Pathogen profile

Bacterial spot of tomato and pepper: diverse *Xanthomonas* species with a wide variety of virulence factors posing a worldwide challenge

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SUMMARY

Taxonomic status: Bacteria; Phylum Proteobacteria; Class Gammaproteobacteria; Order Xanthomonadales; Family Xanthomonadaceae; Genus *Xanthomonas*; Species *Xanthomonas euvesicatoria*, *Xanthomonas vesicatoria*, *Xanthomonas perforans* and *Xanthomonas gardneri*.
Microbiological properties: Gram-negative, rod-shaped bacterium, aerobic, motile, single polar flagellum.
Host range: Causes bacterial spot disease on plants belonging to the Solanaceae family, primarily tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*) and chilli peppers (*Capsicum frutescens*).
Disease symptoms: Necrotic lesions on all above-ground plant parts.
Distribution: Worldwide distribution of *X. euvesicatoria* and *X. vesicatoria* on tomato and pepper; *X. perforans* and *X. gardneri* increasingly being isolated from the USA, Canada, South America, Africa and Europe.

A wide diversity within the bacterial spot disease complex, with an ability to cause disease at different temperatures, makes this pathogen group a worldwide threat to tomato and pepper production. Recent advances in genome analyses have revealed the evolution of the pathogen with a plethora of novel virulence factors. Current management strategies rely on the use of various chemical control strategies and sanitary measures to minimize pathogen spread through contaminated seed. Chemical control strategies have been a challenge because of resistance by the pathogen. Breeding programmes have been successful in developing commercial lines with hypersensitive and quantitative resistance. However, durability of resistance has been elusive. Recently, a transgenic approach has resulted in the development of tomato genotypes with significant levels of resistance and improved yield that hold promise. In this article, we discuss the current taxonomic status, distribution of the four species, knowledge of virulence factors, detection methods and strategies for disease control with possible directions for future research.

Keywords: bacterial spot, pepper, tomato, *Xanthomonas*.

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HOW THE TAXONOMY OF BACTERIAL SPOT XANTHOMONADS HAS CHANGED OVER THE YEARS

Bacterial spot was first discovered on tomato in South Africa in 1914, and named *Bacterium vesicatorium* by Doidge (1920). The disease was later identified in Indiana by Gardner and Kendrick (1921) and they were prepared to propose the name *B. exitiosum*, but deferred to Doidge's designation. The two strains varied noticeably in amylolytic activity, but were treated as one pathogen. Gardner and Kendrick (1923) later discovered that *B. vesicatorium* also caused a leaf spot of pepper (*Capsicum annuum*), but Higgins (1922) was the first to fully describe the disease. After its initial discovery, *B. vesicatorium* was thought to be the only causal agent of bacterial spot when it was reclassified several times to *Pseudomonas vesicatoria*, *Phytomonas vesicatoria* and *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) (Dye, 1978; Jones *et al*., 1998b; Stevens, 1925).

Bacterial spot strains were considered as one group until the mid-1990s, when independent studies demonstrated that *Xcv* consisted of two genetically and phenotypically distinct groups (A and B) (Stall *et al*., 1994; Vauterin *et al*., 1995). These two groups are also diverse in terms of their fatty acid profiles, serology, carbon utilization assays and DNA restriction enzyme digestion profiles. Strains belonging to the same group had DNA homology values of >70% and <46% with strains in the other group (Stall *et al*., 1994). Therefore, Vauterin *et al*. (1995) proposed that the two groups should be reclassified as *X. axonopodis* pv. *vesicatoria* (A) and *X. vesicatoria* (B). Group A strains, unlike group B strains, are

primarily negative for starch hydrolysis and pectolytic activity (Bouzar *et al*., 1996). More recently, two additional genetically distinct xanthomonads have been isolated from tomato. Šutic (1957) isolated an organism in 1953 in former Yugoslavia that he named *Pseudomonas gardneri*. Later, it was suggested to be synonymous with *X. vesicatoria* (Dye, 1966); however, results of DNA–DNA hybridization indicated that these organisms were genetically distinct (DeLey *et al*., 1978). A fourth genetically distinct pathogen was isolated from tomato in Florida in the 1990s and was determined to be a new race of *Xcv* (Jones *et al*., 1995, 2000). These two strains were later characterized as groups D ('*X. gardneri*') and C (*X. axonopodis* pv. *vesicatoria*), respectively, based on DNA–DNA hybridization, pathogenicity tests, 16S rRNA and restriction fragment analysis of the entire genome (Jones *et al*., 2000). Based on the results of DNA–DNA hybridization, Jones *et al*. (2004) determined that strains within groups C and D were distinct *Xanthomonas* species, and that bacterial spot is incited by four distinct species: *X. euvesicatoria* (A), *X. vesicatoria* (B), *X. perforans* (C) and *X. gardneri* (D).

However, the taxonomy for the bacterial spot pathogens may not be fully resolved. Young *et al*. (2008) conducted multilocus sequence analysis (MLSA) using four housekeeping genes (*dnaK*, *fyuA*, *gyrB* and *rpoD*), which revealed that *X. gardneri* appears to be synonymous with *X. cynarae*, which causes a bacterial spot disease on capitulum bracts of artichokes (*Cynarascolymus*) (Trébaol *et al*., 2001). Whole-genome comparison indicated that *X. gardneri* and *X. cynarae* should be considered as a single species (M. Jacques and J. B. Jones, unpublished data). In addition, the MLSA results suggested a close phylogenetic relationship between *X. perforans* and *X. euvesicatoria*. Almeida *et al*. (2010) suggested the use of six housekeeping genes (*fusA*, *gapA*, *gltA*, *gyrB*, *lacF* and *lepA*) to distinguish between *Xanthomonas* species. Using these genes for the comparison of strains, multiple recombination events between *X. euvesicatoria* and *X. perforans* strains were observed (Timilsina *et al*., 2015). Therefore, the taxonomic position of these three bacterial spot species will be further evaluated and could change in the future.

HOST RANGE AND RACE STRUCTURE

The host range of bacterial spot xanthomonads includes a wide range of plants belonging to the Solanaceae family, but mainly tomato (*Solanum lycopersicum*), cherry tomato (*Solanum lycopersicum* var. *cerasiforme*), currant tomato (*L. pimpinellifolium*), pepper (*Capsicum annuum*), chilli peppers (*Capsicum frutescens*), *C. baccatum*, *C. anomalum*, *C. chinensis* and *C. pubescens* (Baker *et al*., 2014; Sahin and Miller, 1998). Additional solanaceous hosts have been suggested (Dye *et al*., 1964; Elliott, 1951); however, at least one study has demonstrated that some of these other genera are not hosts for the bacterium (Laub and Stall, 1967).

