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MINI-REVIEW

Epidemiology, diversity, and management of bacterial spot of tomato caused by *Xanthomonas perforans*

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Abstract

Tomato is an important crop grown worldwide. Various plant diseases cause massive losses in tomato plants due to diverse biotic agents. Bacterial spot of tomato (BST) is a worldwide disease that results in high losses in processed and fresh tomato. *Xanthomonas perforans*, an aerobic, single-flagellated, rod-shaped, Gram-negative plant pathogenic bacterium, is one of the leading causes of BST. Over the past three decades, *X. perforans* has increasingly been reported from tomato-growing regions and became a major bacterial disease. *X. perforans* thrives under high humidity and high temperature, which is commonplace in tropical and subtropical climates. Distinguishing symptoms of BST are necrotic lesions that can coalesce and cause a shot-hole appearance. *X. perforans* can occasionally cause fruit symptoms depending on disease pressure during fruit development. Short-distance movement in the field is mainly dependent on wind-driven rain, whereas long distance movement occurs through contaminated seed or plant material. *X. perforans* harbors a suite of effectors that increase pathogen virulence, fitness, and dissemination. BST management mainly relies on copper-based compounds; however, resistance is widespread. Alternative compounds, such as nanomaterials, are currently being evaluated and show high potential for BST management. Resistance breeding remains difficult to attain due to limited resistant germplasm. While the increased genetic diversity and gain and loss of effectors in *X. perforans* limits the success of single-gene resistance, the adoption of effector-specific transgenes and quantitative resistance may lead to durable host resistance. However, further research that aims to more effectively implement novel management tools is required to curb disease spread.

Key points

- *Xanthomonas perforans causes bacterial spot on tomato epidemics through infected seedlings and movement of plant material.*
- *Genetic diversity plays a major role in shaping populations which is evident in loss and gain of effectors.*
- *Management relies on copper sprays, but nanoparticles are a promising alternative to reduce copper toxicity.*

Keywords Seeds · Transplants · Genomes · Effectors · Phylogenetics · Nanoparticles

Significance of bacterial spot of tomato

Bacterial spot of tomato (BST) is a widespread disease that can affect fresh market and processing tomatoes worldwide. BST is caused by four species of *Xanthomonas*: *X. perforans*, *X. euvesicatoria*, *X. gardneri*, and *X. vesicatoria* (Jones et al. 2004). BST caused by *X. euvesicatoria* can result in up to 50% marketable yield losses (Dougherty 1978; Pohronezny and Volin 1983). Yield losses attributed to BST caused by *X. perforans* are likely similar to *X. euvesicatoria*. BST was initially described to be caused by a single species, *X. campestris* pv. *vesicatoria*, with four distinct races, T1, T2, T3, and T4, identified on differential tomato hosts (Stall et al. 2009). Recent molecular-based studies have proposed changing the

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nomenclature of *X. perforans* to *X. euvesicatoria* pv. *perforans* (Barak et al. 2016; Constantin et al. 2016). In this review, we will follow the nomenclature described by Jones et al. (2004), which defined four BST-causing species.

Historically, *X. euvesicatoria* and *X. vesicatoria* occurred more commonly than *X. perforans* and *X. gardneri* (Bouzar et al. 1994; Potnis et al. 2015). However, *X. perforans* quickly took over since it was first identified in 1991 (Jones et al. 1995) and has been the only species isolated from Florida tomatoes for over a decade (Horvath et al. 2012; Klein-Gordon et al. 2021; Schwartz et al. 2015; Vallad et al. 2013). So far, *X. perforans* has been reported as causing disease within different areas worldwide, such as Canada (Ontario), Australia, Brazil, Ethiopia, Iran, Italy, Korea, Mexico, Nigeria, Southwest Indian Ocean (Mauritius, Mayotte, Seychelles), Taiwan, Tanzania, Thailand, and the USA (Alabama, Florida, Georgia, Illinois, Indiana, Louisiana, Mississippi, North Carolina, Ohio, South Carolina) (Abbasi et al. 2015; Abrahamian et al. 2019a, 2019b; Adhikari et al. 2019; Aiello et al. 2013; Araújo et al. 2017; Burlakoti et al. 2018; Egel et al. 2018; Hamza et al. 2010; Kebede et al. 2014; Khanal et al. 2021; Lewis Ivey et al. 2016; Ma et al. 2011; Mbega et al. 2012; Myung et al. 2009; Osdaghi et al. 2017; Roach et al. 2018; Schwartz et al. 2015; Timilsina et al. 2015, 2016). The geographical expansion of *X. perforans* is likely attributed to long-distance movement, which is commonly associated with movement of infested seeds and transplants (Gitaitis et al. 1992; Kebede et al. 2014; Potnis et al. 2015). Studies that aim to better understand *X. perforans* biology and find new strategies for disease management to reduce the damage caused by this pathogen will continue to be important as this pathogen becomes established in other parts of the world. Therefore, in this review, we provide an overview of recent research and advances in the epidemiology, genome diversity, and disease management specifically for *X. perforans*.

Epidemiology in the greenhouse and field

Symptoms of BST occur on aboveground plant parts such as the foliage, stems, pedicels, sepals, and fruits (Jones and Miller 2014). Foliar and stem lesions start as irregular water-soaked lesions up to 3 mm in diameter. Water-soaked lesions are easily seen when leaves are wet. Lesions become dry, dark, and necrotic and may produce a faint halo (Jones and Miller 2014). During later stages of symptom development, lesions coalesce and plants become blighted and defoliated (Fig. 1). When the center tissue of leaf lesions collapses, a shot-hole appearance can result (Jones and Miller 2014). Although less common, pith necrosis, characterized by vascular browning, has been associated with some *X. perforans* strains in tomatoes (Aiello et al. 2013). Furthermore, co-infections of *Pseudomonas* spp. with *X. perforans* can enhance bacterial population and result in more severe pith necrosis symptoms in tomato stems (Aiello et al. 2017). Interestingly, this observation was not observed with strains isolated from Florida (J.B. Jones, unpublished data). It is possible that pith necrosis symptoms might be cultivar-dependent or endophyte-dependent. Blighting and defoliation can directly reduce fruit yield, as well as indirectly lead to additional losses due to the exposure of fruit to environmental elements (e.g., sun and rain, resulting in sun scald and rain check, respectively). However, several field studies indicate that defoliation alone does not appear to account for the yield losses (G.E. Vallad, unpublished data; Jones 1979; Pohronezny and Volin 1983). *Xanthomonas* spp. can also directly infect fruit. Fruit lesions are initially small, and as symptoms progress, the lesions become dark, scab-like, and often possess a dark green to yellow halo around the lesion (Fig. 1D). *X. perforans* does not appear to be associated with a high frequency of fruit lesions, unlike *X. euvesicatoria* and *X. vesicatoria* (Potnis et al. 2015). Fruit lesions typically render the fruit unmarketable and make the fruit prone to infection by opportunistic pathogens that cause postharvest decays.

