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Survival of *Clavibacter michiganensis* subsp. *michiganensis* in tomato debris under greenhouse conditions

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Bacterial canker, caused by *Clavibacter michiganensis* subsp. *michiganensis*, is one of the most important diseases of tomato worldwide. Once the pathogen has been introduced into an area, i.e. by contaminated seeds or transplants, it survives mainly on host debris. In different geographic areas the survival time of the pathogen in crop residues under field conditions has been very variable, ranging from 2 months in Morocco to 2 years in Iowa (USA). This study took place in the horticultural belt of Buenos Aires – La Plata, Argentina, where greenhouse production prevails, and monoculture with two production cycles per year is a common practice. The aim was to determine the survival time of this pathogen in plant residues left on the soil surface or buried. During three consecutive years, by the end of both production cycles in July (winter) and December (summer), above- (stem, petiole) and belowground (root) tissues were placed into nylon netting bags and left on the soil surface or buried at 10 cm depth. The pathogen population was regularly quantified by dilution plating on semiselective medium. In host debris left on the soil surface, bacteria survived 120–260 days for crop production cycles that ended in winter and 45–75 days for those that ended in summer. In stems or roots buried in winter, this period was 45–75 days. It is concluded that host debris, including roots, might be an important primary inoculum source of the pathogen in greenhouses.

Keywords: bacterial canker, *Clavibacter michiganensis* subsp. *michiganensis*, host residues, roots, *Solanum lycopersicum*

Introduction

Bacterial canker, caused by the Gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* (Davis *et al.*, 1984), is one of the most important bacterial diseases of tomato (*Solanum lycopersicum*) in Argentina (Romero *et al.*, 2014) and the world (de León *et al.*, 2011). Recently, new outbreaks have been reported in several countries including Spain (Canary Islands), France, Japan, Israel, Serbia, Belgium and Argentina (Kleitman *et al.*, 2008; de León *et al.*, 2009; Jacques *et al.*, 2012; Milijašević-Marčić *et al.*, 2012; Zaluga *et al.*, 2013; Kawaguchi & Tanina, 2014; Romero *et al.*, 2014). In the horticultural belt of Buenos Aires – La Plata (Argentina) bacterial canker is a serious problem in greenhouse tomato production (Romero *et al.*, 2003); in field surveys, its incidence ranged from 32 to 92% (A. M. Romero, unpublished data). In this region, there are more than 1000 ha of greenhouses, with soil-based tomato as their main crop (García, 2007).

Once the pathogen has been introduced into an area, by contaminated seeds or transplants, host debris is considered the most important source of inoculum. In field-grown tomatoes, it has been established that an epidemic originating from infested debris can significantly reduce the yield of the subsequent production cycle (Gleason *et al.*, 1991). The pathogen can also survive in volunteer tomato and other host plants, as epiphytic populations on other solanaceous and non-solanaceous species, and even on other surfaces (Chang *et al.*, 1992; Miguel Wruck *et al.*, 2006), but the epidemiological importance of these inoculum sources is as yet unknown.

In different geographic areas, the survival time of the pathogen in host debris (leaves and stems) on field-grown tomatoes was shown to be very variable: from 2 months in Morocco to up to 2 years in Iowa (central USA) (Gleason *et al.*, 1991; Fatmi & Schaad, 2002). In California and Ohio (USA), the pathogen was detected on tomato debris after 10 months (Fatmi & Schaad, 2002) and in Illinois after at least 7 months (Chang & Pataky, 1992), although these experiments ended before the pathogen could no longer be detected.

Not only geographic location, but also host debris placement within the soil profile affected the survival time. In different locations, this period was between 25 and 75% shorter in buried residues than in those left on the soil surface (Gleason *et al.*, 1991; Fatmi & Schaad, 2002). In Illinois the concentration of the pathogen was also an order of magnitude smaller in buried tissues than in those left on the soil surface (Chang & Pataky, 1992).

Differences in the survival time reported in previous studies could also be affected by the technique used for detection. Dilution plating is the most widely used, although when the pathogen population is very low, antagonistic saprophytes can be responsible for false

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negatives. To avoid this, there are selective media that inhibit the growth of most saprophytes, making plating a very sensitive detection method (Fatmi & Schaad, 2002; Ftayeh *et al.*, 2011). Other researchers have reported *in vivo* experiments consisting of the inoculation of a suspension of macerated host debris onto plants and observation of symptom development (Zanón & Jordá, 2008).