As plant resistance was identified in pepper and tomato genotypes, pathogenic races were identified based on differential reactions. Currently, four tomato races and 11 pepper races have been identified (Stall *et al*., 2009). Among the four species, various pepper and tomato races of *X. euvesicatoria*, *X. vesicatoria* and *X. perforans* (tomato only) have been widely reported (Horvath *et al*., 2012; Jones *et al*., 1995; Kebede *et al*., 2014). *Xanthomonas gardneri* has also been associated with pepper and tomato bacterial spot from different regions of the world. In 2010, an *X. perforans* strain was isolated from a pepper field in Florida (J. B. Jones, unpublished data), suggesting recent host range expansion. Strains within *X. euvesicatoria*, *X. vesicatoria* and *X. gardneri* that are pathogenic only on tomato, only on pepper, and on both tomato and pepper have been identified. Avirulence (*Avr*) genes that restrict the strains to pepper or tomato include *avrBs4* (resulting in a hypersensitive response on both tomato and *C. pubescens*) and *avrBsT* (resulting in a hypersensitive response on pepper) (Ballvora *et al*., 2001; Minsavage *et al*., 1990). With both *Avr* genes being plasmid borne, the loss of a plasmid allows a strain to become pathogenic on that host (Canteros *et al*., 1991; Minsavage *et al*., 1990). Apart from specific races, host–pathogen combination is important for pathogen aggressiveness, e.g. *X. euvesicatoria* was found to be more aggressive on pepper than on tomato (Ignjatov *et al*., 2010).

DISTRIBUTION OF XANTHOMONADS

Xanthomonas spp. are widely distributed in different geographical regions probably because of contaminated seed (Kebede *et al*., 2014). Two species, *X. euvesicatoria* and *X. vesicatoria*, historically have had a worldwide distribution and, more recently, the other two species have been increasingly isolated. Different species of bacterial spot-causing xanthomonads have been isolated from tomato-growing regions/countries (Bouzar *et al*., 1999; Jones *et al*., 2005; Hamza *et al*., 2010; 2012; Jibrin *et al*., 2014; Kebede *et al*., 2014; Kim *et al*., 2010; Quezado-Duval *et al*., 2004; Stall, 1995; Stoyanova *et al*., 2014; Timilsina *et al*., 2015; Vauterin *et al*., 1995).

Changes in pathogen populations have been documented. In Florida, bacterial spot of tomato has changed considerably over the years, even though there has been no dramatic change in the tomato cultivars used in commercial production fields during the last decade. Prior to 1991, tomato race 1 (T1) strains of *X. euvesicatoria* were the only known causal agent of bacterial spot of tomato (Jones *et al*., 1995). A field survey in 1991 recovered a low number of tomato race 3 (T3) strains of *X. perforans* in Florida, with the T1 strains being the prevalent bacterial spot pathogen (Jones *et al*., 1995). The prevalence of *X. perforans* T3 strains increased from 1991 to 1994 (Jones *et al*., 1998a) as a probable result of inhibitory bacteriocins produced by *X. perforans* strains that targeted T1 strains of *X. euvesicatoria* (Hert *et al*.,
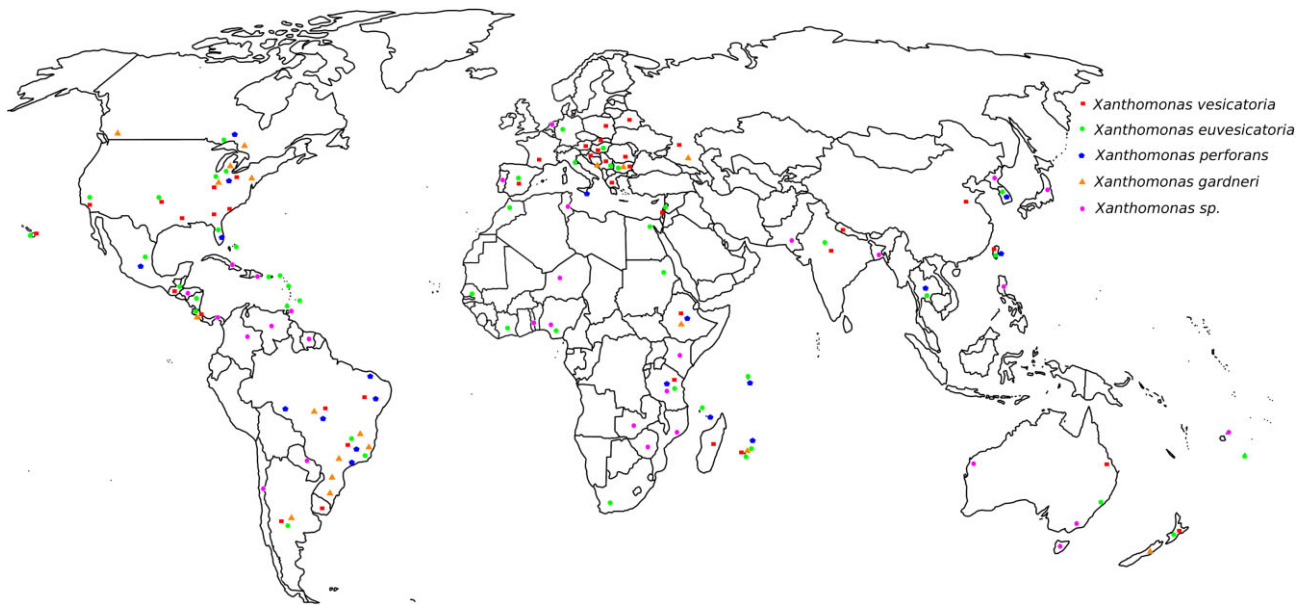
Fig. 1 Current worldwide distribution of *Xanthomonas* causing bacterial spot of tomato and pepper.

2005). In the late 1990s, tomato race 4 (T4) strains of *X. perforans* were identified based on the differential reaction on *Xv3* and *RXopJ4* (Astua-Monge *et al*., 2000a, b; Sharlach *et al*., 2013; Stall *et al*., 2009). A survey conducted in 2006 in different tomato production fields throughout Florida revealed a dramatic shift in pathogen populations, with 100% of the strains being *X. perforans*, 77% of which were T4 and the remainder were T3 (Horvath *et al*., 2012). A similar situation was reported for tomato production areas in Ohio and Michigan, with an increase in *X. gardneri* strains where *X. euvesicatoria* T1 was previously the prevalent bacterial species on tomato (Ma *et al*., 2011). In Canada, *X. euvesicatoria* and *X. gardneri* are the prevalent species and, recently, *X. perforans* strains have been isolated (Cuppels *et al*., 2006; Cândido *et al*., 2008). A recent study by Timilsina *et al*. (2015) has indicated an increase in the geographical distribution of *X. gardneri*.

