, Israel, Italy, Japan, Spain and USA (de León et al. 2009; Kawaguchi et al. 2010; Kleitman et al. 2008; Louws et al. 1998; Nazari et al. 2007; Quesada-Ocampo et al. 2012; Tancos et al. 2015). In some regions, high diversity of Cmm strains was detected by rep-PCR, suggesting that the pathogen was introduced more than once (Ialacci et al. 2015; Quesada-Ocampo et al. 2012). In other regions, cluster analyses of rep-PCR fingerprints grouped Cmm strains by location with no variation between years, which possibly meant that the bacterial strains were persisting locally (de León et al. 2009; Kawaguchi and Tanina 2014).

Although bacterial wilt and canker of tomato can be devastating in Argentina, the pathogen has not been characterized molecularly yet. The aims of this work were: (i) to characterize the genetic diversity and virulence of Cmm strains causing bacterial wilt and canker epidemics in the horticultural belt of Buenos Aires-La Plata, (ii) to establish if there is a correspondence between rep-PCR genetic groups and strain virulence, and (iii) to inquire into the main inoculum sources of bacterial wilt and canker.

Materials and methods

Pathogen isolation and culture media

Strains were isolated from tomato symptomatic plants collected from different greenhouses in five locations in

the Buenos Aires-La Plata horticultural belt between 2000 and 2013 and from an imported commercial seed lot (Table 1). A small portion of the stems was peeled and comminuted in sterile distilled water (SDW) with a sterile pestle and mortar and the resulting suspension was streaked on Yeast Dextrose Carbonate agar plates (YDC; yeast extract 10 g, D-glucose 20 g, calcium carbonate 20 g and agar 15 g, in 1 L of distilled water). Tomato seeds were grinded in a mill and suspended in sterile PBS buffer. The original suspension and a 10-fold dilution were plated in YDC agar (Hadas et al. 2005). After incubation at 28 °C for 48 h to 72 h, *Clavibacter*-like colonies were selected and further purified in YDC (EPPO 2013). A total of 12 local Cmm strains were used for this study (Table 1). Bacteria were grown on YDC for 48 h to 72 h at 28 °C for routine purposes, and stored in nutrient broth with 20% glycerol at –80 °C.

All Cmm-like isolates were initially identified by colony morphology in YDC, Gram reaction, cell morphology and a hypersensitive reaction (HR) on *Mirabilis jalapa* L. (Davis and Vidaver 2001). The identity of isolates was confirmed by PCR using PSA-4/PSA-R primer set as described by Pastrick and Rainey (1999). PSA-4 is a specific primer for Cmm located in the internal transcribed spacer of the 16S–23S rRNA genes, while PSA-R is a universal primer for Gram positive bacteria located in the 23S rRNA gene,

together, they allow the identification of Cmm according to the size of the amplified PCR product (Pastrick and Rainey 1999). NCPPB382, provided by the National Collection of Plant Pathogenic Bacteria (UK), was used as reference strain.

DNA isolation and repetitive sequence-based (rep-PCR) genomic fingerprinting

Total genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega) according to manufacturer instructions and adjusted to 50 ng μl^{-1} . Rep-PCR genomic fingerprints of isolates were performed with BOX-A1R, REP (REP1R-I and REP2-I) and ERIC (ERIC1R and ERIC2) primers with a MJ PTC-100 thermocycler (MJ Research Inc., Waltham, MA) as previously described (Montecchia et al. 2002; Versalovic et al. 1994). Amplification products (8 μl) were separated by electrophoresis on 1.5% agarose gels in TBE buffer at 5 V/cm for 2.75 h. Gels were stained with SYBR Safe DNA Gel Stain (Invitrogen) and photographed under UV light using InGenius LHR2 gel documentation system (Syngene). Fingerprints were analyzed with GelCompar II v. 6.5 (Applied Maths NV) as described by the manufacturer. Dendrograms were elaborated based on Pearson's correlation coefficient and the UPGMA algorithm.

