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EFFECTORS, ELICITOR PROTEINS AND CELL WALL DEGRADING ENZYMES SECRETED FROM *VERTICILLIUM DAHLIAE* (KLEB) AND THEIR ROLE ON VIRULENCE – AN OVER VIEW

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ABSTRACT

Verticillium dahliae, a soil borne pathogen, belongs to the fungal class Deuteromycetes, a group of fungi, which do not have a known sexual stage. It is a hemi-biotrophic pathogenic fungus capable of infecting wide range of hosts. Secretion of virulence determinants referred as effectors proteins that enable the pathogen to suppress plant defense responses and modulate host metabolism are the key governing factor determining the success of infection. Like other plant pathogens, *Verticillium dahliae* secretes a range of effectors, elicitor proteins and cell wall degrading enzymes to promote successful infection and colonization of its hosts. This review focuses on specific effectors proteins secreted by filamentous fungi, *Verticillium dahliae*, the virulence role of effectors in the fungal-host interaction and the molecular mechanism by which the effectors modulate processes in the plant hosts. In addition, proteins secreted during *Verticillium dahliae* infection process and their contributions to virulence are discussed.

Key words: Effectors, Elicitor proteins, Cell wall degrading enzymes, *Verticillium dahliae*, Virulence

INTRODUCTION

Plant pathogens such as fungi, bacteria, viruses and Oomycetes are estimated to cause around 10% of global crop losses annually (Oerke, 2006) and this figure is expected to increase with growing consequences of climate change (Elad and Pertot, 2014). To fulfill the food demands of increasing world population, it is necessary to combat plants diseases through continuous identification and introgression of new resistant traits into crops. To achieve this goal, a fundamental understanding of the molecular basis of host-pathogen interactions is essential.

Despite the diverse strategies adopted by pathogens to invade plants, pathogens are recognized by plant immune systems. Generally, the active immune system of plants is an innate, receptor-based recognition system and is divided into two layers. At the cell surface immune receptors possessing extracellular domains, such as Receptor-Like Proteins (RLPs) and Receptor-Like Kinases (RLKs) survey the environment for conserved Pathogen-Associated Molecular Patterns (PAMPs), and when activated initiate a defense response Pattern-Triggered Immunity (PTI) (Pandey and Chatterjee, 2013). These PTI receptors are termed pattern recognition receptors (PRRs) (Zhang and Zhou, 2010). To prevent pathogen ingress, PTI activates cellular immune signaling that include Ca^{2+} influx, burst of reactive oxygen species (ROS), accumulation of defense hormones, expression of defense-related genes and callose deposition (Couto and Zipfel, 2016).

PTI usually halts or restricts the infection process (Zhang and Zhou, 2010), however, successful pathogens evade or subvert PTI by secretion of so-called “effector” proteins that can inhibit PRR complexes or their downstream signaling events and cause Effector-Triggered Susceptibility (ETS) (Jones and Dangl, 2006; Win *et al.*, 2012). For this purpose, pathogens secrete a range of effectors during the course of an infection and/or are associated with pathogenic transitions (Toruno *et al.*, 2016).

To counteract effectors action, plants have developed a second layer of defense called Effector Triggered Immunity (ETI). This immune system relies on resistant proteins (R proteins), which possess intracellular Nucleotide-Binding (NB) and Leucine-Rich Repeat (LRR) domain architecture. These intracellular immune receptors recognize pathogen effectors or effector activity and subsequently mount immune responses (Nomura *et al.*, 2011). ETI is often observed as Hypersensitive Response (HR) that is associated with stronger immune responses leading to localized cell death at the site of attempted infection (Jones and Dangl, 2006).

In order to facilitate infection, plant pathogens secrete numerous effector proteins into plant cells. Previously, majority of information about plant pathogen effectors comes from studies of prokaryotic pathogens, such as the bacterium *Pseudomonas syringae* (Chang *et al.*, 2005; Axtell *et al.*, 2003). To date, effector proteins have been cloned and characterized from crop infecting fungi

and Oomycetes, which can be used as a model to study effector proteins in eukaryotic plant pathogens. The filamentous fungi, *Verticillium dahliae* (*V. dahliae*), the causal agent of *Verticillium* wilt diseases, represents one of such examples. *V. dahliae*, a soil borne pathogen, belongs to fungal class Deuteromycetes (Fungi Imperfecti), a group of fungi, which do not have a known sexual stage (Agrios, 2005). It is hemi-biotrophic fungi that infect over 200 plant species, including the major economically important crops such as cotton, tomato, potato, tobacco, lettuce, eggplant and strawberry (Inderbitzin and Subbarao, 2014; Short et al., 2014).

Until present time, *V. dahliae* has not been effectively controlled, owing to the production of melanized resting structures microsclerotia that persists for years, ability of infecting an array of hosts and presence in the vascular system (Luo et al., 2014; Klosterman et al., 2009). Moreover, control of *Verticillium* wilt disease through host resistance as well has been constrained due to unavailable resistance genes in many crops (Klosterman et al., 2009). Only from tomato a single dominant locus that confers *Verticillium* resistance *Ve* locus been identified, which controls race 1 *V. dahliae* and *V. albo-atrum* isolates (Song et al., 2018; Fradin et al., 2009).

Now-a-days, due to advances in new technology recent molecular level researches elucidated function of numerous genes and fungal secretomes which has improved our understanding in plant-pathogen interactions and also lead

us to search for alternative disease management practices. In *V. dahliae* genome ~780 potentially secreted proteins have been identified (Klosterman et al., 2011).

This review paper is aimed to discuss specific effector proteins characterized from *V. dahliae* and their virulence role, and to review mechanisms by which these effectors modulate host physiology and alter host metabolism. In addition, *V. dahliae* plant cell wall degrading and secreted elicitor proteins and their pathogenicity roles are discussed.

2. DISCUSSION

2.1. *Verticillium* wilt: pathogen and symptoms

Verticillium wilt disease is one of the most devastating vascular disease infecting a wide variety of herbaceous annual crops to woody perennials (Fig.1). So far, ten *Verticillium* species were distinguished, namely *V. dahliae*, *V. albo-atrum*, *V. alfalfae*, *V. longisporum*, *V. nonalfalfae*, *V. tricorpus*, *V. zaregamsianum*, *V. isaacii*, *V. nubilum* and *V. klebahnii* (Inderbitzin et al., 2013). Among these species within *Verticillium* genus, *V. dahliae* is an economically important wilt causing pathogen with broad host range of over 200 plant species (Inderbitzin and Subbarao, 2014). *V. dahliae* falls into the following taxonomic classification stated below:

Kingdom: Fungi

Phylum: Ascomycota

Class:

Deuteromycetes

Family: *Verticillium dahliae* Species:
Plectosphaerellaceae
Genus: *Verticillium*



FIGURE 1. Microscopic view of *V. dahliae* on culture and symptoms. (a) Microscopic view of verticillate spore bearing structure of *V. dahliae*; (b) culture petri dish showing profuse numbers of microsclerotia and (c) Brown foliage and wilting caused by *V. dahliae* on strawberry and (d) wilting symptoms on tomato.

Even though symptoms vary between hosts, *V. dahliae* infected plants mostly show wilting, chlorosis, stunting, necrosis and vein clearing symptoms. In cross-section of stems tissues, brown vascular discoloration is another symptom caused by *V. dahliae* infection (FIG1A-D).