X. perforans is a well-established pathogen that colonizes different growth stages of tomatoes in greenhouses or fields. Under highly intensive tomato production practices, seedlings are the preferred starting material for growing field tomato. Typically, seedlings are grown under high plant densities creating a favorable environment for disease development. *X. perforans* requires high relative humidity and optimal temperatures ranging from 25 to 28 °C for enhancing disease development (Abrahamian et al. 2021; Obradovic et al. 2008). *X. perforans* colonizes tomato seedlings as an epiphyte and remains for a period of time prior to symptom development (Abrahamian et al. 2021). Furthermore, inside a seedling production facility, latent infections can result in transmission to neighboring plants in a short time period due to overhead irrigation practices (Abrahamian et al. 2021). Irrigation practices such as the overhead irrigation system appears to be a contributing factor in short-distance transmission due to aerosol dispersal which appears to carry *X. perforans* inoculum over short distances (Abrahamian et al. 2021). Also, low temperatures (<24 °C) and lower initial inoculum results in very little dispersal compared to higher temperatures (>24 °C). Based on an epidemiological model, we predicted that at 27 °C and a high inoculum concentration, *X. perforans* can cover a distance of more than 1.5 m within 5 days. As a result, asymptomatic infections can easily be overlooked, and the pathogen can then move long distances from transplant facilities to fields (Abrahamian et al. 2019c). In the field, inoculum sources of *X. perforans* are introduced through initially infected but asymptomatic seedlings or through nearby infected plants, such as volunteer plants or weeds (Abrahamian et al. 2021; Jones et al. 1986). Recently, Sharma et al. (2021)

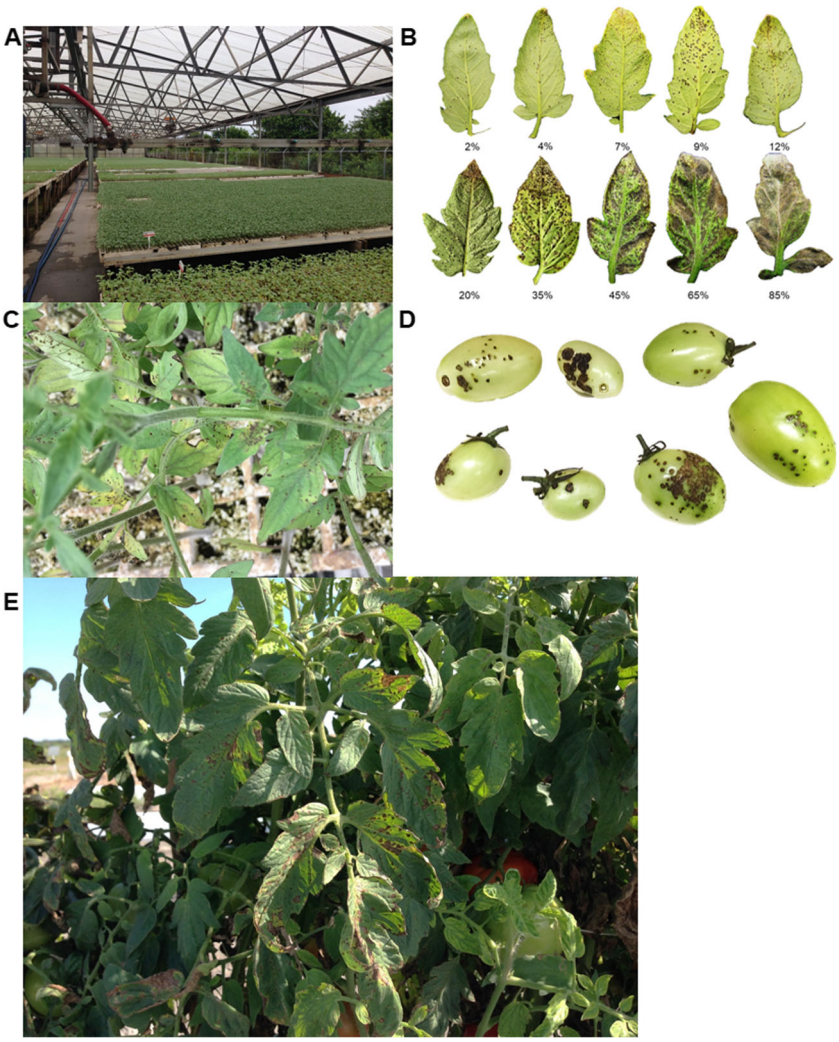

Fig. 1 Bacterial spot symptoms caused by *Xanthomonas perforans* on tomato. **A** Tomato seedlings grown under highly intensive transplant operation which provide conducive conditions for disease development. **B** Variable rates of bacterial spot disease severity on tomato leaves determined using the APS Assess 2.0: Image Analysis Software for Plant Disease Quantification (American Phytopathological Society, Minnesota, USA). **C** Bacterial spot symptoms on tomato seedlings. **D** Necrotic sunken lesions caused by *X. perforans* on tomato fruits under field conditions. **E** Bacterial spot symptoms on field-grown tomato plants.

demonstrated that *X. perforans* was capable of spreading in the field three times faster than a mutant strain lacking an effector gene, *XopJ2*.

Recent surveys in Brazil have shown the presence of *X. perforans* on weeds growing in proximity to tomatoes, such as *Nicandra physalodes* and *Solanum americanum* (Araújo et al. 2015). *X. perforans* has been mostly limited to tomato; however, strains have been isolated from tomato which were capable of infecting pepper plants under natural or experimental conditions (Hernández-Huerta et al. 2021; Newberry et al. 2019; Schwartz et al. 2015). Bophela et al. (2019) also reported *X. perforans* strains causing bacterial blight on nursery plants and young *Eucalyptus pellita* trees. *X. perforans* strains recovered from *Eucalyptus* were not pathogenic on tomato (J.B. Jones, unpublished data). *X. perforans* might also colonize and move on leaf surfaces via hitchhiking with resident microflora. A study by Hagai et al. (2014) showed that *X. perforans* could induce movement of *Paenibacillus vortex*, a soil-dwelling bacterium, and *X. perforans* movement was in turn assisted by *P. vortex*. As a result, several factors play a role in enhancing epidemics and as a result fulfilling the criteria of the disease triangle, such as conducive environmental conditions, time, and pathogen-related factors (e.g., presence/absence of effectors and commensal bacteria).

Pathogen genome

The first publicly available *Xanthomonas* genome for a strain that causes BST was for *X. euvesicatoria*, sequenced by Thieme et al. (2005). The delineation of the BST causal agents into four distinct species by Jones et al. (2004) prompted the need for genome sequences of the other three species, which was conducted by Potnis et al. (2011). The first *X. perforans* strain ever sequenced, strain 91-118, contains a single circular chromosome of 4,898,349 bp with 65% GC content. The genome consists of 4,178 genes, of which 4,084 are coding sequences (CDS) and 94 are ribosomal genes. Ribosomal genes consist of two ribosomal RNA operons having 5S, 16S, and 23S (rRNA). Furthermore, the genome contains 53

transfer RNA (tRNA) and 35 non-coding RNA (ncRNA). Genes and gene clusters of interest are mainly those pertaining to pathogenicity and virulence of the pathogen. For instance, *X. perforans* contains several secretion systems, such as types I, II, III, IV, V, and VI, similar to other pathogenic *Xanthomonas* (Potnis et al. 2011; Alvarez-Martinez et al. 2021). Secretion systems are important in host-pathogen interactions.

