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Review

Opportunities and Challenges to Understand Host-Pathogen Interactions and Management of *Verticillium dahliae* on Tomato

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Abstract

Tomato (*Solanum lycopersicum* L.) is a valuable horticultural crop grown and consumed worldwide. Optimum production is hindered by several factors of which *Verticillium dahliae*, the cause of Verticillium wilt, is one of the major biological constraints in temperate production regions. *V. dahliae* is difficult to manage because it is a vascular pathogen, has a broad host range and worldwide distribution, and can persist in soil for years. Understanding the pathogen virulence and genetic diversity, host resistance, and plant-pathogen interactions can ultimately inform the development of integrated strategies to manage the disease. In recent years, considerable research has focused on providing new insight into these processes as well as the development and integration of environment-friendly management approaches. In this review, we discuss and summarize the recent findings on the race and population structure of *V. dahliae*; pathogenicity factors; host genes, proteins, and enzymes involved in defense; the emergent management strategies, and recent approaches to managing Verticillium wilt in tomatoes.

keywords: Tomato; *Solanum Lycopersicon* L.; *Verticillium dahliae*; plant-pathogen interactions; disease resistance; integrated disease management

1. Introduction

1.1. Tomato

Tomato (*Solanum lycopersicum* L.) is an important fruiting vegetable grown around the world with Asia producing more than 50% of the total production (Fig. 1A). In 2018, 182.3 million tons of tomatoes were produced from 4.8 million ha of land worldwide (FAOSTAT, accessed Jun 2, 2020). Mainland China ranked

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first in tomato production with approximately three times the production of the second-largest producer, India (Fig. 1B). The 3rd largest producer, with 12.6 Mt., is the U.S. Depending on the part of the world where tomato is grown, major constraints in tomato production include lack of quality seeds, labor, and knowledge in optimum agronomic practices; high cost of agricultural inputs, and price fluctuations; Weather constraints; and the serious problem of insect pests and diseases [1-5]. Among the diseases, Verticillium wilt is one of the major constraints of tomato production.

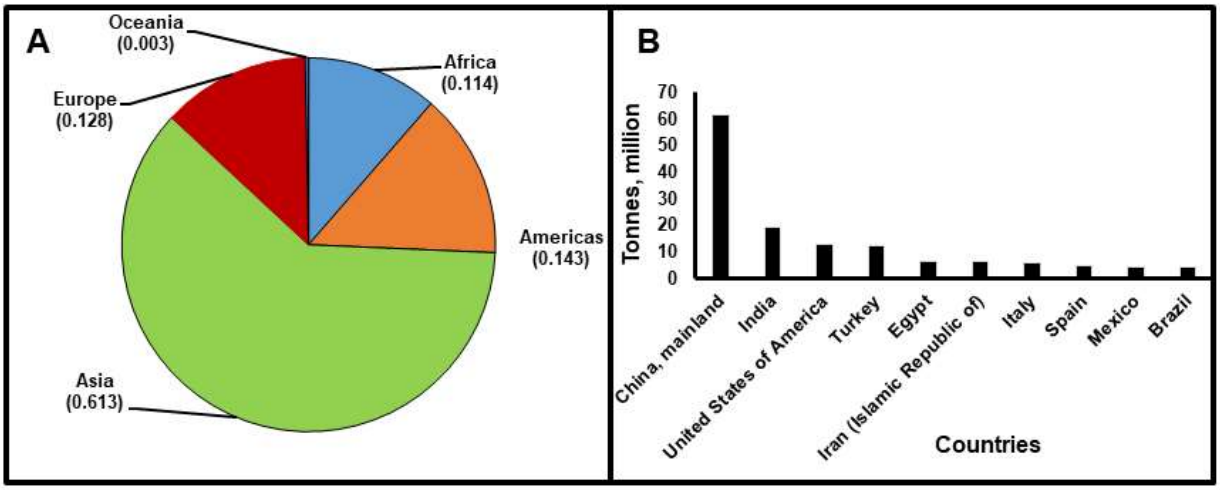

Figure 1. Tomato production by region, **A,** and top 10 tomato-producing countries in the world, 2018, **B** (Source: FAOSTAT, accessed Jun 02, 2020).

1.2. Verticillium Wilt: Economic Importance

Verticillium dahliae is a hemibiotrophic fungal pathogen [6] and has a worldwide distribution (Fig. 2) that causes Verticillium wilt in tomatoes and many other crops [7]. The disease cycle starts with microsclerotia (MS), a resting structure in soil or crop debris that is capable of surviving without a plant host for more than a decade [7,8]. Disease severity is linked to MS density (MS/g soil) and incidence on propagative (e.g. seed) material that can be quantified using several techniques [9-13], but the methods have not been refined for routine use in commercial labs to reliably guide disease management decisions. Inoculum density as low as 0.1 microsclerotia (MS)/ g of soil is sufficient to infect tomato plants but even levels of 9 MS/g of soil do not always yield visible symptoms [14]. Moreover, the level of infection from the same amount of inoculum

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depends on environmental conditions. This complicates the determination of economic thresholds for this pathogen as a basis for the application of integrated disease management approaches.

Figure 2. Worldwide distribution of *Verticillium dahliae*. Circles represent the locations (states and provinces) from where *V. dahliae* was reported.

The microsclerotia germinate in the presence of root exudates [15] and mycelium infect roots through root tips or sites of lateral root formation [16,17]. The typical symptoms of *V. dahliae* in susceptible tomato cultivars start on the lower leaves with chlorosis and V-shaped necrotic lesions at the edges of the leaves with yellow halos that expand to cause browning or purpling of veins and death of leaves [18,19]. The pathogen spreads acropetally through the vascular tissue of the plant, where brown discoloration is visible when incised [18], producing conidia that continue the cycle of germination, infection, and colonization resulting in wilting of branches and/or the entire plant [17]. Prolific conidiation has been correlated with the aggressiveness of the strains [16]. The wilting symptoms in a susceptible tomato cultivar may start at 21 days post-infection (dpi) which is correlated with the accumulation of drought-stress proteins [20].

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The broad host range, which includes annuals, perennials, and woody species, of more than 200 plant species and expanding [7,21] and its ability to persist in soil for a long period renders *V. dahliae* an important and widely studied pathogen. Currently, several studies focused on *V. dahliae* biology, host-pathogen interactions, and contemporary approaches to managing the disease are reported.

2. Current knowledge on *Verticillium dahliae* and host interaction

2.1. *Race structure in Verticillium dahliae*

2.1.1. *Verticillium dahliae* race 1 infecting tomato

The first reported resistance to *Verticillium* was in 1951 [22] conferred by a single dominant gene designated *Ve* [23]. Resistant isolates were discovered within a few years after the deployment of this source of resistance [23-27]. In 1984, Bender and Shoemaker surveyed 96 *V. dahliae* isolates, 89 were designated as race 1, and seven were non-race 1 using differential tomato lines with and without the *Ve* gene [24]. The gene responsible for race 1 resistance in tomatoes, and other hosts, designated as the *Ve1* gene, was described using a combination of whole-genome comparison and gene expression analyses [28]. The *Ve1* gene codes for a cell surface-like receptor which recognizes the *Ave1* effector of the pathogen during the infection process [28,29]. Homologs to *Ave1* exist in *Colletotrichum*, *Fusarium*, and *Cercospora* [28]. *Ve1* can also activate the immune response against *Xanthomonas axonopodis* which causes citrus canker [28]. Interestingly, *Ave1* can also induce defense gene expression in the absence of the Ve1 [30] which suggested other defense responses independent of Ve1 may also be operating.