Molecular characterization has revealed species diversity of bacterial spot xanthomonads in Europe, Asian countries, such as Taiwan and India, and African countries, such as Ethiopia, Nigeria, Tanzania and various islands in the southwest Indian Ocean region (Hamza *et al*., 2012; Kebede *et al*., 2014; Mbega *et al*., 2012; Stoyanova *et al*., 2014). Although there have been reports of bacterial spot from different parts of the world, in many cases, placement into the actual species has not been determined. A map showing the distribution of xanthomonads associated with bacterial spot is presented in Fig. 1.

EPIDEMIOLOGY

Seed can serve as an important inoculum source, especially in seedling production facilities, where high temperatures, high rela-

tive humidity, high plant density and overhead irrigation create an ideal environment for disease development and spread. It has been speculated that even a low incidence of seed transmission can introduce exotic strains (Pohronezny *et al*., 1992). Xanthomonads can also survive epiphytically in the tomato phyllosphere. Epiphytic populations in latent infection under favourable conditions could be an important factor in the dissemination of a pathogen (McGuire *et al*., 1991). The spread of the pathogen on transplants as a result of latent infections or epiphytic populations has been considered as a major source of outbreaks. Epiphytic survival on weeds has been noted, although at low population levels (Jones *et al*., 1986). The bacterium can also persist in crop residue for extended periods of time. In more temperate climates, the bacteria generally survive in crop residue for less than 2 years, but, in tropical and subtropical regions, the bacterium survives only a few months (Jones *et al*., 1986). Bacteria can also survive on volunteer pepper/tomato plants, and in soil in association with crop residue. However, it cannot survive for more than several days in soil after crop residue decomposes (Jones *et al*., 1986).

Disease is favoured by various temperatures and by high precipitation, depending on the particular bacterial species (Araújo *et al*., 2010). *Xanthomonas gardneri* has been found primarily in cooler temperature regions (Jones *et al*., 1998b). In addition, one study has shown that *X. gardneri* caused more disease at 20 °C than did other bacterial spot xanthomonads (Araújo *et al*., 2010).

The bacteria are disseminated within a field by wind-driven rain droplets, wounding caused during field operations (grafting, clipping, tying, harvesting, spraying of pesticides) and aerosols (Lindemann and Upper, 1985; McInnes *et al*., 1988). On
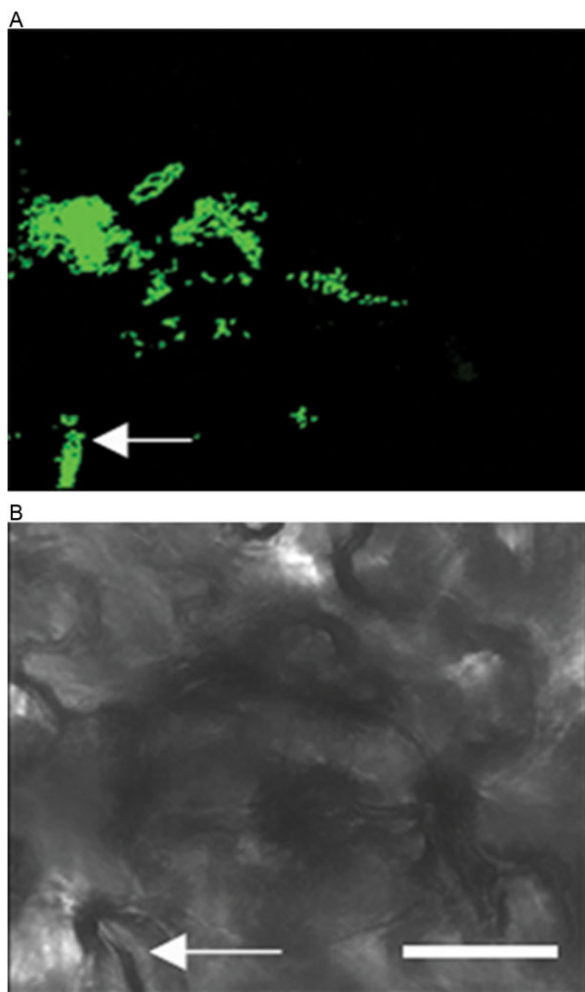
Fig. 2 Colonization of bacterial spot *Xanthomonas perforans* in the tomato phyllosphere. On immigration to the leaf surface, *X. perforans* colonizes around the stomata (indicated by arrow) and in the grooves of the epidermal cells as seen in this confocal laser scanning micrograph. Five-week-old tomato plants were dip inoculated with 10^6 colony-forming units (cfu)/mL of green fluorescent protein (GFP)-labelled *X. perforans*. The images were captured on day 3 post-inoculation. (A) GFP channel. (B) Bright field. White bar, 25 μm.

immigration onto the leaf surface, the bacterium colonizes the phyllosphere, which has been visualized using confocal laser scanning microscopy (Potnis *et al.*, 2014; Zhang *et al.*, 2009). The bacterium can readily be observed in depressions between epidermal cells, around stomata (Fig. 2), trichomes, lenticels and hydathodes, through all of which the bacterium penetrates the host. Zhang *et al.* (2009) observed low-level type III secretion system (T3SS) gene (*hrp*, *hypersensitive response and pathogenicity*) expression on the leaf surface as early as 24 h post-inoculation using an HrpG-GFP (green fluorescent protein) reporter [HrpG is an important regulator of T3SSs as well as other systems, including the type II secretion system (T2SS)], where cells had not yet entered the leaf. This suggests that the HrpG regulon is not up-regulated on

the leaf surface on immigration prior to invasion. Once the bacterium reaches the stomata, a high level of Hrp expression is observed (Wilson *et al.*, 2006; Zhang *et al.*, 2009). The bacterial spot pathogen enters the leaf apoplast through the stomata, followed by growth in the substomatal chamber and multiplication in intercellular spaces (Fig. 3). The later stages of disease involve widespread cell death, tissue necrosis and egress of *Xanthomonas* to the leaf surface, increasing phyllosphere population levels (Fig. 3).