The type I secretion system (T1SS) in *Xanthomonas* is represented by the presence of tyrosine sulfotransferase (RaxST) and three predicted T1SS genes, a membrane fusion protein (RaxA), adenosine triphosphate–binding cassette transporter (RaxB), and an outer membrane protein (RaxC) (Alvarez-Martinez et al. 2021; da Silva et al. 2004). In *X. perforans*, *raxST*, *raxA*, and *raxB* are located in a single operon (Liu et al. 2019). Similar to *X. oryzae* pv. *oryzae* (*Xoo*), *raxC* is present outside the *raxSTAB* operon (da Silva et al. 2004). Furthermore, Ax21 is also present in *X. perforans*, a predicted T1SS protein. In *Xoo*, Ax21 may serve as a quorum sensing molecule and be implicated in rice plant immune response and interaction with the Xa21 resistance locus (da Silva et al. 2004). However, the implications of the T1SS and function of Ax21 in *X. perforans* has not been determined.

The type II secretion system (T2SS) is encoded by the *xps* and *xcs* gene cluster in *X. perforans* (Potnis et al. 2011). Cell wall degrading enzymes, proteases, lipases, and xylanases are secreted by the T2SS. In *X. euvesicatoria*, the T2SS contributes to virulence and is regulated by master regulators HrpX and HrpG, which are also regulators of the type III secretion system (T3SS) (Szczesny et al. 2010). The contribution of T2SS to pathogenicity is not well understood in *X. perforans*, but it is assumed to be similar to *X. euvesicatoria*. A detailed list of the enzymes and their classes is provided by Potnis et al. (2011).

The T3SS or "hypersensitive response and pathogenicity" (*hrp*) cluster in *X. perforans* shows gene synteny compared to the other three *Xanthomonas* species that cause BST and belongs to the hrp2 cluster family (Alvarez-Martinez et al. 2021; Potnis et al. 2011). The T3SS is a major virulence factor that enables host specialization and infection by *X. perforans* through assembly of a pilus structure and translocation of effector proteins into host cells. T3SS assembly is regulated through the HrpX and HrpG master switches. A unique feature of *X. perforans* T3SS is the presence of the x*opAE* effector gene, a fusion of *hpaG* and *hpaF* associated with the *hrp* cluster. Effectors associated with pathogenicity are discussed in the following section. *Hrp* gene clusters are thought to be conserved across strains; however, a recent comparative genomics study revealed that *hrp* genes are not conserved within the same species. For instance, the *hrp* gene cluster in some *X. perforans* strains showed unique sequences and horizontal gene transfer of *hrp* genes from *X. euvesicatoria* (Jibrin et al. 2018).

In addition, two type IV secretion systems (T4SS) are present in *X. perforans*: one located on the chromosome and another on the plasmid, with the latter predicted to be a result of horizontal gene transfer (Potnis et al. 2011). T4SS are known to transport DNA-protein complexes in other plant pathogenic bacteria (Sgro et al. 2019). However, recent studies in *X. citri* have shown that a plasmid-borne T4SS is capable of transferring toxic effectors into competing bacteria resulting in cell death of the other bacterial cells (Sgro et al. 2019). Briefly, VirD4, an inner membrane-associated ATPase, recognizes and translocates toxic substrates through a common C-terminal domain, XVIPCDs, through the T4SS pilus. Comparative genomics revealed the presence of the *X. citri*-like T4SS in *X. perforans* strain Xp4-20 (Alvarez-Martinez et al. 2021). However, implications of the T4SS in *X. perforans* have not been determined.

Type V secretion systems (T5SS) are composed of one or two protein units and are classified into different classes, Va to f (Meuskens et al. 2019). T5SS in bacteria translocate effectors, such as adhesins, enzymes, and toxins which play an important role in adhesion to host and non-host plants, biofilm formation, and cell aggregation (Alvarez-Martinez et al. 2021). Three classes of T5SS, Va (EstA), Vb (FhaB/FhaC), and Vc (XadA), are present in *X. perforans* genomes.

Type VI secretion systems (T6SS) are generally involved in bacterial antagonism through delivery of toxic products. However, there is no evidence that T6SS are involved in direct interference with plants. T6SS are classified into five groups, i1 to i5 (Boyer et al. 2009). In *X. perforans*, the T6SS belongs to the i3 group, and two T6SS operons belonging to two subgroups i3* and i3*** occur in the genome. Recently, a single gene knockout in the T6SS showed reduced virulence of *X. perforans* against *Sphingomonas taxi* (Turner 2020). However, further work is needed to understand the role of T6SS in antagonism and leaf colonization.

In addition, other significant genomic features occur in *X. perforans*, such as a lipopolysaccharide (LPS) cluster which plays a role in eliciting plant defense. The LPS cluster in *X. perforans* is unique in comparison to the other *Xanthomonas* species and is 17.3 kb in length with 12 coding sequences (Potnis et al. 2011). This unique LPS cluster might be attributed to host specificity of *X. perforans* (Potnis et al. 2011). Furthermore, diffusible signaling factor (DSF), a mediator of cell-to-cell signaling, is synthesized, perceived, and transduced by RPF proteins. In *X. perforans*, the *rpf* gene cluster contains *rpfB*, *rpfF*, *rpfC*, *rpfH*, and *rpfG*. Furthermore, a unique feature of xanthomonads is the presence of the gum operon which produces the extracellular polysaccharide xanthan. The *X. perforans* gum operon contains 14 ORFs, *gumA* through *gumN*. Copper tolerance genes are also present within the *X. perforans* genome. The copper tolerance operon contains three copper homeostasis (*coh*) genes, *cohL*, *cohA*, and *cohB*. CohL serves as a regulatory element of the

copper operon, whereas CohA and CohB are involved in copper binding (Behlau et al. 2011). Chromosomally encoded copper genes can provide *X. perforans* tolerance against copper up to concentrations of 75 ppm on mannitol glutamate yeast agar (Potnis et al. 2011). Bacteriocins are also important features of *X. perforans* due to their involvement in bacterial antagonism against *X. euvesicatoria* (Hert et al. 2005). Three bacteriocin loci, BCN-A, BCN-B, and BCN-C, have been shown to contribute to antagonism (Hert et al. 2009; Tudor-Nelson et al. 2003). BCN-A is part of a five gene operon encoded by *ORFA*, two additional genes *ORF2* and *ORF4* are involved in bacteriocin delivery, ORF3 is involved in production of BCN-A, and ORF5 as an immunity factor against its own bacteriocin (Marutani-Hert et al. 2020). On the other hand, BCN-B and BCN-C are encoded by single genes, *bcnB* and *bcnC*, respectively, and show protease activity (Marutani-Hert et al. 2020). Also, more recently, *X. perforans* strains were shown to harbor transcription activator-like (TAL) effectors, such as PthXp1, which promote bacterial virulence (Newberry et al. 2019). Overall, *X. perforans* genome organization is more similar to *X. euvesicatoria* than *X. gardneri* or *X. vesicatoria*, which is evident through its genetic diversity (further discussed in the following sections).