2.1.2. *Verticillium dahliae* "race 2" infecting tomato

Isolates pathogenic on race 1 resistant plants are present on every continent on earth, except Antarctica [24,25,31-35]; these have been classified as "race 2" in the past; however, it is actually "non-race 1" and comprise race mixtures. In 2017, the cultivars 'Aibou' and 'Ganbarune-Karis' were shown to be resistant to some non-race 1 strains of *V. dahliae* [35]. 'Aibou' and 'Ganbarune-Karis' are F1 hybrids. F2 progeny of these lines segregated into a 3:1 resistant to susceptible ratio suggesting the presence of a single dominant resistance gene. Isolates non-pathogenic on 'Aibou' and 'Ganbarune-Karis' were termed race 2, while isolates pathogenic on these cultivars were termed race 3 [35]. Further research has shown that knocking out the race 1 effector in isolate Vdp4 (a race 1 strain of *V. dahliae*) was pathogenic on 'Aibou', which contains both race 1 and race 2 resistance [36]. Kano and Usami [33,34] also showed that one isolate (Vdp4) was

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pathogenic on race 1 resistant plants, but not on tomatoes containing just the race 2 resistance gene. Furthermore, they demonstrated that some race 1 isolates were non-pathogenic on the cultivar that was susceptible to race 1 but resistant to race 2, suggesting that some race 1 isolates also contains the race 2 effector present. Ingram et al. (unpublished data) showed that race 2 and race 3 isolates were present in the USA in isolates from tomatoes in North Carolina and California (Supplementary Table 1). An analysis of the genomes of Japanese and USA races 1, 2, and 3 isolates showed that there were 3 candidate secreted effectors that may be responsible for the race 2 phenotype [37], demonstrated that one of these secreted effectors was responsible for the race 2 phenotype. The race 2 secreted effector was introduced into race 3 strains, which then exhibited the race 2 phenotype when inoculated onto tomato lines containing the V2 locus [37]. The host resistance gene responsible for the race 2 resistance phenotype is currently unknown.

2.2. Influence of genetics on *V. dahliae* pathogenicity

2.2.1. Defoliating (DF) vs. non-defoliating (NDF) strains on Tomato

In cotton, there are two radically different pathotypes of *V. dahliae*, NDF and DF strains [38,39]. DF strains of *V. dahliae* cause a massive amount of damage to cotton plants [40]. While primers exist to differentiate these pathotypes, some strains that are PCR positive (such as VdLs17) for DF do not have the DF phenotype [40]. The DF strains, however, do not increase pathogenicity on tomatoes [41]. The DF phenotype is caused by the presence of a *VdDf5* and *VdDf6* genes which are contained in a lineage-specific region that was horizontally transferred from *Fusarium oxysporum* f. sp. *vasinfectum* to *V. dahliae [41].*

2.2.2. Vegetative compatibility of *V. dahliae* isolates

Verticillium dahliae is an asexually reproducing haploid ascomycete fungus in the class sordariomycetes, which is a class that contains many plant pathogenic fungi [6,42,43]. Despite the presence of two mating types (MAT1-1 and MAT1-2), there is very little evidence of recombination [44,45]. However, there are vegetative compatibility groups (VCGs) which may allow for some parasexual exchange of genetic material [46,47]. Because of the highly clonal nature of *V. dahliae*, VCGs were used in the past to differentiate isolates, although it is unclear whether there are any direct links to pathogenicity [46,48]. In 2017, isolates from strawberry containing the race 1 effector *Ave1* were grouped into two different VCGs, and these two race 1 VCG groups were also phylogenetically different from each other [49]. Overall, there is very little information to suggest *V. dahliae* isolates exchange any genetic material at all, claims of VCGs affecting pathogenicity should be examined on a case by case basis.

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2.2.3. Chromosomal rearrangement and its influence on pathogenicity in *V. dahliae*.

To date, considerable variation in pathogenicity of *V. dahliae* isolates has been attributed to either chromosomal rearrangement or horizontal gene transfer events from other organisms [50-52]. The absence of *Ave1* in non-race 1 strains of *V. dahliae* is due to the absence of a large region on the chromosome where the effector is located [51]. *Ave1* is homologous to plant natriuretic peptides (PNPs) which are secreted peptides that regulate abiotic stress in plants [51,52]. Similarly, the absence of the Av2 locus in race 3 is the result of a large deletion on chromosome 5 (JR2 reference genome) [37]. A large number of insertions and deletions in the *V. dahliae* have led to the hypothesis of a two-speed genome, where vital genes are kept in specific regions, while pathogenicity related genes are located on more flexible regions with transposable elements (TEs) [53-55]. Genomic plasticity appears to be a major force driving the host-pathogen evolution of *V. dahliae* and other fungal pathogens [54-56].

2.2.4. Phylogenetic analysis of *V. dahliae* isolates

Microsatellite data and whole-genome sequencing have been effective ways to differentiate *V. dahliae* populations [6,42,45]. The largest most comprehensive phylogenetic analysis of *V. dahliae* isolates to date was conducted by Short et al. [57] on 1100 *V. dahliae* isolates from a wide range of hosts and continents using microsatellite genotyping. The study indicated that there are 7 distinct clusters, and isolates from tomato were present in clusters 1, 2, and 7 [57] which included the sequenced tomato *V. dahliae* isolates, Le1811 and Le1087, and the lettuce isolate VdLs17 (alternatively labeled as PD322). In 2020, Ingram et al. (unpublished data) have circumscribed at least two supergroups, and 4 sub-groups of *V. dahliae* isolates infecting tomato. Whole-genome analysis has yielded a great deal of information into this pathogen evolution [41,42,57].

Phylogenetic analysis is complicated by the existence of a diploid hybrid, *Verticillium longisporum*, a long-spore hybrid between *V. dahliae* and a cryptic *Verticillium* species (A1) [58-60]. The merging of *V. dahliae* and the cryptic *Verticillium* appears to be associated with three independent events[59]. Each of these three lineages (termed A1/D1, A1/D2, A1/D3) are genetically distinct, and some isolates showed reduced pathogenicity on tomatoes compared to *V. dahliae* isolates [61].

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2.3. Molecular insights into *Verticiliium dahliae* pathogenicity

Molecular genetics and other "omics" technologies have been widely used to uncover the molecular basis of pathogenicity in *V. dahliae* in recent years. *V. dahliae* is phylogenetically closely related to other foliar and soilborne pathogens. Consequently, homology-based approaches have been exploited in several instances to identify and characterize genes and pathways, known to be involved in development and pathogenicity in other pathogens. For example, hydrophobin, a small secreted hydrophobic protein is known to be essential for fungal development and pathogenicity [62]. The study of the *V. dahliae* homolog (VDH1) showed that hydrophobin is essential for microsclerotia formation, but is not required for host colonization and pathogenicity [63].

Several proteins involved in core fungal processes such as cell wall modification play crucial roles in cell wall integrity and pathogenicity. Mannoproteins, which are rich in fungal cell walls (in the range of 30-50% in yeast cell walls), are connected to the cell wall via either non-covalent connections or covalent linkages to β-1,6-glucans. Alpha-1,6-mannosyltransferase (OCH1) is required for the production of yeast mannoproteins [64]. In *V. dahliae*, an OCH1 homolog is required for both microsclerotia formation and pathogenicity [65].

Genes for energy metabolism have been characterized in *V. dahliae* and in some instances play a role in pathogenicity. The enzyme alpha-oxoglutarate dehydrogenase (OGDH) catalyzes the oxidative decarboxylation of alpha-ketoglutarate to succinyl-CoA in the tricarboxylic acid (TCA) cycle. VdOGDH in *V. dahliae* is not only involved in energy metabolism but also affects the expression of melanin biosynthesis and is required for full virulence [66].

2.3.1. Signal transduction pathways

Signaling pathways play multiple roles in fungal development and pathogenicity. G-protein regulated cyclic AMP signaling pathway, MAP Kinase cascades, and Ca2+/Calmodulin signaling pathways are highly conserved in phytopathogenic fungi and have been studied in *V. dahliae* in recent years. In the G-protein coupled cyclic AMP signaling pathway, extracellular signals are transmitted via membrane binding G-protein coupled receptors (GPCRs). G protein-mediated signaling is involved in virulence, development, and hormone production of *V. dahliae* [67,68]. Gene knock out of the G protein β subunit gene (*VGB*) resulted in reduced virulence, increased microsclerotia formation and conidiation, and decreased ethylene production [67]. Mutants lacking *VdPKAC1*, the catalytic subunit of the cAMP-dependent protein kinase are unable to form microsclerotia, produced high amounts of ethylene, and exhibited reduced virulence towards tomato

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[68]. Both VGB and VdPKAC1 regulate other signal pathway genes including the MAP kinase, VMK11, and hydrophobin, VDH1 [67].