In recent decades, greenhouse production systems of the horticultural belt of Buenos Aires – La Plata experienced a period of specialization and land use intensification (García, 2007). Nowadays, monoculture and two tomato cycles per year (spring and summer) have become common practices. The short or nonexistent time lag between successive crops suggests that any infested host tissue left is potentially an important source of inoculum. This is a serious threat, because it has been suggested that in many instances the primary source of inoculum in greenhouse crops is mostly local; in different locations in Okayama, Japan, all the strains of the pathogen isolated from the same greenhouse belonged to the same haplotype, regardless of the cultivar or year of isolation (Kawaguchi *et al.*, 2010). de León *et al.* (2009) arrived at a similar conclusion in the Canary Islands. Moreover, the pathogen can be transmitted, and cause disease, from infested debris buried in the soil to tomato plants grown in a greenhouse (Zanón & Jordá, 2008; Kawaguchi & Tanina, 2014). Despite the evidence suggesting that host debris might be of great epidemiological importance for disease development, the survival time of this pathogen on host debris under greenhouse conditions remains unknown. *Clavibacter michiganensis* subsp. *michiganensis* can colonize roots, which might therefore be another local source of inoculum (Xu *et al.*, 2012). Nevertheless, the roots have been largely overlooked in previous survival studies. The aim of the present study was to determine the survival time of *C. michiganensis* subsp. *michiganensis* in stems and petioles left on the soil surface and in buried stems and roots, under greenhouse conditions. Because the use of semiselective media, as used in this study, can result in false negatives due to the antagonistic effect of saprophytes, results were compared with those of a recently developed, allegedly better, semiselective medium and an *in vivo* detection method. In addition, the interference by saprophytes was evaluated.

Materials and methods

The experiments were carried out in a greenhouse at Facultad de Agronomía, University of Buenos Aires, Argentina (34°35′36·121″S, 58°29′10·172″W). In 2009, 2010 and 2011, leaves or 10-cm long stem sections of naturally infected tomato plants, recently harvested from a commercial crop, were placed on the soil surface of the greenhouse in July (Southern hemisphere winter) and December (summer), at the end of the summer or spring crop, respectively; roots and another set of stems were buried 10 cm deep in the soil in July. There were six replicates per treatment each year, but only three replicates of buried stems in 2009 and three replicates of roots in 2011. In order to facilitate recovery, plant material was put into nylon netting bags (2 mm mesh) (Gleason *et al.*, 1991; Fatmi & Schaad, 2002), each of which represented a replicate and contained four leaves, four stem sections (subsamples) and the plant's whole root system. Previously, a section of each piece of tissue from each bag (replicate) was cut to verify the presence of *C. michiganensis* subsp. *michiganensis* and determine its initial concentration (CFU g^{-1}) and the ability of these isolates to grow on a semiselective medium. This was carried out employing the same isolation technique and medium (MSCM) used for pathogen detection described below.

Assessment of survival time

Bacterial populations in plant residues were determined every 30–45 days, until the pathogen could not be further detected. One section of each petiole (0·5 cm), stem (0·5 cm) and four sections of adventitious roots (1 cm) from each bag (replicate) at each time point was used to determine dry weight (after drying at 70°C for 7 days). Another section (same size per tissue) was comminuted in sterile distilled water (SDW, 5 mL) with a sterile pestle and mortar, serially diluted and plated on a modified semiselective agar medium (MSCM). This growing medium is based on mSCM (Waters & Bolkan, 1992), with the only difference that mannose was replaced by sucrose, as in the original SCM medium developed by Fatmi & Schaad (1988). The plates were incubated at 28°C, and after 7–10 days putative *C. michiganensis* subsp. *michiganensis* colonies were counted and purified by streaking onto plates of YDC agar medium (Ritchie & Dittapongpitch, 1991). At least 30 putative colonies from each plate were streaked. All the colonies identified as positive, based on colony colour and morphology, were tested for Gram reaction and microscopically checked; 10–30% of these colonies were further tested for pathogenicity either by inoculating susceptible tomato plants, or by observation of a hypersensitive response on *Mirabilis jalapa* leaves (Gleason *et al.*, 1993; Alarcón *et al.*, 1998). In order to confirm the identity of the pathogen, one isolate from the debris of each plant (replicate) of the experiments initiated in winter of 2010 and 2011 and in summer of 2011/12 were tested by PCR using the primers CMM-5 and CMM-6 (Dreier *et al.*, 1995).