Plasmids

Plasmids are DNA elements that can harbor important genes affecting virulence and fitness of *X. perforans*, such as effectors, secretion systems, and drug resistance loci. Strain LH3, from Mauritius, contains four plasmids, pLH3.1, 3.2, 3.3, and 3.4, of different sizes, 222 kb, 69 kb, 45 kb, and 38 kb, respectively (Richard et al. 2017). In a survey of *X. perforans* strains from Australia, a plasmid of 41 kb was found in one strain and plasmid sizes of less than 10 kb in nine strains (Roach et al. 2019). In Alabama, *X. perforans* strains contained plasmids of 43 to 45 kb in size (Newberry et al. 2019). pLH3.1 has a copper resistance operon encoding the genes *copL*, *copA*, and *copB*, which are not identical to *coh* genes. Three plasmids harbor a T4SS cluster. pLH3.2 carries a T3SS effector gene *xopE2*, similar to plasmids isolated from strains in Alabama (Newberry et al. 2019). Also, another important plasmid-borne T3SS effector is *avrBsT* (*xopJ2*), this gene encodes an effector which plays a role in host range and fitness (Abrahamian et al. 2018; Schwartz et al. 2015; Timilsina et al. 2016). Other plasmid-borne T3SS genes, such as *xopAQ*, *xopE3*, *xopH*, *xopJ6*, and *xopAO*, were observed in diverse *X. perforans* strains (Iruegas-Bocardo et al. 2018; Newberry et al. 2019, 2020; Roach et al. 2019). Interestingly, Newberry et al. (2019) showed the presence of a TAL effector, *avrHah1*, with high similarity from *X. gardneri*, indicating potential horizontal gene transfer.

Pathogenicity and molecular mechanisms of host infection

Multi-faceted host-pathogen interactions occur during *X. perforans* colonization of tomato plants. *X. perforans* possesses a multitude of enzymes, adhesins, effectors, and other virulence factors that are delivered by specialized secretions systems that enable it to cause disease (delivery systems discussed in the "Pathogen genome" section). One of the most important secretions systems is the T3SS encoded by the hypersensitive response and pathogenicity-2 (hrp-2) genes. The hrp-2 genes of *X. perforans* are similar to the gene cluster found in *Ralstonia*, *Acidovorax*, *Burkholderia*, and other *Xanthomonas* species (Tampakaki et al. 2010). T3SS are complex membrane-spanning proteins, also referred as injectisomes due to their ability to deliver type III secreted effectors (T3SE). T3SS in plant pathogenic bacteria are thoroughly reviewed elsewhere (Büttner and He 2009; Büttner 2012; Chang et al. 2014). The total number of effectors of *X. perforans* is difficult to enumerate due to the continuous loss and gain of effectors in the population. For instance, in a comparative genomics study, Potnis et al. (2011) identified 11 core effectors across all bacterial spot-causing xanthomonads and 12 unique or shared effectors in *X. perforans* strain 91-118. However, the actual number of core effectors is unknown as more genome sequences reveal a high amount of effector profile diversity. For instance, several effectors have been identified in more recently recovered *X. perforans* strains that are missing from 91-118, such as AvrBsT, XopJ6, XopE2, and XopE4 (Klein-Gordon et al. 2021; Newberry et al. 2019; Schwartz et al. 2015). Furthermore, many strains of *X. perforans* have lost the function of the *avrXv3* gene found in 91-118 either due to a transposon insertion or frameshift (Klein-Gordon et al. 2021; Newberry et al. 2019; Timilsina et al. 2016). Table 1 shows the total number of effectors reported to date in *X. perforans* genomes.

Effectors have multiple functions, sometimes redundant, inside the plant cell which determines the outcome of the interaction, such as enhanced disease symptoms and colonization or limited disease and resistance. Incompatible reactions, i.e., disease progression, occurs when effectors suppress effector-triggered immunity (ETI) or pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Yuan et al. 2021). On the other hand, limited disease occurs when effectors are recognized by a cognate resistance gene resulting in a gene-for-gene interaction. The functions of several effectors have been characterized in *X. euvesicatoria* (Kim and Mudgett 2019; Popov et al. 2016; Teper et al. 2014). However, effector function differs between hosts; for instance, some effectors might trigger an HR in pepper but enhance pathogenicity and fitness in tomato (Abrahamian et al. 2018; Schwartz et al. 2015). Even though similarities in effector functions across pathogen species

Table 1. *Xanthomonas perforans* type III secreted effectors, function, and associated phenotypes.

Effector	Synonym	Function/feature	Notes	Reference
AvrBs2		Glycerolphosphoryl diester phosphodiesterase	Fitness factor	Kearney and Staskawicz 1990
AvrBsT	XopJ2	Acetyltransferase, C55 cysteine protease	Virulence and fitness factor; host range determinant	Abrahamian et al. 2018; Kim et al. 2010; Schwartz et al. 2015
AvrHah1		Transcription activator-like	Virulence and fitness factor	Newberry et al. 2019
PthXp1		Transcription activator-like	Enhanced chlorosis and lesion	Newberry et al. 2019
XopA		Unknown	Involved in effector translocation; virulence factor	Noël et al. 2002
XopAD		SKWP repeat protein		Potnis et al. 2011
XopAE		LRR protein	Inhibits flg22-induced callose deposition	Popov et al. 2016
XopAF	AvrXv3	Transcription activator-like	Absent in most *X. perforans* race T4 strains	Timilsina et al. 2016
XopAK		Unknown		
xopAO		Unknown		
XopAP		Unknown	Inhibits flg22-induced callose deposition	Popov et al. 2016
XopAR		Unknown		
xopAQ		Unknown		
XopC2		Haloacid dehalogenase-like hydrolase		Potnis et al. 2011
XopD		C48-family SUMO cysteine protease	Suppress plant defenses	Kim and Mudgett 2019
XopE1		Putative transglutaminase		Potnis et al. 2011
XopE2		Putative transglutaminase	Inhibits flg22-induced callose deposition	Popov et al. 2016
XopE4		Unknown		
XopF1		Unknown		
XopF2		Unknown	Inhibits flg22-induced callose deposition	Popov et al. 2016
XopI		F-box domain		Potnis et al. 2011
XopJ6		Acetyltransferase		Iruegas-Bocardo et al. 2018
XopJ4	AvrXv4	SUMO protease; Acetyltransferase		Roden et al. 2004
XopK		Unknown		
XopL		LRR protein		Potnis et al. 2011
XopN		14–3–3 binding protein	Inhibits host defense	Taylor et al. 2012
XopP1		Unknown		
XopP2		Unknown		
XopP3		Unknown		
XopQ		Nucleoside hydrolase; 14–3–3 binding protein	Host range determinant	Teper et al. 2014
XopR		Unknown		
XopV		Unknown		
XopX		Unknown	Virulence factor and modulate host defenses	Stork et al. 2015
XopZ		Unknown		

and hosts can occur, in this review, we will only discuss characterized effectors of *X. euvesicatoria* and *X. perforans* in relation to tomato (Figs. 2 and 3).