The mitogen-activated protein (MAP) kinase signaling pathway plays a major role in transducing external signals into the cell to invoke biological responses. In budding yeast, *Saccharomyces cerevisiae*, distinct MAP kinase pathways are required for mating, morphological changes, osmoregulation, and cell wall integrity [69,70]. In fungal pathogens, MAP kinase signal pathways are also involved in fungal pathogenicity [71,72]. Functional studies of the components of different MAP kinase pathways have confirmed the role of these proteins in the pathogenesis in *V. dahliae* including a surface sensor (VdMsb) [73], an osmosensor (VdSho1) in the high osmolarity glycerol (HOG)-MAP kinase signaling pathway [74,75], a Hog1 MAP kinase (VdHog1) [76], VdPbs2, an upstream component of VdHog1 [77], Verticillium MAP Kinase 1 (Vmk1) [78] and MAPKKKs (VdSsk1, VdSsk2, and VdSte11) [79,80]. The deletion of these genes has shown that these MAP kinase cascades are involved in stress adaptation, plant root penetration, and microsclerotia formation in *V. dahliae*.

The Ca^{2+}-calcineurin signaling pathway is conserved in eukaryotes and is involved in several biological processes including Ca^{2+} homeostasis and stress responses. In pathogenic fungi, the Ca^{2+}-calcineurin signaling cascades are involved in host and environment adaptation, infectious structure formation, virulence, and antifungal drug resistance [81]. In response to external or internal signals, intracellular calcium concentrations increase, and calcium ions bind to the calcium-binding protein calmodulin, which in turn binds to and activates calcineurin, a serine-threonine phosphatase. Activated calcineurin dephosphorylates various target proteins, including a transcription factor Crz1 [81]. The Crz1 homolog in *V. dahliae*, VdCrz1 is required for cell wall integrity, microsclerotia development, and full virulence *[82]*. It has also been shown that reactive oxygen species (ROS) production elevates intracellular Ca^{2+} levels in specialized hyphal branch cells (hyphopodia) and activates VdCrz1, which induces penetration peg formation during early colonization in cotton roots [83].

The “target of rapamycin” (TOR) signaling pathway is also evolutionarily conserved in eukaryotes and regulates cell growth, proliferation, and metabolism from yeasts to humans [84]. The putative components of TOR signaling pathways in *V. dahliae* (VdTOR) were recently identified [85]. When mycelia were treated with the TOR inhibitor, rapamycin, growth, and pathogenicity were significantly reduced and genes involved in various cellular processes, including ribosome biogenesis and cell wall degrading enzymes (CWDEs) were

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differentially downregulated. This suggests that VdTOR plays an essential role in hyphal growth, development, and pathogenicity [85].

During growth, development, and host infection in *V. dahliae*, these signaling pathways are under the control of complex regulatory networks that are governed by central transcriptional regulators. To date few are known in *V. dahliae*, however, Vst1 is involved in sporulation, melanin biosynthesis, and microsclerotia formation [86].

2.3.2. Secondary metabolism and melanin biosynthesis

Fungi produce an extensive array of secondary metabolites (SM) derived from several biochemical pathways including the polyketides, non-ribosomal peptides, terpenes, and indole alkaloids. These metabolites are mediated by the core enzymes named polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), terpene cyclases, and prenylation synthetases, respectively [87]. Also, Polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) hybrid enzymes have been identified and can be linked to the structurally diverse and complex SMs and further to their diverse biological activities in fungi [88]. Typically, genes involved in SM biosynthesis are clustered together [87]. In *V. dahliae,* 25 potential secondary metabolite gene clusters were identified and 36% of those clusters were located in sub-telomeric regions close to the chromosomal end [89]. The phylogenetic and comparative genomic analysis suggested clusters in *V. dahliae* are linked to the biosynthesis of two putative siderophores, ferricrocin and triacetylfusarinine C (TAFC), 1,8-dihydroxynaphthalene (DHN)-melanin and fujikurin [89]. Melanin, a polyketide is one of the most thoroughly studied SMs because it is directly linked to fungal cell wall stability and pathogenicity [74,80,90-92]. In *V. dahliae*, black melanin granules are heavily deposited in the cell wall of the survival structure, microsclerotia [93]. Many of the genes in a melanin biosynthesis gene cluster are highly induced during microsclerotia formation [94]. Functional analyses of these genes showed that both the central polyketide synthase VdPKS1 (VDAG_00190) gene and a transcription factor VdCmr1 (VDAG_00194) are required for melanin biosynthesis with VdCmr1 being involved in the regulation of gene expression of VdPKS1 [90,91]. VdPKS1 is involved in *V. dahliae* virulence, conidiation, and ethylene production even though microsclerotia production itself is not affected in VdPKS1 mutant strain [91]. VdPKS1 is also regulated by MADS-Box transcription factor VdMcm1 (VDAG_01770), which is a key regulator in *V. dahliae* and is involved in melanin biosynthesis, conidiation, microsclerotia formation and virulence [95]. VdMcm1 also controls PKS/NRPs hybrid-cluster gene expression. Deletion of Nag-1 in this cluster results in defects in

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growth, virulence and melanin biosynthesis [96]. Vayg1 gene, a homolog of Aayg1 from *Aspergillus fumigatus* (Aayg1) is also required for melanin production and microsclerotia formation in *V. dahliae* [92].

2.3.3. Cell wall degrading enzymes, carbohydrate modifying enzymes

Comparative genomics studies revealed that *V. dahliae* has developed enhanced carbohydrate degrading machinery of potential value for weakening plant cell walls [42]. Polysaccharide lyase (PL) families including PL1, PL3, PL4, and PL11 directly target different forms of pectins. Also, glycoside hydrolase (GH) families hydrolyze the glycosidic bond between carbohydrate compounds generated by PL are significantly enriched in *V. dahliae* compared to other ascomycete fungi. Besides, *V. dahliae* has 30 proteins that contain the conserved carbohydrate-binding module 1 (CBM1), generally known as a fungal specific cellulose-binding domain. CBM1 is widespread in fungal enzymes including PL proteins. *V. dahliae* has 3 CBM1-containing PL proteins [42]. Pectin degrading enzymes, which are highly secreted during fungal infection play a key role in pathogenesis. Gene knock-out mutants lacking pectin lyase genes, *VdPL3.1* , and *VdPL3.3* were unable to develop wilting symptoms in cotton *[97].* High levels of pectin lyase activity occurred during the compatible interaction between tomato and *Verticillium spp.* before disease symptoms appeared *[98].* Furthermore, VdPEL1 triggered plant immunity responses and was involved in *V. dahliae* virulence. This implies that during infection, the pectin hydrolysis products may function as damage-associated molecular patterns (DAMPs) to elicit plant defense response [99]. Similarly, *V. dahliae* cutinase, VdCUT11, acts as a virulence factor and can induce plant defense responses mediated by the leucine-rich repeat (LRR)-RLP/SOBIR1/BAK1 receptor complex in tobacco [100]. This response can be further suppressed by VdCBM1, a member of the carbohydrate-binding module family 1 (CBM1) in *V. dahliae* [100,101].

2.3.4. Effector proteins in *V. dahliae*

Fungal effector proteins are typically secreted proteins that are involved in host determination and colonization of the host plants [102]. In *V. dahliae*, about 700 proteins contain a signal peptide that guides the protein into the extracellular plant spaces. Typically, known effector proteins are small with a high cysteine content in addition to a signal peptide. Studies have suggested *V. dahliae* contains ~ 150 small secreted effector proteins that are less than 400 aa with more than 4% cysteine content [42]. Recently, combining SignalP and EffectorP effector searching tools, we have predicted about 200 core effector proteins among 19 sequenced *V. dahliae* genomes (unpublished data). Also, *V. dahliae* isolates possess lineage-specific (LS) regions that contain predicted effectors, in many cases, such regions contain avirulence or virulence factors [73,77,102-104] including currently known two avirulence factors, VdAve1 and VdAv2. These LS

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effectors are surrounded by transposable elements such as LTR type transposons, which has been suggested to provide opportunities for rearrangement and possibly originate through horizontal gene transfer from other organisms including plants, bacteria, and fungi [50,89,105].

The *Ve1* gene-mediated resistance against *V. dahliae* had been employed for many years in tomato breeding programs but elucidating the corresponding avirulence factor was only possible after the advent of modern molecular technologies, whole-genome sequencing, and transcriptome analysis. Using a whole-genome comparison between avirulent (race1) and virulent (non-race1) *V. dahliae* isolates against Ve1 tomato lines, a 50kb of race 1 lineage-specific region was identified. Further gene expression profiling and transgenic expression led to the identity of the small secreted effector VdAve1 [51]. Recently, a similar approach was applied to identity another avirulence factor VdAv2, which governs resistance to tomato lines which contain the V2 resistance locus [37]. Both VdAve1 and VdAV2 fall into the typical effector category; small cysteine-rich effector protein located in lineage-specific chromosomal regions that are highly expressed during host colonization [106]. These characteristics could be particularly useful for the future discovery of new avirulence factors. In both cases, loss of recognition of these effectors by the host occurred through the deletion of DNA segments rather than single nucleotide polymorphisms (SNPs) [37,51].

