Leandro de León
Instituto Canario de Investigaciones Agrarias (ICIA). Tenerife, Canary Islands, Spain

Felipe Siverio
Laboratorio de Sanidad Vegetal de la Consejería de Agricultura, Ganadería, Pesca y Alimentación del Gobierno de Canarias. Tenerife, Canary Islands, Spain

María M. López
Instituto Valenciano de Investigaciones Agrarias (IVIA). Moncada, Valencia, Spain

Ana Rodríguez
Instituto Canario de Investigaciones Agrarias (ICIA). Tenerife, Canary Islands, Spain, and Universidad de La Laguna, Tenerife, Canary Islands, Spain

Clavibacter michiganensis subsp. *michiganensis*, a Seedborne Tomato Pathogen: Healthy Seeds Are Still the Goal

Clavibacter michiganensis subsp. *michiganensis* (22), causal agent of tomato bacterial canker, is a seedborne pathogen and is considered one of the most destructive bacterial diseases of this crop. For this reason, in the European Union (EU) and in many other countries, *C. michiganensis* subsp. *michiganensis* is a quarantine pathogen (6). It was first reported by E. F. Smith (93) at the beginning of the twentieth century in Michigan (USA), and currently it is present worldwide. Its movement over long distances is facilitated by traded seeds, which explains its distribution throughout all of the tomato-growing regions of the world, but its spread differs widely among countries (75). However, it can also survive in plant debris and on volunteer plants or alternative hosts that can act as local sources of inoculum (45).

In general, this disease shows a typical start/stop pattern, with devastating outbreaks that cause major economic losses but appear unpredictably in time (95). According to Gleason et al. (45), the sporadic nature of bacterial canker helps to explain the slow pace of progress in improving the efficacy of management practices. Once the bacterium is present in a production area, changes in cultural practices are the only advised measures that can be adopted to reduce the risk of dissemination and of new outbreaks (44,45). Unfortunately, resistant or highly tolerant cultivars are still not commercially available, and assays focusing on the chemical control of *C. michiganensis* subsp. *michiganensis* are scarce and have shown variable results (25,45,48,96,104). Treatments with copper and streptomycin can reduce epiphytic populations and disease symptoms, and a synergistic effect has been observed when copper is combined with the fungicide mancozeb and other copper-chelating compounds such as 8-hydroxy-quinoline (25,48,104). Other control methods, such as biological control by use of antagonistic bacteria (5,13,32,99), compost (106), plant activators for induction of resistance (94,100,104), plant essential oils (21,56) or vegetable extracts (10), as well as the use of soil solarization (7), have been investigated, but at present they are still far from providing universally successful control of the disease in tomato crops.

In the current situation, the most efficient control of bacterial canker is prevention, based on the use of pathogen-free seeds. To this end, quarantine regulations have been adopted by different countries to guarantee that commercial seed lots are *C. michiganensis* subsp. *michiganensis*–free, or at least that the contamination is below an acceptable threshold. Phytosanitary regulations can have a profound impact on the international trade of seeds (72) and have forced seed companies to implement seed sanitation and health control. However, despite the efforts made by plant health authorities, researchers, seed industry, and plant production companies, many new outbreaks of bacterial canker have been recorded in the last decade, some of them affecting areas in which *C. michiganensis* subsp. *michiganensis* had not been reported previously.

Previous reviews regarding tomato bacterial canker were published by Strider in 1969 (95) and Gleason et al. in 1993 (45). Therefore, it is necessary to provide a current comprehensive review, and this article discusses the disease situation, integrating previous data with the most recent findings and new information available. Thus, the objectives of this article are: (i) to review the progress on tomato bacterial canker, the role of infected or infested seeds, and of local sources of inoculum in disease outbreaks, highlighting recent records; (ii) to provide an overview of plant health regulations, especially with respect to standard protocols for testing seeds for *C. michiganensis* subsp. *michiganensis*; and (iii) to update information regarding research innovations and future perspectives on new, useful tools for detecting seed contamination by *C. michiganensis* subsp. *michiganensis* that can aid in designing new strategies to improve its control.

Bacterial Canker Symptoms

Tomato plants affected by bacterial canker show a variety of symptoms (Table 1; Figs. 1 and 2), depending on cultivar susceptibility, conditions of tomato cultivation, time and type of infection (systemic or localized according to Gleason et al. [45]), and other factors. In systemic infections, the pathogen invades the vascular

Corresponding author: Ana Rodríguez, Departamento de Protección Vegetal. Instituto Canario de Investigaciones Agrarias (ICIA). Apdo. 60, 38200 La Laguna, Tenerife, Canary Islands, Spain;
E-mail: arguezp@icia.es; anarguez@ull.es

Current address of L. de León: Department of Molecular and Structural Biochemistry. North Carolina State University, Raleigh NC 27695-7622.

doi:10.1094/PDIS-02-11-0091

Table 1. Symptoms caused by *Clavibacter michiganensis* subsp. *michiganensis* in tomato

Infection	Symptoms	Mode of infection
Systemic	Wilt; leaves and leaflets wilt unilaterally; common at early stages (Fig. 1A and B)	Infected or infested seeds transmit disease to emerging seedlings
	Yellow-brown vascular discoloration (Fig. 1C)	Invasion of vascular tissues through seeds or wounds
	Cankers formed on stems and petioles (Fig. 1D)	
Localized	Marginal necrosis of leaflets which appear dried and curl upward (Fig. 2B)	Infection through broken trichomes or natural openings such as stomata and hydathodes
	"Bird's-eye spots" develop on fruit (Fig. 2C)	
	Small white blister-like spots on stems (Fig. 2A)	

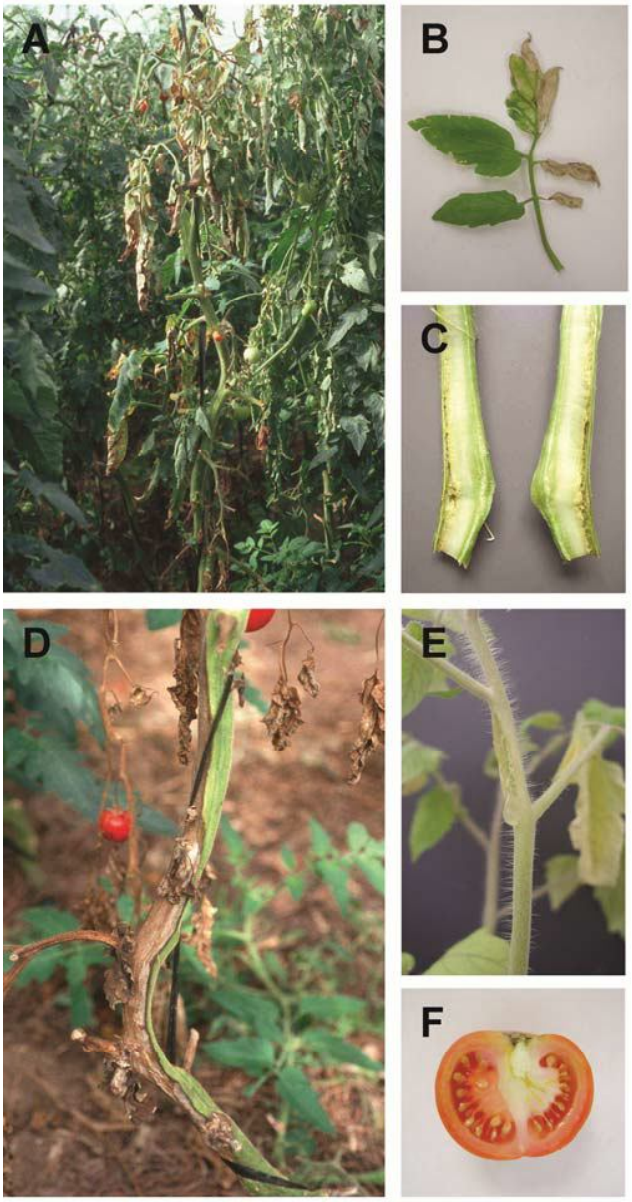

Fig. 1. Systemic infection on tomato plants by *Clavibacter michiganensis* subsp. *michiganensis*. **A,** Generalized wilting, **B,** unilateral leaflet wilt and necrosis, **C,** part of the vascular system invaded by the pathogen causing yellow-brown discoloration, **D,** cankers on stems in later stages of disease development, **E,** droplets of bacterial ooze observed when the stem splits open at the beginning of canker formation, **F,** pathogen reaching the fruit and infecting the seeds through the vascular tissues.

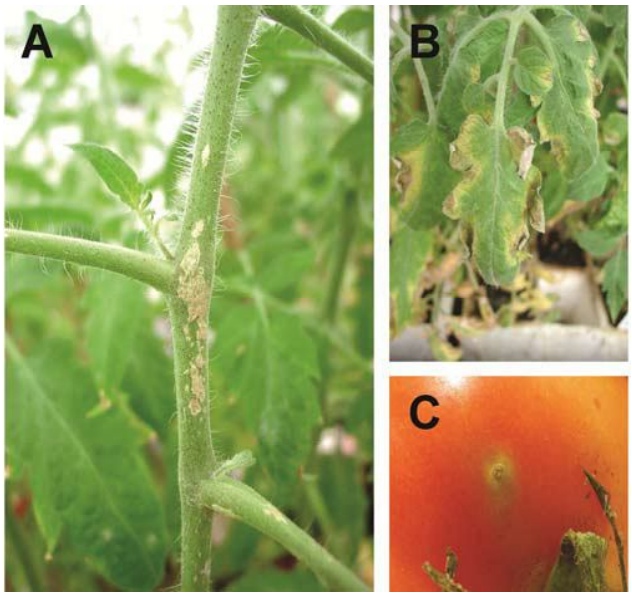

Fig. 2. Localized infection by *Clavibacter michiganensis* subsp. *michiganensis*. **A,** Small white blisterlike lesions in stem and petiole, **B,** marginal necrosis of leaflets, and **C,** typical spots with whitish halos on fruits, also called "bird's-eye spots".

tissues (Fig. 1C), and plants wilt (Fig. 1A); frequently the wilt is observed on one side of the leaves at early stages (Fig. 1B), and, less frequently, cankers appear on stems (Fig. 1D). In tomato plants inoculated under greenhouse conditions, the stem splits open, and a flow of droplets containing masses of bacteria can be observed (Fig. 1E) when the relative humidity is high. In fruit, *C. michiganensis* subsp. *michiganensis* can be transmitted to the developing seeds through the vascular tissues of the mother plant (Fig. 1F). After external infections, when the bacterium enters through broken trichomes or natural openings, leaves, flowers, and fruits can show localized infections (15,68). Marginal necrosis of leaflets (Fig. 2B) is the most frequent symptom during field epidemics. Blister lesions on stems (Fig. 2A) and fruit spots called "bird's eye" lesions (Fig. 2C), arising from local infections, are less frequently observed.