Some effectors have dual roles as suppressors and activators of plant immunity. For instance, XopD is a non-TALE that localizes to the nucleus and induces strong activation of a basic helix-loop-helix transcription factor, bHLH132. Activation of bHLH132 results in slower symptom development of *X. euvesicatoria* and delayed chlorosis in tomato (Kim and Mudgett 2019). XopX is another effector with a

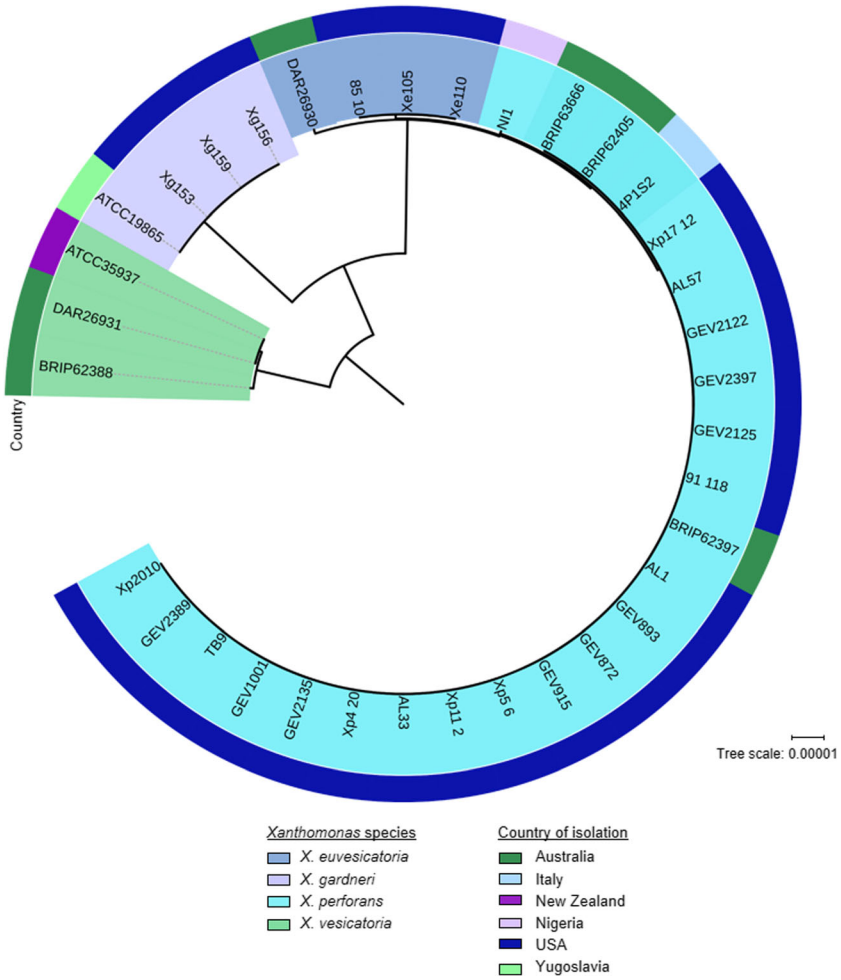

Fig. 2. Phylogenetic tree of 35 bacterial leaf spot-associated *Xanthomonas* strains based on genetic distance of core gene SNPs and recombination correction. Species identity is denoted by highlights overlaid on strains. The colored block within the ring surrounding the phylogenetic tree denotes the country of isolation for the respective strain. The phylogenetic tree was constructed with methods from Klein-Gordon et al. (unpublished). Briefly, core genes were identified via Roary (v. 3.12.0), specifying core genes as those with a 75% minimum percentage identity for BLASTp and present in all genomes. 2,341 core genes were identified. ModelTest-NG was used to identity the appropriate substitution model (i.e., GTR+I+G). RAxML (v. 8.2.10) was used with 1,000 rapid bootstraps to conduct phylogenetic analyses, and then the best-scoring multilocus tree was corrected to account for recombination with ClonalFrameML (v. 1.0). iTOL (v. 6) was used to visualize the subsequent phylogenetic tree. Strains included in the phylogenetic analysis were originally reported by Abrahamian et al. (2019c), Jibrin et al. (2018), Newberry et al. (2019), Potnis et al. (2011), Roach et al. (2019), Schwartz et al. (2015), Thieme et al. (2005), and Torelli et al. (2015).

dual role that modulates PTI responses in plants. XopX was found to increase ethylene-related genes, plant cell death, and PTI-related genes (Stork et al. 2015). XopN was shown to interact with the 14-3-3 host protein, SlTFT1, which is required to inhibit bacterial growth (Taylor et al. 2012). Also, XopQ was found to have a 14-3-3 binding domain which interacts with 14-3-3 host isoforms, TFT4, and possibly modulates host defenses related to ETI (Teper et al. 2014). XopQ was found to be a host range determinant in *X. perforans*. The knockout of *xopQ* and *avrBsT* resulted in the expanded host range of *X. perforans* onto *N. benthamiana* and pepper (Schwartz et al. 2015). AvrBsT (XopJ2) suppresses early defense signaling, such as callose deposition and defense-marker gene expression, in tomato plants (Kim et al. 2010). Furthermore, AvrBsT appears to contribute to increased spread of *X. perforans* under field conditions, possibly by increased egress of bacteria in infected plants (Abrahamian et al. 2018). Several other effectors, such as XopAE, XopAJ, XopE2, XopF2, XopN, and XopX, have been shown with *X. euvesicatoria* to inhibit PTI responses (Popov et al. 2016).