DISEASE SYMPTOMS

Bacterial spot of tomato and pepper is characterized by necrotic lesions on the leaves, stems, petals and flowers, and fruit (Jones *et al.*, 1991; Fig. 4A). During the initial stages of symptom development, circular water-soaked lesions appear, which later dry and turn dark brown to black with a wet to greasy appearance (Vallad *et al.*, 2004). Sometimes, halos are present around the spots. Primary lesions coalesce, resulting in extensive necrosis and a blighted appearance. The lesions may have a water-soaked appearance during favourable conditions with rainy weather and when dew is present. In the case of *X. perforans*, lesions on the leaves often develop a shot-hole appearance when conditions favour rapid bacterial growth (Stall *et al.*, 2009, Fig. 4A). Lesions caused by *X. gardneri* have a characteristic water-soaked appearance. On fruit, the lesions have a raised, scabby appearance (Fig. 4B). *Xanthomonas euvesicatoria* and *X. vesicatoria* have been associated with fruit lesions. For years, *X. perforans* has not been associated with fruit lesions although, recently, strains have been isolated from fruit (G. E. Vallad, University of Florida, unpublished data). Recently, *X. gardneri* has also been associated with deep, large fruit lesions that are problematic for the processing of tomatoes. Fruit lesions caused by *X. gardneri* begin as large, water-soaked spots that become crusty and may take on a star-shaped appearance as the epidermal tissue surrounding the lesion cracks (Ma *et al.*, 2011; Miller, 2012). In pepper production fields where bacterial spot is prevalent, defoliation is commonly observed; furthermore, shedding of blossom and young fruit often occurs. Fruit quality is affected because of the presence of lesions and sunscald as a result of defoliation (Ritchie, 2000). A reduction in fruit quality and additional fruit loss caused by secondary post-harvest pathogens can result in significant yield reductions.

MOLECULAR ASPECTS OF HOST–PATHOGEN INTERACTION

Bacterial spot xanthomonads possess a collection of virulence factors that are involved in different stages of disease development. On contact with host surfaces, external signals activate two-component signalling cascades in xanthomonads. A few of
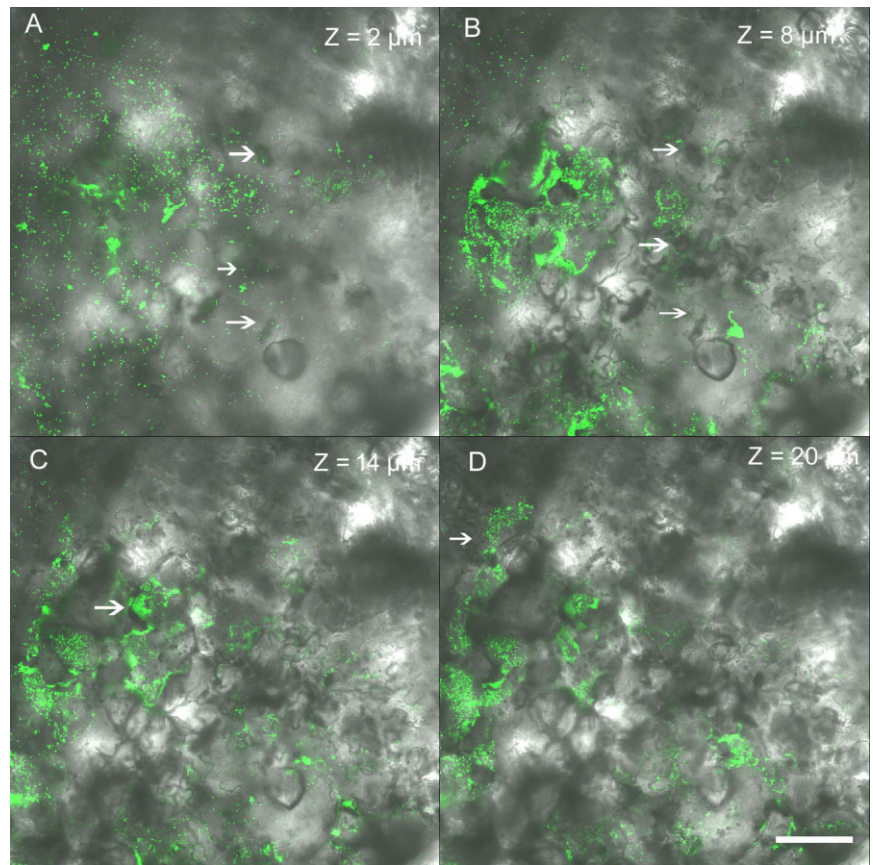
Fig. 3 Colonization of bacterial spot *Xanthomonas perforans* in the tomato phyllosphere. Bacterium enters though stomata (A), followed by growth in the substomatal chamber (B) (indicated by arrow). Bacterium multiplies in the intercellular spaces (C and D). Representative photomicrographs showing green fluorescent protein (GFP)-labelled virulent *X. perforans* aggregates that are part of the lesion along a Z stack of 20 μm overlaid with Nomarski differential interference contrast images (40× magnification). Z values represent the distance in micrometres from the abaxial (lower) leaf surface. White bar, 25 μm.

Fig. 4 Bacterial spot disease symptoms on the leaves (A) and fruits (B) of pepper and tomato. On the leaves, dark brown to black lesions with a necrotic blighted appearance are seen. Classic shot-hole lesions are observed on tomato leaves infected with *Xanthomonas perforans* (shown as enlarged image). Scabby and raised lesions are seen on the fruit.

these, such as RpfC/RpfG (quorum sensing), RavS/RavR (He and Zhang, 2008; He *et al*., 2009), CorS/CorR (Qian *et al*., 2008a, b) and PhoPQ (Lee *et al*., 2008) have been studied for their role in virulence in xanthomonads (Qian *et al*., 2008a). The HrpG/HrpX response regulator system, which controls the activation of T2SS and T3SS, has been very well studied in *X. euvesicatoria* (Koebnik *et al*., 2006). These systems orchestrate virulence gene expression.

The Hrp cluster encoding the T3SS of *Xanthomonas* was first characterized in *X. euvesicatoria* (Bonas *et al*., 1991). The protein effectors that are secreted and translocated by T3SS into the host cell are determinants of pathogenicity and host range for the pathogen. Bacterial spot xanthomonads contain at least 45 effectors based on computational analysis, a few of which have been experimentally confirmed to be secreted by T3SS (Potnis *et al*., 2011) and studied for their role in disease development. Core effectors that play essential roles at different stages of disease development include AvrBs2, XopD, XopF1, XopK, XopL, XopN, XopQ, XopR, XopX, XopZ1 and XopAD. Some of these core effectors, XopD, XopL and XopN, are virulence factors that interfere with pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Kim *et al*., 2009, 2013; Singer *et al*., 2013). PTI is activated early in response to infection and is a major contributor in restricting the growth of a pathogen. XopN suppresses PTI responses by interacting directly with tomato 14-3-3 isoform TFT1 and TARK1 (tomato atypical receptor-like kinase), and influences symptom development and pathogen growth. XopN mutants of *X. euvesicatoria* display attenuated growth *in planta* as well as impaired symptom development (Kim *et al*., 2009). The effector AvrBs2 contributes to the virulence and fitness of the pathogen (Gassmann *et al*., 2000; Kearney and Staskawicz, 1990). Two other core effectors, XopX and XopZ, have been identified as virulence factors in other xanthomonads (Kim *et al*., 2009; Metz *et al*., 2005).

