

The secretome of *Verticillium dahliae* in collusion with plant defence responses modulates *Verticillium* wilt symptoms

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ABSTRACT

Verticillium dahliae is a notorious soil-borne pathogen that enters hosts through the roots and proliferates in the plant water-conducting elements to cause *Verticillium* wilt. Historically, *Verticillium* wilt symptoms have been explained by vascular occlusion, due to the accumulation of mycelia and plant biomacromolecule aggregation, and also by phytotoxicity caused by pathogen-secreted toxins. Beyond the direct cytotoxicity of some members of the secretome, this review systematically discusses the roles of the *V. dahliae* secretome in vascular occlusion, including the deposition of polysaccharides as an outcome of plant cell wall destruction, the accumulation of fungal mycelia, and modulation of plant defence responses. By modulating plant defences and hormone levels, the secretome manipulates the vascular environment to induce *Verticillium* wilt. Thus, the secretome of *V. dahliae* colludes with plant defence responses to modulate *Verticillium* wilt symptoms, and thereby bridges the historical concepts of both toxin production by the pathogen and vascular occlusion as the cause of wilting symptoms.

Key words: vascular pathogen, *Verticillium dahliae*, secretome, toxins, vascular occlusion, *Verticillium* wilt.

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I. INTRODUCTION

Verticillium dahliae is a well-known hemi-biotrophic soil-borne pathogen that infects over 200 hosts and causes billions of dollars in losses annually (Inderbitzin & Subbarao, 2014). The pathogen attacks plants through the roots and colonizes the unique ecological niche of the plant xylem, resulting in Verticillium wilt, the symptoms of which include foliar wilting, chlorosis, stunting, necrosis and vein clearing (Fradin & Thomma, 2006; Chen *et al.*, 2021). Over the past 60 years, two main mechanisms that may explain the induced wilt symptoms, i.e. vascular occlusion and through the involvement of toxins have been advanced in the literature (Fradin & Thomma, 2006; Báidez *et al.*, 2007). Following successful infection of the plant, *V. dahliae* reaches the xylem and increases its biomass concurrent with plant defence responses that aim to restrict the pathogen, including the production of tyloses and accumulation of a colloidal matrix (Robb, Powell & Street, 1989; Báidez *et al.*, 2007; Yadeta & Thomma, 2013). This eventually leads to vascular occlusion and plant wilting. An alternative view argues that the pathogen delivers toxins, which in this case refers broadly to a wide-array of molecules and complex compounds such as large polysaccharides, protein-lipopolysaccharides, glycoproteins, and enzymes including some that act as effectors that induce plant wilting (Fradin & Thomma, 2006; de Sain & Rep, 2015). Both hypotheses are equally credible (Pegg & Brady, 2002; Fradin & Thomma, 2006; Báidez *et al.*, 2007; de Sain & Rep, 2015), and in fact, the two are tightly interlinked as the secretome, including these toxins, colludes with plant defence responses to cause Verticillium wilt symptoms. Here, we review recent findings and the mechanistic actions of the secretome of *V. dahliae*, which causes Verticillium wilt symptoms *via* both a contribution to vascular occlusion and *via* cytotoxicity.

(1) The role of vascular occlusion in Verticillium wilt

Following successful breaching of the endodermis, *V. dahliae* generally is confined to the fluid environment of the xylem cells and exerts its effects on host physiology indirectly (Yadeta & Thomma, 2013). Unlike prokaryotic vascular pathogens that break out of the vascular system and spread indiscriminately in the root and shoot parenchyma, *V. dahliae* seldom leaves the confines of the xylem until the death of the surrounding tissue or the host (Pegg & Brady, 2002). Key steps in the pathogenesis of *V. dahliae* include germination of microsclerotia, hyphal penetration of the root epidermis, and growth in the cortex that crosses the endodermis finally breaching the highly structured and rigid secondary xylem walls to enter vessels of the root (Zhao *et al.*, 2014). Once in the xylem, the pathogen

produces conidia, which germinate, ramify and penetrate adjacent vascular cells to continue colonization (Fradin & Thomma, 2006; Yadeta & Thomma, 2013).