Table 1 *Clavibacter michiganensis* subsp. *michiganensis* strains used in this study. Year, origin of isolation, and summary of results of BOX-PCR fingerprints cluster analysis, PCR-based identification and detection of pathogenicity genes

Strain	Year	Origin		BOX-PCR group	PCR ITS 16S–23S rRNA	PCR detection of virulence genes					
		Location	Greenhouse			<i>pat-1</i>	<i>celA</i>	<i>tomA</i>	<i>chpC</i>	<i>chpG</i>	<i>ppaA</i>
Cm7	2000	Florencio Varela	A	III	+	+	+	+	+	+	+
Cm11	2000	Florencio Varela	A	I	+	+	+	+	+	+	+
Cm26	2011	Florencio Varela	A	I	+	+	+	+	+	+	+
Cm27	2011	Florencio Varela	A	II	+	+	+	+	+	+	+
Cm34	2011	Florencio Varela	B	II	+	+	+	+	+	+	+
Cm38	2013	Florencio Varela	B	II	+	+	+	+	+	+	+
Cm54	2002	Arana	C	I	+	+	+	+	+	+	+
Cm58	2009	Berazategui	D	II	+	+	+	+	+	+	+
Cm64	2011	Olmos	E	I	+	+	+	+	+	+	+
Cm66	2012	Berazategui	D	I	+	+	+	+	+	+	+
Cm99	2011	Florencio Varela	A	I	+	+	+	+	+	+	+
Cm97	2011	Seeds	-	I	+	+	+	+	+	+	+
NCPPB382	1956	UK	-	IV	+	+	+	+	+	+	+

Detection of pathogenicity genes

The presence of plasmid-encoded pathogenicity genes *celA* and *pat-1* were evaluated by PCR in all strains using the primer pairs PFC3/PFC5 and CMM-5/CMM-6, respectively (Dreier et al. 1995; Kleitman et al. 2008). Chromosomal genes *tomA*, *ppaA*, *chpC* and *chpG*, involved in bacterial virulence and fitness, were also evaluated by PCR in all strains using primers based on the sequence of the NCPPB382 strain (*tomA*-F/*tomA*-R, *ppaA*-F/*ppaA*-R, *chpC*-F/*chpC*-R and *chpG*-F/*chpG*-R) as described (Kleitman et al. 2008; Yim et al. 2012).

Pathogenicity and virulence tests

Tomato plants (var. ACE 55, Asgrow Seed Co.) were grown individually in 1-L pots containing 4:1 v/v compost:perlite and were fertilized twice a week (0.4 g calcium nitrate, 0.27 g potassium nitrate, 0.27 g monopotassium phosphate and 0.24 g magnesium sulfate per liter of tap water).

Cmm strains were cultivated on YDC agar plates at 28 °C for 72 h. Bacterial cells were suspended in SDW and adjusted at $A_{590} = 0.3$ (Jenway 7315 spectrophotometer®), which corresponds to $\sim 10^8$ CFU ml⁻¹, to use as inoculum for pathogenicity tests (Romero et al. 2014) and 1:10 diluted for virulence tests.

The pathogenicity of each strain was tested by cutting the second true leaf of a tomato plant with a scalpel immersed in a bacterial suspension ($\sim 10^8$ CFU ml⁻¹). Three plants were inoculated for each strain. Symptoms were evaluated two weeks later. For virulence tests plants were inoculated when they had six expanded leaves, by placing 20 µl of the bacterial suspension ($\sim 10^7$ CFU ml⁻¹) in the node of the second true leaf and pricking the stem with a sterile insulin needle (de León et al. 2008). There were five plants inoculated per Cmm strain. Control plants were mocked inoculated with SDW. Nine strains were evaluated, five from BOX-PCR group I, three from group II and one from group III. The experiment was repeated three times. In both tests, plants were maintained in the greenhouse under natural temperature (18 °C night–28 °C day) and light (~14 h) conditions.

The first day post inoculation (DPI) on which bacterial canker symptoms appeared was registered for each strain. Leaf incidence was evaluated every 3–4 days as the proportion of the leaves that were wilting per plant,

and data were used to calculate the area under disease progress curve (AUDPC) (Campbell and Madden 1990). At the end of the experiment, the stems were cut longitudinally and the extent of vascular discoloration was determined as a proportion of the total length of the stems.