Disease Cycle

In the cycle of *C. michiganensis* subsp. *michiganensis* (Fig. 3), there are three environments in which the pathogen is present: (i) seeds, in which the bacteria survives inconspicuously; (ii) nurseries for the production of transplants; and (iii) tomato production areas (Fig. 4).

In seed production crops, *C. michiganensis* subsp. *michiganensis* can reach the fruit and consequently infest (externally) or infect

(internally) the seeds, where it is able to survive for long periods (14,52), allowing its long-distance dissemination and its introduction into tomato bacterial canker-free areas through traded seeds or tomato transplants produced from such seeds. It is also possible that, like many other bacteria, the pathogen could become quiescent or enter the viable but nonculturable state (VBNC) induced by dry conditions, lack of available nutrients, chemical treatments, or other conditions (83), but this topic has not yet been explored.

The amount of *C. michiganensis* subsp. *michiganensis* on naturally infected seeds can be variable, but it has been reported to be between 10^2 and 10^4 CFU/seed (33,47), and a population of 10^2 CFU/seed has been suggested as the probable threshold level for transmission of the pathogen from naturally infected seeds (53). Seed transmission of *C. michiganensis* subsp. *michiganensis* was followed by Chang et al. (17), who described incidence rates of systemic infection below 1% after transplanting seedlings grown from infected seeds containing 10^4 CFU/g seed. More recently, Hadas et al. (47) described from 0.05 to 4% incidence of bacterial canker in tomato seedlings grown from seed lots containing from 58 to 1,000 CFU/g seed, finding a high correlation between CFU/g seed and disease incidence. However, disease incidence does not depend solely on the inoculum concentration present in seeds because *C. michiganensis* subsp. *michiganensis* can be mechanically transmitted by cultural practices during transplant production, with a subsequent strong effect on disease incidence in the field.

In nurseries, the risk of disease transmission by clipping tomato seedlings in transplant beds is well documented (17,42). Seedling harvest practices and other factors also contribute to the spread of *C. michiganensis* subsp. *michiganensis,* particularly with sprinkler or fog irrigation (17). The importance of latently infected transplants has been observed in practice, and several methods have been evaluated for detecting this pathogen in symptomless transplants, pointing out that bacterial populations increase rapidly in stems after plant infection (42).

Grafting is a relatively recent innovation in tomato nursery production (61), requiring wounding of both rootstock and scion, providing a quick way for *C. michiganensis* subsp. *michiganensis* to spread from plant to plant. A study using bioluminescent mutants allowed the visualization of bacterial colonization dynamics in seed transmission and the translocation of *C. michiganensis* subsp. *michiganensis* in grafted plants (105). The results showed that bacteria were aggregated on hypocotyls and cotyledons and were then translocated in both directions from the graft union in grafted seedlings, in which either the rootstock or scion was exposed to contamination via grafting knives.

Once the seedlings are transplanted into the greenhouse or production field, the presence of a few systemically infected plants can result in high leaf epiphytic populations of *C. michiganensis* subsp. *michiganensis,* which might serve as inocula that cause secondary infections. The relative weight of contaminated seed as a

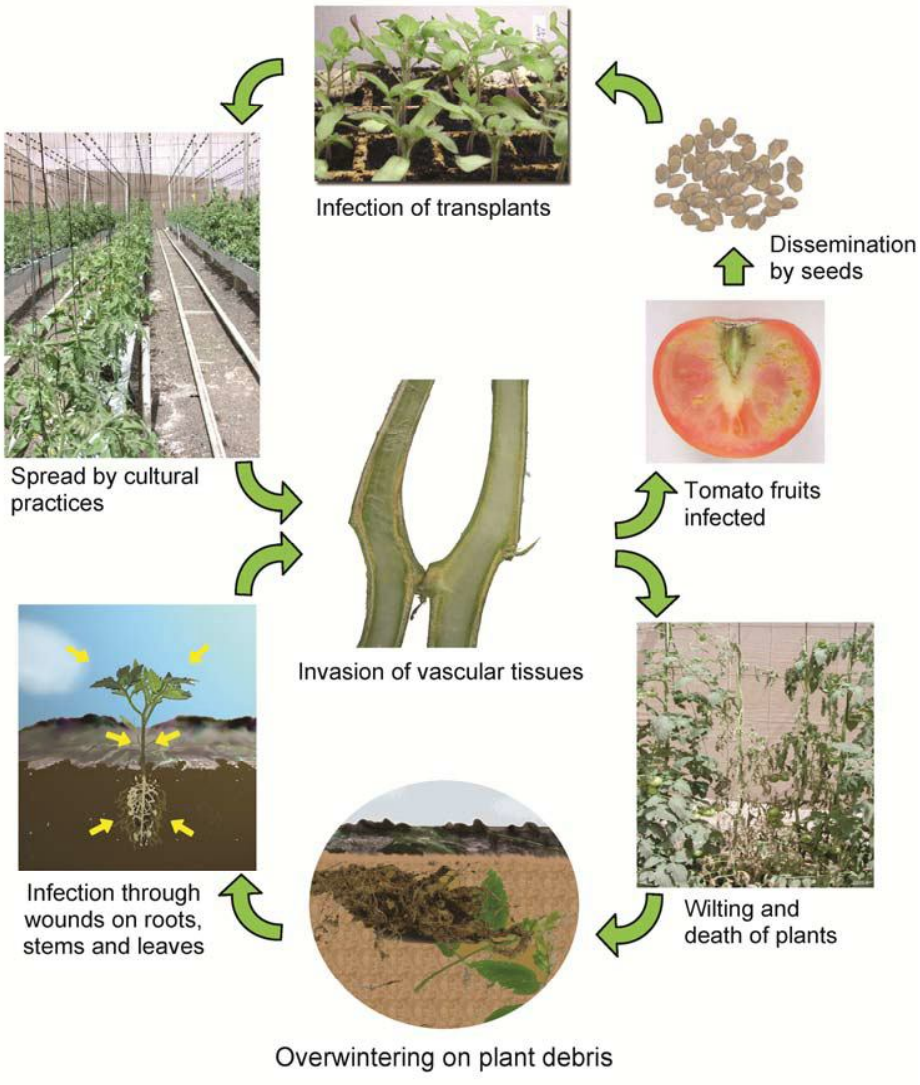

Fig. 3. Disease cycle of *Clavibacter michiganensis* subsp. *michiganensis*, adapted from Eichenlaub et al. (31).

source of inoculum depends upon the secondary spread of the pathogen, which is influenced greatly by cultural practices and environmental conditions (16,18). This fact can help to explain the apparent erratic behavior of bacterial canker outbreaks, but it has been assumed that seed contamination rates as low as 0.01% (one seed per 10,000) could be enough to initiate an epidemic of bacterial canker in production fields (17,42).

C. michiganensis subsp. *michiganensis* could also have an epiphytic phase on an alternative host, in the absence of tomato, which could serve as a continuous source of inoculum (15,18,44). Several solanaceous and nonsolanaceous species have been described as hosts (62,74,95,97), although for most of them only results of artificial inoculation have been reported, showing symptoms less severe than tomato plants. In addition, *C. michiganensis* subsp. *michiganensis* can persist from several months to more than one year when associated with tomato plant debris (18,30,34, 44,70), which could also serve as a local source of inoculum and initiate new outbreaks (44). However, as most of these reports were based only on data from isolation of *C. michiganensis* subsp. *michiganensis* onto different culture media, survival of the pathogen could be underestimated, because stressed, injured, or VBNC bacterial cells (83) could be unable to form visible colonies.

Molecular Epidemiology of Bacterial Canker

In the last decade, epidemiological studies using molecular typing of natural populations of *C. michiganensis* subsp. *michiganensis* have provided valuable information for understanding the roles of infected seeds and local sources of inoculum in recent bacterial canker outbreaks. Although not an exhaustive list, Table 2 compiles recent information from several countries to help understand the disease epidemiology. In Israel, where the presence of *C. michiganensis* subsp. *michiganensis* was known since 1963, a severe epidemic occurred in 2000 in the southern region, and since then, the disease has been found in most greenhouses in the same area. The inoculum might have originated from infected tomato seeds or seedlings, but the presence of two persistent, genetically different groups suggested that the primary inoculum originated each year from residual plant matter in the soil rather than from contaminated seeds (57).

The situation seems similar in Japan, where the pathogen was first reported in 1958 and where tomato bacterial canker has emerged in Okayama Prefecture in recent years. In this area, molecular analysis showed that bacterial isolates from individual greenhouses remained invariable from 2005 through 2008 (55).

In the Canary Islands (Spain), where *C. michiganensis* subsp. *michiganensis* was reported for the first time in 2002 and caused severe yield losses, the high homogeneity observed among bacterial strains isolated from 2002 to 2007 suggests a single introduction of the bacterium into the region through infected seeds in 2002, whereas plant debris could be the prime inoculum source in the sporadic appearance of the disease in following years (23).

Although plant debris can explain the persistence or even new outbreaks of bacterial canker in a region where the disease was already known to occur, the 13 reports of *C. michiganensis* subsp. *michiganensis* from 2000 to 2010 in different countries of Asia, Europe, and North and South America due to infected or infested seeds (Table 2) suggest that tomato seed sanitation remains an elusive goal in the control of bacterial canker.