Genetic diversity and evolution

X. perforans strains are genetically diverse, and new genome sequences continue to reveal new phylogenetic groups within the *X. perforans* species. The first two sequenced strains of *X. perforans*, isolated in Florida in the 1990s, represented a

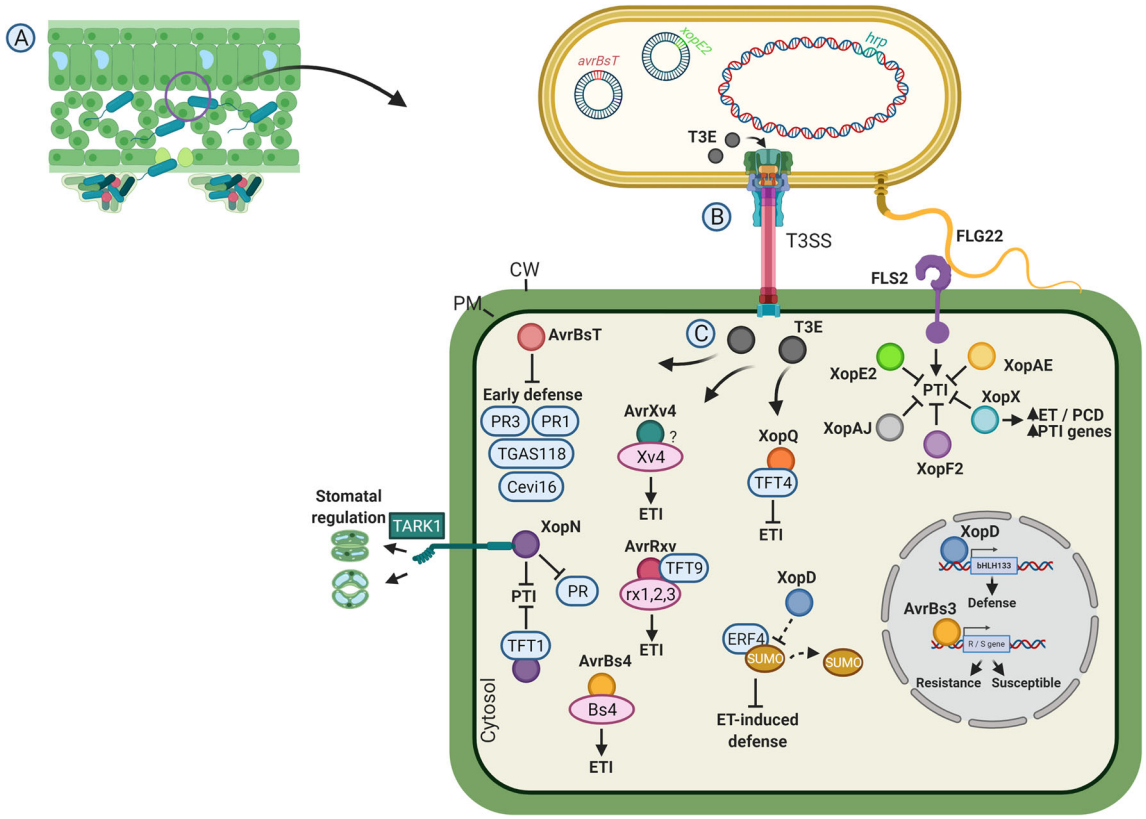

Fig. 3 *Xanthomonas perforans* molecular interactions in tomato cells. (A) A cross-section of a bacterial biofilm, which ensures pathogen survival in the phyllosphere. External stimuli (e.g., conducive conditions) triggers bacterial colonization and invasion through the stomata. (B) *X. perforans* produces several virulence genes and effectors. The type III secretions system (T3SS) is encoded by the *hrp* genes. Most type III secretion effector (T3SE) genes are chromosomally encoded and some plasmid-borne, such as *avrBsT*, *avrBs4* or *xopE2*. (C) Specialized interactions of T3SEs occur through delivery of effectors by the T3SS into the host cell. Exterior host-pathogen interactions occur through recognition of bacterial flagella (FLG22) triggering pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) inside the cell to suppress bacterial growth. Several *Xanthomonas* effectors suppress early FLG22-triggered immunity. Effector-triggered immunity (ETI) occurs through recognition of specific effectors by cognate resistance genes (pink ovals). ETI also occurs through non-conventional gene-for-gene interactions, such as transcriptional activator-like (TAL) effectors in the nucleus. Suppression of ETI-triggered immunity occurs through interaction with host factors. Abbreviations: CW cell wall, PM plasma membrane, hrp hypersensitive response and pathogenicity, ET ethylene, PCD plant cell death. Figure is not drawn to scale.

single phylogenetic group (Schwartz et al. 2015; Timilsina et al. 2019). Strains isolated from Florida in 2006 were split into two phylogenetic groups (Schwartz et al. 2015) and later split into three phylogenetic groups (Timilsina et al. 2019). Subsequent whole genome sequencing efforts in Florida from 2010 to 2016 further supported the presence of three phylogenetic groups (Abrahamian et al. 2019c; Timilsina et al. 2019), although a recent collection by Klein-Gordon et al. (unpublished) in 2017 uncovered as many as six phylogenetic groups. An Alabama-based study by Newberry et al. (2019), which compared the genomes of strains isolated from Alabama with previously reported Florida strains, revealed six sequence clusters, two of which were unique to Alabama-associated strains. An Australian-based genomic study by Roach et al. (2019) revealed additional unique clusters of strains within *X. perforans*. An atypical Nigerian strain (NI1) was also found to be genetically unique compared to previously isolated strains from Florida and Italy, likely representing yet another phylogenetic group (Jibrin et al. 2018). At least six phylogenetic groups exist within the *X. perforans* species, albeit further study is needed to compare genomes of all available genomic sequences of global *X. perforans* strains and determine whether the number of phylogenetic groups present across *X. perforans* is actually greater than six. However, we note that while this would be useful to assess the overall diversity across *X. perforans*, the number of groups is likely to continue to increase as additional strains are sequenced and previously polyphyletic or diverse monophyletic groups are split up. As many previous *X. perforans* genetic studies were based on comparisons across a limited number of genes, it is difficult to ascertain the true genetic diversity across the *X. perforans* species for all reported strains. However, we anticipate that continued whole genome sequencing efforts, enabled by the increasing affordability of genome sequencing, will likely reveal much greater genetic diversity than is currently reported in the *X. perforans* worldwide population.

Constantin et al. (2016) and Barak et al. (2016) proposed that *X. perforans* be grouped within the *X. euvesicatoria* genome due to their high average nucleotide identity (ANI) in whole genome sequence analyses. Within these studies, ANI values were greater than 98.1% between strains that were previously designated as *X. euvesicatoria* and *X. perforans*, which each argued is well above the proposed species delineation value of 95% (Konstantinidis and Tiedje 2005; Richter and Rosselló-Móra 2009). The genetic similarity of *X. euvesicatoria* and *X. perforans* is also observed in the core-gene-based phylogenetic tree in Figure 2, where these two species form a monophyletic group which is genetically distinct from *X. gardneri* and *X. vesicatoria*. In addition, other studies have provided evidence for multiple recombination events throughout the genome between *X. euvesicatoria* and *X. perforans*, which was also found to have a higher impact than mutations on sequence variation for *X. perforans* and were proposed to be responsible for shaping the observed phylogenetic diversification (Jibrin et al. 2018; Newberry et al. 2019; Timilsina et al. 2019). Some of the key genetic differences between *X. euvesicatoria* and *X. perforans* are the presence of bacteriocins (discussed in previous section). The presence of bacteriocins in *X. perforans* led to a population shift of the dominant BST-causing *Xanthomonas* sp. in Florida (Hert et al. 2009; Timilsina et al. 2016; Tudor-Nelson et al. 2003). Furthermore, the absence of certain effectors in *X. euvesicatoria* and *X. perforans* population shapes the host range. For instance, the presence of AvrBst effector in *X. perforans* limits its pathogenicity to tomato plants (Schwartz et al. 2015). However, recent studies have shown host expansion of *X. perforans* onto pepper through acquisition of novel effectors and its occurrence in pepper fields albeit at very low incidence (Newberry et al. 2019, 2020). A metagenomic study revealed the presence of up to three unique genotypes, or phylogenetic groups, of *X. perforans* across several tomato fields across Alabama in the USA (Newberry et al. 2020). However, *X. perforans* co-occurrence with *X. euvesicatoria* was extremely low and restricted to pepper, raising questions as to the exact ecological habitat were genetic exchange might take place between both species (Jibrin et al. 2018; Newberry et al. 2020). The loss and acquisition of effectors and emergence of T3SS allelic variants is widely prevalent across *X. perforans* and *X. euvesicatoria* populations (Barak et al. 2016; Jibrin et al. 2018). Overall, *X. perforans* appear to have an open pan-genome as evident by the high variability across sequenced strains mainly driven by recombination (Jibrin et al. 2018; Newberry et al. 2019; Timilsina et al. 2019).