Confirmation of survival data

Isolation on MBCT

During the last evaluation of the experiments initiated in the winter of 2011, a new semiselective growing medium, BCT, was used in addition to MSCM. This medium was developed by Ftayeh *et al.* (2011) and was reported to have a higher selectivity and recovery rate than other media for *C. michiganensis* subsp. *michiganensis*. The fungicides epoxiconazole and fenpropimorf were replaced by cycloheximide (20 mg L^{-1}, dissolved in 1 mL methanol) and this modified medium was named MBCT. This experiment was repeated in the summer of 2011/12.

Comparison of in vitro *and* in vivo *detection methods*

Plant material from the experiment initiated in the winter of 2011 was used to compare an *in vivo* detection method to the *in vitro* method described above. The experiment was conducted with stems that had been buried for 70 days and repeated with stems left on the soil surface that experienced a decomposition time of 150–165 days. The same suspensions obtained from the comminuted tissues were used to evaluate both methods. For the *in vivo* detection, tomato plants of the fresh-market cultivar Ace 55 (Asgrow Seed Co.) were inoculated when they had 4–5 true leaves by removing the first true leaf of each plant with a flame-sterilized scalpel, which had been immersed in the different suspensions of comminuted tissues. At least two plants per replicate were inoculated. Disease symptoms were evaluated 8 weeks after inoculation by cutting the stems longitudinally and observing the browning of vascular tissues. Negative and positive control plants were treated with a scalpel immersed in SDW or a suspension of Cm9, a virulent strain of *C. michiganensis* subsp. *michiganensis*, respectively. Cm9 was from a collection of strains maintained at −80°C and was cultured in YDC agar at 28°C for 48 h, suspended in SDW and then the concentration was adjusted to OD_{600} 0·3 (*c.* 10^8 CFU mL^{-1}). A positive control for both detection methods consisted of the addition of Cm9 (*c.* 10^8 CFU mL^{-1}) to the suspension of the stems left on the soil; 100 μL of this suspension was added to 900 μL of the stem suspension to reach a concentration equivalent to 10^8 CFU g^{-1}.

Assessment of interference caused by saprophytes

The ability of *C. michiganensis* subsp. *michiganensis* to grow alone or in the presence of saprophytes in decomposing tissues was tested using stems left on the soil surface in the winter of 2011 for 150 days. A pure culture of Cm9, grown as previously described, was suspended in SDW or in comminuted stem tissues (10^7 CFU mL^{-1}) and plated on MSCM. The difference between the concentration of the pathogen growing alone or with saprophytes from the stem suspensions was calculated (CFU mL^{-1}), and statistically assessed with a Student's *t*-test. In order to determine whether there was a relationship between pathogen recovery and the abundance of saprophytes in a plate, the number of saprophyte colonies isolated from stems buried for 30–75 days or left on the soil surface for 60–165 days in winter of 2011were counted; counts were compared between plates that were negative or positive for pathogen detection. A Kruskal–Wallis analysis of variance was performed on these data because they were not normally distributed.

Results

Survival on crop residues

The survival time of *C. michiganensis* subsp. *michiganensis* in tomato plant residues left on the soil surface in experiments set up in winter, after summer crops, was 120–260 days, depending on the year. In buried stems this period was 70–75% shorter: 45–75 days (Table 1). In 2010 and 2011, the pathogen was detected in decomposing roots after 45 or 45–75 days, respectively, despite a negative result in the initial root evaluation (Table 1).