Chitin is a major structural component of the fungal cell wall. When a host plant is attacked by a fungal pathogen, chitin-degrading enzymes are released by the host into apoplastic space to release chitin oligomers which activate pattern triggered immunity (PTI). These fungal chitin oligomers are recognized by lysin motif (LysM)-containing receptors in the plant membrane. LysM effectors that also contain chitin-binding motifs are ubiquitously found in phytopathogenic fungi and mammalian fungi. These effectors function by sequestering fungal chitin fragments and prevent their recognition by host LysM receptors blocking the chitin triggered plant immunity. In *V. dahliae*, the family of LysM effectors has expanded to contain six to seven LysM effectors [42]. Functional analysis of three core LysM effectors showed that they are not expressed during host colonization nor are they involved in pathogenicity or fungal development. In contrast, a lineage-specific LysM effector (Vd2LysM) in the strain VdLs17 functions as a virulence factor [107,108]. Similar to LysM effectors, a secreted polysaccharide deacetylase (PDA1) in *V. dahliae* targets fungal chitin oligomers for the successful fungal colonization. Rather than physically sequestering chitin, VdPDA1 converts chitin oligomers into chitosan and prevents activation of chitin triggered immunity. VdPDA1 does not inhibit host chitinases activity nor is it involved in fungal development [109].

2.3.5. Genome-wide analysis of host-pathogen interaction with *Verticilliium dahliae*

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The development of large-scale transcriptomic, proteomic and metabolomic technologies and availability of functional databases such as Gene Ontology(GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and Plant Resistance Genes database (PRGdb), are providing opportunities to gain more detailed insight into pathogenicity and host defense responses.

In *V. dahliae*, genome-wide RNA-sequencing (RNA-seq) expression analyses have revealed important biological pathways during microsclerotia formation including ubiquitin-mediated protein degradation and melanin biosynthesis [94,110]. Gene expression is regulated not only at the mRNA level but also alternative splicing based on the fact that about 50% of intron-containing genes were possibly regulated by alternative splicing [111]. Other gene expression studies showed that *V. dahliae* responded more strongly to root exudates from a susceptible cultivar than tolerant and resistant cultivars as evidenced by increased gene expression for hydrolase activity; particularly genes involved in hydrolyzing O-glycosyl compounds at early stages of the interaction [112]. Differential root exudate profiles were also associated with tomato rootstock, grafted to eggplant scions, compared to non-grafted eggplants and this was associated with suppression of mycelial growth and enhancement of mycelial growth, respectively [113]. Suppression of mycelial growth was associated with delayed onset of *V. dahliae* colonization and symptom development.

Defense-related gene expression responses are activated by various abiotic and biotic stresses in plants. In tomato, physical wounding induced defense-related genes including the *Ve1* gene in both susceptible and resistance lines [114]. During the compatible interaction between tomato and *V. dahliae*, compared to the non-inoculated control plant, 1,953 significantly differentially expressed genes (DEGs) were identified in the root samples two days after inoculation. Most of the DEGs were associated with phenylpropanoid metabolism and plant-pathogen interaction pathways [103]. Comparative proteomic and metabolomics of compatible and incompatible interactions with *V. dahliae* provided differential profiles in tomato stem tissues. During the incompatible interaction between Beefsteak (Ve+) tomato and Le1087 (race 1) *V. dahliae*, higher levels of phenolic compounds responsible for plant defense mechanisms and enzymes involved in plant defense responses including phenylalanine ammonia-lyase(PAL) and lignin biosynthesis were significantly induced [115]. These resistance related responses were consistent across the entire host plant because similar groups of genes were found to be induced in the *V. dahliae*-inoculated root tissues during incompatible interactions in a separate study [116]. Similarly, transcriptional profiles of sunflower infected with *V. dahliae* revealed that a large group of genes responsible for plant defense was induced in both resistant and susceptible hosts with higher induction in resistant host lines compared to susceptible ones. Genes involved

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in hypersensitive response and the salicylic and jasmonic acid-mediated signaling pathways were linked to *V. dahliae* resistance [117].

RNA-seq analyses upon *V. dahliae* infection have confirmed previously known common resistance-associated biological pathways in host plants. Temporal transcriptional analysis from *V. dahliae* inoculated *Arabidopsis thaliana* revealed 13,916 differentially expresses genes (DEGs) including 401 transcription factors compared to mock-treated plants [103]. Gene ontology (GO) functional classification of DEGs identified a total of 2,308 genes involved in the stress response which were subcategorized to 453 DEGs associated with defense response, 369 with the regulation of the plant-type hypersensitive response, and 358 with the defense response to fungi. Pathway analysis of DEGs showed that the genes involved in the biosynthesis of secondary metabolism are greatly enriched and a group of genes related to plant-pathogen interaction, plant hormone signal transduction, phenylalanine metabolism, flavonoid biosynthesis was highly enriched. Genes (413) involved in the SA hormone signaling pathway and 404 genes involved in JA signaling were differentially expressed during the infection process [103]. Similar gene expression patterns were exhibited during the interaction between the wild type resistant eggplant and *V. dahliae*: 17,645 DEGs were identified and genes involved in the phenylpropanoid pathway, lignin biosynthesis, and plant hormone signal transduction and genes encoding pathogenesis-related proteins(PRs) and transcription factors were induced during this incompatible interaction [118].

Currently, cross talk analysis between *V. dahliae* and tomato gene expression remains challenging because the majority of the transcripts from infected tissue samples are mapped to the host genome; less than 1% of the reads mapped to fungal genomes [119]. Likewise, only a few proteins from infected plant tissue have been mapped to *V. dahliae* proteome [115]. This lack of fungal information greatly impedes attempts to link fungal gene/protein patterns with corresponding host responses.

3. Current measures and limitations of Verticillium wilt management

3.1. The genetic basis of plant disease resistance

Broadly, the interactions between tomato and *V. dahliae* resulted in three states: (i) susceptibility (compatible interaction), (ii) resistance (incompatible interaction), and (iii) tolerance (intermediate interaction). In susceptibility, the fungus proliferates systematically throughout the plant leading to symptom expression and disease development. Disease resistance is further grouped into qualitative and quantitative resistance.

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3.1.1. *Qualitative disease resistance*. As detailed above, resistance to *V. dahliae* (race 1) is conferred by a single dominant *Ve* locus and incorporated into a tomato breeding programs [22] The *Ve* locus contains two closely linked inversely oriented genes, *Ve1* and *Ve2,* and are mapped on tomato chromosome 9 [29]. Intriguingly, only *Ve1*, but not *Ve2*, conferred resistance to *V. dahliae* in tomatoes [120]. The interfamily transfer of *Ve1* to *Arabidopsis thaliana* provided fully functional *Verticillium* wilt resistance [121]. Comparative genomic analysis of race 1 identified an effector, Ave1, which was a small secreted protein with four cysteines that contributed to virulence in tomato plants lacking *Ve1* [50]. The *Ve* locus encodes the extracellular leucine-rich repeat receptor-like protein class of *R* protein and triggers effector-triggered immunity in the host [29].

3.1.2. *Quantitative disease resistance (QDR)*. QDR is conditioned by multiple genes or quantitative trait loci (QTL) of small effects and may interact with the environment [122]. Generally, QDR is race non-specific and provides partial resistance, which reduces pathogen multiplication, plant colonization, and disease severity [123]. The effects of QDR are often additive and more durable than *R* gene-mediated resistance [123]. We have found variation in the level of resistance to *V. dahliae* races 2 and 3 in the tomato germplasm, however, the biological and molecular basis of resistance to these races in tomatoes is still unclear and needs further research.

3.1.3. *Plant tolerance.* Hosts that are tolerant reduce levels of symptoms and produce higher yields compared to susceptible ones [124,125]. Host tolerance can be quantified using pathogen biomass, disease severity, and yield impacts in the host cultivar [125]. In *V. dahliae*, hyphae colonize internal tissues and rapidly spread systemically [124]. Vascular wilt severity is often assessed using a disease index [126,127] or percentage of chlorosis and necrosis of leaves [124] while plant growth is quantified by measuring stem height [124][127] or by fresh weight [126]. Although host tolerance to *V. dahliae* was controlled by polygenes in cotton and potato [128,129], a single dominant gene, *VET* was found to enhance tolerance in *Arabidopsis thaliana* L. [127]. The use of tolerant cultivars as rootstocks or source of resistance in breeding programs may be a productive pursuit as a component of an IPM strategy to manage Verticillium wilt.