Quarantine Regulations for *C. michiganensis* subsp. *michiganensis*

To prevent the introduction and dissemination of new or existing pathogens, governments have historically applied quarantine regulations in an effort to limit the import of plants or plant products

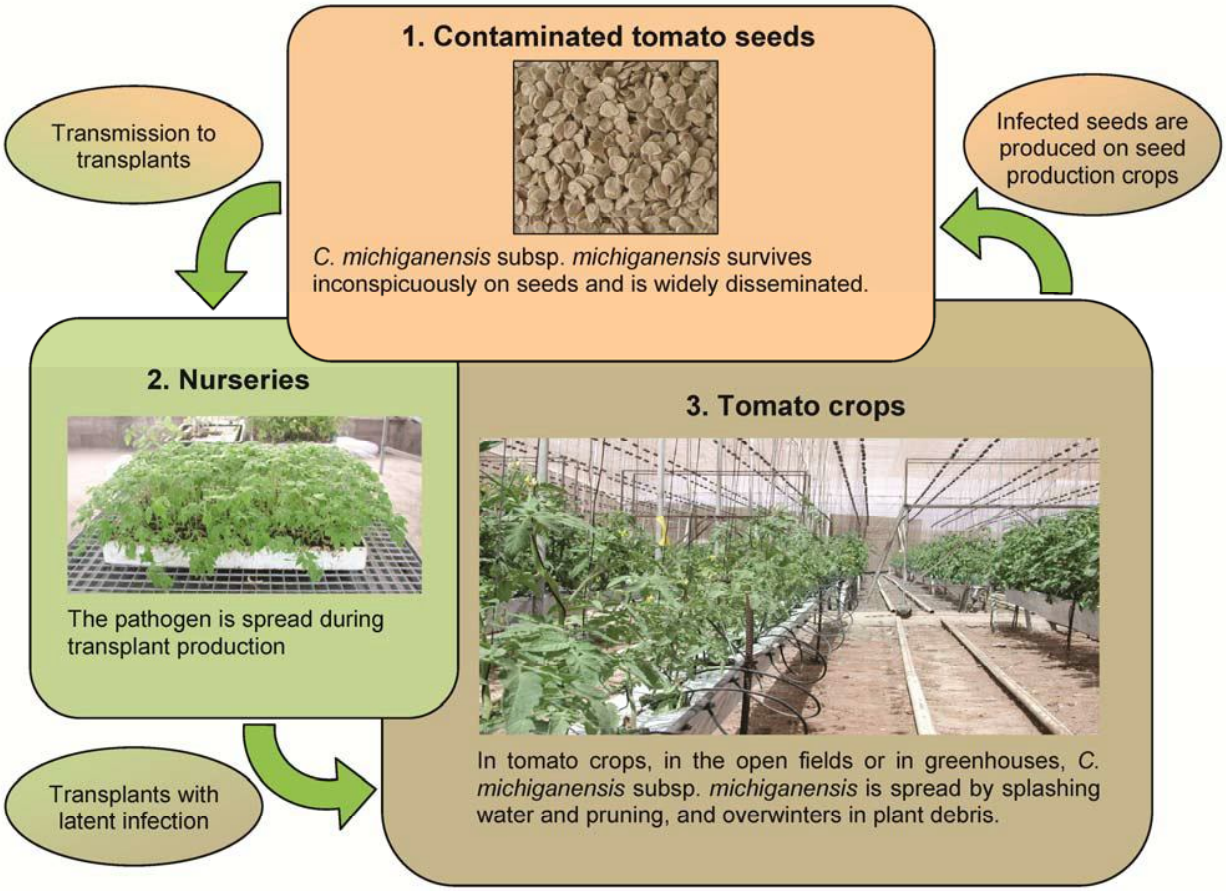

Fig. 4. Tomato bacterial canker feature and environments in which *Clavibacter michiganensis* subsp. *michiganensis* is present: 1, contaminated tomato seeds; 2, nurseries; and 3, tomato crops.

contaminated with pathogens. In this context, seedborne bacterial pathogens are of particular concern because successful strategies for the management of most bacterial diseases are not available, and use of clean seeds and/or transplants seems to be the most obvious measure for effective control (43). Thus phytosanitary regulations are needed to ensure that commodities are pathogen-free. In the past, different countries have developed their regulations independently, and the principles generally had little technical justification. To avoid confusing regulations and unjustified trade barriers, international organizations, including the Food and Agricultural Organization (FAO) of the United Nations that supports the International Plant Protection Convention (IPPC), and the Regional Plant Protection Organizations (RPPOs), have provided standards for principles and procedures that should guide the development of plant health measures. RPPOs coordinate and harmonize the phytosanitary actions of their member countries on a regional basis and also advise them about the pathogens that should be considered quarantine organisms, dividing them into A1 quarantine pests (those absent from the region) and A2 quarantine pests (those present in some parts of the region) (51). Today, *C. michiganensis* subsp. *michiganensis* is included in the A2 list by the European Plant Protection Organization (EPPO), the Asia and Pacific Plant Protection Commission (APPPC), the Caribbean Plant Protection Commission (CPPC), and the Inter-African Phytosanitary Council (IAPSC) (75).

The EU has also included *C. michiganensis* subsp. *michiganensis* in the list of quarantine organisms for all member states (6). The current plant health regulations of the EU indicate that tomato transplants originating in third countries require a phytosanitary certificate to be introduced into the EU. Transplants produced within the EU are subject to control during their production and are accompanied by a plant passport that indicates that the material has successfully undergone the national checking system and ensures their free movement throughout the EU. However, there is no official detection protocol for all EU countries, and in most of them, the passports are based on visual inspections. In addition, the introduction and movement of tomato seeds into and within all member states requires an official statement that the seeds have been obtained by an appropriate acid extraction or equivalent method and that either (i) they originated in areas where bacterial canker is not known to occur, (ii) the plants were free from bacterial canker symptoms at the place of production during their entire growth cycle, or (iii) the seeds were certified free from *C. michiganensis* subsp. *michiganensis* after official testing on a representative sample using appropriate methods (6). In this case, the problem is related to the availability of true "appropriate methods" of sufficient sensitivity to detect low levels of seed contamination.

Tomato Seed Production and Sanitation

The first, and perhaps only, opportunity to fully control bacterial canker is to avoid its presence in the seed production fields. Unfortunately, *C. michiganensis* subsp. *michiganensis* is broadly disseminated, and there are difficulties in finding pathogen-free areas for production of *C. michiganensis* subsp. *michiganensis*–free seeds. The dry climate of western states of the United States was thought to limit the development of bacterial diseases in seed production crops, but it has been shown that seeds produced in these areas may be latently infested with seedborne phytopathogenic bacteria, even in the absence of symptoms in seed crops (43). In addition, the seed industry has moved its seed production fields to developing countries to take advantage of low labor costs. This outsourcing is considered to be a risk for the re-emergence of seedborne diseases and for the introduction of diseases into new areas (43,45). In Europe, notifications to the EPPO, during the last decade, of noncompliance owing to the detection of *C. michiganensis* subsp. *michiganensis* in tomato seeds has given rise to concern regarding lots originating mainly from Southeast Asia (India, China, Thailand, and Taiwan) and, to a lesser extent, from South America (Bolivia and Brazil) (EPPO reporting service: http://www.eppo.org/PUBLICATIONS/reporting/reporting_service.htm). However, it is not always easy to obtain detailed information from seed companies about the location of their different seed-producing areas, which hinders the appropriate monitoring of the seed lots.

Methods for seed extraction and chemical or thermal seed treatments have been investigated to provide an effective procedure to obtain seeds free of this pathogen. Some methods are effective for reducing *C. michiganensis* subsp. *michiganensis* populations, but there is no available method that ensures the complete eradication of this pathogen from naturally infected seeds without reducing seed germination. Fermentation used to extract tomato seeds from fruit pulp reduces populations of *C. michiganensis* subsp. *michiganensis*, but relatively long fermentation periods, up to 96 h, are necessary to eradicate the pathogen (28,88,95). Acid extraction of seeds, or chemical treatments of dry seeds mainly with HCl at different concentrations and exposure times, greatly reduces the pathogen populations of tomato seeds (18,35,88,92,98) and disease incidence in seedlings (28). However, both fermentation and HCl treatments can reduce seed germination (28,35,92,95). Most of the published studies on seed disinfection were performed more than 20 years ago, and unfortunately no comprehensive systematic studies for comparative purposes are currently available. It is likely that

Table 2. Worldwide bacterial canker reports since 2000

Year	Country	Observations	Reference
2000	Chile	High incidence in transplant production for exportation attributed to cultural practices and infected seeds	102
2000	Israel	Severe epidemic in southern region; since then primary inoculum each year originated from plant debris	57
2001	Turkey	First report in eastern Anatolia region causing heavy losses, attributed to contaminated seeds and/or transplants	89
2002	Spain	First report in the Canary Islands causing severe losses, attributed to infected seeds	23
2002	Indonesia	First report in Indonesia, pathogen isolated from commercial seeds produced in Java	8
2005	Cyprus	Severe wilt on tomato crops	86
2005-06	Mexico	Incidence of 4-46% in San Quentin and San Simón area, Baja California Península of Mexico, and first report in Sonora	12, 49
2007	Korea	First report in Korea causing severe wilt in greenhouses in Cheorwon and Iksan provinces	73
2005-08	Japan	Emergence in greenhouses in Okayama prefecture, primary inoculum each year originated from plant debris	55
2007	Syria	First report in Syria, affecting up to 7% of surveyed greenhouses in Latakia and Tartous, incidence up to 70% at the end of July	37, 38
2007	The Netherlands	Two outbreaks in tomato plant propagation companies, most likely related to contaminated seeds	76, 77
2007-09	Austria	Isolated outbreaks, pathogen detected in seeds locally produced	78, 79, 80
2010	Italy	Severe outbreaks in Viterbo and Puglia regions, attributed to infected seeds	63, 81

seed companies have much more information for their own use, but reports of new disease outbreaks suggest that the methods they use are still imperfect.

Tomato Seed Testing

Seed testing is an essential tool for the control of this seedborne pathogen, through phytosanitary certification and quarantine programs in domestic and international seed trade (43,67,71,72). In the case of *C. michiganensis* subsp. *michiganensis,* infected tomato seeds are visually indistinguishable from healthy seeds (18,42), and therefore, seed testing is necessary. However, only by the impractical task of testing all of the seeds by a currently nonexistent protocol with 100% accuracy can a seed lot be guaranteed to be pathogen-free (67).