Several plant pathogen effectors are known to exhibit mimicry of host proteins to interfere with eukaryotic cellular processes. Examples include XopD, XopL and AvrBs3. In *X. euvesicatoria*, XopD mimics enzymes of the small ubiquitin-related modifier (SUMO) pathway. This effector is a SUMO protease that suppresses host transcription, promotes pathogen growth and suppresses host defence responses at the late stages of tissue colonization. XopD suppresses ethylene levels by desumoylating the ethylene-responsive transcription factor, SIERF4 (Kim *et al*., 2013). Another effector, XopL, exhibits E3 ubiquitin ligase activity and contains a novel structural fold that interacts with plant ubiquitination system components (Singer *et al*., 2013) and suppresses PTI. It interferes with ubiquitination, a key eukaryotic cell function, with an XL box and leucine-rich repeat (LRR) domain, and subverts plant immunity. AvrBs3, which belongs to the transcription activator-like (TAL) effector family, mimics a host transcription activator by binding and activating upa20, a master cell size

regulator (Kay *et al*., 2007). A homologue of *avrBs3*, *avrHah1*, which is found in *X. gardneri*, is responsible for enhanced water-soaking phenotype and virulence (Schornack *et al*., 2008). These TAL effectors have been well studied for their contribution to virulence by activating host genes, resulting in pathogen spread and disease (Marois *et al*., 2002).

Comparative genomic analyses of the four *Xanthomonas* spp. associated with bacterial spot have revealed unique variable effectors. These four *Xanthomonas* spp. all contain XopJ family effectors (i.e. *avrBsT*, *xopJ*, *avrXv4* and *avrRxv*) that might be redundant in function. *Xanthomonas euvesicatoria* contains two effectors belonging to the *XopJ* family, *xopJ1* and *xopJ3* (*avrRxv*). *Xanthomonas vesicatoria* and *X. perforans* possess *avrBsT* and *avrXv4*, respectively; recently collected *X. perforans* contains both effectors, whereas *X. gardneri* has none (Potnis *et al*., 2011; Thieme *et al*., 2005). The effector AvrXv4 is involved in PTI suppression, whereas the effector *avrBsT* encodes acetyl transferase that targets microtubule-associated protein, interferes with lipid signalling and activates the hypersensitive response. AvrBsT has also been shown to interfere with effector-triggered immunity activated by AvrBs1 through an SNF1-related kinase (Szczesny *et al*., 2010). Effector XopJ from *X. euvesicatoria* is involved in the inhibition of protein secretion by targeting vesicle trafficking, thereby interfering with cell wall-based defences (Bartetzko *et al*., 2009). *Xanthomonas vesicatoria* and *X. gardneri* may have acquired unique effectors via horizontal gene transfer, possibly from other *Xanthomonas* species or other genera. Next-generation sequencing analyses have revealed that the two tomato pathogens, *X. gardneri* and *Pseudomonas syringae* pv. *tomato*, which both prefer lower optimal temperature for infection (Araújo *et al*., 2010), share a number of type III effectors, including *xopAO*, *xopAS* and *avrBs1*, which are absent from other bacterial spot xanthomonads. The acquisition of these effectors in *X. gardneri* might be one of the factors causing the unusual aggressiveness of these strains on tomato (Potnis *et al*., 2011). Interestingly *X. gardneri* is more closely related to *X. campestris* pv. *campestris*, a weed and cruciferous pathogen, based on whole-genome phylogeny, as well as phylogeny based on important pathogenicity clusters (Potnis *et al*., 2011), suggesting the possible evolution of this tomato pathogen from wild populations found on weeds or the plant-associated environment.

Cell wall-degrading enzymes, such as cellulases, polygalacturonases, xylanases and proteases, are secreted by the T2SS. The role of the T2SS in contributing to virulence has been studied in *X. euvesicatoria*. Xylanases and proteases, under the control of HrpG and HrpX, are secreted via the T2SS Xps system (Szczesny *et al*., 2010). Another T2SS cluster, known as the Xcs system, does not contribute to virulence, but can partially complement *xps* mutants. Substrate specificities for T2SS differ among xanthomonads (Szczesny *et al*., 2010), possibly based on the preference of host tissues.

The lipopolysaccharide (LPS) biosynthesis cluster, defined by a 14–26-kb region flanked by *etfA* and *metB*, is variable in terms of identity and size across the sequenced xanthomonads (Patil *et al*., 2007). However, in a comparative study for this cluster among sequenced xanthomonads, variation in LPS cluster was not associated with host or tissue specificity (Lu *et al*., 2008). Interestingly, among bacterial spot xanthomonads, pepper pathogens possess an identical LPS cluster, whereas *X. perforans* has a hybrid LPS cluster, sharing similarity with *X. citri*. The possible role of LPS O-antigen in pepper pathogenicity has been studied, although it contributes to pepper pathogenicity by increasing *in planta* growth when expressed with two other pepper pathogen-specific genes, XCV1839 and XopG effector (Potnis *et al*., 2011). A secreted lipolytic enzyme encoded by *lipA* of *X. euvesicatoria* has been shown to be involved in virulence during interaction of the pathogen with tomato (Tamir-Ariel *et al*., 2012).

Recently, a transcriptomics approach has been used in *X. euvesicatoria* 85-10 to identify the transcriptional start sites and the small RNAs that could be involved in virulence. This study suggested a role for the sx12 small RNA in the virulence of *X. euvesicatoria* in pepper, proposing that it interfered with the complex interactions between the pathogen and the host (Schmidtke *et al*., 2012). More recently, a regulatory small RNA, sx13, has been shown to be involved in the regulation of HrpG/HrpX. The small RNA sx13 has also been shown to regulate motility and signal transduction pathways, thereby contributing to the virulence of *X. euvesicatoria* (Schmidtke *et al*., 2012).