In the experiments initiated in the summer of 2009/10 and 2011/12 the pathogen was detected after 45–75 days in stems and petioles left on the soil surface (Table 2). In 2010/11, despite high initial concentrations (*c.* 10^{10}

Table 1 Survival of *Clavibacter michiganensis* subsp. *michiganenesis* (Cmm) under greenhouse conditions in stems and petioles left on the soil surface and in buried stems and roots in experiments initiated in winter, from 2009 to 2011

	Soil surface				Buried (~10 cm)			
	Stems		Petioles		Stems		Roots	
Time (days)[a]	Prop.[b]	Conc. (log$_{10}$ CFU g^{-1})[c]	Prop.	Conc. (log$_{10}$ CFU g^{-1})	Prop.	Conc. (log$_{10}$ CFU g^{-1})	Prop.	Conc. (log$_{10}$ CFU g^{-1})
2009								
Initial	5/6	7·9 ± 0·32	–[d]		3/3	7·8 ± 0·56	4/6	6·7 ± 0·45
30–45	–		–		1/3	6·8	0/6	
60–75	3/6	4·9 ± 1·42	–		0/3		–	
105–120	1/6	7·6	–		–		–	
150–165	1/6	7·3	–		–		–	
2010								
Initial	5/5	10·4 ± 0·21	6/6	9·5 ± 0·74	5/5	10·4 ± 0·21	0/7	
30–45	–		–		1/5	7·1	1/7	6·7
60–75	–		–		–		0/2	
105–120	1/5	5·0	0/6		0/5		–	
150–165	0/5		0/6		–		–	
2011								
Initial	6/6	10·2 ± 0·11	6/6	10·9 ± 0·71	6/6	10·2 ± 0·11	0/7	
3–45	–		6/6	8·7 ± 0·15	3/6	6·2 ± 0·19	1/3	6·5
60–75	2/6	7·5 ± 0·46	–		2/6	6·6 ± 0·21	1/3	5·7
15–165	1/6	6·8	3/6	7·0 ± 0·33	0/6		0/3	
245–260	1/6	7·2	2/6 (3/3)	8·1 ± 1·05	–		–	
335–350	0/2 (0/1)		0/3 (0/3)		–		–	

[a] Days after initiation during which the Cmm population was quantified.

[b] Proportion of positive replicates (positive samples/total samples). Data in parentheses are from the plates containing MBCT medium.

[c] Mean bacterial concentration per tissue dry weight of positive replicates (± standard error). CFU, colony-forming units.

[d] No data.

Table 2 Survival of *Clavibacter michiganensis* subsp. *michiganenesis* (Cmm) in a greenhouse in stems and petioles left on the soil surface in experiments initiated in summer, for two different years

	Stems		Petioles	
Time (days)[a]	Prop.[b]	Conc. ($\log_{10}$ CFU g^{-1})[c]	Prop.	Conc. ($\log_{10}$ CFU g^{-1})
2009/10				
Initial	6/6	10·7 ± 0·14	6/6	10·6 ± 0·17
30–45	1/6	7·6	3/6	8·2 ± 1·21
60–75	0/6		0/6	
2011/12				
Initial	6/6	10·8 ± 0·15	6/6	10·3 ± 0·21
30–45	5/5 (4/5)	7·7 ± 0·29	0/5	
60–75	1/5 (0/5)	7·3	0/5 (0/3)	
150–165	0/5 (0/5)		–[d]	

[a] Period after initiation during which the Cmm population was quantified.

[b] Proportion of positive replicates (positive samples/total samples). Data in parentheses are from the plates containing MBCT medium.

[c] Mean bacterial concentration per tissue dry weight of positive replicates (± standard error). CFU, colony-forming units.

[d] No data.

Table 3 Saprophyte populations on plates from the winter experiment of 2011, on which *Clavibacter michiganensis* subsp. *michiganensis* was (positive) or was not (negative) detected

Result	*n*[a]	Mean (CFU/plate)[b]	Median (CFU/plate)[b]
Negative	20	1840 a	1360 a
Positive	10	842 a	862 a

The same letters indicate no significant differences between categories ($P = 0{\cdot}05$).

[a] Number of results (*n*).

[b] Mean and median number of saprophytes. CFU, colony-forming units.

CFU g^{-1}), no replicates were positive at any subsequent sampling date (data not shown).

Confirmation of survival data

The results obtained with the MSCM medium were verified when simultaneously plating onto MBCT for the last sampling dates of 2011 (Table 1) and the summer of 2011/12 (Table 2). On MBCT, *C. michiganensis* subsp. *michiganensis* colonies were larger and easier to distinguish from saprophytes than on MSCM, as reported by Ftayeh *et al.* (2011). The majority of the *in vitro* results corresponded with those *in vivo*, with the exception of 3 out of 27; two of the assessment dates were negative using the *in vivo* method, but positive for the *in vitro* method, and vice versa for another measurement.