Commercially, seed tests are performed on samples taken from seed lots. The first matter is whether the samples are representative of the lot as a whole, but there are practical limitations in determining this information. First, as the seed lot size increases, so does the difficulty of obtaining a truly representative sample, and tomato lot sizes may range from less than a kilogram to several thousand kilograms. In addition, seed lots should be composed of seeds harvested from a single seed production field or site, but in practice, they may be composed of seeds from different lots that share acceptable quality traits but in which the distribution and frequency of infected seeds is far from uniform. However, practical experience with small-seeded crops suggests that the lack of seed lot uniformity has seldom limited the accuracy of seed health test results, as long as the sampling was done carefully and consistently (71). Seed companies use indirect methods, in which samples with a certain number of seeds are randomly taken and extracted and the extracts are tested for the pathogen. Two elements dictate the result: whether the sample contains any infected seed, and whether the assay technique or techniques will detect such contamination (41,71). In this context, both sample size and protocol sensitivity and selectivity are important (43). Tests should be able to detect the targeted seedborne pathogen at the level known as the inoculum threshold, which can be defined as the amount of seed infection or infestation that will cause a disease in the field under a conductive environment and lead to economic losses (67). As it has been pointed out previously, available information indicates that at least one infested seed in 10,000 can lead to an epidemic under favorable conditions (17,42). According to the experience of several laboratories in the EU (82), this sensitivity is not always achieved by the standard protocols, which are based on only one or a few techniques, as discussed below.

During the past decade, several organizations have made a systematic effort to agree on standardized seed health protocols that will be accepted internationally (72). For *C. michiganensis* subsp. *michiganensis*, recent standard seed test protocols are available from the EPPO (74; a new version is currently under preparation) and the International Seed Federation (ISF) through the International Seed Health Initiative for Vegetable Crops (ISHI-Veg) (50). Currently, the ISHI-Veg protocol for *C. michiganensis* subsp. *michiganensis* is approved as a temporary standard by the National Seed Health System (NSHS) of the United States (http://www.seedhealth.org/files/xls/NSHSMethodCodes.xls). Both protocols are based on the plating of seed extracts on semi-selective media and adopt the standard sample size recommended by the International Seed Testing Association (ISTA) of 10,000 seeds, which statistically implies a 95% probability of detecting a 0.03% level of contamination in the seed lot (74). The seeds can be analyzed in subsamples of 2,000 seeds. The recommended method for obtaining the seed extracts is soaking the seeds and blending them with a Stomacher Laboratory Blender (Seward, London, UK) or similar apparatus. There is evidence that extraction methods that include grinding the seeds are significantly better than methods that use only soaking (33,47), probably because the need for recovery of both external and internal contamination. Previous treatments of seeds with fungicides or other chemicals can affect the accuracy of the analysis, and there are not always ways to avoid their negative influence. A nondestructive tomato seed assay, extracting the bacteria from seed fiber, was developed by Biggerstaff et al. (11), although this method has not yet been included in standard protocols.

Standard Protocols for Seed Testing Based on Plating on Semi-selective Media

Following the standard protocols (50,74), once seed extracts are obtained, they should be plated on semi-selective media such as SCM (33), mSCM (a modification of SCM medium [103]), D2ANX (20), KBTS (27), or CNS medium (46). Currently, new media for *C. michiganensis* subsp. *michiganensis* isolation are under development (58). Isolation is a robust technique, cheap and easy to perform, does not need specialized equipment, and can isolate living pathogenic bacteria. However, isolation of *C. michiganensis* subsp. *michiganensis* from seed samples is often difficult because this bacterium grows slowly and the colonies are frequently inhibited by other microorganisms, even in the available semi-selective media. Following the EPPO protocol, seed extracts can also be tested by immunofluorescence (IF) or by polymerase

Table 3. Sensitivity of isolation and serological techniques described for detection of *Clavibacter michiganensis* subsp. *michiganensis*

Method	Culture media / antibodies[a]	Sensitivity reported	Reference
Isolation[b]	SCM	1 contaminated seed[c]/10,000 seeds	33
	KBTS-based medium	1 contaminated seed[d]/10,000 seeds	74
	mSCM; D2ANX; CNS	1 contaminated seed[e]/5,000 seeds	47
	mSCM; D2ANX; mCNS	1 contaminated seed[f]/5,000 seeds	69
	mSCM	10-10^2 CFU/ml seed extract	24
	SCM; D2ANX	1-10 CFU/ml seed extract	82
IMS-plating[g]	YPGA / PAbs from PRI	1-10 CFU/ml seed extract	24
IF	PAbs from PRI	10^3 CFU/ml seed extract	24
	PAbs from PRI	10-10^2 CFU/ml seed extract	82
	PAbs from Neogen	10^3-10^4 CFU/ml pure culture	59
ELISA	PAbs obtained by authors	1 contaminated seed/50 seeds	60
	PAbs from Agdia	10^4-10^5 CFU/ml seed extract	24
	PAbs from Neogen	10^5-10^6 CFU/ml pure culture	59

[a] PAbs: polyclonal antibodies. PRI: Plant Research International.
[b] Isolation by plating seed extracts on semi-selective medium.
[c] At minimum contamination level of 50 CFU.
[d] At minimum contamination level of 8×10^2 CFU.
[e] Contamination rate of 10^2-10^3 CFU/seed.
[f] Contamination rate of 10^8 CFU/seed.
[g] Immunomagnetic separation followed by plating in nonselective YPGA (yeast-peptone-glucose agar) medium.

chain reaction (PCR) after enrichment in liquid semi-selective media (enrichment-PCR) (74). However, these two tests are considered only presumptive, and seed extracts that tested negative by plating on semi-selective media but positive by IF or enrichment-PCR should be subjected to bioassay in tomato plantlets, trying to multiply the target bacteria *in planta*. According to EPPO (74), the identity of putative *C. michiganensis* subsp. *michiganensis* isolates should then be confirmed by a pathogenicity test and a biochemical, serological, or PCR test. Confirmation of suspected colonies by pathogenicity assay is also required by the ISHS-Veg protocol (50). If no typical colonies can be isolated from seeds by selective plating or after tomato bioassay, the detection is finally considered negative because the presence of the pathogen is not confirmed (74).

The sensitivity level described by different authors for detection of *C. michiganensis* subsp. *michiganensis* by plating on semi-selective media is shown in Table 3. It has been estimated that plating on SCM can detect one contaminated seed containing as few as 50 CFU in samples of 10,000 seeds (33). Similarly, according to information from the EPPO, one contaminated seed (8×10^2 CFU) added to a sample of 10,000 seeds was consistently detected by plating seed extracts on KBTS-based medium (74). However, in routine analyses, seed-testing laboratories and industrial seed-screening programs have not observed such high sensitivity, probably because of the effect of extraction procedures, variation in the levels of seed contamination (47), and/or the presence of large numbers of nontarget bacteria that can also grow in these media (36). The detection threshold observed by Hadas et al. (47) by agar plating was one infected seed in 5,000, with an infection rate of 10^2 to 10^3 CFU per seed, but they were unable to detect one infected seed in a sample of 10,000, even with a low level of saprophytic bacteria in the lots (47). Detection of 1 to 10^2 CFU/ml seed extract can be achieved in clean seed lots in which the growth of the saprophytic microbiota was inhibited (24,82). Detection was less reliable in seed lots with $>10^5$ CFU background population (74), and results obtained by De León et al. (24) are in agreement with that conclusion. Furthermore, a recent EU cooperative study showed that none of the eight laboratories that took part in the study was capable of isolating *C. michiganensis* subsp. *michiganensis* from naturally infected seeds using SCM or D2ANX media (82). Both seed microbiota and the possibility that a non-growing state of *C. michiganensis* subsp. *michiganensis* could occur were pointed out as hypotheses to explain this fact. Kaneshiro and Alvarez (52) suggested that quiescent cells in stored seed could show an increased susceptibility to antibiotics, leading to an underestimation of viable pathogen populations.

Serological Methods

The most frequently used serological test for the detection of *C. michiganensis* subsp. *michiganensis* in the EU is still immunofluorescence (IF) with commercially available polyclonal antibodies (PAbs), for example, from Loewe (http://www.loewe-info.com), Neogen Europe-Adgen Phytodiagnostics (http://www.neogeneurope.com), or Plant Research International (http://www.pri.wur.nl/UK). Particularly in France, IF is commonly used to assay tomato seeds for *C. michiganensis* subsp. *michiganensis* (1,43), although positive IF results are obtained for both viable and nonviable bacteria. The conventional detection limit of this technique is known to be approximately 10^3 cells/ml, and results obtained by De León et al. (24) and Kokosková et al. (59) are in agreement with this threshold (Table 3). Olivier et al. (82) improved the IF detection limit up to 10 to 10^2 cells/ml by soaking the samples for 3 days at room temperature because the bacteria could multiply during soaking. In an EU inter-laboratory test, IF showed better sensitivity in the analysis of naturally infected seeds than did plating on semi-selective media, although plating was 10-fold more sensitive than IF in experimentally contaminated seed extracts (82) (Table 3). The IF technique has been widely criticized for showing too many false positives when using PAbs, due to cross-reactions (36,59,82). Seed extract preparation by soaking seeds instead of grinding or Stomacher blending can reduce this problem and is the recommended extraction procedure (36,74).

Monoclonal antibodies (MAbs) have shown higher titer and specificity against *C. michiganensis* subsp. *michiganensis* than PAbs (3). In particular, the MAb Cmm1 showed high specificity and near universality because of the presence of the reactive antigen among geographically diverse *C. michiganensis* subsp. *michiganensis* strains (54). It is available in commercial kits for enzyme-linked immunosorbent assay (ELISA) and immunostrip tests from Agdia (http://www.agdia.com) and was also successfully integrated into IF procedures (54) and indirect immunofluorescence colony staining (IFC) (101). However, according to Olivier et al. (82), commercial MAbs from Agdia were unable to detect *C. michiganensis* subsp. *michiganensis* strains by IF at usable titer. The detection limit for ELISA was 10^4 to 10^5 CFU/ml (24,59) (Table 3). For immunostrip (Agdia), the level of sensitivity was around 10^5 to 10^6 CFU/ml, and agglutination tests (Neogen Europe-Adgen Phytodiagnostics) require even more bacterial cells, making these methods useful as confirmatory tools for the identification of colonies showing *C. michiganensis* subsp. *michiganensis*–like morphology or as a field test with extracts of typical symptomatic plants, but not for seed testing.

Other serological techniques able to detect viable bacteria, such IFC (101) and a filtration and colony blot immunoassay (4), were developed for the detection of *C. michiganensis* subsp. *michiganensis* in tomato seeds but are not currently routinely used. Flow cytometry has also been applied for the detection of the pathogen in seed extracts (2) and can be used in conjunction with appropriate fluorescence probes (19) to assess intracellular pH in *C. michiganensis* subsp. *michiganensis* cells as an indicator of the viability of the target cells.