Since *V. dahliae* enters the xylem tissue, it can be trapped in the pit cavities at the xylem perforation plates. Because the nutritional value of the xylem fluid is limited, the pathogen draws its nutrition by degrading the vascular cell wall using an abundance of diverse extracellular enzymes encoded within its genome (Klosterman *et al.*, 2011; Chen *et al.*, 2016). The insufficient degradation of the xylem walls and pit membranes causes the deposition of polysaccharides (Fradin & Thomma, 2006; Kubicek, Starr & Glass, 2014). In addition, this triggers plant defences to produce tyloses and other biomacromolecules (resins, gums, gels, etc.) from neighbouring parenchyma cells, which act as a defensive barricade to restrict pathogen spread (Benhamou, 1995; Yadeta & Thomma, 2013). Analyses of the plant vascular system using electron microscopy during Verticillium wilt of tomato (*Lycopersicon esculentum* L.) has indicated that terminal occlusion leading to sealing of entire veins is a limiting factor preceding the irreversible appearance of vascular wilt (Robb, Powell & Street, 1989). Thus, the pathogen rapidly proliferates, accompanied by continuous deposition of polysaccharides and plant defence-driven structures and biomacromolecules (tyloses, suberin, etc.) that interfere with the flow of water and nutrition, ultimately leading to plant wilting (Pegg & Brady, 2002; Báidez *et al.*, 2007; Yadeta & Thomma, 2013).

(2) The role of toxins in Verticillium wilt

The concept that toxins could underlie *Verticillium*-induced foliar wilting was first proposed from results obtained using components from culture filtrates that induced wilt symptoms (Stoddart & Carr, 1966; Meyer & Dubery, 1993). Since then, efforts have been made to elucidate the role of toxins in the mechanisms of foliar wilting (Pegg & Brady, 2002). While some evidence linking toxins to foliar wilting is undeniable, other investigations have yielded ambiguous results (Fradin & Thomma, 2006). For instance, a low molecular weight phytotoxic polypeptide that exhibited toxicity to potato (*Solanum tuberosum* L.) leaves was detected using immunofluorescent antibodies in the xylem of infected potato stems (Nachmias, Buchner & Burstein, 1985). A similar toxin induced the typical foliar wilting when injected into *Arabidopsis thaliana* seedlings (Jing, Fan & Wu, 2005). Incremental progress in understanding the toxins involved has occurred with advances in genomics and metabolomics technologies, and some toxin components have been verified, including a range of proteins with enzymatic or effector activity (Klimes *et al.*, 2015), metabolites (Zhang *et al.*, 2016; Zhang *et al.*, 2019a), and volatile compounds

(Li & Kang, 2018). Proteins secreted from *V. dahliae* that serve as toxins have been shown to play critical roles in its pathogenesis resulting in foliar wilting through plant cell wall destruction, manipulation of host responses, or cytotoxicity (de Sain & Rep, 2015; Klimes *et al.*, 2015; Chen *et al.*, 2016). The secreted proteins have been suggested to target various sites, including the cell wall, plasma membrane, and other intracellular components (Meyer & Dubery, 1993), and also to induce changes in the microfilaments and microtubules in plant cells (Zhao *et al.*, 2020), leading to rapid cell lesions.