DETECTION AND IDENTIFICATION

The bacteria are readily isolated from infected tissue by streaking onto nutrient agar, sucrose peptone or yeast–dextrose–calcium carbonate (YDC) agar, and are characterized by yellow, mucoid and convex colonies (Doidge, 1921). The yellow colour is caused by the presence of xanthomonadins, a unique class of brominated, aryl-polyene, water-insoluble pigments present in the outer membrane (Stephens and Starr, 1963). The bacteria are straight, Gram-negative rods with single polar flagella. Various biochemical and physiological tests have been used to characterize these bacteria. The bacteria are catalase positive and oxidase negative, and can be identified to species by various molecular techniques. During the reclassification of *Xanthomonas* species, Jones *et al*. (2004) used several techniques other than DNA sequencing to characterize the species. These techniques included the determination of amylolytic and pectolytic activity, protein patterns with sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bouzar *et al*., 1994), serological reactions with a panel of monoclonal antibodies using an enzyme-linked immunosorbent assay (Bouzar *et al*., 1994) and pulsed-field gel electrophoresis patterns (Jones *et al*., 2000). Strains of *X. euvesicatoria* and *X. gardneri* are weakly amylolytic and pectolytic, whereas *X. vesicatoria* and

X. perforans strains show strong amylolytic and pectolytic activity (Jones *et al*., 2004). All four species exhibit distinct reaction patterns with the selected monoclonal antibodies, together with distinct protein profiles (Jones *et al*., 2004). A 25–27-kDa protein was present in three *Xanthomonas* species, except for *X. euvesicatoria* strains. In addition, *Xanthomonas* strains can also be differentiated by inoculation onto race-specific resistant tomato and pepper genotypes. Moreover, various research studies on the diagnosis and identification of xanthomonads causing bacterial spot of pepper and tomato have been carried out. Kuflu and Cuppels (1997) developed a diagnostic DNA probe, but only for the diagnosis of *X. euvesicatoria* and *X. vesicatoria*. Later, Cuppels *et al*. (2006) developed PCR primer sets based on the probes that can distinguish between the four species of bacterial spot xanthomonads.

Researchers have evaluated many different methods to diagnose and identify the bacterial spot pathogens. Rademaker *et al*. (2000) compared the results of amplified fragment length polymorphism (AFLP) analysis with those of DNA–DNA hybridization involving 80 *Xanthomonas* strains belonging to 20 DNA–DNA homology groups. The AFLP analysis provided phylogenetic results that were comparable with the DNA–DNA hybridization studies. Therefore, AFLP analysis was useful in differentiating species of *Xanthomonas*, such as the bacterial spot pathogens. Koenraadt *et al*. (2009) designed polymerase chain reaction (PCR) primers specific to the bacterial spot pathogens based on unique banding patterns resulting from AFLP analysis. DNA was extracted from strains representing the four bacterial spot species and digested with restriction enzymes. Only DNA fragments that were present in every strain belonging to one bacterial species and not in any other species were selected for sequencing. This allowed for four species-specific PCR primers to be designed. These primers were able to accurately amplify all of the tested bacterial spot strains. However, further validation of the primers for *X. perforans* and *X. gardneri* was necessary because the number of strains tested for these two species was limited. These primers are particularly useful in seed assays (Koenraadt *et al*., 2009). Araújo *et al*. (2012) further validated these primers using DNA extracted from 30 *X. euvesicatoria*, 30 *X. vesicatoria*, 50 *X. gardneri* and 50 *X. perforans* strains collected in Brazil. After validation, they designed a multiplex PCR assay involving Koenraadt's four species-specific primer sets. This multiplex PCR assay effectively differentiates and identifies the tested Brazilian strains from the four bacterial spot species.

Since the mid-1990s, researchers have explored the use of diagnostic methods based on regions of *hrpB*, specifically *hrpB2*, for the bacterial spot pathogens. *hrpB* is part of the *hrp* gene cluster (Bogdanove *et al*., 1996; Noël *et al*., 2002). In addition, *hrpB2* is essential for type III protein secretion and pathogenicity (Rossier *et al*., 2000). Leite *et al*. (1994) were the first to explore a diagnostic method based on the *hrp* gene cluster. PCR primers

based on different regions of the *hrp* gene cluster were created, and restriction enzyme analysis (REA) was used to detect 28 different *X. campestris* pathovars. Unique banding patterns were observed for different xanthomonads. In addition, this method was also effectively utilized to detect the bacterial spot pathogen *Xcv* from tomato and pepper seed washings (Leite *et al*., 1995). Obradovic *et al*. (2004b) expanded on this work by developing PCR primers designed to amplify a 420-bp fragment of *hrpB2* from the four *Xanthomonas* spp. associated with bacterial spot. Following digestion of the amplified PCR products with three restriction enzymes (*Cfo*I, *Taq*I and *Hae*III), each species produced unique gel patterns that were used for their identification. Strayer *et al*. (2014) also utilized *hrpB2* by developing a multiplex quantitative PCR to simultaneously detect all four bacterial spot pathogens. Four species-specific probes and two primer sets were created based on the *hrpB2* sequence of each species. This method effectively and accurately identified pure cultures of 72 strains representing all four pathogens. It is an improvement over the other gel-based detection techniques because it saves time and materials when processing large sample numbers.

As *Xanthomonas* spp. can be disseminated via contaminated seed and plant material, the detection of bacterial spot in tomato or pepper seeds and seedlings is critical for the reduction of potential inoculum sources. Bacterial spot pathogens can be isolated and detected in soil, seed and plant material via a semi-selective culture medium called Tween. The pathogen's lipolytic enzymes utilize the fatty acid ester, Tween-80, as a substrate, which causes the formation of a white crystalline halo or precipitate around yellow bacterial spot colonies (McGuire and Jones, 1989; McGuire *et al*., 1986). In addition, the International Seed Federation (International Seed Federation, 2011; 2013) describes similar protocols for the detection of *Xanthomonas* spp. in tomato and pepper seed. These protocols involve the use of a modified Tween Medium B (mTMB) in combination with another semi-selective medium (CKTM) to isolate xanthomonads from seed (Sijam *et al*., 1991, 1992). Identification of the bacterial spot pathogens is completed on the basis of colony morphology on YDC and pathogenicity assays (ISF, 2011; 2013).

INTEGRATED PEST MANAGEMENT OF BACTERIAL SPOT

Xanthomonas strains are either endemic or introduced into field production areas via infested seed or contaminated transplant material (Jones *et al*., 1986; Sijam *et al*., 1991). Thus, the primary management strategy should include the use of pathogen-free, certified seed or disease-free transplant material (Ritchie, 2000). Preventative measures, such as treating seed with hot water, can significantly reduce disease inoculum in seeds. In addition, removal of potential inoculum sources, such as volunteer plants and infected host plants, should be carried out on a timely basis.