3.2. Grafting as a measure to combat Verticillium wilt

Grafting tomato has been documented as a tool to manage important soilborne diseases [130,131]. However, durable success has not been found using a diversity of rootstocks to manage Verticillium wilt of tomato. As expected, *V. dahliae* race 1 resistance provides a high degree of protection to susceptible

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scions in the regions where race 1 predominates [130-134]. Likewise, it is reasonable to anticipate race 2 resistance would protect susceptible scions in the field where race 2 predominates; currently, there is only lab and greenhouse evidence that race 2 resistance protects tomato plants [35,36]. Since pathogen races can only be discerned as resistance genes are deployed, the durability of any single gene is uncertain. For example, the discovery of "race 2" resistance enabled elucidation that "race 3" (non-race 1 and non-race 2 isolates) is widespread throughout Japan and North America, [35][unpublished]. . The long-term success of any given rootstock with race-specific resistance will rely on widespread pathogen screening in regions where those rootstocks are deployed.

Varieties developed from interspecific hybrids between *Solanum lycopersicum* and *S. hirsutum* may protect the wilting symptoms of *V. dahliae* [134]. The interspecific tomato hybrid rootstock "Beaufort" reduced disease in eggplant scions in field trials [130] although the reduction in disease and subsequent yield increases may simply be the result of higher vigor [134,135], possibly a form of tolerance as discussed above. Future studies will need to address whether increases in plant health from rootstocks are due to resistance or vigor.

3.3. Chemicals in use for Verticillium wilt management

With the phase-out of methyl bromide due to environmental concerns, exploration for effective alternate soil fumigants and other chemicals, and biologically based methods to manage *V. dahlia* has been implemented. Even though some alternative biological methods have been identified (see below), no new fungicide chemistry is available for use against *V. dahliae.* The most common fumigants: 1,3-dichloropropene (1,3-D), trichloronitromethane (chloropicrin), 3,5-dimethyl-(2H)-tetrahydro-1,3,5-thiadiazine-2-thione (dazomet), dimethyl disulfide (DMDS), sodium and potassium- N-methyldithiocarbanate (metam sodium, metam potassium) and their combinations have been widely evaluated for their efficacy to manage soilborne pathogens including *V. dahliae* [136-138]. These fumigants, singly (not 1,3-D) or in combination, are effective in reducing wilt incidence and *V. dahliae* microsclerotia in soil [137,139,140]. In an experiment using bell pepper, the use of chloropicrin at 30 and 40 g m^{-2}, applied by drip irrigation reduced verticillium wilt disease incidence significantly and reduction in disease progress rate was better than dazomet at 40 g m^{-2} [141] while the systemic fungicide, thiophanate-methyl, was found to be effective against *V. dahliae* in potato but only partially and when disease pressure was not high [142]. In another experiment with Chrysanthemum, DMDS, chloropicrin,

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and metam sodium had similar effects in significantly reducing Verticillium wilt incidence compared to the control [137]. When fumigants (DMDS, chloropicrin, 1,3-D) were used alone, a higher dosage was required for *V. dahliae* and *Meloidogyne incognita* suppression but when any two of these fumigants were combined, the lower dosage was effective [143]. Dazomet was also reported to promote phosphorus mineralization and allowed crops to absorb and use phosphorus [144].

Fumigants have sometimes been integrated with non-chemical approaches such as using resistant rootstocks and bio fumigants to improve disease control, reduce the use of chemicals, and protect the soil environment. When chloropicrin was alternated with bio-fumigation (fresh chicken manure + wheat straw), the nutrient availability in soil was improved and increased the strawberry marketable yield and microbe genetic diversity in the soil [145].

However, soil treated with some of these fumigants was reported to have a detrimental effect on the soil biochemical properties and microbiome. For instance: Chloropicrin inhibited conversion of ammonia to nitrite in five different soil types [146], chloropicrin and dazomet treatments lowered microbial activities and soil microbiome biomass, decreased alkaline phosphatase harboring microbes, and also resulted in different microbiomes as compared to those of anaerobic soil disinfestation (ASD) treatments [136,144,147]. However, a study with metam-sodium showed a mixed effect where it inhibited substrate-induced respiration, microbial biomass nitrogen, and accumulated ammonium ion in the soil in short term, but reduced the population of bacteria and fungi in the soil and shifted soil bacterial population to plant growth-promoting bacteria and biodegrading bacteria [148]. The negative impact of these chemical fumigants on the soil physicochemical properties and microbiome has provided an impetus to advance the science of non-chemical alternatives to manage *V. dahliae*.

3.4. Biocontrol agents and biologicals to manage Verticillium wilt

Biocontrol agents (BCAs) are microorganisms that are used to manage several pests including insects and plant pathogens of agriculturally important crops either by reducing pathogen inoculum or its ability to cause disease [149] while biologicals are products obtained from living organisms. Biocontrol is a tool in an integrated management strategy that is environmentally friendly and is viewed as a potential alternative to chemical pesticides to prevent their side effects [149]. Parasitism, competition for nutrients and space, antibiosis, and induction of systemic resistance (ISR) are major mechanisms of biocontrol [149,150]. The desirable traits of BCAs and their uses against Verticillium wilt are discussed elsewhere

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[151]. However, several recent studies have broadened the scope of BCAs with some new candidates within the well-established genus of *Bacillus, Pseudomonas,* and *Trichoderma* and beyond (Supplementary Table 2). Currently, studies involving BCAs do not only look for organisms with antagonistic properties, using the available genetic tools, their mode of action, genes, proteins, and metabolites involved has also been characterized. For instance: In *Bacillus velezensis* AL7, a biocontrol agent isolated from cotton soil that synthesizes antifungal antibiotics, 3,706 protein-coding genes, 86 tRNAs, and 27 rRNAs were predicted which can help identify the candidate genes involved; transcriptomic analysis of *Trichoderma atroviridae* T11 identified *cpa1* gene, whose increased level of expression and protease activity was associated with higher antifungal activity against *V. dahliae* V-138I [152,153]. These findings open avenues for further understanding of these BCAs to increase their efficacy for commercialization.

In some cases, mixing different BCAs or their extracts among themselves or with organic amendments have provided better management of *V. dahliae* [149,154,155] since mixing increased the biological activities of microbes and/ or their extracts. Little information is available regarding the use of biologicals to manage *V. dahliae* but oils, derivatives, and extracts from medicinally important plants and some algae are being tested and with encouraging results [156,157], however, more research is required to explore new sources and mechanisms of action before further use. Even though studies on the potential use of BCAs against *V. dahliae* have increased, the majority of the research on BCAs are conducted in vitro or greenhouses under controlled conditions. A major problem in the widespread use of BCAs is their inconsistent efficacy when tested under field conditions. However, some BCAs have shown promising results when experimented in the fields against *V. dahliae* with olives [158] and cotton [159], and combining BCAs with different modes of action has offered some efficacy [149,160]. Ensuring the long-term viability of BCAs and biologicals for storage is another problem that needs to be taken into consideration for commercialization and their practical application in the fields.

3.5. Organic amendments

For soilborne pathogens such as *V. dahliae*, chemical-based suppression has not proven sustainable and the use of organic amendments (OAs) has been explored to design suppressive conditions to limit pathogen infestation levels or onset of disease. OAs include materials that are worked into the soil or applied on the surface to improve the physical properties of the soil and by fostering living microorganisms that are present in the soil to directly or indirectly impact disease incidence [149]. Some

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examples of OAs used to manage *V. dahliae* in various crops comprise of plant and animal-based composts and manures; green manure/cover crop; and other industrial co/by-product wastes (Supplementary Table 3).