Disease management

Chemical control

Antibiotics were initially used for controlling BST (Thayer and Stall 1962). Prior to the presence of *X. perforans*, streptomycin resistance was observed in the *X. euvesicatoria* populations (Thayer and Stall 1962). Nevertheless, streptomycin resistance in *X. perforans* has been reported from different tomato fields in the USA, such as in Florida and North Carolina but not in Mississippi, even though spray applications are only limited to transplant production (Abrahamian et al. 2019b; Adhikari et al. 2019; Klein-Gordon et al. 2021; Strayer-Scherer et al. 2019). In other countries, including Iran, Ethiopia, and the Caribbean Islands, resistance to streptomycin has not yet been observed in the *X. perforans* population (Bouzar et al. 1999; Osdaghi et al. 2017; Kebede et al. 2014). This might be due to limited sampling or due to limited use of antibiotics in other parts of the world. For many decades the tomato industry relied on the use of copper and copper-mancozeb tank mixes (Strayer-Scherer et al. 2019). The heavy use of copper and copper-based compounds resulted in very high tolerance or complete resistance among worldwide populations of *X. perforans* (Abbasi et al. 2015; Araújo et al. 2012; Klein-Gordon et al. 2021; Martin et al. 2004; Mirik et al. 2007). Continued foliar applications of copper can lead to high copper levels in soils that can be phytotoxic to tomato, leading to reduced growth and fruit yields (Rhoads et al. 1989; Sonmez et al. 2006). A field and greenhouse study by Abrahamian et al. (2019a) showed that copper sprays were not effective in reducing disease severity. In vivo studies comparing the aggressiveness of copper tolerant and sensitive strains showed a higher virulence for the latter, suggesting a fitness cost of copper tolerance (Araújo et al. 2012). Strains recovered in Ethiopia and Iran were copper sensitive, which is likely related to fewer copper sprays in those areas (Osdaghi et al. 2017; Kebede et al. 2014). Copper and copper-mancozeb sprays show variable efficacy even when bacterial population are resistant to copper (Abrahamian et al. 2019a).

Environmentally friendly alternatives to copper and streptomycin, such as acibenzolar-S-methyl (ASM) and bacteriophages, are reported to reduce disease severity (Abrahamian et al. 2019a; Jones et al. 2012; Louws et al. 2001; Pontes et al. 2016). ASM, a synthetic compound, induces systemic acquired resistance (SAR) against a broad range of pathogens (Vallad and Goodman 2004). SAR is accompanied with an increase in salicylic acid and upregulation of pathogenesis-related genes (Durrant and Dong 2004). ASM showed significant disease reduction compared to copper-based sprays (Abrahamian et al. 2019a; Huang et al. 2012; Louws et al. 2001; Pontes et al. 2016). Furthermore, weekly application of ASM was significantly better at reducing disease compared to biweekly applications (Huang et al. 2012). Nevertheless, ASM applications did not improve tomato yield (Abrahamian et al. 2019a; Huang et al. 2012; Louws et al. 2001; Pontes et al. 2016). Bacteriophages also showed to be efficient in reducing BST (Obradovic et al. 2004). Single phage applications, or applications made in combination with ASM, significantly reduced disease severity (Obradovic et al.

2004, 2005). However, formulation and environmental conditions (ultra-violet rays, temperature) play a major role in phage survival on the leaf surface (Jones et al. 2012). Phage survival is dependent on the application time, with the highest efficacy being observed for evening applications (Jones et al. 2012). Furthermore, fungicides, such as cymoxanil and famoxadone or quinoxyfen, were evaluated for control of copper-tolerant *Xp* strains but were not very effective in reducing disease severity (Abrahamian et al. 2019a; Fayette et al. 2012; Roberts et al. 2008). However, cymoxanil or famoxadone combined with copper hydroxide or ASM significantly reduced disease (Abrahamian et al. 2019a; Fayette et al. 2012).

Recently, several experimental nanomaterials have shown some success at controlling copper-tolerant *X. perforans* strains in the field (Paret et al. 2013; Strayer-Scherer et al. 2018; Strayer et al. 2016; Liao et al. 2019a, 2019b). Paret et al. (2013) evaluated photocatalytic titanium dioxide (TiO_2) with silver (Ag) and zinc (Zn) against *X. perforans* in the greenhouse. TiO_2/Zn and TiO_2/Ag showed significant reduction in vitro, and TiO_2/Zn showed reduced bacterial spot in field trials (Paret et al. 2013). Ocsoy et al. (2013) developed DNA-directed Ag nanoparticles (NPs) grown on graphene oxide (GO) which showed antibacterial activity against *X. perforans* in vitro and on tomato seedlings. The Ag-dsDNA-GO composite was further evaluated at different concentrations against copper-tolerant *X. perforans* under greenhouse conditions. At 75 μg/ml, low phytotoxicity with acceptable disease reduction was achieved, comparable to the grower's standard of copper (Strayer et al. 2016). Nano-magnesium oxide (MgO) (Liao et al. 2019a) and other copper-based nanomaterial formulations were also developed, such as a core-shell copper (CS-Cu), a multivalent copper (MV-Cu), and a fixed quaternary ammonium copper (FQ-Cu) (Strayer-Scherer et al. 2018). The formulated CS-Cu and FQ-Cu showed 100% inhibition of a copper-tolerant *X. perforans* strain in vitro but no significant difference compared to the grower standard under field conditions (Strayer-Scherer et al. 2018). Furthermore, MgO nanoparticles provided significant reduction in disease compared to a non-treated control, but did not differ from the grower copper standard (Liao et al. 2019a, 2019b). Another noteworthy variable affecting chemical performance is the addition of surfactants. Jibrin et al. (2021) recently showed that certain surfactants affect bacterial severity either by suppressing or promoting disease development.