Field isolation from infected host plants in close proximity, accompanied by sanitation, physical removal and disposal of diseased crop material, and crop rotation with non-hosts, should be followed for disease management (Goode and Sasser, 1980; Ritchie, 2000).

Chemical control has been a primary focus in pest management strategies for bacterial spot of tomato and pepper. Copper-based bactericides have been used extensively for disease control. The grower standard for many years has been copper-based bactericides in combination with mancozeb or maneb (ethylene-bis-dithiocarbamates) (Conover and Gerhold, 1981; Marco and Stall, 1983). Copper-based bactericides became the standard in part because of the emergence of streptomycin resistance in xanthomonads in the 1960s (Thayer and Stall, 1961). Unfortunately, copper-tolerant xanthomonads have been present since the 1960s and were described by Marco and Stall (1983). In the 1980s, it was observed that copper–mancozeb combinations resulted in improved bacterial spot control because of the increased release of soluble copper when compared with copper alone (Conover and Gerhold, 1981; Marco and Stall, 1983). Therefore, it became the grower standard. However, disease control with copper–mancozeb is not effective when copper-tolerant strains are present together with optimal weather conditions for disease development (Jones and Jones, 1985; Obradovic *et al*., 2004a).

As indicated, disease management strategies using chemicals composed of metal ions such as copper have been explored. More recently, researchers have evaluated the use of different types of nanomaterials to control bacterial spot of tomato caused by *X. perforans*. Paret *et al*. (2013) evaluated a nanoscale version of the light-activated molecule TiO$_2$. TiO$_2$ was evaluated alone or doped with either silver (TiO$_2$/Ag) or zinc (TiO$_2$/Zn). TiO$_2$/Ag and TiO$_2$/Zn significantly reduced the bacterial populations *in vitro*. In glasshouse and field trials, TiO$_2$/Zn-treated plants showed significantly reduced disease severity when compared with those treated with copper–mancozeb. Unfortunately, phytotoxicity was observed, which limits the commercialization of this product. In addition, Ocsoy *et al*. (2013) developed a silver-based nanocomposite, termed Ag-dsDNA-GO. In this composite, silver ions accumulate in the major groove of dsDNA attached to graphene oxide (GO). Ag-dsDNA-GO showed high antibacterial activity at low concentrations (16 ppm) *in vitro*. In this study, Ag-dsDNA-GO at 100 ppm provided bacterial spot control at levels comparable with those of copper–mancozeb-treated plants. This was also confirmed in multiple glasshouse studies conducted by A. Strayer *et al*. (University of Florida, unpublished data). However, these results need to be further validated in field trials before this composite can be considered as a feasible alternative.

The plant activator Acibenzolar-*S*-methyl (ASM) (Actigard 50WG, Syngenta Crop Protection, Greensboro, NC, USA), which activates systemic acquired resistance (SAR), has been extensively

studied. Although ASM treatment for the first 6 weeks reduced disease symptoms, application throughout the season at weekly intervals was not efficient (Roberts *et al*., 2008). Effective disease control was reported when ASM was applied in combination with other biological and chemical control measures (Graves and Alexander, 2002; Obradovic *et al*., 2005), and has been suggested as a possible alternative to excessive reliance on copper bacteri-cides for rotation (Louws *et al*., 2001). In addition, ASM is not recommended for a full season application because of its effects on the plant and associated decreased yield (Graves and Alexander, 2002; Romero *et al*., 2001). It has been suggested that there is a fitness cost in expressing SAR by plants in the absence of a pathogen (Louws *et al*., 2001). Recent field studies examining ASM application rates and frequency have led to improved usage patterns to maximize disease control, whilst minimizing the impact on plant yields (Huang *et al*., 2012).

Antibiotics and molecular additives have also been evaluated for bacterial spot disease control. Streptomycin and kasugamycin have been labelled for use against bacterial spot in the glass-house. Streptomycin-resistant xanthomonads were reported very early after extensive use in the field (Thayer and Stall, 1962). A comparative study showed that kasugamycin was as effective as standard copper–mancozeb treatment for the control of bacterial spot (Vallad *et al*., 2010). It also significantly increased the total marketable yield of tomato when used together with other bactericides, such as Kocide (Ivors *et al*., 2006). Worthington *et al*. (2012) examined the use of a small molecule additive, 2-aminoimidazole (2AI), in combination with copper for the man-agement of bacterial spot. It is an analogue of the marine sponge natural product oroidin and lacks bactericidal activity alone. *In vitro*, 2AI, in combination with copper, was shown to suppress copper resistance in *X. euvesicatoria* and decreased biofilm for-mation. In field experiments, this combination reduced bacterial spot disease and increased bell pepper fruit yields.

Efforts to identify biological control strategies for the control of bacterial spot have been extensive. Bacteriophages have been widely studied for the control of bacterial spot disease in tomato and pepper and other crops (Fig. 5; Balogh *et al*., 2003). Foliar applications of bacteriophage have provided effective control of disease when compared with a copper–mancozeb standard (Flaherty *et al*., 2000; Momol *et al*., 2008; Obradovic *et al*., 2004a). However, the efficacy of bacteriophage depends on the environmental factors that determine phage survival in plants (Iriarte *et al*., 2007). Other biological agents have shown limited efficacy in controlling the disease when applied alone, but research has begun to focus on improved integration into disease management programmes (Fravel, 2005). During an evaluation of 50 different biological agents, *Cellulomonas turbata* BT1 showed the highest reduction in disease severity and *Pseudomonas syringae* Cit7 gave the most consistent control (Byrne *et al*., 2005). In addition to these two bacterial species, various plant growth-
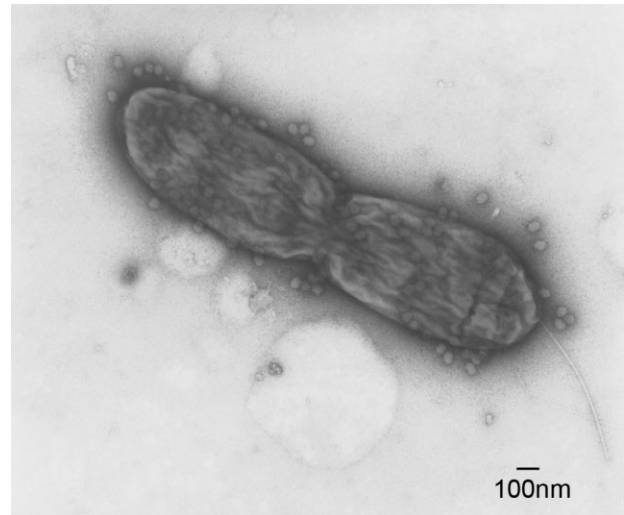

Fig. 5 Foliar applications of bacteriophage have provided effective control of bacterial spot disease on tomato and pepper. The figure shows bacteriophage of *X. vesicatoria* attached to dividing cell of *Xanthomonas*. Photograph courtesy of Dr Botond Balogh, Nichino America, Inc.

promoting rhizobacteria (PGPRs) and *Bacillus pumilis* have shown effectiveness in suppressing bacterial spot in some field trials (Ji *et al*., 2006).