2.2. *V. dahliae* host root colonization

The life cycle of *Verticillium*-wilt fungal pathogen is very similar and can be divided into three phases: a dormant, parasitic and saprophytic (Fradin and Thomma, 2006). A melanized resting structure, microsclerotia, either as dispersed propagules or embedded within plant debris represents the dormant stage of *V. dahliae* life cycle (Klosterman et al., 2009). To initiate the parasitic phase, root exudates stimulate germination of the dormant microsclerotia driving emerging

infectious hyphae to grow towards host roots (Mol, 1995). Upon contact of hyphae with host roots, penetration takes place through the root tips or sites of lateral root by forming an infection structure known as hyphopodium (Prieto et al., 2009). Recent information on *V. dahliae* host colonization process which also indicates its life cycle is shown using Green fluorescent protein (GFP)-tagged assay and *Arabidopsis thaliana* and sunflower roots as a model (Zhao et al., 2014; Zhang et al., 2018).

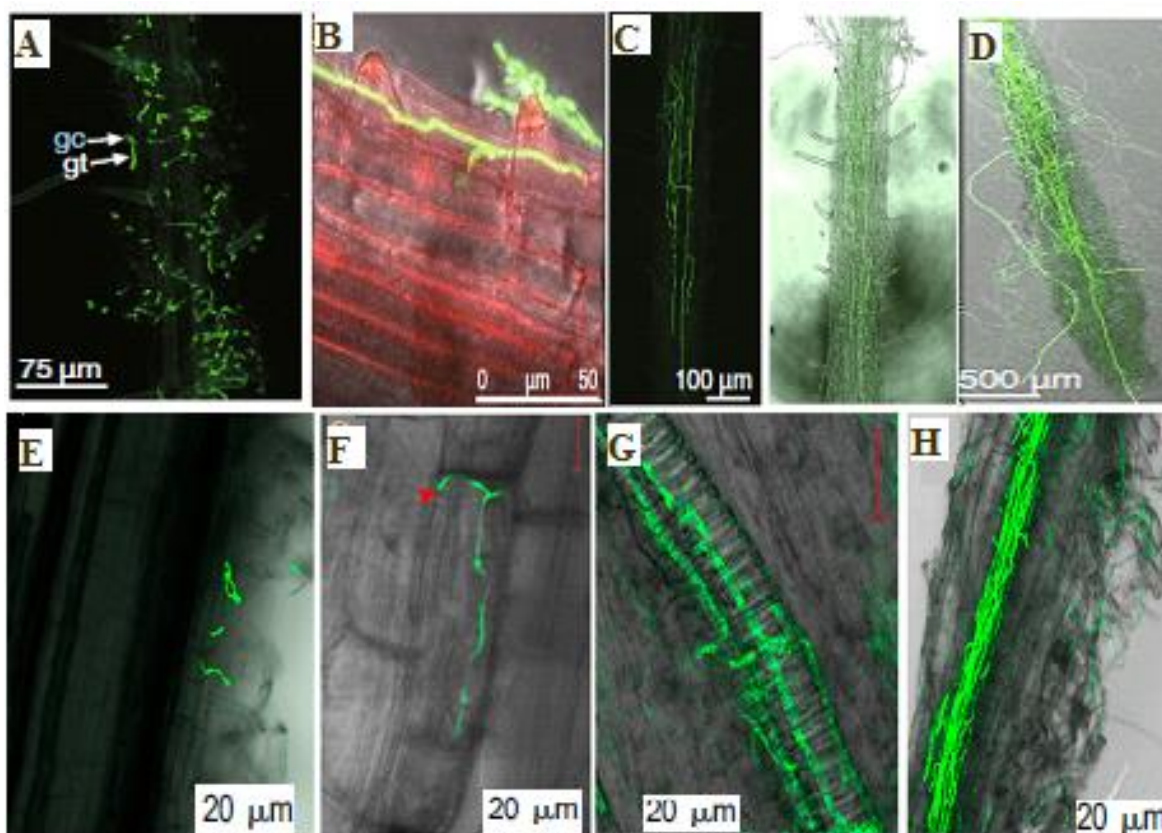


FIGURE 2. *V. dahliae* caused wilt disease cycle as observed by GFP-tagged V592 and VdBM9-6 strain on *Arabidopsis* (A-D) and safflower (E-H) roots, respectively. (a) Hyphae growth on a root of *Arabidopsis* around the root hair zone 24 hpi; (b) Hyphae tightly adhered on the root surface and grew along the longitudinal junction and extended across the transverse to penetrate into epidermal cells intercellularly 2 dpi; (c) Hyphal net within the xylem vessel 5 dpi, (d) Hyphae extended acropetally up the xylem vessels to the above-ground tissues 10 dpi, (e) Germinated conidia on the root hairs 24 hpi; (f) Mycelium extended along the longitudinal junction between root epidermal cells on the elongation zone of lateral root 48 hpi; (g) Mycelia in vascular vessels of lateral roots 5 dpi and (h) Mycelium extended along the vascular bundles of tap root 7 dpi. Images A-D from Source: Zhao *et al.* (2014) and E-H from Zhang *et al.* (2018).

Images under Confocal Laser Scanning Microscopy (CLSM) of *Arabidopsis thaliana* roots infected with GFP-tagged *V. dahliae* V592-strains detected massive germinated conidia and extended hyphae around root zone by 24 hours post inoculation (hpi) (FIG2A). By 48 hpi, subsequent penetration of the hyphae into the *Arabidopsis* epidermal cells has been evident, followed by vast colonization near root tips (FIG2B). Soon after penetration, the hyphae crossed epidermal and cortex cells intercellularly to reach xylem cells within 5 dpi (FIG2 C). In this study, even though successful vascular

infection of the pathogen has been confirmed within 5 days after inoculation, additional 5 days are required for hyphae to reach at the xylem of above ground *Arabidopsis* tissues (FIG2 D). As the infection progresses, the above ground *Arabidopsis* organs developed foliar wilt symptoms by 14 dpi (Zhao *et al.*, 2014). Similar initial colonization process of *V. dahliae* has been reported on sunflower roots infected by VdBM9-6 strain at both 24 and 48 hpi as shown in FIG2 E-H (Zhang *et al.*, 2018). Further multiplication of the *V. dahliae* in the xylem vessels blocked water transport, which ultimately lead to wilting

and dying of plant tissue signifying the saprophytic stage and accumulation of microsclerotia.

2.3. Effector repertoire of *Verticillium* species

The inclusive effector definition includes all pathogen proteins and small molecules that modify host cell structures, metabolism and functions to promote the success of a pathogen (Kamoun, 2006). These proteins often interfere with signal pathways either those required by host invasion (virulence factors or toxins) or those that trigger host resistance (avirulence factors: Avr) (Selin et al., 2016; Wawra et al., 2012). Effectors are grouped into apoplastic and cytoplasmic based on their target sites in the host plant (Kamoun, 2006). Apoplastic effectors exert their function in the extracellular space, while cytoplasmic effectors are translocated into inside the plant cell where they target different subcellular compartments (Pelgrom and Van den Ackerveken, 2016).