The concentration of the pathogen recovered on semiselective growth medium was two orders of magnitude larger when plated from a SDW suspension (10^7 CFU mL^{-1}) than when suspended with stem tissues (10^5 CFU mL^{-1}; $P = 0{\cdot}0001$). In pathogen-positive suspensions, the mean and median numbers of saprophytes were smaller than from pathogen-negative suspensions (Table 3; Kruskal–Wallis, $P = 0{\cdot}06456$).

Discussion

Survival times of *C. michiganensis* subsp. *michiganensis* in tomato debris under greenhouse conditions in the horticultural belt of Buenos Aires – La Plata, Argentina, were similar to, or shorter than, those reported in other countries for field production systems. In the conditions of this study, the pathogen was detected for up to 120–260 days in residues left on the soil surface in winter, depending on the year. These periods are similar to those observed in Morocco, where the pathogen survived 132–194 days (Fatmi & Schaad, 2002). In the USA, longer survival times were observed, ranging from 7 to 24 months, depending on the location (Gleason *et al.*, 1991; Chang & Pataky, 1992; Fatmi & Schaad, 2002); in all these cases, the experiments were finished when the pathogen could still be detected, hence these periods might have been even longer.

In agreement with the results of other authors, survival times of the pathogen were longer in debris left on the soil surface than in buried tissues. Depending on the year, in the present experiments bacteria were isolated for up to 45–75 days in buried debris. Similar results were obtained in Morocco (Fatmi & Schaad, 2002), where survival time was two to three times longer in residues left on the soil than in buried debris. In Iowa, *C. michiganensis* subsp. *michiganensis* (Rif$^+$) was isolated after 180 days in buried leaves and stems, whereas it could still be recovered in tissues left on the soil surface after 730 days (Gleason *et al.*, 1991). In California and Ohio, *C. michiganensis* subsp. *michiganensis* was still isolated after 314 days on surface residues, when the experiment finished; in contrast the pathogen was not detected in buried debris after 180–240 days in California and 240–314 days in Ohio (Fatmi & Schaad, 2002). In Illinois, the population of the pathogen was approximately an order of magnitude smaller in tissues that had been buried than in those left on the soil, at all times (Chang & Pataky, 1992).

Besides location in the soil, climatic conditions of the sites could explain differences observed between results of studies on the survival of *C. michiganensis* subsp. *michiganensis* in host debris. In field-grown tomatoes in Ohio, Illinois, Iowa or California pathogen populations may have been exposed to cooler conditions than in Morocco or in a greenhouse. In the north-central region of the USA (Iowa, Illinois or Ohio), soils can remain frozen several months of the year, and it has been demonstrated that *C. michiganensis* subsp. *michiganensis* populations do not decrease greatly under these conditions (Basu, 1970). Similarly, in the present study in the temperate region of Buenos Aires – La Plata, the survival of the pathogen in host debris was affected by the crop season; the survival period and population size of the pathogen were shorter when decomposition started in summer than in winter (the mean and standard deviation temperatures were 24·1°C ± 0·2 and 11·9°C ± 0·4, respectively). In fact, the survival times of the pathogen in the summer greenhouse experiments were shorter than any previously published, suggesting that temperature could play an important role in determining the survival of this pathogen in its host debris. However, in the present study, the variation in survival times between different years cannot be explained by temperature, as the seasonal temperatures were similar each year.