Statistical analyses

Strains virulence variables were analyzed using a non-parametrical analysis of variance and a multivariate analysis of variance using R 3.0.1 (R Core Team, 2014).

Results

Identification of isolates

All isolated strains showed typical yellow mucoid colonies on YDC medium, were Gram positive, rendered an HR on *M. jalapa* and yielded the expected size PCR product (270 bp) for *C. michiganensis* subsp. *michiganensis* with the PSA-4/PSA-R primer set.

Rep-PCR genomic fingerprinting and pathogenicity genes

rep-PCR genomic fingerprints with BOX, ERIC, and REP primers were generated from all isolates and NCPPB382 reference strain. The cluster analysis of fingerprints showed the same clustering of local strains in all cases (Supplemental material, Fig. S1), therefore, for now on we will refer our results to BOX-PCR clustering (Fig. 1). Group I clustered 7 of the 12 strains, group II contained 4 strains and group III included only one strain. NCPPB382 formed a separate cluster (group IV) with an 88% similarity with the local strain of group III (Fig. 1).

Group I clustered strains isolated in four years in different locations and the strain isolated from commercial seeds. Group II included strains obtained from three greenhouses in two locations and three different years, while group III contained only one strain (Cm7) isolated in 2000 (Table 1). In some instances, strains with different fingerprint types were isolated from the same greenhouse at the same sampling time, i.e. Cm11 (group I) and Cm7 (group III) in 2000, or Cm99 and Cm26 (group I) and Cm27 (group II) in 2011. In other cases, strains belonging to the same genetic group were

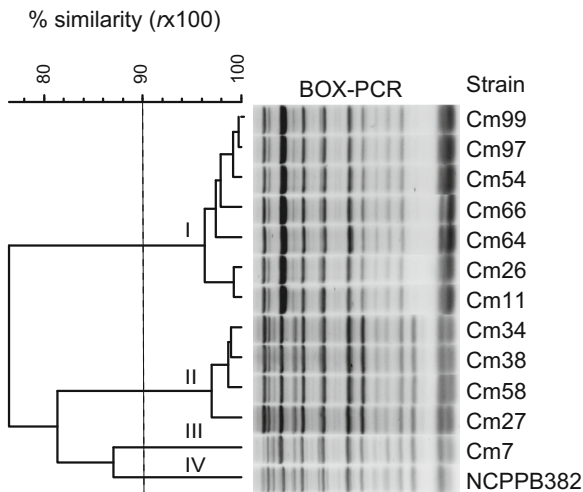


Fig. 1 UPGMA dendrogram based on the Pearson correlation coefficient (r) obtained from BOX-PCR genomic fingerprints analysis of *Clavibacter michiganensis* subsp. *michiganensis* strains isolated in Buenos Aires-La Plata horticultural belt. The groups indicated by I to IV were defined at the 90% similarity level (vertical dashed line). Tags at the right show strains denomination. *C. michiganensis* subsp. *michiganensis* NCPB382 was used as reference strain

isolated from the same greenhouse in different years, i.e. strains Cm34 and Cm38 (group II), which were obtained from greenhouse B in Florencio Varela in 2011 and 2013, respectively (Table 1), and Cm99, Cm26 and Cm11 (group I) obtained from greenhouse A in Florencio Varela in 2011 and 2000.

Pathogenicity genes were detected in all strains (Table 1). They all yielded the expected PCR products for *celA* (551 bp) and *pat-1* (614 bp) genes encoding essential pathogenicity determinants, and for those genes involved in virulence and fitness; *tomA* (529 bp), *chpC* (639 bp), *chpG* (394 bp) and *ppaA* (587 bp) (Table 1).