From the total genome size ~33.8 Mb of *V. dahliae*, 10,535 genes are encoding 780 secreted proteins. Of these 127 are conserved hypothetical proteins designated as small, cysteine-rich candidate effector proteins (Klosterman et al., 2011). In a closely related *V. dahliae* species, *V. albo-atrum*, a similar number of total secretome and secreted effectors proteins have been predicted based on subcellular localization and presence of signal peptide. Moreover, from total 246 proteins conserved hypothetical protein between *V. dahliae* and *V. albo-atrum*, 119 proteins have been postulated as effectors based on the criteria that fungal

effectors are small (less 400 amino acids) and cysteine-rich (greater than 4 cysteine residues) (Klosterman et al., 2011). Likewise, in silico secretomes analysis of *V. nonalfalfae* has shown a higher number of classically secreted proteins (944) and fewer set of small cysteine-rich effector proteins than other plant pathogenic *Verticillium* species (Marton et al., 2018).

From the effector repertoire of the three *Verticillium* species such as *V. dahliae*, *V. albo-atrum* and *V. nonalfalfae*, LysM and the necrosis and ethylene-inducing-like protein (NLP) families of proteins are more expanded, which might have contributed to a broad host range among dicotyledonous plant hosts (Klosterman et al., 2011). Currently, very few *V. dahliae* effector proteins have been characterized and shown to play significant role to virulence. In this seminar, the functions of effector proteins that have been characterized so far from *V. dahliae* are reviewed. In addition, the mechanisms the effectors proteins use to modulate host physiology and exploit plants nutrition are also discussed.

2.3.1. Apoplastic *V. dahliae* effectors

Apoplastic effectors interact with extracellular targets and surface receptors and functions several ways to deactivate plant defenses (Kamoun, 2006). From the apoplastic effectors *V. dahliae* secretes LysM to block chitin induced immunity.

Chitin-binding lysine motif (LysM) effectors are versatile secreted proteins that contain no annotated protein domains other than LysM domains, which are found in a fungal species with extremely divergent lifestyles (Kombrink

and Thomma, 2013; de Jonge and Thomma, 2009). Investigation of effector proteins in strains of the vascular wilt pathogen *V. dahliae* showed a significantly expanded LysM effector family of six to seven members (Kombrink and Tahoma, 2013). Among these effectors, comparative genomics revealed that three core LysM effector genes conserved in the genome of *V. dahliae* collections (Kombrink et al., 2017). However, core LysM effectors did not show any role in virulence, as lack of expression was observed in *planta* in a taxonomically diverse panel of host plants. Moreover, targeted deletion of single core LysM effector genes in *V. dahliae* strain JR2 did not show altered virulence during infections on *Arabidopsis*, tomato or *Nicotiana benthamiana* (Kombrink et al., 2017). Surprisingly, in addition to the core genome, all *V. dahliae* strains carry up to ~4 Mb genome sequence which is unique or shared by only a subset of strains composing a highly

dynamic lineage-specific (LS) region of the genome. Four effectors candidates were found among in planta-induced genes in this LS regions of strain JR2 (de Jonge et al., 2013; Klosterman et al., 2011). Deletion of two of the four genes resulted in significantly reduced disease development, suggesting that the genes having significant contribution to virulence (Kombrink et al., 2017). Likewise, VdLs17 strain contains additional in planta-induced one LysM effector gene (VDAG_05180) within a LS region, encoding a LysM effector that contains two LysM domains (Kombrink et al., 2017; de Jonge et al., 2013). Remarkably, of the seven LysM effectors in the VdLs17 genome, only this LysM effector is required for full disease development and host colonization (FIG3 A and B). Intriguingly, apart from VdLs17 strain, none of the other sequenced *V. dahliae* strains carry lineage-specific LysM effector genes (Kombrink et al., 2017).

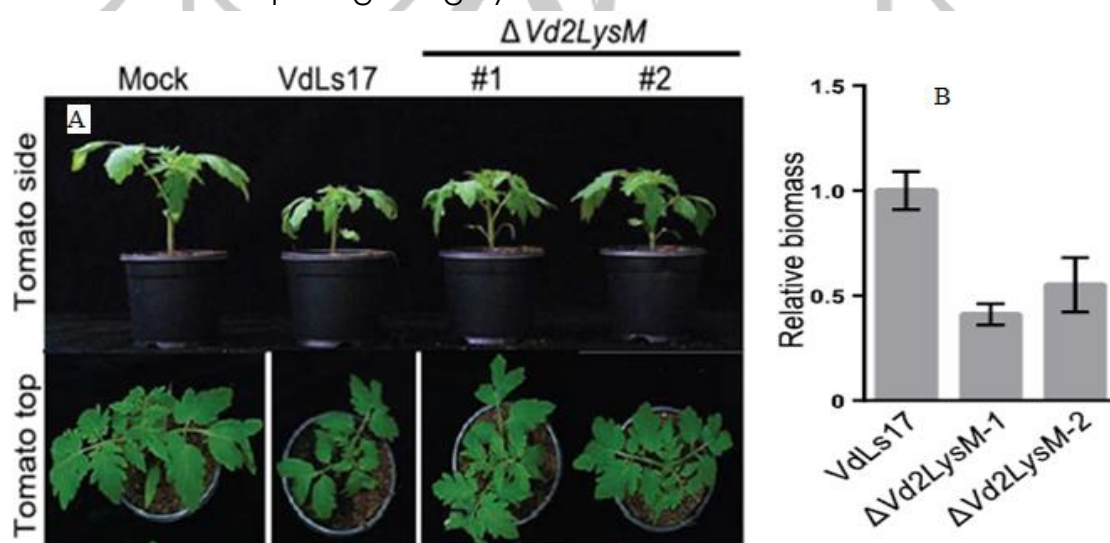


FIGURE 3. Disease reaction of tomato and fungal biomass inoculated with deletion strain of Vd2LysM (a) Photographs of tomato plants mock-inoculated, wild-type *V. dahliae* strain VdLs17 or two deletion strains of Vd2LysM at 14 dpi and (b) Fungal biomass accumulation in tomato plants inoculated with wild-type strain VdLs17 or Vd2LysM deletion strains at 14 dpi (Source: Kombrink et al., 2017).

Generally, apoplastic LysM domain containing effectors are responsible for hiding the pathogen from detection by the host immune systems (Doehlemann and Hemetsberger, 2013). A previously characterized LysM effectors Avr4 from biotrophic fungal pathogen, *Cladosporium fulvum*, protects fungal hyphae against hydrolysis by chitinases during plant colonization (Van den Burg et al., 2006). Whereas, the other identified *Cladosporium fulvum* effectors named as extracellular protein 6 (Ecp6) prevent chitin triggered immunity through sequestering of chitin oligosaccharides and preventing chitin binding to chitin PAMP (de Jonge et al., 2010; Bolton et al., 2008). Furthermore, homologous of *Cladosporium fulvum* Lysin (LysM) domain-containing effector Ecp6, have been identified in *Mycosphaerella graminicola*, the causal agent of Septoria tritici blotch disease of wheat (*Triticum aestivum*) leaves. Of the three LysM effector homologs present in the *M. graminicola* genome, Mg3LysM and Mg1LysM binds chitin, however only Mg3LysM blocked the elicitation of chitin-induced plant defenses. In addition, both Mg1LysM and Mg3LysM protect fungal hyphae against plant-derived hydrolytic enzymes in the same way as Avr4 of *C. fulvum* (Mentlak et al., 2012).