Composts not only add organic matter to the soil but also serve as the reservoir to foster a microbiome that can protect crops through increased soil microbial activities against soil-borne pathogens [161]. Compared to animal-based amendments (dairy and horse manure), plant-based amendments can impact pathogen success due to deleterious chemicals introduced from the plants, in addition to beneficial microbial activities [162]. Cover/Green manure crops are rotated with main crops to cover the soil surface that improves the physical, chemical, and biological properties of soil [163]. Furthermore, they can be incorporated into the soil to suppress soil-borne pathogens [164]. Crops in the Brassicaceae family are a good example of green manure often used in crop rotation to reduce soil-borne pests and pathogens. They are rich in glucosinolates, the precursors of isothiocyanates that produce volatile sulfur compounds, known for fungicidal, nematocidal, and allelopathic properties through bio-fumigation [164]. When green manures were polyethylene-covered, the toxic effects on pathogens were greater compared to their application in open soil.

As with biocontrol agents, single OA may not provide sufficient pathogen suppression, hence, when applied as a mix of OAs or with biocontrol agents efficacy was better [149,165,166]. However, factors such as the type of amendments; the lack of standardization of application rates; the inconsistency in their efficacy; and phytotoxic effects of released toxic compounds on crops limit the practical applications and widespread use of OAs for disease control [167] and requires further attention.

3.6. Anaerobic soil disinfestation (ASD)

Anaerobic soil disinfestation (also known as reductive soil disinfestation or biological soil disinfestation) is an organic amendment-based pre-plant soil-borne disease management tool [168,169]. For ASD, the soil is first amended with a carbon source, irrigated to field capacity to fill soil pore spaces with water, and covered with an impermeable plastic tarp, or surface-sealed using other methods, to limit gas exchange for several weeks to complete the ASD treatment [170,171]. Some examples of carbon sources from recent studies of ASD used in various crops include rice-bran, molasses, ethanol, and others (Supplementary Table 4).

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ASD has proven effective against a wide range of soil-borne pathogens in many different cropping systems, however, the efficacy against a target pathogen depends on the carbon-source used, tarp type, soil type, soil microbiome, and soil temperature retained during ASD [169,171]. Ebihaha and Uematsu [172] tested the survival of three strawberry pathogens under anaerobic conditions and found that *V. dahliae* could not grow under anaerobic conditions at 22.5^{o} C, indicating that anaerobic conditions obtained during ASD can have a fungistatic effect on *V. dahliae*. ASD treatments also induced changes in soil microbial communities and increased the soil microbial activity and the populations of Bacteroidales, Clostridiales, Selenomonadales, Enterobacteriales, Sphingobacteriales, Bacillales, and Burkholderiales that antagonize plant pathogens [136,168,173]. The change in the bacterial communities and composition increased denitrification, nitrogen fixation, and produced organic acids that influenced disease suppressiveness [174]. Optimizing the carbon source for ASD can improve the effectiveness of ASD and affordability for growers [168]. An economic analysis of ASD for open-field fresh-market tomato production using molasses and composted poultry litter showed that ASD requires higher labor costs for land preparation and treatment application but the yield increase from ASD treatment was enough to cover the increased labor cost [175]. Similarly, in the studies with strawberry, where different carbon sources of ASD were compared to chemical treatment (PicChlor 60), the net return and marketable yield were either similar or increased due to ASD (e. g. Rice bran) [139,176]. Even though the issues related to efficacy, cost, and standardized application rates of a carbon source need attention [168,169,171], the results obtained from ASD studies are encouraging and is gaining popularity.

Most of the current studies on utilizing non-chemical-based approaches to manage *V. dahliae* have focused on cotton, olive, and eggplant, and little information is available using tomatoes. Hence, experimenting with the potential BCAs, OAs, and carbon sources from other crops with the tomato-*Verticillium dahliae* pathosystem could help identify the candidates that may benefit tomato growers.

4. Novel approaches and Future Directions

4.1. Advances in plant microbiomes: applications to *Verticillium dahliae* system

Microbiomes are composed of numerous individuals (e. g., bacteria, fungi, actinomycetes, virus, and protists) of diverse species [177]. All tissues of a plant harbor microbiomes including roots, leaves, shoots, flowers, and seeds. Based on the association with habitats in the host plants, microbiomes are classified as the rhizosphere, phyllosphere, and endosphere microbiomes [178]. The rhizosphere is a rich and soil-derived microbial diversity zone, which is influenced by plant roots through the

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rhizodeposition of exudates, and mucilages [179]. Although some work is available elucidating rhizosphere microbiomes with antagonistic activity towards *V. dahliae* in cotton, oilseed rape, potato, and strawberry [180,181], it is not known how these microbes play beneficial roles in tomato-microbiome interactions. Phyllosphere microbes residing on the leaf surface are mainly epiphytes and are influenced by leaf structures such as veins, hairs, and stomata [182]. Tomato rootstocks have differential impacts on tomato scion phylospheres [183], but again, to our knowledge, there are no published reports on tomato × Verticillium interactions. Endosphere microbiomes reside within the intracellular apoplast and in the xylem vessels, which may enter through natural breaks in root and root tips and translocate to the aerial parts of the plant [178]. Endosphere microbes are typically latent and non-pathogenic and can influence host metabolism and plant immunity [184]. The antagonistic activity of *B. amyloliquefaciens* from different cultivars and regions against the olive-pathogenic *V. dahliae* also showed a close functional relationship [185]. Interestingly, an endophytic, non-pathogenic *Fusarium solani* (strain CEF559) also conferred protection against *V. dahliae* [159]. To date, microbiomes of tomato plants growing under field conditions remain poorly characterized and many of the roles and interactions of diverse disease-resistant rootstocks and the field environments remain to be elucidated. We hypothesize that many of the benefits of rootstocks are mediated by soil and rhizosphere microbiomes and that intra- and inter-specific genetic variation can impact the structure and composition of the microbial community and suppress *V. dahliae* and enhance plant health. Moreover, plant root exudates may contain signal molecules that may influence species composition in the rhizosphere. Increasing evidence supports the hypothesis that the association between grafted tomato rootstocks and rhizosphere microbiomes can improve plant growth and inhibit *V. dahliae* pathogen [186]. Beneficial microbiomes also activated immune systems such as induced systemic resistance (ISR) [187] and systemic acquired resistance (SAR) to plant pathogens [188]. To this end, mapping populations and innovative grafting experiments can be conducted to test ecological hypotheses and devise prescriptive approaches to manage microbiomes to suppress Verticillium wilt problems in tomato.

4.2. Exploiting knowledge of microbiomes – *Verticiliium dahliae* interactions to enhance plant health

To date, microbiomes of tomato plants growing under field conditions remain poorly characterized and many of the roles and interactions of diverse disease-resistant rootstocks and the field environments remain to be elucidated. We hypothesize that many of the benefits of rootstocks are mediated by soil

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and rhizosphere microbiomes and that intra- and inter-specific genetic variation can impact the structure and composition of the microbial community and suppress *V. dahliae* and enhance plant health. Moreover, plant root exudates may contain signal molecules that may influence species composition in the rhizosphere. Increasing evidence supports the hypothesis that the association between grafted tomato rootstocks and rhizosphere microbiomes can improve plant growth and inhibit *V. dahliae* pathogen [186]. Beneficial microbiomes also activated immune systems such as induced systemic resistance (ISR) [187] and systemic acquired resistance (SAR) to plant pathogens [188]. To this end, mapping populations and resistant scion onto resistant rootstocks can be used to test the ecological hypotheses and for the discovery of new molecules or compounds in the rhizosphere and phyllosphere microbiomes using new approaches (Fig. 3).