Pathogenicity and virulence tests

Pathogenicity tests were positive for all strains; plants had their leaves wilted two weeks after inoculation. In the virulence assay symptoms began to appear five days after inoculation. First, plants exhibited the wilting of one or two leaflets, progressing to a generalized wilting of the plants by the end of the experiment. Strains differed in AUDPC and the extent of the stem discoloration they induced ($p < 0.05$ for both variables) while there were no significant differences for DPI ($p > 0.05$). There were no differences between genetic groups

neither when the variables were evaluated separately nor when a multivariate analysis was performed ($p > 0.05$). However, Cm7 from group III was consistently one of the strains with the lowest AUDPC and stem severity values (Fig. 2a and b). On the other hand, strains from group II induced intermediate levels of disease for both variables, while strains from group I were associated with the highest and lowest AUDPC and stem severity values (Fig. 2a and b). The average of

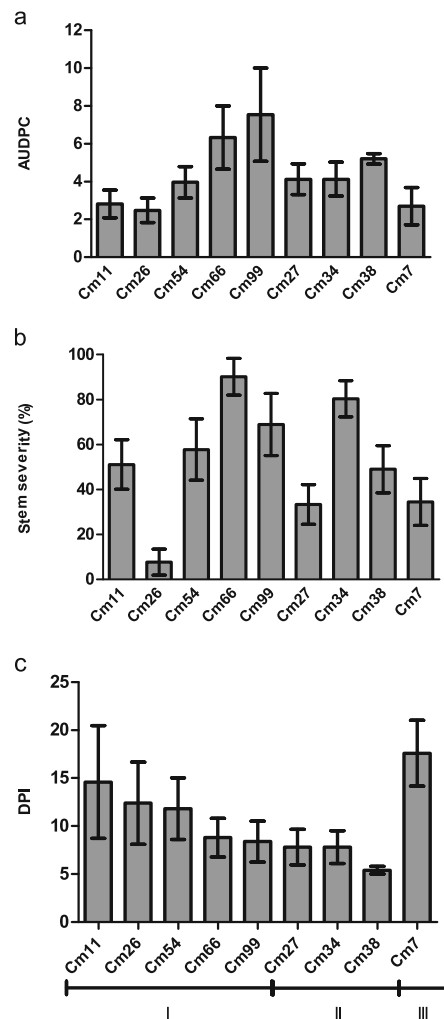


Fig. 2 Virulence characterization of *Clavibacter michiganensis* subsp. *michiganensis* strains used in this study. **a** Area under the disease progress curve (AUDPC) **b** Stem severity (percentage of discolored stem length / total stem length) and **c** First day post inoculation (DPI) on which plants showed symptoms. Statistical analysis was done using Kruskal-Wallis non-parametric analysis of variance; it was significant for AUDPC and stem severity ($p < 0.05$). Data are mean of five plants, bars are standard error of the mean. Lower horizontal axis indicates the BOX-PCR group to which each strain belongs

days for showing symptoms ranged from 7 to 18 DPI; plants inoculated with strains of group II were the first to express disease symptoms, with a mean of seven DPI, while it took nearly 18 days for those inoculated with Cm7 (Fig. 2c). Results were more variable for plants inoculated with strains from group I.

Discussion

Tomato bacterial wilt and canker, caused by Cmm, has produced severe outbreaks in greenhouses in the horticultural belt of Buenos Aires-La Plata, Argentina, during the last 15 years. However, up to now, no study has focused on the genetic diversity of local Cmm strains, strain virulence or the source of inoculum. This knowledge could help producers to improve disease management. Cluster analyses of rep-PCR genomic fingerprints generated using BOX, ERIC or REP primers resulted in the same grouping of Cmm strains from Buenos Aires-La Plata. In previous works, each set of primers resolved different number of groups (Louws et al. 1998) or BOX-PCR yielded the most informative profiles (de León et al. 2009; Zaluga et al. 2013), therefore, we decided to continue our study with BOX-PCR.

In other tomato growing regions of the world three to six Cmm BOX-PCR groups were detected (Ialacci et al. 2015; Kawaguchi et al. 2010; Kleitman et al. 2008; Louws et al. 1998; Nazari et al. 2007; Tancos et al. 2015). Similarly, we identified three genetic groups among our local strains, revealing a moderate genetic diversity in the pathogen population present in the horticultural belt of Buenos Aires-La Plata. Most of the strains collected corresponded to group I; those strains were isolated in all locations and in different years. Likewise, in other studies one or two Cmm genetic groups were prevalent, while the others were rare (Ialacci et al. 2015; Milijašević-Marčić et al. 2012; Nazari et al. 2007; Quesada-Ocampo et al. 2012; Tancos et al. 2015). Knowledge of the prevalence of certain strain types may be useful for choosing strains for future disease management studies.