Combination of Isolation and Serological Techniques

Immunomagnetic separation (IMS) permits the specific serological capture of target cells, allowing the removal of nontarget bacteria, seed debris and inhibitory compounds from seed extracts as well as the subsequent concentration of target cells. For *C. michiganensis* subsp. *michiganensis*, IMS was applied to seed extracts prior to plating in nonselective medium, in which colonies of the pathogen appear in fewer days than on semi-selective media (26). IMS-plating has provided better sensitivity results than direct plating onto mSCM medium, IF, or PCR (24), showing a detection threshold of 1 to 10 CFU/ml seed extract (Table 3). This technique has isolated the pathogen from IF-positive samples of naturally infected seeds for which semi-selective media and Bio-PCR screen had failed (82). Consequently, it is an interesting alternative for accurate, sensitive, and rapid seed detection of *C. michiganensis* subsp. *michiganensis*, allowing confirmation of the pathogenicity or other strain characteristics.

Molecular Methods

In the last two decades, PCR has provided promising diagnostic tools because of the high sensitivity and specificity (85). Despite the advances made in PCR protocols, their implementation for routine analyses has been slow, especially in commercial seed testing programs. PCR-based methods require specialized equipment and better-trained personnel, and maybe for these reasons, some laboratories still prefer conventional techniques (1,43,72). However, in recent years, PCR-based techniques have been implemented successfully, and there is currently no reason for not introducing them in integrated protocols, for routine analysis.

For PCR tests, several pairs of primers (Table 4) have been specifically designed for *C. michiganensis* subsp. *michiganensis*, most of them based on the 16S-23S rDNA intergenic region. According to the EPPO protocol (74), identification of presumptive *C. michiganensis* subsp. *michiganensis* isolates can be achieved using two pairs of primers: CMM5/CMM6, developed by Dreier et al. (29); and PSA-4/PSA-R, developed by Pastrik and Rainey (87). Other primer pairs, such as CM3/CM4 (90) and Cmm1F/Cmm1R (59), have also been developed specifically for *C. michiganensis* subsp.

michiganensis. However, false negatives have been reported with primers CMM5/CMM6, which reacted with only 75% of tested *C. michiganensis* subsp. *michiganensis* strains (65). Similarly, Hadas et al. (47) found that four out of 23 *C. michiganensis* subsp. *michiganensis* strains assessed did not react with the primers CM3/CM4 and that two did not react with the primers CMM5/CMM6. It is therefore advisable to use more than one set of primers to obtain more reliable PCR results.

Although PCR is theoretically able to detect DNA from a single cell, the small volume (1 to 5 µl) of sample used as template in each reaction, the presence of inhibitors in seed extracts, and other unknown factors make the indicated protocols no more sensitive than other techniques (43). Thus, in practice, the detection limit of *C. michiganensis* subsp. *michiganensis* by PCR is about 10^3 CFU/ml (24,59); but more concerning is the frequency of false negatives due to the presence of PCR inhibitors and its ability to detect DNA from nonviable cells (43,72).

Several strategies have been developed to overcome these disadvantages and increase the sensitivity of PCR-based methods shown in Table 5. The PCR inhibitors present in seed extracts can be removed by different DNA extraction protocols, facilitating *C. michiganensis* subsp. *michiganensis* detection by conventional PCR (24,29,69). Enrichment of seed extracts on semi-selective liquid media followed by PCR (enrichment-PCR) has been included in the EPPO protocol and has consistently detected one contaminated seed (4×10^2 CFU) added to a sample of 10,000 seeds (74). Similar sensitivity was reported in tomato plant homogenates, using DNA extraction and combining PCR and Southern hybridization (29), but was not confirmed by Milijasevic et al. (69). Another possibility of enrichment, called Bio-PCR, that involves previous multiplication of the putative pathogen on solid media and subsequent PCR amplification (43,72), was able to detect one seed contaminated in 10,000, with an infection rate of 10^2 to 10^3 CFU/seed, and showed higher sensitivity than plating on semi-selective media or direct PCR according to Hadas et al. (47) (Table 5). The results reported by Milijasevic et al. (69) and Olivier et al. (82) are in agreement with this efficiency. In all of these enrichment protocols, the amplification of DNA from living cells can be assumed if PCR is negative before enrichment and positive after it.

Real-time PCR enables the detection and quantification of pathogens without post-PCR processing, providing data in real-time (91), and is becoming the gold standard for the diagnosis of plant pathogens (85). Its advantages over conventional PCR warrant its use for seed health testing (72) because the developed protocols have, in general, higher sensitivity and specificity than conventional PCR, especially those using TaqMan probes. These protocols developed for *C. michiganensis* subsp. *michiganensis* (9,66,107) are also shown in Table 4. The detection threshold reported for real-time PCR in pure cultures of *C. michiganensis* subsp. *michiganensis* was 10^3 CFU/ml (66,107), but data about its application in seed testing are still lacking. Only Zhao et al. (107) have reported the successful detection of *C. michiganensis* subsp. *michiganensis* by real-time PCR in samples prepared by adding 10 infected seeds to 1,000 healthy seeds (Table 5), but this sensitivity is not high enough for routine seed testing. In addition, Luo et al. (66) used DNA-binding ethidium monoazide (EMA) in combination with a real-time PCR assay for quantification of viable cells, which can be discriminated from dead cells because EMA selectively penetrates dead bacteria and binds to DNA, inhibiting amplification of the target sequence. Other variants of PCR recently developed for other plant pathogens, such as IMS-PCR, co-operational PCR (Co-PCR), or loop-mediated isothermal amplification (LAMP) (85), have not yet been developed for *C. michiganensis* subsp. *michiganensis*.

Multiplex PCR allows the amplification of more than one target region in one PCR mixture, detecting several pathogens or several targets of the same pathogen in a single test by conventional or real-time PCR. In the future, seed health tests should be based on multiplex real-time PCR assays able to detect all of the seedborne pathogens relevant for phytosanitary purposes. A first approximation for the simultaneous detection of *C. michiganensis* subsp. *michiganensis* together with other seedborne tomato bacteria such as *Pseudomonas syringae* pv. *tomato* and *Xanthomonas* spp. was carried out by the development of a multiplex PCR using pure cultures and previously published primers (84), but the protocol was not assayed with seeds.

Closing Remarks and Perspectives

Successful control of tomato bacterial canker remains a serious problem for tomato cultivation worldwide because new outbreaks and first reports of its causal agent are still periodically noticed in different regions. There are probably many other unpublished results on all continents and new outbreaks that can be masked in regions that were previously affected. The current losses due to this pathogen have not been accurately evaluated, but although variable among years, may be very high at the global level.

Current knowledge supports the belief that the use of pathogen-free seeds is of prime importance for bacterial canker control. The bottom lines to achieve this objective are: (i) pathogen-free seed production fields; (ii) seed sanitation; and (iii) seed health testing. Phytosanitary measures should address these goals in a global mode in order to control tomato seed production with suitable procedures at all the process stages. Careful inspections of fields and analyses prior to their selection for seed production are required, mainly in developing countries in which available information about the presence and degree of spread of *C. michiganensis*

Table 4. Polymerase chain reaction (PCR) primers and TaqMan probes designed for *Clavibacter michiganensis* subsp. *michiganensis*

PCR	Primers probe	Sequence 5′ - 3′	Target DNA	Reference
Conventional	CMM5	GCGAATAAGCCCATATCAA	pCM2 plasmid gen pat-1	29
	CMM6	CGTCAGGAGGTCGCTAATA		
	PSA-4	TCATTGGTCAATTCTGTCTCCC	16S–23S rDNA intergenic region	87
	PSA-R	TACTGAGATGTTTCACTTCCCC		
	CM3	CCTCGTGAGTGCCGGGAACGTATCC	Chromosomal DNA	90
	CM4	CCACGGTGGTTGATGCTCGCGAGAT		
	Cmm1F	GACAAGCACCTCTACACCTGG	Cmm tomatinase gen	59
	Cmm1R	TTGATCCCTGACTTCAGCGT		
Real-time	FP Cm	TGTCGAGGGCATGTTGCACG	16S–23S rDNA intergenic region	9
	RP Cm	GGAGACAGAATTGACCAATGAT		
	Cmm probe[a]	TTCCGTCGTCCTGTTGTGGATG		
	ITSYG-1	CGCGTCAGGCGTCTGTT	16S–23S rDNA intergenic region	107
	ITSYG-2	AGTGGACGCGAGCATC		
	Cmm probe[a]	TGGCGGTGGCGCTCATGG		
	Spm4f	TCAGGCGTCTGTTCTGGC	16S–23S rDNA intergenic region	66
	Spm2r	CCCACCACCATCCACAAC		
	Cmm probe[a]	CCTTCTGGGTGTGTCTGGTTTC		

[a] 5′ end labeled with FAM and 3′ end labeled with TAMRA (TaqMan probe).

subsp. *michiganensis* could be insufficient. Moreover, strict sanitary measures must be maintained in seed production fields to minimize the risk of pathogen introduction.

Research on seed extraction and sanitation procedures is still needed because at the moment, a safe method that ensures the eradication of *C. michiganensis* subsp. *michiganensis* from naturally infected seeds without reducing seed germination is not available. Pathogen populations can be reduced greatly but not eradicated entirely (28,35,88,92,98), making the detection of remaining viable bacteria more difficult. Moreover, seed treatments could also induce the appearance of injured or VBNC bacteria, preventing their growth on semi-selective media on which standard seed testing protocols for *C. michiganensis* subsp. *michiganensis* are based. Therefore, the influence on pathogen viability of seed treatments currently used by the seed industry also requires further investigation.

Standard protocols for detecting *C. michiganensis* subsp. *michiganensis* in tomato seeds are based on pathogen isolation and confirmation of pathogenicity (50,74), but true progress to improve pathogen isolation from seeds has been scarce in the last two decades (26). Research on nonisolation methods, serological and mainly PCR-based techniques, has intensified in recent years (9,47,54,59,66,82,84,107), but in many cases they are only used as presumptive tests because of their inability to confirm the presence of viable and pathogenic cells required for a positive diagnosis (74). However, there is a risk in using a commercial seed lot in which the pathogen has been detected by different techniques even though viable bacteria cannot be isolated. Accordingly, it seems necessary to improve and review the test schemes for diagnosis and detection of *C. michiganensis* subsp. *michiganensis* in seeds.