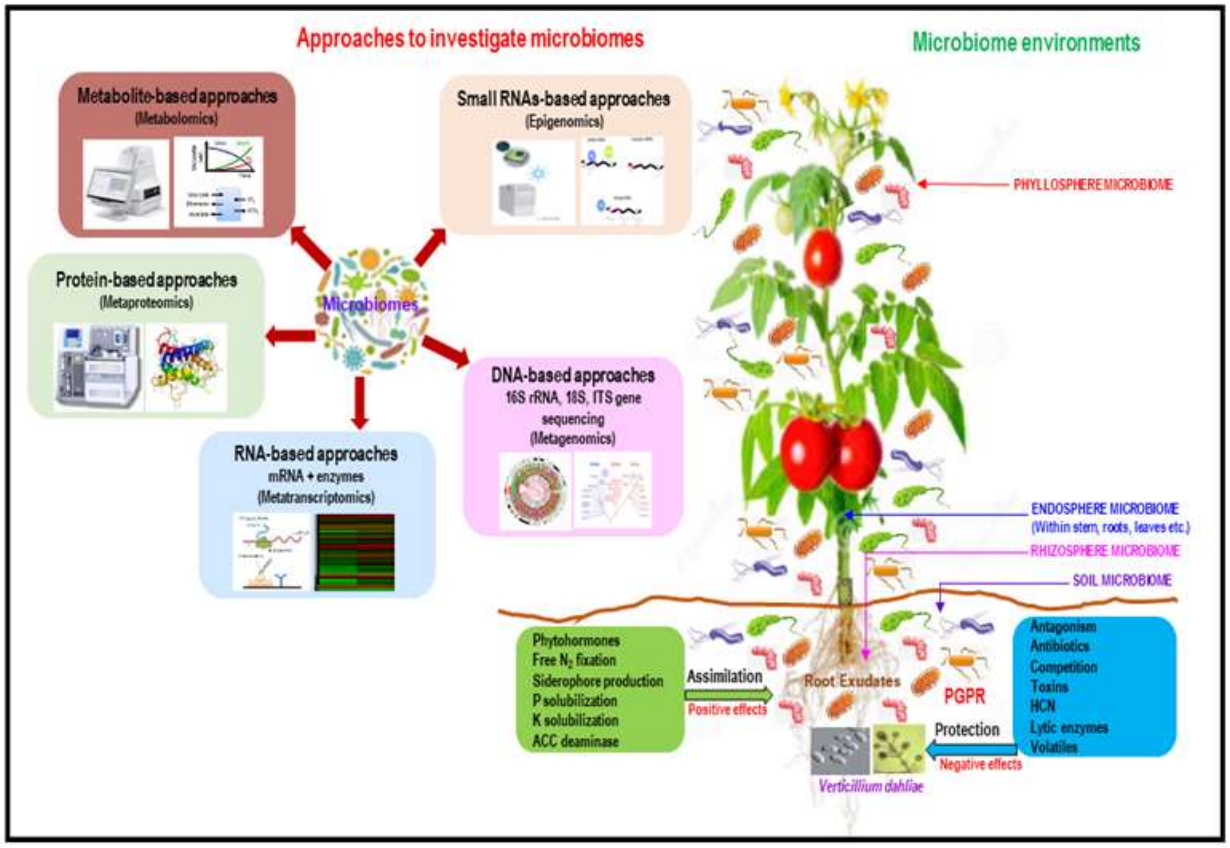

Figure 3. A proposed schematic illustration of the plant microbiome impact on *Verticillium dahliae* protection and crop productivity crop yield [180,181].

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4.3. Novel molecular and genomic approaches to enhance Verticillium wilt resistance

Although conventional breeding plays an important role in developing and testing several tomato lines in the field, traditional improvement methods are time-consuming and troublesome. However, breeding efforts to exploit the genetic variability in the cultivated and wild relatives of tomato and utilize resistance to *V. dahliae* and other pathogens in tomato breeding programs have been achieved with some successes. For example, wild tomato relatives such as *L. pimpinellifolium*, *L. peruvianum*, and *L. hirsutum* have been utilized as sources of resistance to develop segregating breeding populations to test against *V. dahliae* and other pathogens [189]. Other populations developed are recombinant inbred lines (RILs), near-isogenic lines (NILs), and multiparent advanced generation inter-cross (MAGIC) populations [189,190]. Although conventional breeding has been successfully used to improve yield and quality and meet consumer requirements, the introgression of *R* genes or QTL and examination of large populations is time-consuming and labor-intensive [191]. Molecular markers such as cleavage-based cleaved amplified polymorphic sequences (CAPS), kompetitive allele-specific PCR (KASP), simple sequence repeats (SSR), single nucleotide polymorphisms (SNPs), and InDels [192] have been developed and used to locate and tag genes or QTLs for disease resistance and other traits in tomato via marker-assisted selection (MAS) [193-195]. Whole-genome resequencing approaches such as QTL-seq [196], genetic mapping and mutant identification (MutMap) [197] and bulked-segregant analysis based on RNA-seq (BSR-seq) [198] and specific locus amplified fragment sequencing (SLAF-seq) [199], and genome-wide association studies (GWAS) [200] have also been utilized to identify candidate genes or markers linked to the gene of interests in tomato.

Plants have developed sophisticated defense mechanisms to fight pathogen attacks [201]. Plasma membrane-bound and intracellular immune receptors initiate innate defense responses upon the perception of pathogens either directly interacting with pathogen-derived immunogens or indirectly by monitoring modifications of host targets incurred by pathogens [201,202]. Plant-derived antimicrobial peptides and other compounds such as *FLS2*, *LecRK-VI.2*, *EFR*, *CERK1*, *Ve1,* and *PERPs* all belong to Receptor-like Kinases (RLKs) [203] that inhibit pathogen virulence [204,205]. On the other hand, plant pathogens have evolved some strategies to overcome the defense responses of their hosts. These offensive weapons include cell-wall degrading enzymes (CWDEs) that degrade the plant cell wall for successful infection [206] or secretion systems to deliver effectors into the host cytoplasm to suppress host defense and promote colonization [207,208]. The recent advances in biotechnological innovations

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and the rapid development of high-throughput sequencing technologies, and some aspects of host-microbe provide opportunities to greatly enhance functional investigations and deployment of useful disease resistance genes [209] portends a promising future toward managing Verticillium wilt of tomato.

4.4. Identifying quantitative disease resistance (QDR) and pyramiding for broad-spectrum and durable resistance

Durable disease resistance refers to the resistance that remains effective over a prolonged period [210]. A deeper understanding of pathogen biology, population structure, epidemiology, and mechanism of genetic variation help predict the durability of disease resistance [211]. To develop broad-spectrum and durable resistance, gene pyramiding (also known as gene stacking) strategy has been used to deploy multiple *R* genes into a single cultivar simultaneously [212]. For instance, resistance to multiple races of rice blast and bacterial blight was achieved by stacking genes using MAS [213,214]. Similarly, broad-spectrum resistance to late blight pathogen was achieved by molecular stacking of three *R* genes in two separate occasions, (*Rpi-sto1*, *Rpi-vnt1.1*, and *Rpi-blb3*) [215] and (*RB*, *Rpi-blb2*, and *Rpi-vnt1.1*) [216], at a single genetic locus in potato using *Agrobacterium* transformation [217]. Hence, these new advancements offer an opportunity to rapidly identify several small effect alleles through genomics-enabled newer breeding approaches [122,209], and stacking them for broad-spectrum resistance to *V. dahliae* in tomato (Fig. 4).

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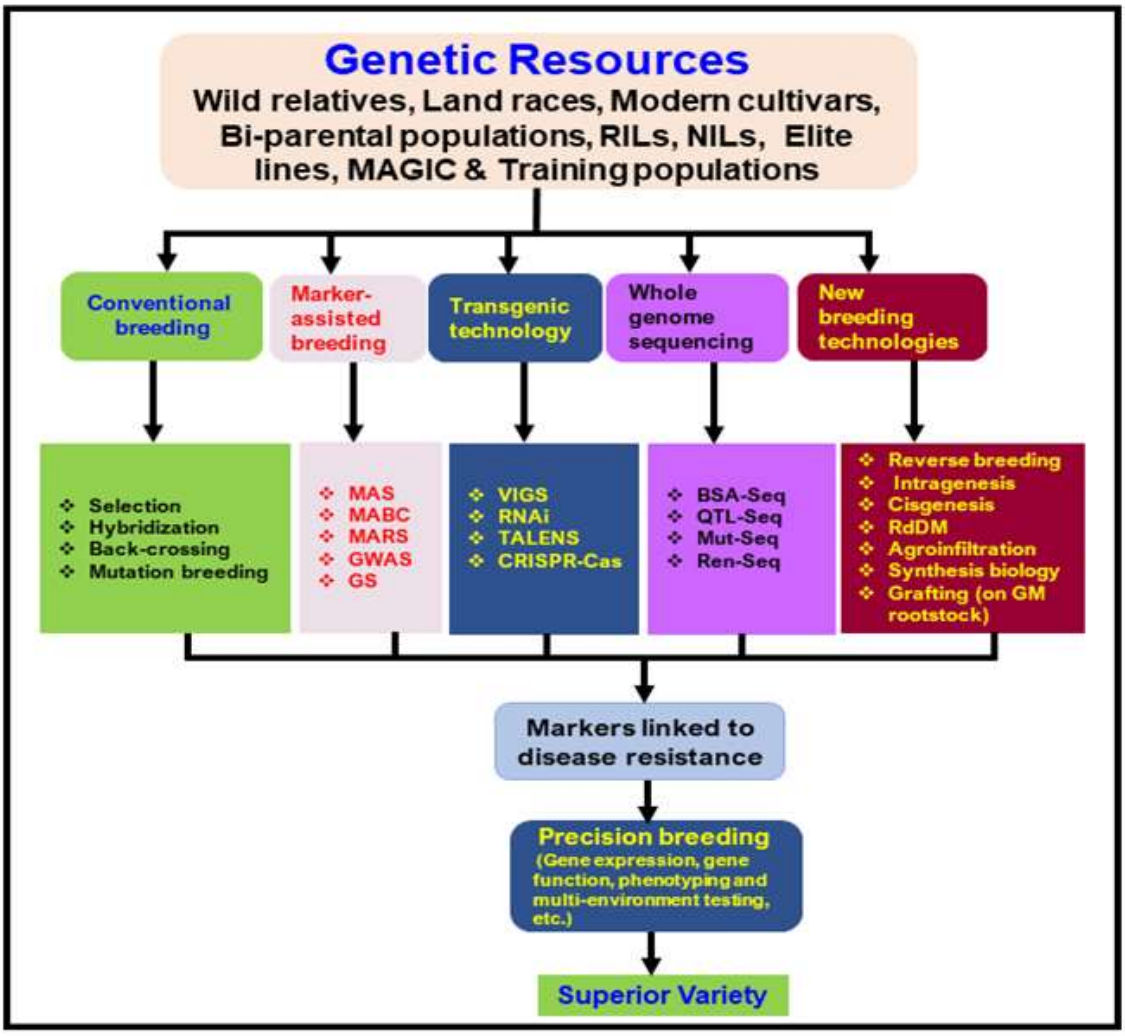