False negatives in *Clavibacter* population counts can occur because of growth inhibition of the pathogen by saprophytes. Detection sensitivity of semiselective media depends on the concentrations of saprophytes and the pathogen, and saprophyte identity (Ftayeh *et al.*, 2011). An inhibitory effect by saprophytes was described by Fatmi & Schaad (2002) in Morocco, and recently by Zanón & Jordá (2008) and Ftayeh *et al.* (2011), who reported an antagonistic effect of some saprophyte isolates on *C. michiganensis* subsp. *michiganensis*. In the present investigation, there was a significant difference, of two orders of magnitude, between the concentration of the pathogen growing alone or with the debris suspension, and a trend of a higher concentration of saprophytes in plates where the pathogen was not isolated than on those with a positive result. Thus, the larger population size of saprophytes might have reduced detection sensitivity, increasing the chances of false negatives. However, it is difficult to determine whether this occurred and what the magnitude of this effect was. Nevertheless, there are indications that this growth inhibition effect might not have interfered with the evaluation of survival time of the pathogen in this study; the negative results obtained by the *in vivo* method corresponded with those *in vitro*, suggesting that the pathogen populations were either below a pathogenic threshold to cause disease or not present. In addition, the negative results obtained in evaluations from experiments of winter of 2011 and summer of 2011/12 were corroborated using MBCT, which is considered to be more sensitive than other media; for example, Ftayeh *et al.* (2011) isolated 8 CFU per agar plate of *C. michiganensis* subsp. *michiganensis* growing with 12 750 CFU of saprophytes on BCT, a medium that differs from MBCT only in the fungicides used. The high correlation of results obtained on the less and more sensitive media used in the present investigation might be related to the high sampling intensity: at least 30 colonies of each replicate suspected to be *C. michiganensis* subsp. *michiganensis* were isolated and tested in order to reduce the chances of false negatives. PCR techniques could be used to directly detect the pathogen in host debris, preventing false negatives caused by the presence of saprophytes. However, this might lead to false positives, because of the detection of nonviable cells, or false negatives, if the effect of PCR inhibitors were not successfully controlled (Munkvold, 2009).

As far as the authors are aware, all previously published investigations of *C. michiganensis* subsp. *michiganensis* survival on host debris refer to stems and leaves, despite the fact that the pathogen can also colonize roots (Xu *et al.*, 2012). In the present study, the pathogen was able to survive for 45–75 days in root tissues in winter under greenhouse conditions, similar to buried stems. It is surprising that in many cases no pathogen was initially detected in the roots, considering the high concentrations detected in stems or petioles. It is possible that, under natural conditions, the pathogen might not invade all adventitious roots, leading to high variability among the roots, and thus reducing the probability of detection. Nevertheless, when seedlings are transplanted to soil their roots become very close to those remaining from the previous crop, which could lead to a high transmission rate of the pathogen.

It has been suggested that the soil could be a source of inoculum of *C. michiganensis* subsp. *michiganensis*, where the pathogen could survive indefinitely (Fatmi & Schaad, 2002). Until the 1970s, the methods used to determine the survival of the pathogen in the soil were based on the evaluation of symptoms on tomato plants seeded or transplanted into soils that were thought to be infested; however, this did not guarantee the absence of other sources of inoculum. Later, based on pathogen isolation using semiselective culture media, it was determined that this bacterium could only survive in soil for short periods ranging from 12 to 40 days depending on the soil biological activity (Trevors & Finnen, 1990; Yogev *et al.*, 2009). Chang & Pataky (1992) recovered the pathogen from soil next to infested tomato debris 210 days after the samples were buried, but this soil was very close to host residues, and the bacterial concentration was smaller than in the tissues; hence, the authors inferred that the pathogen could be released from the infested debris. The same authors determined that the pathogen died very quickly in non-sterile soil. Thus, so far, there is no evidence to support the survival of *C. michiganensis* subsp. *michiganensis* in soil for long periods of time under natural conditions, without being in close association with its host debris.

In conclusion, the present study has shown that tomato debris can be an important source of primary inoculum in greenhouse production systems. *Clavibacter michiganensis* subsp. *michiganensis* can survive not only in aerial tissues but also in the roots. The longest period the pathogen could be detected in tomato debris in a greenhouse in winter, after a summer crop, was about two and a half months on buried plant residues and almost 9 months on plant material left on the soil surface. For spring crops, ended in summer, this survival time was shorter, lasting up to one and a half months in petioles and two and a half months in stems, both left on the soil surface. Based on these results, if tomatoes are to be grown in greenhouses affected by bacterial canker in an area where two crops per year are possible, it is advisable to plant in spring and rotate with non-susceptible crops in summer, when the pathogen can only survive a short time in host debris. On the other hand, if tomatoes are planted in summer, rotations should be longer: tomato should not be cultured for at least a year in the same place. In addition, sanitation measures, such as the removal of as much host residue as possible from the greenhouse, would contribute to a reduction of the source of inoculum.

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