The recent advances in *C. michiganensis* subsp. *michiganensis* genomics (39,40) and proteomics, together with microarray possibilities, could lead to the discovery of new targets for detection and diagnosis and, hopefully, innovative methods (64). Furthermore, new data should be generated to provide a more complete picture of the life cycle of this tomato pathogen to help in developing more appropriate sampling and integrated methodologies for seed analysis.

Literature Cited

1. Alvarez, A. M. 2004. Integrated approaches for detection of plant pathogenic bacteria and diagnosis bacterial diseases. Annu. Rev. Phytopathol. 42:339-366.
2. Alvarez, A. M., and Adams, P. D. 1999. Flow cytometry: A promising tool for seed health testing. Pages 93-97 in: Proc. 3rd Int. Seed Health Sympos. International Seed Testing Association.
3. Alvarez, A. M., Derie, M., Benedict, A., and Gabrielson, R. 1993. Characteristics of a monoclonal antibody to *Clavibacter michiganensis* subsp. *michiganensis*. (Abstr.) Phytopathology 83:1405.
4. Alvarez, A. M., Kaneshiro, W. S., and Vine, B. G. 2005. Diversity of *Clavibacter michiganensis* subsp. *michiganensis* populations in tomato seed: What is the significance? Acta Hortic. 695:205-214.
5. Amkraz, N., Boudyach, E. H., Boubaker, H., Bouizgarne, B., and Ben Aoumar, A. A. 2010. Screening for fluorescent pseudomonades, isolated from the rhizosphere of tomato, for antagonistic activity toward *Clavibacter michiganensis* subsp. *michiganensis*. World J. Microbiol. Biotechnol. 26:1059-1065.
6. Anonymous. 2000. Council Directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. Office for Official Publications of the European Communities. Consolidated Text, pages 1-159. http://eur-lex.europa.eu/LexUriServ/site/en/consleg/2000/L/02000L0029-20060414-en.pdf
7. Antoniou, P. P., Tjamos, E. C., and Panagopoulos, C. G. 1995. Use of soil solarization for controlling bacterial canker of tomato in plastic houses in Greece. Plant Pathol. 44:438-447.
8. Anwar, A., van der Zouwen, P. S., Ilyas, S., and van der Wolf, J. M. 2004. Bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*) of tomato in commercial seed produced in Indonesia. Plant Dis. 88:680.
9. Bach, H. J., Jessen, I., Schloter, M., and Munch, J. C. 2003. A TaqMan-PCR protocol for quantification and differentiation of the phytopathogenic *Clavibacter michiganensis* subspecies. J. Microbiol. Methods 52:85-91.
10. Balestra, G. M., Heydari, A., Ceccarelli, D., Ovidi, E., and Quattrucci, A. 2009. Antibacterial effect of *Allium sativum* and *Ficus carica* extracts on tomato bacterial pathogens. Crop Prot. 28:807-811.
11. Biggerstaff, C. M., Gleason, M. L., and Braun, E. J. 2000. Refinement of a nondestructive tomato seed assay for *Clavibacter michiganensis* subsp. *michiganensis* using seed fiber. Seed Sci. Technol. 28:261-269.
12. Borboa Flores, J., Rueda Puente, E. O., Acedo Félix, E., Ponce, J. F., Cruz, M., Grimaldo Juárez, O., and García Ortega, A. M. 2009. Detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato of the state of Sonora, Mexico. Rev. Fitotec. Mex. 32:319-326.
13. Boudyach, E. H., Fatmi, M., Akhayat, O., Benizri, E., and Ait-Ben-Aoumar, A. 2001. Selection of antagonistic bacteria of *Clavibacter michiganensis* subsp. *michiganensis* and evaluation of their efficiency against bacterial canker of tomato. Biocontrol Sci. Technol. 11:141-149.
14. Bryan, M. K. 1930. Studies on bacterial canker of tomato. J. Agric. Res. 41:825-851.
15. Carlton, W. M., Braun, E. J., and Gleason, M. L. 1998. Ingress of *Clavibacter michiganensis* subsp. *michiganensis* into tomato leaves through hydathodes. Phytopathology 88:525-529.
16. Carlton, W. M., Gleason, M. L., and Braun, E. J. 1994. Effects of pruning on tomato plants supporting epiphytic populations of *Clavibacter michiganensis* subsp. *michiganensis*. Plant Dis. 78:742-745.
17. Chang, R. J., Ries, S. M., and Pataky, J. K. 1991. Dissemination of *Clavibacter michiganensis* subsp. *michiganensis* by practices used to produce tomato transplants. Phytopathology 81:1276-1281.
18. Chang, R. J., Ries, S. M., and Pataky J. K. 1992. Local sources of *Clavibacter michiganensis* subsp. *michiganensis* in the development of bacterial canker on tomatoes. Phytopathology 82:553-560.
19. Chitarra, L. G., Breeuwer, P., van den Bulk, R. W., and Abee, T. 2000. Rapid fluorescente assessment of intracellular pH as a viability indicator of *Clavibacter michiganensis* subsp. *michiganensis*. J. Appl. Microbiol. 88:809-816.
20. Chun, W. W. C. 1982. Identification and detection of *Corynebacterium michiganense* in tomato seed using the indirect enzyme-linked immunosorbent assay. M.S. thesis. University of Hawaii, Honolulu.
21. Daferera, D. J., Ziogas, B. N., and Polissiou, M. G. 2003. The effectiveness of plant essential oils on the growth of *Botrytis cinerea*, *Fusarium* sp. and *Clavibacter michiganensis* subsp. *michiganensis*. Crop Prot. 22:39-44.
22. Davis, M. J., Gillaspie, A. G., Jr., Vidaver, A. K., and Harris, R. W. 1984. *Clavibacter*: A new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *cynodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and Bermudagrass stunting disease. Int. J. Syst. Bacteriol. 34:107-117.
23. De León, L., Rodríguez, A., Llop, P., López, M. M., and Siverio, F. 2009. Comparative assessment of genetic diversity of *Clavibacter michiganensis* subsp. *michiganensis* isolates from the Canary Islands by RAPD, BOX, and AFLP. Plant Pathol. 58:862-871.
24. De León, L., Rodríguez, A., López, M. M., and Siverio, F. 2008. Evaluation of the efficacy of immunomagnetic separation for the detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds. J. Appl. Microbiol. 104:776-786.
25. De León, L., Siverio, F., López, M. M., and Rodríguez, A. 2008. Comparative efficiency of chemical compounds for *in vitro* and *in vivo* activity against *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of tomato bacterial canker. Crop Prot. 27:1277-1283.
26. De León, L., Siverio, F., and Rodríguez, A. 2006. Detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds using immunomagnetic separation. J. Microbiol. Methods 67:141-149.
27. Dhanvantari, B. N. 1987. Comparison of selective media for isolation of *Clavibacter michiganensis* subsp. *michiganensis*. (Abstr.) Phytopathology 77:1694.
28. Dhanvantari, B. N. 1989. Effect of seed extraction methods and seed treatments on control of tomato bacterial canker. Can. J. Plant Pathol. 11:400-408.
29. Dreier, J., Bermpohl, A., and Eichenlaub, R. 1995. Southern hybridization and PCR for specific detection of phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*. Phytopathology 85:462-468.
30. Echandi, E. 1971. Survival of *Corynebacterium michiganense* in soil as free cells and in infected tomato tissue. Phytopathology 61:890-891.
31. Eichenlaub, R., Gartemann, K. H., and Burger, A. 2006. *Clavibacter michiganensis*, a group of gram-positive phytopathogenic bacteria. Pages 385-421 in: Plant-Associated Bacteria. S. S. Gnanamanickam, ed. Springer, The Netherlands.
32. El-Abyad, M. S., El-Sayed, M. A., El-Shanshoury, A. R., and El-Sabbagh, S. M. 1993. Towards the biological control of fungal and bacterial diseases of tomato using antagonistic *Streptomyces* spp. Plant Soil 149:185-195.
33. Fatmi, M., and Schaad, N. W. 1988. Semiselective agar medium for isolation of *Clavibacter michiganensis* subsp. *michiganensis* from tomato seed. Phytopathology 78:121-126.
34. Fatmi, M., and Schaad, N. W. 2002. Survival of *Clavibacter michiganensis* subsp. *michiganensis* in infected tomato stems under natural field conditions in California, Ohio and Morocco. Plant Pathol. 51:149-154.
35. Fatmi, M., Schaad, N. W., and Bolkan, H. A. 1991. Seed treatments for eradicating *Clavibacter michiganensis* subsp. *michiganensis* from naturally infected tomato seeds. Plant Dis. 75:383-385.
36. Franken, A. A. J. M., Kamminga, G. C., Snijders, W., van der Zouwen, P. S., and Birnbaum, Y. E. 1993. Detection of *Clavibacter michiganensis* ssp. *michiganensis* in tomato seeds by immunofluorescence microscopy and