The recently identified LysM effector protein of *V. dahliae*, Vd2LysM, for which a role in virulence has been investigated, is able to perturb chitin-centred host immune responses like described for Ecp6, Mg1LysM and Mg3LysM (Kombrink et al., 2017; Mentlak et al., 2012; de Jonge et al., 2010). Thus, *V.*

dahliae employs LysM effectors to protect itself from secreted plant chitinases and increase its virulence on host plants (Kombrink et al., 2017; Mentlak et al., 2012; de Jonge et al., 2010). Apoplastic effector proteins like Pit2 with inhibitory activities for protection against host proteases (Mueller et al., 2013) and effector Pep1 that targets peroxidase 12 to inhibit oxidative burst triggered during *Ustilago maydis* infection (Hemetsberger et al., 2015) have not been reported in *V. dahliae*.

2.3.2. V. dahliae AVR Effectors

AVR effectors are a special class of pathogen effectors encoded by the avirulence (AVR) genes, which could be recognized by host resistant (R) proteins resulting in an immune response from the host leading to resistance (de Wit et al., 2011). In the absence of R protein recognition, Avr effectors may assist in virulence of the pathogen by manipulating the host immune system (Vleeshouwers and Oliver, 2014). In tomato, *V. dahliae* interaction, only one functional AVR genes, Ave1, have been identified. **Ave1** (for Avirulence on Ve1 tomato) encodes a predicted 134 amino acid secreted protein and is induced by *V. dahliae* during host colonization (de Jonge et al., 2012). Comparative genomics analysis combined with transcriptome sequencing of *Verticillium*-infected *Nicotiana benthamiana* plant by race 1 and 2 *V. dahliae* strains identified Ave1 on a single 50-kb race 1-specific region (de Jonge et al., 2012). Interestingly, homologs of Ave1 were identified in the genome of *Fusarium oxysporum* f. sp. *lycopersici* (designated FoAve1), *Colletotrichum higginsianum*

(ChAve1) and *Cercospora beticola* (CbAve1) pathogens. Furthermore, Ave1 is homologous to a widespread family of Plant Natriuretic Peptides (PNPs) (de Jonge et al., 2012). In the study conducted by Song et al. (2017) prediction of signalP 4.0 of Ave1 homologs illustrated that presence of N-terminal signal peptide and a disulphide bridges forming four conserved cysteine residues with the functionality to direct secretion into the extracellular space and contribute to protein stability upon secretion, respectively.

During tomato infection with *V. dahliae*, Ave1 is recognized by the immune receptor Ve1, plasma membrane localized extracellular Leucine-Rich Repeat (eLRR)-containing cell surface receptor of the Receptor-Like Protein (RLP) class (Zhang et al., 2014; Fradin et al., 2009; Fradin and Thomma, 2006). Thus, the presence of Ave1 in *V. dahliae* isolate conveyed an [avirulence phenotype](#) when infecting a [tomato](#) host that possesses Ve1, whereas expression Ave1 enhances full virulence on susceptible tomato cultivars (de Jonge et al., 2012).

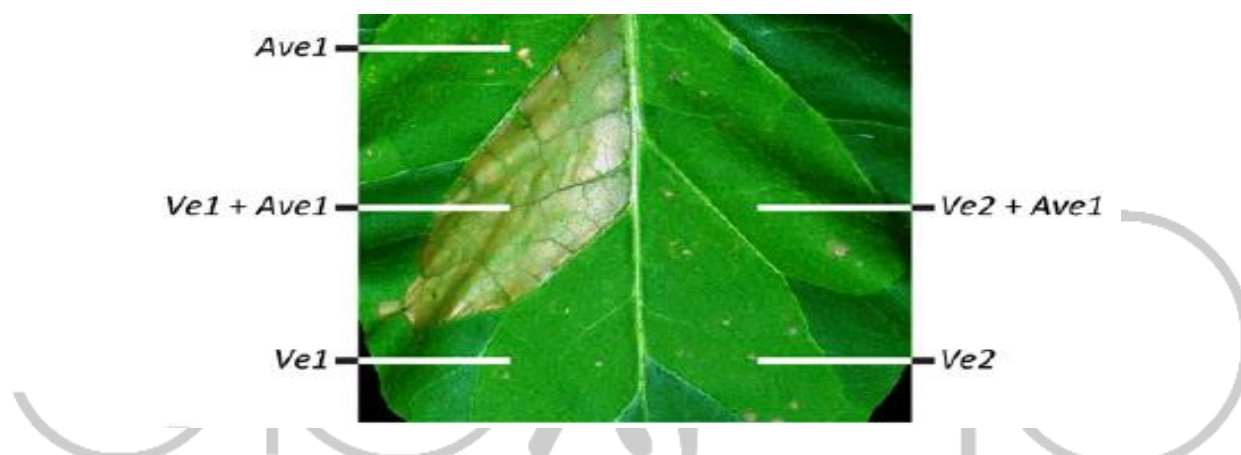


FIGURE 4. Coexpression of Ave1 with tomato Ve1 and Ve2 in *N. tabacum* (Source: de Jonge et al., 2012).

Pathogenicity assays upon *Agrobacterium tumefaciens*-mediated transient expression demonstrated that co-expression of Ve1 and Ave1 induced hypersensitive response (HR) in tomato and tobacco shown in FIG. 4 (Song et al., 2018; Zhang et al., 2014; de Jonge et al., 2012), while finding by Zhang et al. (2013) has shown that Ave1/Ve1-induced immune signaling did not lead to an HR in *Arabidopsis*. Moreover, induction of defense related genes such as PR-1 (P6), PR-2 (β beta-1, 3-glucanase) and peroxidases has been induced by

transgenic Ave1 expression in tomato (Castroverde et al., 2016). Though not well functionally characterized, Ave1 may play role in the regulation of water and ion homeostasis and consequently can affect many downstream processes including photosynthesis based on its homologous to natriuretic peptides (Wang et al., 2011).

2.3.3. Cytoplasmic (translocated) effectors

Generally, intracellular (translocated) effectors affect host defense mechanisms inside plants by

targeting proteins involved in plant immune signaling cascades. Based on their localization or site of action this class of effector can further be divided into cytoplasmic and nuclear effectors (Chaudhar et al., 2014). Cytoplasmic effectors target different cytosolic components of the cell to interfere with plant defense systems. On the other side, nuclear effectors are assumed to be efficient to manipulate host transcription or directly target essential nuclear host components for the benefit of the pathogen (Rivas and Genin, 2011). Unlike the presence of oomycete like RXLR and dEER motifs (McGowan and Fitzpatrick, 2017), there are no obvious conserved translocation motifs among fungi intracellular effectors, which impacted identification of new effectors and their target proteins. Up to date, few *V. dahliae* translocated effectors interfering with plant immunity and SA synthesis were identified and characterized.