Host resistance has been a major goal of pepper and tomato breeding programmes. Conventional resistance (*R*) gene-mediated bacterial spot resistance relies on known *Avr* gene–*R* gene inter-actions. However, genetic resistance may be ineffective because of shifts in the bacterial populations that emerge even before resist-ant cultivars are deployed (Gassmann *et al*., 2000). These race shifts may impact the durability of plant resistance. Quantitative or multigenic resistance to bacterial spot has also been identified, and appears to be more durable than plant resistance associated with hypersensitive reactions.

Six dominant *R* genes have been identified in pepper: *Bs1*; *Bs2*; *Bs3* (Stall *et al*., 2009); *Bs4C* (Strauß *et al*., 2012); *Bs7* (Potnis *et al*., 2012); and BsT (Minsavage *et al*., 1990). They inter-act with *avrBs1*, *avrBs2*, *avrBs3*, *avrBs4C*, *avrBs7* and *avrBsT*, respectively, to provide a hypersensitive type of resistance. Although other *R* genes have been identified using classical *Avr*–*R* gene studies, *Bs4C*, present in pepper (*C. pubescens* PI235047), was identified on the basis of transcriptome profiling with next-generation sequencing (RNA-seq), and was expressed only in the presence of *avrBs4* (Strauß *et al*., 2012). The *Bs4C* gene was determined using bioinformatics to encode a structur-ally unique R protein. Among quantitative resistances, various levels of resistance to bacterial spot have been found in pepper accessions with two to three recessive *R* genes (Jones *et al*., 2002; Riva *et al*., 2004). Efforts to transfer these quantitative resistances from various PI accessions into commercial varieties led to backcross populations with various levels of resistance;

additional intercrossing of plants specifically from PI163192 and PI271322 backcross lines resulted in a level of resistance higher than either parent under field conditions (Jones *et al*., 2002). This additive resistance was found to be governed by the recessive genes *bs5* and *bs6*. Several seed companies are currently incorporating these recessive genes into the commercial cultivars (Stall *et al*., 2009).

Four sources of resistance have been identified in tomato: Hawaii 7998 derived *Rx1*, *Rx2* and *Rx3* (Yang *et al*., 2005); *Xv3* (Astua-Monge *et al*., 2000a); *RXopJ4* (Sharlach *et al*., 2013); and *Bs4* (Bonas *et al*., 1993). These four sources interact with *AvrRxv* (Whalen *et al*., 1993), *AvrRxv*, *AvrXv3*, *XopJ4* and *AvrBs4*, respectively, to elicit a hypersensitive response. These tomato resistance genes were first identified in Hawaii 7998 (H7998), Hawaii 7981 (H7981) and *S. pimpinellifolium* accession PI128216, respectively (Wang *et al*., 2011; Whalen *et al*., 1993; Yang and Francis, 2007). Another approach in tomato has been to identify genes that confer resistance to multiple bacterial spot races. Two loci, *QTL-11* (from H7998, on chromosome 11) and *QTL-3* (from PI114490), are associated with resistance to multiple races (Hutton *et al*., 2010; Yang *et al*., 2005). Quantitative resistance to bacterial spot in tomato lines has been found to various degrees and against various races. PI114490 has shown resistance against races T1, T2 and T3, which represent three bacterial spot species; therefore, there is considerable interest to introgress this quantitative resistance into commercial lines (Scott *et al*., 2006).

Transgenic resistance offers another promising means for the control of bacterial spot. Recent technologies, such as TALEN-based genome editing, hold the potential to engineer disease resistance given the well-studied interactions of AvrBs3-like proteins with pepper *Bs3* alleles (Romer *et al*., 2009). Transgenic tomato plants expressing the Arabidopsis pattern recognition receptor (PRR) EF-Tu receptor (EFR), a class of immune receptor, conferred resistance to *X. perforans* strains (Lacombe *et al*., 2010). The search for a core effector essential to pathogen virulence/fitness and its corresponding plant target led to the deployment of the *Bs2* gene from pepper, a very close relative of tomato that interacts in a gene-for-gene manner, with the AvrBs2 effector, and resistance against bacterial spot was observed in tomato expressing *Bs2* originally cloned from the pepper gene (Tai *et al*., 1999). Recently, a tomato transgenic *Bs2* overexpression line expressed in VF 36, and designated VF 36-BS2, and other transgenic lines carrying *Bs2*, have been tested for durable resistance in field trials against bacterial spot pathogens. These lines conferred a high level of resistance against field populations of *X. perforans* in Florida, under high disease pressure, in the absence of copper pesticides. In addition, these transgenic lines had double the fruit yields of those lines lacking the Bs2 transgene (Horvath *et al*., 2012). However, some rare mutant bacterial strains with mutations in *avrBs2* were isolated that could overcome the resistance by losing the ability to be recog-

nized by the Bs2 transgene. In the future, transgenic plants with multiple targets for such core effectors might reduce the rate of mutations (Dangl *et al*., 2013).

FUTURE PROSPECTS

Diversity among the bacterial spot xanthomonads has been observed in various studies. Given this diversity, very little is known about the pathogenicity factors of the strains from a worldwide collection. Cost-effective, next-generation sequencing methods would be useful in providing a genetic snapshot of these diverse strains from different parts of the world. Recent MLSA studies have indicated differences within a single species. A genome of a single strain cannot provide the correct representation of an entire species and, in some cases, not even of a regional population; therefore, sequencing many bacterial spot strains would also help in better defining the core virulence factors of bacterial spot xanthomonads. Finding targets for these core factors would offer candidates for the development of transgenic varieties. Current efforts for control of the disease have pointed towards several strategies that could be employed in conjunction. Rotation and a combination of various chemical bactericides have been suggested in response to the current expansion of chemically tolerant bacterial spot populations. The identification of potential new bactericides or chemical enhancers to manage bacterial spot disease is equally important. Nanoparticles, such as TiO_2 and Ag-dsDNA-GO, appear to have the potential to effectively manage bacterial spot disease. The nano-form of other bactericidal heavy metals, such as copper, could be an area to explore in the future.

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