Figure 4. A proposed flow-chart to develop Verticillium wilt resistant tomato variety by genome-based approaches and new plant breeding technologies.

4.5. Exploring and exploiting the intracellular immune receptors

Resistance gene enrichment sequencing (Ren-Seq) [218,219] has been used to identify regulatory elements and nucleotide-binding leucine-rich repeat (*NRL*) family proteins from uncharacterized germplasms [50]. Recently, Ren-Seq with single-molecule real-time (SMRT) has been successfully utilized to rapidly identify and clone anti-potato late blight *NLR* genes from wild potato [220], and four stem rust (*Sr*) genes for resistance to *Puccinia graminis* f. sp. *tritici* from wild accessions (*Aegilops tauschii* spp. *strangulata*) [221]. Besides, two stem rust *NLR* genes, *Sr22* and *Sr45* from hexaploid bread

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wheat have been discovered and these genes conferred resistance to multiple races of stem rust pathogen [222]. Potentially, Ren-Seq can be a powerful tool to rapidly uncover novel *NLR* genes for resistance to races 2 and 3 of *V. dahliae* from wild tomato species and utilize them in breeding programs.

4.6. Modulating microRNAs and improving plant disease resistance

Plants carry two major classes of small RNAs, namely microRNA (miRNA) and small interfering RNA (siRNA), which are endogenous, single-stranded non-coding RNAs molecules (21-24 nucleotides in length) that bind to partially complementary sequences in target messenger RNAs (mRNAs) [223]. RNA interference (RNAi) technique has been used to suppress the expression of a gene by the host- or the pathogen-induced gene [224]. Extensive studies have demonstrated that miRNAs play important roles in plant growth and development, and tolerance to abiotic and biotic stresses [225,226]. Available evidence suggests that miRNAs also play critical roles in plant immune systems [227-229]. For example, the miR393 has been implicated in pathogen-associated molecular pattern-triggered immunity (PTI) [229]. miRNAs are considered master regulators of the *NLR* defense gene family [230-232]. Importantly, miR482-mediated silencing cascade in *Arabidopsis,* cotton, potato, and eggplant enhanced plant defense against *V. dahliae* [232-234]. Two *V. dahliae* genes, *Clp-1* (encodes a Ca2+-dependent cysteine protease) and *HiC-15* (encodes an isotrichodermin C-15 hydroxylase) were targeted by miR166 and miR159, respectively, and silencing of these two fungal virulence genes conferred resistance to *V. dahliae* [235]. In this scenario, the modulation of miRNAs by RNA silencing [236] offers a powerful strategy to improve our understanding of tomato - *V. dahliae* interactions and to enhance plant defense.

4.7. Harnessing gene-editing technologies

More recently, genome-editing based on CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) technologies have revealed a breakthrough for miRNA fine-tuning [237]. In this process, Cas9 protein (an RNA-guided nuclease) can be cleaved at a specific desired sequence on the substrate viral DNA or RNA, generating DNA double-strand breaks that usually result in gene silence due to their degradation [238]. CRISPR–Cas9 and -Cas13a mediated single or multiple protein-coding gene knockouts have been successfully developed in several crops and utilized to engineer resistance to DNA or RNA plant virus diseases [202]. The CRISPR/Cas9 genome-editing platform has also been used to enhance resistance to *V. dahliae* in cotton. The indels of the *Gh14-3-3d* gene (signaling receptor proteins) were generated in the At and Dt sub-genomes of tetraploid cotton

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(*Gossypium hirsutrum)* and transgene-clean edited T2 plants showed enhanced plant defense against *V. dahliae* [239]. Using the CRISPR–Cas9 system, multiple genes, *lig1*, *ms26,* and *ms45* were stacked in a single chromosomal location in corn [240]. Other major genome editing and new plant breeding techniques (NPBTs) developed are homologous recombination (HR), meganucleases (MNs), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), pentatricopeptide repeat proteins (PPRs), Oligonucleotide directed mutagenesis (ODM) cisgenesis, and intragenesis [209,241]. Besides, RNA-directed DNA methylation (RdDM), reverse breeding, grafting on genetically modified rootstock, agro-infiltration, and synthetic genomics have been employed to develop desirable plant material for sustainable food production [209,241-244]. Although these techniques have been applied for altering important phenotypic traits, their utilization to ensure resistance to *V. dahliae* in tomatoes needs to be explored.

5. Concluding remarks

Recently, numerous studies have emerged relating to the biology of *V. dahliae* and its management in different crop systems. This indicates that *V. dahliae* is a notorious pathogen and management of this pathogen is complicated. This review sought to provide recent information and discussions on *V. dahliae*, its importance in tomato production, molecular mechanisms involved to make it a successful pathogen, and an overview of current management tactics. The recent reports of the discovery of race 3 of *V. dahliae,* prediction of about 200 core effector proteins, and others in lineage-specific regions has opened up a lot of opportunities for downstream research and to elucidate the mechanisms and genes involved. For Verticillium wilt management, several non-chemical methods are being explored. This could be due to the reduction in the number of available chemical alternatives and their harmful impacts on the environment and human health. More recently, studies involving biocontrol agents, organic amendments, and anaerobic soil disinfestation to manage *V. dahliae* has increased substantially, however, their efficacy for use in the field needs additional optimization. Recent advances in molecular and sequencing technologies are providing a better understanding of the mechanisms for disease suppression conferred by these tactics informing the future prescriptive implementation of these methods. For genetic resistance, the *Ve1* gene had been identified and deployed in tomatoes to manage race 1 in the early 1950s but was defeated in a few years by the resident or evolved non-race 1 strains. A recent study suggests that resistance to race 2 in tomato is conferred by a single gene as well. However, the presence of partial resistance and tolerance to *V. dahliae* is predicted in tomatoes as variation in the level of

resistance to race 2 and 3 have been observed in tomato germplasm, when tested under lab, greenhouse, and field conditions. The mechanism of resistance and the genes involved in this type of resistance is still to be explored. In addition to these methods, recent studies suggest the utilization of microbiomes present in crop organelles can enhance resistance and protect the crop from pathogens. Microbiomes associated with tomato-*V. dahliae* pathosystem has not been characterized and needs attention in this new area of research. Furthermore, in the era of "omics", there are several molecular and genomic breeding as well as genome editing tools available for a rapid and in-depth understanding of the mechanisms of resistance to Verticillium wilt in tomato, as discussed in this review, that can lead to a fast-pace development of resistant cultivars.

Author contributions

All the authors conceptualized this review manuscript; B. A., T. I., Y. O., and T. B. A. contributed equally in writing different sections of the original draft of the manuscript; all the authors contributed to the reviewing and editing; R. D. and F. J. L. provided the oversight in preparing this manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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