dilution plating. Neth. J. Plant Pathol. 99:125-137.
37. Ftayeh, R., 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. Phytopathol. Mediterr. 49:172-178.
38. Ftayeh, R., von Tiedemann, A., Koopmann, B., Rudolph, K., and Abu-Ghorrah, M. 2008. First record of *Clavibacter michiganensis* subsp. *michiganensis* causing canker of tomato plants in Syria. Plant Dis. 92:649.
39. Gartemann, K.-H., Abt, B., Bekel, T., Burger, A., Engemann, J., Flügel, M., Gaigalat, L., Goesmann, A., Gräfen, I., Kalinowski, J., Kaup, O., Kirchner, O., Krause, L., Linke, B., McHardy, A., Meyer, F., Pohle, S., Rückert, C., Schneiker, S., Zellermann, E.-M., Pühler, A., Eichenlaub, R., Kaiser, O., and Bartels, D. 2008. The genome sequence of the tomato-pathogenic actinomycete *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382 reveals a large island involved in pathogenicity. J. Bacteriol. 190:2138-2149.
40. Gartemann, K.-H., Kirchner, O., Engemann, J., Gräfen, I., Eichenlaub, R., and Burger, A. 2003. *Clavibacter michiganensis* subsp. *michiganensis*: First steps in the understanding of virulence of a Gram-positive phytopathogenic bacterium. J. Biotechnol. 106:179-191.
41. Geng, S., Campbell, R. N., Carter, M., and Hills, F. J. 1983. Quality-control programs for seedborne pathogens. Plant Dis. 67:236-242.
42. Gitaitis, R., Beaver, R. W., and Voloudakis, A. E. 1991. Detection of *Clavibacter michiganensis* subsp. *michiganensis* in symptomless tomato transplants. Plant Dis. 75:834-838.
43. Gitaitis, R., and Walcott, R. 2007. The epidemiology and management of seedborne bacterial disease. Annu. Rev. Phytopathol. 45:371-397.
44. Gleason, M. L., Braun, E. J., Carlton, W. M., and Peterson, R. H. 1991. Survival and dissemination of *Clavibacter michiganensis* subsp. *michiganensis* in tomatoes. Phytopathology 81:1519-1523.
45. Gleason, M. L., Gitaitis, R. D., and Ricker, M. D. 1993. Recent progress in understanding and controlling bacterial canker of tomato in eastern North America. Plant Dis. 77:1069-1076.
46. Gross, D. C., and Vidaver, A. K. 1979. A selective medium for isolation of *Corynebacterium nebraskense* from soil and plant parts. Phytopathology 69:82-87.
47. Hadas, R., Kritzman, G., Kleitman, F., Gefen, T., and Manulis, S. 2005. Comparison of extraction procedures and determination of the detection threshold for *Clavibacter michiganensis* ssp. *michiganensis* in tomato seeds. Plant Pathol. 54:643-649.
48. Hausbeck, M. K., Bell, J., Medina-Mora, C., Podolsky, R., and Fulbright, D. W. 2000. Effect of bactericides on population sizes and spread of *Clavibacter michiganensis* subsp. *michiganensis* on tomatoes in the greenhouse and on disease development and crop yield in the field. Phytopathology 90:38-44.
49. Holguín-Peña, R. J., Vázquez-Juárez, R. C., and Rueda-Puente, E. O. 2006. Bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* on tomato in the Baja California Peninsula of Mexico. Plant Dis. 90:1550.
50. ISHI. 2008. Method for the detection of *Clavibacter michiganensis* subsp. *michiganensis* on tomato seed. Version 3, Jan 2008. http://www.worldseed.org/isf/ishi_vegetable.html
51. Janse, J. D. 2004. Management of bacterial diseases of plants: Regulatory aspects. Pages 669-674 in: Encyclopedia of Plant and Crop Science. R. M. Goodman, ed. Marcel Dekker, Inc., New York.
52. Kaneshiro, W. S., and Alvarez, A. M. 2003. Variability and survival of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in seed. (Abstr.) Phytopathology 93:S43.
53. Kaneshiro, W. S., Ingram, D. M., and Alvarez, A. M. 2008. *Clavibacter michiganensis* subsp. *michiganensis* threshold levels required for transmission by naturally-infested tomato seed. (Abstr.) Phytopathology 98:S78.
54. Kaneshiro, W. S., Mizumoto, C. Y., and Alvarez, A. M. 2006. Differentiation of *Clavibacter michiganensis* subsp. *michiganensis* from seed-borne saprophytes using ELISA, Biolog and 16S rDNA sequencing. Eur. J. Plant Pathol. 116:45-56.
55. Kawaguchi, A., Tanina, K., and Inoue, K. 2010. Molecular typing and spread of *Clavibacter michiganensis* subsp. *michiganensis* in greenhouses in Japan. Plant Pathol. 59:76-83.
56. Kizil, S., Uyar, F., and Sagir, A. 2005. Antibacterial activities of some essential oils against plant pathogens. Asian J. Plant Sci. 4:225-228.
57. Kleitman, F., Barash, I., Burger, A., Iraki, N., Falah, Y., Sessa, G., Weinthal, D., Chalupowicz, L., Gartemann, K. H., Eichenlaub, R., and Manulis-Sasson, S. 2008. Characterization of a *Clavibacter michiganensis* subsp. *michiganensis* population in Israel. Eur. J. Plant Pathol. 121:463-475.
58. Koenraadt, H., van Vliet, A., Neijndorff, N., and Woudt, B. 2009. Improvement of semi-selective media for the detection of *Clavibacter michiganensis* subsp. *michiganensis* in seeds of tomato. Phytopathology (Abstr.) 99:S66.
59. Kokosková, B., Mráz, I., and Fousek, J. 2010. Comparison of specificity and sensitivity of immunochemical and molecular techniques for determination of *Clavibacter michiganensis* subsp. *michiganensis*. Folia Microbiol. 55:239-244.
60. Krämer, I., and Griesbach, E. 1995. Use of ELISA for detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato. Bull. OEPP/EPPO Bull. 25:185-193.
61. Kubota, C., McClure, M. A., Kokalis-Burelle, N., Bausher, M. G., and Rosskopf, E. N. 2008. Vegetable grafting: History, use and current technology status in North America. HortScience 43:1664-1669.
62. Lai, M. 1976. Bacterial canker of bell pepper caused by *Corynebacterium michiganense*. Plant Dis. Rep. 60:339-342.
63. Lamichhane, J. R., Balestra, G. M., and Varvaro, L. 2011. Severe outbreak of bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* on tomato in central Italy. Plant Dis. 95:221.
64. López, M. M., Llop, P., Olmos, A., Marco-Noales, E., Cambra, M., and Bertolini, E. 2008. Are molecular tools solving the challenges posed by detection of plant-pathogenic bacteria and viruses? Curr. Issues Mol. Biol. 11:13-46.
65. Louws, F. J., Rademaker, J. L. W., and de Bruijn, F. J. 1999. The three Ds of PCR-based genomic analysis of phytobacteria: Diversity, detection, and disease diagnosis. Annu. Rev. Phytopathol. 37:81-125.
66. Luo, L. X., Walters, C., Bolkan, H., Liu, X. L., and Li, J. Q. 2008. Quantification of viable cells of *Clavibacter michiganensis* subsp. *michiganensis* using a DNA binding dye and a real-time PCR assay. Plant Pathol. 57:332-337.
67. Maddox, D. A. 1998. Implication of new technologies for seed health testing and the worldwide movement of seed. Seed Sci. Res. 8:277-284.
68. Medina-Mora, C. M., Hausbeck, M. K., and Fulbright, D. W. 2001. Bird'eye lesions of tomato fruit produced by aerosol and direct application of *Clavibacter michiganensis* subsp. *michiganensis*. Plant Dis. 85:88-91.
69. Milijasevic, S., Todorovic, B., Potocnik, I., and Stepanovic, M. 2009. Comparison of different methods in detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds. Acta Hortic. 808:103-109.
70. Moffett, M. L., and Wood, B. A. 1984. Survival of *Corynebacterium michiganense* subsp. *michiganense* within host debris in soil. Australas. Plant Pathol. 13:1-3.
71. Morrison, R. H. 1999. Sampling in seed health testing. Phytopathology 89:1084-1087.
72. Munkvold, G. P. 2009. Seed pathology progress in academia and industry. Annu. Rev. Phytopathol. 47:285-311.
73. Myung, I. S., Kim, D. G., An, S. H., Lee, Y. K., and Kim, W. G. 2008. First report of bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis* in Korea. Plant Dis. 92:1472.
74. OEPP/EPPO. 2005. EPPO Standards PM 7/42 (1) Diagnostic. *Clavibacter michiganensis* subsp. *michiganensis*. Bull. OEPP-EPPO Bull. 35:275-283. http://archives.eppo.org/EPPOStandards/diagnostics.htm
75. OEPP/EPPO. 2007. EPPO Plant Quarantine Data Retrieval System, version 4.6. July 2007. http://www.eppo.org/DATABASES/pqr/pqr.htm
76. OEPP/EPPO. 2007. *Clavibacter michiganensis* subsp. *michiganensis* found in the Netherlands on tomato plants for planting. EPPO Reporting Service No. 5. Rep. No. 2007/090. http://archives.eppo.org/EPPOReporting/2007/Rse-0705.pdf
77. OEPP/EPPO. 2008. *Clavibacter michiganensis* subsp. *michiganensis* found in the Netherlands. EPPO Reporting Service No. 1. Rep. No. 2008/005. http://archives.eppo.org/EPPOReporting/2008/Rse-0801.pdf
78. OEPP/EPPO. 2008. Outbreak of *Clavibacter michiganensis* subsp. *michiganensis* in Austria. EPPO Reporting Service No. 2. Rep. No. 2008/035. http://archives.eppo.org/EPPOReporting/2008/Rse-0802.pdf
79. OEPP/EPPO. 2008. Outbreak of *Clavibacter michiganensis* subsp. *michiganensis* in Austria. EPPO Reporting Service No. 11. Rep. No. 2008/214. http://archives.eppo.org/EPPOReporting/2008/Rse-0811.pdf
80. OEPP/EPPO. 2009. Outbreak of *Clavibacter michiganensis* subsp. *michiganensis* in Burgenland, Austria. EPPO Reporting Service No. 6. Rep. No. 2009/118. http://archives.eppo.org/EPPOReporting/2009/Rse-0906.pdf
81. OEPP/EPPO. 2010. *Clavibacter michiganensis* subsp. *michiganensis* detected in Puglia (IT). EPPO Reporting Service No. 9. Rep. No. 2010/157. http://archives.eppo.org/EPPOReporting/2010/Rse-1009.pdf
82. Olivier, V., Baloche, A., Drouin, A., Audusseau, C., Paillard, S., and Soubelet, H. 2010. Internal methods comparison study and inter-laboratory study on *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds. Bull. OEPP/EPPO Bull. 40:248-256.
83. Ordax, M., Marco-Noales, E., Lopez, M. M., and Biosca, E. G. 2006. Survival strategy of *Erwinia amylovora* against copper: Induction of the viable-but-nonculturable state. Appl. Environ. Microbiol. 72:3482-3488.
84. Özdemir, Z. 2009. Development of a mulitplex PCR assay for the simultaneous detection of *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. *tomato* and *Xanthomonas axonopodis* pv. *vesicatoria* using pure cultures. J. Plant Pathol. 91:495-497.
85. Palacio-Bielsa, A., Cambra, M. A., and López, M. M. 2009. PCR detection and identification of plant-pathogenic bacteria: Updated review of protocols (1989-2007). J. Plant Pathol. 91:249-297.
86. Papayiannis, L. C. 2008. Detection and identification of bacterial diseases infecting tomato crops in Cyprus. Phytopathol. Mediterr. 47:147.
87. Pastrik, K. H., and Rainey, F. A. 1999. Identification of *Clavibacter michiganensis* subspecies by polymerase chain reaction-based techniques. J. Phytopathol. 147:687-693.
88. Pradhanang, P. M., and Collier, G. 2009. How effective is hydrochloric acid treatment to control *Clavibacter michiganensis* subsp. *michiganensis* contamination in tomato seed? Acta Hortic. 808:81-85.
89. Sahin, F., Uslu, H., Kotan, R., and Donmez, M. F. 2002. Bacterial canker,