While the aforementioned chemicals, biologicals, and nanomaterials were able to reduce disease relative to a non-treated control, few performed better than the grower standard that typically consisted of a copper-based product under field conditions. In addition, rarely did improved disease control translate into a significant yield improvement. These shortcomings do not negate the overall benefits of reducing grower reliance or copper loads in agricultural soils. Rather, it stresses the challenge and need for continued research to improve disease control using chemicals, biologicals, and nanomaterials and to develop novel materials and agents.

Cultural practices and resistance breeding

Management of BST mainly relies on a combination of cultural practices and chemical sprays (Potnis et al. 2015; Stall et al. 2009). However, the absence of a "silver bullet" management approach for bacterial diseases makes disease control difficult in the field. Exclusion of disease is the primary method to avoid disease introduction into the field. A recent study showed asymptomatic colonization of *X. perforans* and lag periods of symptom development on tomato seedlings, which emphasizes the importance of proper sanitation measures prior to movement of plant material (Abrahamian et al. 2021). *X. perforans* can survive on tomato seeds; therefore, disease-free seeds should be used to grow plants. However, research in seed colonization is still lacking for *X. perforans*. Furthermore, sanitation plays an important role in reducing inoculum load in the field. Practices such as removing volunteer crops and weeds and the use of resistant cultivars are effective in reducing disease pressure (Gitaitis et al. 1992; Jones et al. 1986).

Development of host resistance to BST has been a challenge. Initial studies showed a high level of field resistance to the BST pathogen on the tomato genotype 'Hawaii 7998' (H7998) (Scott and Jones 1989). The resistance was determined to be associated with elicitation of a hypersensitive response (HR) by *X. euvesicatoria* strains (Jones and Scott 1986). In the late 1980s, *X. vesicatoria* strains from Brazil (Jones et al. 2004) produced a susceptible reaction in H7998 (Wang et al. 1990). The strains that were originally determined to elicit an HR were designated as race T1 strains, and the strain from Brazil was identified as race T2 (Bouzar et al. 1994). The HR elicited by T1 strains was determined to be associated with the avirulence gene, *avrRxv* (Whalen et al. 1993). The resistance in H7998 was not associated with a single dominant gene, typical of most hypersensitive resistance reactions (Wang et al. 1994a, 1994b; Stall et al. 2009). Yu et al. (1995) identified three loci in Hawaii 7998, which they designated Rx1, Rx2, and Rx3. The former two are located on chromosome 1 at the top and bottom, respectively, whereas Rx3 is located on chromosome 5. The Rx3 locus was the only locus determined to be required for resistance in the field (Yang et al. 2005).

Following the discovery of resistance in H7998, a breeding program was begun to breed commercial tomato varieties with resistance to T1 strains. However, strains were identified in 1991 that were able to overcome resistance in H7998 (Jones et al. 1995), rendering the resistance developed to tomato T1 strains ineffective against this new group. These strains were later characterized as *X. perforans* and designated into a new

race, T3. The new strains were shown to elicit an HR in the two plant introductions (PIs), PI 128216 and PI 126932. Resistance derived from these two PIs was utilized in breeding programs. The HR resistance to the T3 strain in at least one tomato line (H7981) was inherited as a single gene (Scott et al. 1996). A near-isogenic line was developed by incorporating Xv3 from PI 128216 into FL 7060 seven times. This line was designated as FL 216 and was useful as a tomato race differential (R.E. Stall, unpublished data), as well as for identifying the avirulence gene, which was identified as *avrXv3* (also referred to as *xopAF*) (Astua-Monge et al. 2000a; White et al. 2009). *Xv3* was later designated as *RX4*, which was later fine-mapped to chromosome 11 (Pei et al. 2012) and recently cloned and characterized (Zhang et al. 2021).

In 1998 a *X. perforans* strain was isolated that contained a mutation in *xopAF* and was designated as a T4 strain. As a result, the breeding program for developing resistance based on H7998 and *Xv3* was problematic in terms of continuing to incorporate this resistance into tomato varieties. Another source of resistance to *X. perforans* was identified in *S. pennellii* LA716 (Astua-Monge et al. 2000b). The resistance was determined to be associated with elicitation of an HR following infiltration of a bacterial suspension of *X. perforans* into the intercellular spaces of S. *pennellii* LA716 leaves, but not when infiltrated with an *X. euvesicatoria* strain expressing *xopAF*, indicating a possible other *avr* gene. Molecular characterization of *X. perforans* to identify the HR-eliciting gene revealed the effector gene, *avrXv4*, also referred to as *xopJ4* (White et al. 2009). Genetic analysis revealed that the resistance gene, *RXopJ4*, may be semi-dominant as there was an intermediate level of resistance in F1 inoculated plants (Sharlach et al. 2013). There has been significant linkage drag associated with the *S. pennellii* LA716 introgression lines 6-2 and 6-2-2 that contain the *RXopJ4* resistance locus, which results in low fruit yield, small fruit, and an autogenous leaf necrosis. More advanced lines carrying the *RXopJ4* locus may still have a disadvantage in the field. Recently, five tomato lines derived from *S. pimpinellifolium* L3707 showed partial resistance against *X. perforans* race T4 (Bhattarai et al. 2017). As a result, prospects for using these sources of resistance have not panned out. Future efforts should be focused on identifying recessive resistance similar to what was identified in pepper for control of bacterial spot (Stall et al. 2009) and effector-specific resistance (Timilsina et al. 2016).

Concluding remarks and future concerns

Currently, tomato production is threatened by various biotic and abiotic stresses. Abiotic factors are relatively easier to manage or predict than biotic factors. The future of tomato production is constantly threatened by new and emerging pathogens, such as *X. perforans*. In order to achieve sustainable tomato production and meet the tomato demand of an ever-increasing population, further research is needed for proper disease management. For instance, the constant emergence of exotic strains and horizontal gene transfer of novel effectors into the *X. perforans* population is challenging to meet durable genetic resistance in plants. Therefore, continuous surveys of bacterial spot and sequencing of strains are needed in tomato production areas to understand the genetic diversity and identify potential targets for resistance breeding. Further research is needed to understand the impact of contaminated seeds on long-distance movement, the emergence of novel strains through recombination, and introduction of exotic strains. For instance, the recent capability of strains to acquire or lose effector genes to enable host expansion, such as onto pepper, is intriguing and alarming. The presence of novel effector genes, such as TAL effectors, should be further characterized and investigated with respect to host range and increased virulence. Novel resistance breeding strategies should in particular avoid single-gene resistance to avoid resistance breakdown which is evident in *X. euvesicatoria* on pepper. Breeding programs should incorporate gene insertions and pyramiding through novel techniques, such as CRISPR, to produce durable resistance.

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Author contribution PA conceived the manuscript. JKG conducted phylogenetic analysis. All authors wrote and reviewed the manuscript. All authors read and approved the manuscript.

Data availability Not applicable.

Declarations

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Conflict of interest The authors declare no competing interests.

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