3.3.1. Effectors interfering immune systems

VdSCP7 (*V. dahliae* secreted cysteine-containing proteins): By analyzing secretome of cotton roots infected by *V. dahliae* strain V592 identified the novel small secreted cysteine-containing effector VdSCP7. This effector protein is predicted to carry a characteristic bipartite nuclear localization signal (NLS) sequence (Zhang et al., 2017). Expression of green fluorescent protein (GFP) and VdSCP7 fusion protein demonstrated that NLS mediates translocation of the protein from the apoplast to the nucleus following extracellular secretion. Remarkably, VdSCP7 was detected inside the plant

nucleus by Confocal Laser Scanning Microscopy, providing the evidence that translocation this protein into host nucleus is indispensable for induction of plant immunity during *V. dahliae* cotton root colonization shown in FIG6 B (Zhang et al., 2017). Gene-knockout of VdSCP7 enhanced the *V. dahliae* virulence on the host cotton, whereas complementation of the VdSCP7 attenuated the virulence, which is associated with failure to trigger host immunity in the absence of this gene (Zhang et al., 2017). Immunity response of *N. benthamiana* leaves infiltrated with GFP-VdSCP7 has been elucidated by production of ROS, callose deposition, HR and activation of SA and JA signaling pathways (Zhang et al., 2017). Altogether, similar to other plant pathogenic fungi effector like Uf-RTP1 *Uromyces fabae* (Kemen et al., 2005) and CgEp1 of *Colletotrichum graminicola* (Vargas et al., 2016), *V. dahliae* effector VdSCP7 targets host nucleus to modulate plant immunity (or activate ETI). However, as this pathogen does not form haustorium-like structures to transport effector protein, the mechanism of VdSCP7 transport to host cell is unknown.

3.3.2. Effectors interfering hormone signaling

Plants synthesize salicylic acid (SA) in response to pathogen infection commonly for large scale transcriptional induction of defense-related genes and establishment of systemic acquired resistance (Dempsey et al., 2011; Strawn et al., 2007). SA is synthesized in chloroplast by two enzymatic pathways: from phenylalanine via cinnamate and from

chorismate via isochorismate (Wildermuth *et al.*, 2001). Like other filamentous fungi pathogens, *V. dahliae* has been found to exploit isochorismate synthase (designated as VdIscl1) as virulence effectors to hydrolyze isochorismate (the direct precursor of SA) and suppress host SA biosynthesis (Zhu *et al.*, 2017; Liu *et al.*, 2014). VdIscl1 (encoding isochorismatase) is a *V. dahliae* secreted isochorismatase without classical N-terminal signal peptides, but exhibiting characteristics that lead to unconventional secretion. Cotton plants that are infected with VdIscl1-mutated strain accumulated significantly higher level of SA than plants that are

infected with the corresponding wild-type strain (FIG5 A) (Liu *et al.*, 2014). Similarly, silencing and overexpression of VdIscl1 in highly aggressive isolate of *V. dahliae* increased and reduced SA level, respectively during host tomato infection (shown in FIG5 B) (Zhu *et al.*, 2017). It has been proposed that VdIscl1 depleted SA levels by hydrolyzing isochorismate into 2, 3-dihydro-2, 3-dihydroxybenzoate (DDHB) and pyruvate (shown in FIG6 A) (Liu *et al.*, 2014). Altogether, these results suggest that perturbation of SA-mediated immunity is crucial for *V. dahliae* host root colonization.

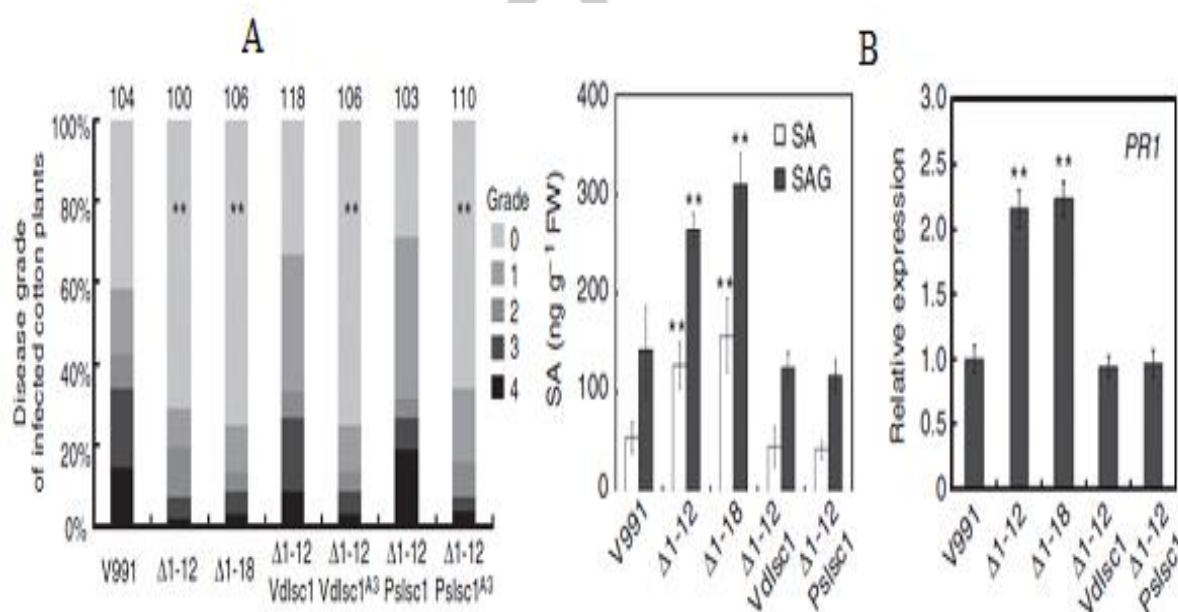


FIGURE 5. Disease symptoms and concentration of SA, SAG and PR1 transcripts (a) Disease symptoms on cotton plants inoculated with indicated strains of *V. dahliae* and scored at 15 dpi; (b) Free SA and SAG levels in cotton roots at 14 dpi and (c) Relative levels of cotton PR1 transcripts measured using qRT-PCR at 12 hpi. (Source: Liu *et al.*, 2014).

Though SA is synthesized in chloroplast, co-localization of VdIscl1 has not been detected in plastids of host plants. However, when VdIscl1 is delivered into host plant cells by the pathogens, reduction of

cytosolic concentrations of isochorismate was observed, which strongly suggest that the unconventional secreted effector protein might be able to affect the cellular homeostasis of isochorismate to suppress

SA synthesis (Liu *et al.*, 2014). Similarly, *Phytophthora sojae* secretes virulence promoting effectors Pslsc1 which acts on isochorismate following the same mechanism described for VdIscl to promote infection (Liu *et al.*, 2014). Alternatively, maize smut fungus *Ustilago maydis* actively suppresses defense related hormone synthesis by secreting chorismate

mutase, Cmu1, effector into plant cell. Cmu1 appears to benefit the pathogen by redirecting the shikimate pathway towards production of prephenate by catalyzing chorismate to suppress SA-induced defense responses FIG6 A (Djamei *et al.*, 2011).