caused by *Clavibacter michiganensis* ssp. *michiganensis*, on tomatoes in eastern Anatolia region of Turkey. Plant Pathol. 51:399.
90. Santos, M. S., Cruz, L., Norskov, P., and Rasmussen, O. F. 1997. A rapid and sensitive detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds by polymerase chain reaction. Seed Sci. Technol. 25:581-584.
91. Schaad, M. W., and Frederick, R. D. 2002. Real-time PCR and its application for rapid plant disease diagnostics. Can. J. Plant Pathol. 24:250-258.
92. Shoemaker, P. B., and Echandi, E. 1976. Seed and plant bed treatments for bacterial canker of tomato. Plant Dis. Rep. 60:163-166.
93. Smith, E. F. 1914. The Grand Rapids tomato disease. Pages 161-165 in: Bacteria in Relation to Plant Diseases, Vol. 3. Carnegie Institute, Washington, DC.
94. Soylu, S., Baysal, Ö., and Soylu, E. M. 2003. Induction of disease resistance by the plant activator, acibenzolar-S-methyl (ASM), against bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*) in tomato seedlings. Plant Sci. 165:1069-1075.
95. Strider, D. L. 1969. Bacterial canker of tomato caused by *Corynebacterium michiganense.* A literature review and bibliography. N.C. Agric. Exp. Stn. Tech. Bull. 193.
96. Theodoro, G., and Maringoni, A. C. 2000. *In vitro* and *in vivo* action of chemicals on *Clavibacter michiganensis* subsp. *michiganensis*, causal agent of the bacterial canker of tomato. Sci. Agric. 57:439-443.
97. Thyr, B. D., Samuel, L. J., and Brown, P. G. 1975. New solanaceous host records for *Corynebacterium michiganense*. Plant Dis. Rep. 59:595-598.
98. Thyr, B. D., Webb, R. E., Jaworski, C. A., and Ratcliffe, T. J. 1973. Tomato bacterial canker: Control by seed treatment. Plant Dis. Rep. 57:974-977.
99. Umesha, S. 2006. Occurrence of bacterial canker in tomato fields of Karnataka and effect of biological seed treatment on disease incidence. Crop Prot. 25:375-381.
100. Ustun, N., Ulutas, E., Yasarakinci, N., and Kilic, T. 2009. Efficacy of some plant activators on bacterial canker of tomato in Aegean region of Turkey. Acta Hortic. 808:405-408.
101. Veena, M. S., and van Vuurde, J. W. L. 2002. Indirect immunofluorescence colony staining method for detecting bacterial pathogens of tomato. J. Microbiol. Methods 49:11-17.
102. Vega, E., Ureta, T., and Campos, G. 2001. Aumento de la incidencia de *Clavibacter michiganensis* subsp. *michiganensis*, agente causal de la enfermedad cancro bacteriano del tomate, en el cultivo de tomate de la última temporada. Simiente 71:33-73.
103. Waters, C. M., and Bolkan, H. A. 1992. An improved selective medium and method of extraction for detecting *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds. Phytopathology 82:1072.
104. Werner, N. A., Fulbright, D. W., Podolsky, R., Bell, J., and Hausbeck, M. K. 2002. Limiting populations and spread of *Clavibacter michiganensis* subsp. *michiganensis* on seedling tomatoes in the greenhouse. Plant Dis. 86:535-542.
105. Xu, X., Miller, S. A., Baysal-Gurel, F., Gartemann, K. H., Eichenlaub, R., and Rajashekara, G. 2010. Bioluminescence imaging of *Clavibacter michiganensis* subsp. *michiganensis* infection of tomato seeds and plants. Appl. Environ. Microbiol. 76:3978-3988.
106. Yogev, A., Raviv, M., Kritzman, G., Hadar, Y., Cohen, R., Kirshner, B., and Katan, J. 2009. Suppression of bacterial canker of tomato by composts. Crop Prot. 28:97-103.
107. Zhao, W. J., Chen, H. Y., Zhu, S. F., Xia, M. X., and Tan, T. W. 2007. One-step detection of *Clavibacter michiganensis* subsp. *michiganensis* in symptomless tomato seeds using a TaqMan probe. J. Plant Pathol. 89:349-351.

Leandro de León

Felipe Siverio

María M. López

Ana Rodríguez

Dr. de León received his B.S. in biology (2000) and his M.S. (2003) and Ph.D. (2008) in microbiology from the University of La Laguna (ULL), Spain, having conducted the predoctoral studies in the Department of Plant Protection, Instituto Canario de Investigaciones Agrarias. His Ph.D. dissertation dealt with diagnosis, epidemiology, and control of phytopathogenic bacteria (*Clavibacter michiganensis* subsp. *michiganensis*) on tomato crops. During his university and Ph.D. studies, he also joined the Department of Cellular Biology and Microbiology (ULL), where he investigated the antimicrobial activity and mechanism of action of phenolic substances obtained from natural sources. Since 2010, he has been a postdoctoral Fulbright scholar at the Department of Molecular and Structural Biochemistry at North Carolina State University. Currently, his research interests focus on geminivirus replication, cassava mosaic geminiviruses (CMGs), and host interaction.

Dr. Siverio is a plant pathologist in the Plant Health Laboratory of the Consejería de Agricultura, Ganadería, Pesca y Aguas del Gobierno de Canarias since 2002. He received his degree in agricultural biology in 1986 and his Ph.D. in 1994 from the University of La Laguna after a stage in the Bacteriology Laboratory of Instituto Valenciano de Investigaciones Agrarias studying pith necrosis of tomato plant caused by *Pseudomonas corrugata*. He also worked with *Phytophthora cinnamomi* in avocado plants at the Department of Plant Protection of the Instituto Canario de Investigaciones Agrarias (19951999). He has followed programs for the control of different diseases as brown rot of potato caused by *Ralstonia solanacearum*, bacterial canker of tomato by *Clavibacter michiganensis* subsp. *michiganensis,* and currently collaborates to avoid the entrance of bacteria transmitted by psyllids.

Prof. López is the head of the Bacteriology group at the Instituto Valenciano de Investigaciones Agrarias (IVIA) in Moncada, Valencia, Spain, where she has been working with several plant pathogenic bacteria since 1978. She graduated and received her Ph.D. degree at the Polytechnic University of Valencia, Spain. She was research visitor at INRA, Angers and INRA, Bordeaux, France, SCRI, Dundee, Scotland and ARS-USDA, Orlando, USA. She is responsible for the Spanish Reference Laboratory for Plant Pathogenic Bacteria of the Ministry of Agriculture (MARM) since 1993 and president of the Spanish Phytopathological Society (SEF) since 2009. Her research focuses on the detection, biology, epidemiology, and biocontrol of plant pathogenic bacteria with emphasis on *Agrobacterium* spp., *Erwinia amylovora*, *Ralstonia solanacearum,* and *Clavibacter michiganensis* subsp. *michiganensis*.

Dr. Rodríguez is a researcher in the Department of Plant Protection of the Instituto Canario de Investigaciones Agrarias (ICIA), Canary Islands, Spain, since 1999. She obtained her degree in molecular biology in 1987 and her Ph.D. in 1994 from the University of La Laguna, Canary Islands, Spain. Since her incorporation to the ICIA, she has worked on fungal and bacterial diseases affecting tomato, avocado, and potato crops in the Canary Islands. Her research interests mainly include the control of soilborne pathogens by soil solarization, selection and evaluation of avocado rootstocks tolerant–resistant to root-rot caused by *Phytophthora cinnamomi*, fungicide resistance of *Botrytis cinerea* in tomato crops, and the diagnosis and control of tomato bacterial canker caused by *Clavibacter michiganensis* subsp. *Michiganensis*. She is also an associate professor of microbiology at the University of La Laguna since 2001, teaching agricultural microbiology at the Agricultural Engineering Faculty.

Table 5. Sensitivity of polymerase chain reaction (PCR)-based methods described for detection of *Clavibacter michiganensis* subsp. *michiganensis*

Method	Primers	Sensitivity reported	Reference
PCR	CMM5/CMM6	10^3 CFU/50 seeds	29
	CMM5/CMM6	10^3 CFU/ml seed extract	24
	CMM5/CMM6	10^4 CFU/ml pure culture	59
	CM3/CM4	4×10 CFU/ml seed extract	90
	CMM5/CMM6; CM3/CM4	1 contaminated seed[a]/5,000 seeds	47
	CMM5/CMM6; PSA-4/PSA-R	1 contaminated seed[b]/2,000 seeds	69
	Cmm1F/Cmm1R	10^3 CFU/ml pure culture	59
PCR + hybridization	CMM5/CMM6	2×10^2 CFU/ml plant homogenate	29
Enrichment PCR	PSA-4/PSA-R	1 contaminated seed[c]/10,000 seeds	74
	PSA-4/PSA-R	5 contaminated seeds[b]/5,000 seeds	69
Bio-PCR	CMM5/CMM6; CM3/CM4	1 contaminated seed[a]/10,000 seeds	47
	CMM5/CMM6; PSA-4/PSA-R	1 contaminated seed[b]/5,000 seeds	69
Real-time PCR	ITSYG-1/ITSYG-2	10^3 CFU/ml pure culture	107
	ITSYG-1/ITSYG-2	10 contaminated seeds/1,000 seeds	107
	Spm4f/Spm2r	10^3 CFU/ml pure culture	66

[a] Contamination rate of 10^2-10^3 CFU/seed.
[b] Contamination rate of 10^8 CFU/seed.
[c] At minimum contamination level of 4×10^2 CFU.

Table 5 was inadvertently omitted from this article when it first appeared. It was added on 1 December 2011.