Grouping of strains based on genomic fingerprints was not related to location, greenhouse or year of isolation, which suggests different sources of inoculum. Moreover, strains from all genetic groups were isolated in the same greenhouse (A) over the years. High genetic diversity in Japan, Sicily in Italy, and New York in the USA, was proposed to be related to the reintroduction of

the pathogen with imported seed (Ialacci et al. 2015; Kawaguchi and Tanina 2014; Tancos et al. 2015). This could also be the case in our area, given that tomato seeds, considered Cmm primary source of inoculum (Gleason et al. 1993; Kleitman et al. 2008), are imported each year. Reinforcing this idea, one of the strains included in this study was isolated from imported commercial seeds. However, strains from the same genetic group were detected in a greenhouse in different years, i.e. strains from group I were collected in 2000 (Cm11) and 2011 (Cm26 and Cm99) in one greenhouse (A), and the same happened in another greenhouse (B) with strains of group II over a period of two years (Cm34 and Cm38). A similar situation was observed in other countries, where Cmm presence was proposed to be associated with strain persistence in plant debris rather than reintroductions (de León et al. 2009; Kawaguchi et al. 2010; Kleitman et al. 2008). It is possible that in our study dominant strains persisted on host debris. Depending on debris position in the soil and time of the year, Cmm can survive from 45 to 260 days in tomato debris in greenhouse conditions in our area (Vega and Romero 2016), and it has been demonstrated that Cmm can be transmitted from tomato residues to growing plants and cause disease (Kawaguchi and Tanina 2014). On the contrary, strain Cm7, the only representative of group III in this study, was isolated in 2000 and was not detected afterwards. This strain might have a low fitness or ability to survive in host debris.

Our study shows that strains from different sources might coexist in the same greenhouse, i.e. the ones that survived in plant debris from previous production cycles and those introduced later with seeds. The same situation was described for New York isolates (Tancos et al. 2015). In both cases, only the most dominants might be persisting in the greenhouse.

All the strains evaluated in this study were pathogenic, but varied in virulence. Virulence was assessed in a comprehensive way by considering the first day post inoculation when symptoms were observed, the AUDPC calculated from leaf incidence data, and the proportion of the stems with visible vascular tissue discoloration. Group II strains were the first to induce symptoms, with moderate values of AUDPC and stem severity. The incubation period for systemic symptoms is very variable for bacterial canker, and it is related to temperature, plant age, inoculum concentration, and cultivar (Chang et al. 1992; Gleason et al. 1993; Sharabani et al. 2013). In this study, it is shown that it

also depends on the pathogen strain. Tancos et al. (2015) and Ialacci et al. (2015) also detected differences in strain virulence in New York and Sicily, respectively. The six genes related to virulence evaluated were detected in all the strains included in this study, suggesting that a difference in virulence may also depend on other factors (Savidor et al. 2014). However, it is worth noting that we did not analyze the sequences of the detected genes.

In conclusion, three BOX-PCR fingerprint types of Cmm strains were detected in tomato crops from the Buenos Aires-La Plata horticultural belt. Strain NCPPB382 was only similar to Cm7, with an 88% similarity. The virulence of the strains was highly variable; Cm7 was less virulent than strains from groups I and II. In this area, new strains seem to be introduced every year with imported seeds, which might be coexisting with others surviving locally on debris from previous crops.

The information presented here highlights the importance of prevention management strategies to control tomato bacterial wilt and canker in the horticultural belt of Buenos Aires-La Plata. It would be advisable for growers to use pathogen free seeds and seedlings to avoid the introduction of the pathogen into new areas, or new strains where the disease is already established. This would minimize damage and economic losses to the present crop, and reduce the inoculum left for the following campaign. All infected material should be removed and destroyed in order to prevent pathogen persistence on crop residues. Crop rotation could also help disease management.

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