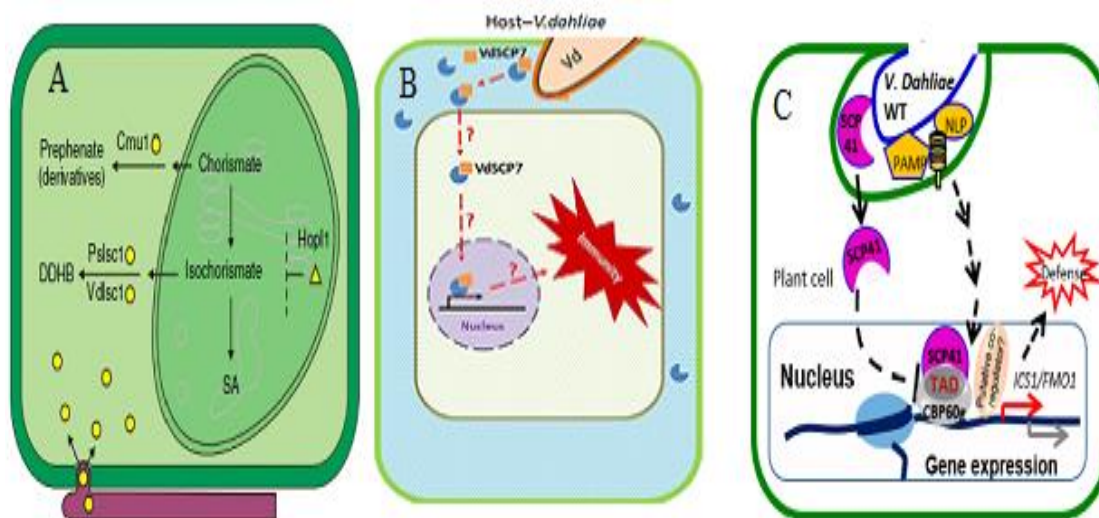


FIGURE 6. Models for functionally characterized *V. dahliae* effector proteins. (a) VdIscl decreases SA levels in plastids by reducing the level of SA precursor isochorismate and increasing the production of 2, 3-dihydro-2, 3-dihydroxybenzoate (DDHB) and pyruvate (Source: Djamei *et al.*, 2011; Liu *et al.*, 2014); (b) VdSCP41-mediated suppression of defense in Arabidopsis during *V. dahliae* infection. VdSCP7 localizes to the plant nucleus and activates plant immunity (Source: Zhang *et al.*, 2017) and (c) VdSCP41 translocates into nucleus and interacts with CBP60g transcription activation domain (TAD) interrupting its activity and with plant immunity (Source: Qin *et al.*, 2018).

On the other side, initiation of SA biosynthesis requires transcriptional regulation of isochorismatase by Ca^{2+} sensor proteins, such as calmodulin (CaM) and Ca^{2+} dependent protein kinases (CDPKs) (Zhang *et al.*, 2010). Upon pathogen infection, calmodulin (CaM)-binding transcription factor CBP60g (CALMODULIN BINDING PROTEIN 60g) and its homolog SARD1 (SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1) bind to the isochorismatase promoter and activate its expression (Seyfferth and Tsuda, 2014). In

this condition, *V. dahliae* secretes cysteine protein **VdSCP41** (*V. dahliae* secreted cysteine protein) that interferes with the transcription activity of CBP60g and SARD1 to inhibit SA-dependent and SA-independent regulators to suppress plant immunity (shown in FIG6 C) (Qin *et al.*, 2018). This effector protein contains a signal peptide and localizes to the base of the hyphopodium and forms the septin-organized apparatus (a septin-like ring) for secretion purpose upon plant infection at the fungus-host interface. Localization

study using GFP-tagged VdSCP41 in onion epidermal cells and mCherry-tagged VdSCP41 in *Arabidopsis* cells revealed that it is the nuclear localized effector protein (Qin et al., 2018).

2.4. Proteins associated with cell wall and defense systems

The plant cell wall consisting predominantly of cellulose, hemicellulose and pectin is a natural barrier, which provides mechanical strength and rigidity to prevent pathogen infection (Quoc and Chau, 2017). In contrast, pathogenic fungi secrete an array of cell wall-degrading enzymes (CWDEs) that allow them to invade host tissues through the degradation of cell wall components of plants (Kubicek et al., 2014). Comparative genomic showed that extensive diversification of *V. dahliae* genome by plant cell wall degrading enzymes than other fungi studied (Klosterman et al., 2011). Among these diverse CWDEs, pectate lyases and glycoside hydrolase family 12 (GH12) proteins have been identified to contribute directly to the advancement of *V. dahliae* within plant xylem vessel (Yang et al., 2018; Gui et al., 2017).

Recently, a 255 amino acid protein **VdPEL1** (*V. dahliae* pectate lyases) was isolated from *V. dahliae* culture filtrates (Yang et al., 2018). Infiltration of this purified secreted protein elicits local cell death in tomato, soybean, and cotton plants. Analysis of VdPEL1 protein sequence revealed that it belongs to the Pectate lyase PL3 subfamily having a conserved domain (39-229 amino acids)

with the first 20 N-terminal amino acids encoding a signal peptide (Yang et al., 2018). Previously, it was reported that PL3 subfamily are able to cleave poly-1, 4- α -D-galacturonan (pectate, the backbone of pectin) to 4-(4-deoxy- α -D-gluc-4-enuronosyl)-D-galacturonate at the non-reducing end (Yang et al., 2018).

VdPEL1 was able to induce resistance in *N. benthamiana* and cotton against *V. dahliae*. Deletion mutant of VdPEL1 in *V. dahliae* lead to significantly reduced virulence on *N. benthamiana* (Yang et al., 2018) and Cotton (Chen et al., 2016) as compared to the wild-type strain. Similarly, disruption of pectate lyase gene in *Colletotrichum coccodes* (CcpelA) and *Colletotrichum gloeosporioides* (pelB), causal agent of black dot on potato and anthracnose on tomato (Ben-Daniel et al., 2012); and avocado fruit (Yakoby et al., 2001), respectively reduced the virulence of these fungi on their host. Pretreating cotton and tobacco with VdPEL1 showed reduced virulence and induced strong basal defense responses, suggesting that VdPEL1 has a role in virulence, possibly through the degradation of pectin component of the plant cell wall by its pectate lyase enzymatic activity. Consequently, immunity triggered by VdPEL1 is mediated by the degradation of plant cell wall polymers that release pectin hydrolysis products (DAMPs), which in turn, trigger defense responses in plants (Yang et al., 2018).

Glycoside Hydrolase 12 (GH12):

Six GH12 domain containing proteins (VdEG1 to VdEG6) were isolated from the

highly virulent *V. dahliae* isolate Vd991 from cotton (Gui et al., 2017). Among these proteins, only VdEG1 and VdEG3 triggered cell death in *N. benthamiana* leaves. Amino acid sequence alignment indicated that VdEG1 and VdEG3 belong to typical GH12 family proteins. In soybean infecting fungi, *Phytophthora sojae*, glycoside hydrolase family 12 (GH12) protein, XEG1, is able to induce cell death in tobacco, tomato, pepper, and soybean but not in maize and cotton (Ma et al., 2015). XEG1 exhibits xyloglucanase and β -glucanase activity (Ma et al., 2015), that are able to degrade hemicellulose plant cell wall components (xyloglucans and β -glucans). Site directed mutagenesis of catalytic residues of both VdEG1 and VdEG3 resulted in complete loss of cellulase activity. In contrast, VdEG1 and VdEG3 non-enzymatically active mutant proteins were able to induce cell death after infiltration with recombinant protein, strongly suggesting that cellulase activities was not required for cell death-inducing activities in *N. benthamiana* (Gui et al., 2017). Moreover, purified active and inactive recombinant proteins were able to induce PTI by acting as PAMPs to induce cell death on *N. benthamiana* tissues.

Silencing as well as reintroduction of either VdEG1 or VdEG3 genes in *V. dahliae* resulted in differential virulence levels on *N. benthamiana* and cotton through distinct mechanism. Deletion of VdEG1 or VdEG3 resulted in enhanced virulence of *V. dahliae* on *N. benthamiana*, while reintroduction resulted in reduced virulence and triggered defense responses, confirming the idea

that VdEG1 and VdEG3 act as PAMPs to trigger PTI response. In contrast to *N. benthamiana*, VdEG1 and VdEG3 contributed to virulence on cotton due to their enzymatic activities, but did not act as PAMPs to induce the PTI response (Gui et al., 2017). In the same way, *Phytophthora sojae* XEG1 is required for virulence, but also triggers PAMP-triggered immunity during soybean infection (Ma et al., 2015). Altogether, *V. dahliae* GH12 proteins, VdEG1 and VdEG3, cellulase activity is required for virulence and elicitor activity that induces immunity during plant infection, depending on the host (Gui et al., 2017).

2.5. Secreted elicitor proteins

Plant pathogens, including *V. dahliae*, secreted elicitor proteins that may contribute to virulence. These include proteins that target induced defenses responses and cause cell death. In *V. dahliae*, an important role of secreted proteins as elicitors of hypersensitive responses-like necrosis and plant defense responses has been identified.

NLP1 and NLP2 (Nep 1-like proteins) that form a family of secreted proteins are well known to induce necrosis and ethylene production in dicot plants (Oome et al., 2014; Oome and Ackerveken, 2014). A 24-kDa proteins inducing ethylene biosynthesis and causing necrosis (necrosis-and ethylene-inducing-like protein [Nep-1]) was first purified from cultures filtrates of *Fusarium oxysporium* (Bailey, 1995). These results provided a base for identification of a group of proteins by presence of common Necrosis-inducing *Phytophthora* Protein

(NPP1) domain collectively named as Nep1-Like Proteins (NLP) (Gijzen and Nurnberger, 2006; Fellbrich *et al.*, 2002). All NLP proteins family identified so far shares common conserved seven amino acid (heptapeptide) motif, GHRHDWE, which is not present in other proteins (Zhou *et al.*,

2012). NLPs are divided into two groups based on the presence of two conserved cysteine residues in type 1 NLP and four in type 2 NLP, and both types may occur in a single species (Gijzen and Nurnberger, 2006).

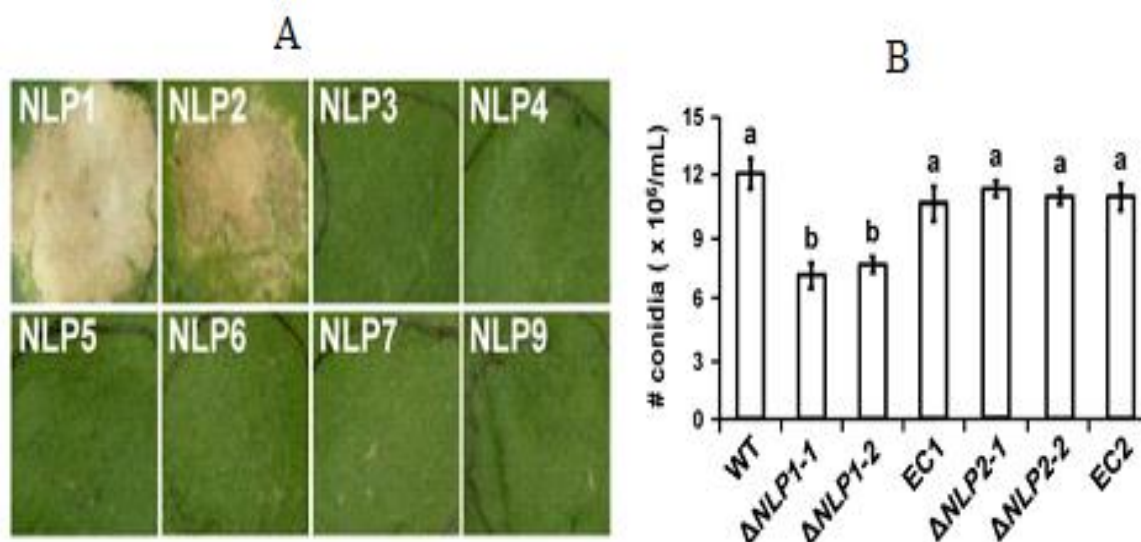


FIGURE 7. *V. dahliae* necrosis and ethylene-inducing proteins (a) Only *V. dahliae* NLP1 and NLP2 induces cell death in *N. benthamiana* and (b) Average number of conidia produced after 7 days of growth on PDA (Source: Santhanam *et al.* (2013).

A 233 amino acids necrosis and ethylene-inducing protein, VdNEP, is the first elicitor protein identified from cultured mycelium of *V. dahliae* strain Vd-8 based on sequencing of expressed sequence tags (ESTs) (Wang *et al.*, 2004). Infiltration of VdNEP using a His-tag fusion protein (His-VdNEP) resulted in clear necrotic lesions on *Nicotiana benthamiana* leaves, and production of ROS and induced expression of *PR* (*PR1* and *PDF1.2*) genes on *Arabidopsis thaliana*. Remarkably, VdNEP caused wilting on the host cotton leaves and cotyledons, whereas on the non-host plants, it caused necrosis but not wilting (Wang *et al.*, 2004).

A decade later, eight and nine potential NLP family has been reported from genome of cotton-

pathogenic *V. dahliae* isolate V592 (Zhou *et al.*, 2012) and lettuce-pathogenic VdLs.17 (Klosterman *et al.*, 2011), respectively. These nine VdNLP proteins are named as VdNLP 1 to VdNLP 9 (Wang *et al.*, 2004). In search of the function of the nine VdNLPs in *V. dahliae* using agrobacterium mediated plant expression method, it was found that only VdNLP1 and VdNLP2 exhibit cytotoxic activity and trigger plant immunity in *Nicotiana benthamiana*, *Arabidopsis* and cotton shown in FIG7 A (Santhanam *et al.*, 2013; Zhuo *et al.*, 2012; Qutob *et al.*, 2006). In addition to the virulence roles, targeted deletion of *V. dahliae* NLPs affected conidiospore production (FIG7 B) and

induced formation of aerial hyphae (Santhanam *et al.*, 2013). The NLP gene family is expanded in the *V. dahliae* genome, compared to most fungal genomes, which suggests that NLP genes may contribute to the broad host range among dicotyledonous plant hosts (Klosterman *et al.*, 2011).

PevD1 is a novel protein elicitor secreted from the pathogenic *V. dahliae* which induces a typical hypersensitive responses-like necrosis and systemic acquired resistance (SAR) in cotton and tobacco (Bu *et al.*, 2014; Wang *et al.*, 2012). The protein-encoded by PevD1 gene consists of a 468-bp open reading frame that produces a polypeptide of 155 amino acids, with a theoretical molecular weight of 16.23 kDa (Liu *et al.*, 2014; Wang *et al.*, 2012). Subsequently, only the C-terminal 57 amino acids of PevD1 have been responsible for HR activity while N-terminal 98 amino acids for induction of systemic acquired resistant (SAR) devoid of HR against tobacco mosaic virus (TMV) (Liu *et al.*, 2014).

A Yeast Two-Hybrid (Y2H) and Bimolecular Fluorescence Complementation (BiFC) assay identified the interaction between PevD1 and an Asparagine rich protein Nbnrp1 (homologous gene of AtNRP from *N. benthamiana* genome). After binding, it was shown that PevD1-induced necrotic cell death and disease resistance activity on *N. benthamiana*, confirming the idea that binding of Nbnrp1 to PevD1 is critical for induction of *N. benthamiana* defense responses (Liang *et al.*, 2018). Like Alt a 1-like protein, PevD1 contributes to *V.*

dahliae pathogenicity on cotton via its interaction with the protein GhPR5 (pathogenesis-related protein 5 (PR5)-like protein from cotton) (Zhang *et al.*, 2019). PevD1 knockout mutants displayed attenuated pathogenicity compared with the wild-type (WT) strain. Remarkably, deletion of GhPR5 from cotton and re-introduction of the PevD1 gene into a PevD1 knockout mutant resulted in restoration of the virulence phenotype to WT levels. These results suggest that PevD1-GhPR5 interaction resulted in a decrease in the antifungal activity of GhPR5 in cotton as a strategy to resist the plant defense system and promote *V. dahliae* infection (Zhang *et al.*, 2019). The authors also proved that PevD1 contributes to *V. dahliae* virulence by preventing the recognition of degraded β -1, 3- glucan by the plant thereby allowing fungal growth to be maintained (Zhang *et al.*, 2019). Furthermore, PevD1 overexpression can enhance cotton growth and development by decreasing the time required for the developmental stages (Zhang *et al.*, 2019).

VdCP1 (*V. dahliae* cerato-platanin protein): is a 138 amino acids protein consisting of signal peptide at the cleavage site between amino acids 18 and 19 (Zhang *et al.*, 2017). The amino acid sequence analysis revealed that VdCP1 is **SnodProt1-like protein**. SnodProt1 belongs to typical cerato-platanin protein family (CPP) homologs, exhibiting a low molecular weight, four conserved cysteine residues and high hydrophobicity (Zhang *et al.*, 2017). CPP are produced by all types filamentous fungi and plants have developed the ability to recognize CPPs to

activate defense responses (Gaderer *et al.*, 2014). In *V. dahliae*, the virulence role of VdCPI has been elucidated by low disease severity exhibited on cotton by VdCPI mutants as compared to the wild. Treating plants with purified recombinant VdCPI protein expressed in *Pichia pastoris* elicited plant immune system. Moreover, pretreatment with purified VdCPI conferred resistance to *Botrytis cinerea* and *Pseudomonas syringae* pv. *tabaci* in tobacco and to *V. dahliae* in cotton (Zhang *et al.*, 2017). Like other CPP, VdCPI has shown to bind to chitin and displayed an expansin-like activity on cellulosic materials. In *V. dahliae*, VdCPI contributes to virulence by protecting cell wall from enzymatic degradation by chitinases, thereby attenuating host plant defense responses (Zhang *et al.*, 2017).

2.6. Regulation of effector protein expression in *V. dahliae*

Transcription factors (TFs) are essential in regulation of target gene expression and signal transduction pathways (Shelest, 2008). Though, researchers have identified and elucidated the functions of several fungal effectors, how phytopathogenic fungi regulate the expression of effector genes is unknown for majority of plant pathogens.

TABLE 1. Characterized *V. dahliae* effectors and proteins

Effector/ protein	N-terminal signal motives or conserved domains	Localization	Known targets; associated functions	References/Sources
VdLysM	LysM domain	Apoplastic	Chitin	[Kombrink <i>et al.</i> , 2017]
Ave1	Expansin-like EG45 domain	Apoplastic	Protect against chitinases Ve1	[de Jonge <i>et al.</i> , 2013]

Presently, 12 TFs from four families have been characterized for their roles in the regulation of effector or candidate effector gene expression in fungi (Tan and Oliver, 2017). Thus far, **VdSge1** (encoding a transcriptional regulator), *V. dahliae* TFs regulating the expression of effector protein expression has been identified (Santhanam and Thomma, 2013). The *VdSge1* gene consisted of an open reading frame of 1,533 nucleotides, and encodes a protein of 510 amino acids. Even though, *Ave1* regulation has not been detected, *VdSge1* is suggested in differentially regulating *in planta* expression of effector proteins in *V. dahliae* (Santhanam and Thomma, 2013).

Similarly, in vascular invading tomato wilt fungus, *F. oxysporum* f. sp. *lycopersici*, the transcriptional regulator (SIX Gene Expression 1 (SGE1) has been found required for *in planta* expression of various *SIX* effector genes (van der Does *et al.*, 2016; Michielse *et al.*, 2009). Previously, *SIX* effector proteins were reported to contribute to *F. oxysporum* virulence (Thatcher *et al.*, 2012; Takken and Rep, 2010). The *V. dahliae* *VdSge1* is orthologous of *Wor1* (White-opaque regulator 1) identified as a master regulator of white-opaque switching in human pathogen *Candida albicans* (Huang *et al.*, 2006).

NLP1 and NLP2	Seven amino acid (heptapeptide) motif, “GHRHDWE”	Apoplastic	Unknown Induce necrosis and wilting	[Wang et al., 2004]
VdSCP7	Bipartite nuclear localization signal	Nucleus	Required for full virulence	[Zhang et al., 2017]
VdIsc1	Non-classically secreted	Cytoplasm	Affects host SA synthesizes via hydrolysis of isochorismate	[Liu et al., 2014]
PevD1	Unknown	Apoplastic	GhPR5	[Zhang et al., 2019]
VdSCP41	Unknown	Nucleus	Decrease anti-fungal CBP60g and SARD1	[Qin et al., 2018]
VdSge1	Unknown	Nucleus	Regulate effector proteins expression	[Santhanam and Thomma, 2013]

3. CONCLUDING REMARKS

This review paper showed that *V. dahliae* deployed effectors, elicitor proteins and plant CWDEs to manipulate its hosts and enhance disease susceptibility (Table 1). In conclusion, many of these effectors and other proteins had a measurable effect on virulence in *V. dahliae*-host system. Therefore, such detailed study on functional characterization of secreted proteins in *V. dahliae* and defining their interacting domains in the host could thus be translated into developing new strategies to control *V. dahliae* caused wilt diseases in crops.

4. RECOMMENDATIONS

- ❖ In future as a recommendation research on *V. dahliae*-host interaction at molecular level should be carried out for the identification of more *V. dahliae* effector proteins and their target host processes.
- ❖ As reported in other plant pathogens, presence of fungal structures enables the pathogen to deliver effectors and to

successfully colonize their hosts. In this respect, investigation of *V. dahliae* structural information and the mechanism that underlies effector delivery into host cells should be prior area for researchers.

❖ Finally, further molecular level investigation needs to be undertaken to identify *V. dahliae* effectors involved and how this pathogen utilizes these effector proteins for enhancing its biotrophic and necrotrophic phase.

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