II. FUNCTIONS OF THE *VERTICILLIUM DAHLIAE* SECRETOME

Fungal pathogens secrete numerous proteins (i.e. their secretome) to modulate plant defences by altering host cellular structure and physiology, and to facilitate colonization (Hogenhout *et al.*, 2009). Since *V. dahliae* mainly resides in the vascular tissue during its disease cycle, understanding the role of the secretome in contributing to vascular occlusion or producing toxins could offer a better understanding of the mechanisms that underlie foliar wilting. Genomics-driven research has shown that *V. dahliae* encodes more than 700 potential secreted proteins (Klosterman *et al.*, 2011; Chen *et al.*, 2018), and hundreds are delivered into the extracellular space with generalist functions (Chen *et al.*, 2016). Currently, many secreted proteins have been shown to contribute to pathogenesis, resulting in Verticillium wilt *via* diverse modes of action (Table 1). Thus, *V. dahliae* employs its secretome to degrade plant cell walls, manipulate host immunity, exert cytotoxicity, neutralize host oxidative stress, compete with its host for nutrients, and even to affect the host microbiome to facilitate Verticillium wilt development (Fig. 1).

Verticillium dahliae employs its secretome to proliferate in the low-nutrient confines of the xylem. Its secretome encodes a large arsenal of enzymes that allow the complete degradation of many plant polysaccharides (Klosterman *et al.*, 2011). These processes inevitably induce xylem collapse and the deposition of polysaccharides. The host plants respond by producing biomacromolecules (gums, gels, and other resins) and tyloses as defensive structures which protrude into the vascular tissue to block progress of the pathogen. Together, the massive propagation of hyphae, the deposition of polysaccharides and the production of biomacromolecules by the plant cause xylem occlusion and plant wilting (Fig. 2).

(1) Proliferation in the plant xylem

(a) Cell wall degradation

During the infection process, *V. dahliae* must break through plant root cell wall barriers to reach the xylem. During this process, *V. dahliae* produces an array of cell wall degrading enzymes (CWDEs) (Kubicek, Starr & Glass, 2014). Some of these CWDEs destroy the highly structured and rigid

secondary xylem walls, and also enable breaches of the pectin-enriched pit cavities or the vascular end walls (Zhao *et al.*, 2014). Additional secreted enzymes include two members of the glycoside hydrolase 12 family, the endoglucanases VdEG1 and VdEG3, which participate in cellulose/hemicellulose degradation (Gui *et al.*, 2017) similar to the CWDEs involved in degradation of the plant cuticle and xylan (Gui *et al.*, 2018; Yin *et al.*, 2021; Wang *et al.*, 2021a). Furthermore, transcription regulators of CWDEs such as the sucrose non-fermenting protein kinase VdSNF1 (Tzima *et al.*, 2021) and fungal-specific transcription factor VdFTF1 (Zhang *et al.*, 2018) regulate CWDEs expression or secretion, and significantly contribute to pathogenesis. The superabundance of secreted carbohydrate-active enzymes suggests an extraordinary capacity of *V. dahliae* to degrade plant cell wall barriers during early infection stages and to degrade plant xylem walls during proliferation in these vessels.

(b) Scavenging host reactive oxygen species

Plants respond to pathogen attack with a transient burst of reactive oxygen species (ROS) that play a central role in plant immune responses (Qi *et al.*, 2017). Pathogens can counteract this by secreting ROS-scavenging proteins to neutralize or degrade ROS, such as superoxide dismutases (SODs), catalases, and peroxidases (Broxton & Culotta, 2016; Khademian & Imlay, 2021). Comparative analyses of the *V. dahliae* secretome have revealed that many secreted proteins with ROS-scavenging and oxidative stress response functions are activated when the pathogen is cultured on host tissue or under nutrient-starvation conditions (El-Bebany, Rampitsch & Daayf, 2010; Chu *et al.*, 2015; Chen *et al.*, 2016). *V. dahliae* secretes SODs to detoxify host-generated extracellular ROS, contributing significantly to virulence during plant infection (Tian *et al.*, 2020, 2021a,b). *V. dahliae* ROS production facilitates penetration peg formation during the initial colonization of cotton (*Gossypium hirsutum* L.) roots (Zhao, Zhou & Guo, 2016). Thus, the deployment of ROS during infection combined with counterprovisions for neutralization of host-generated oxidative stress is key to successful infection and proliferation in the vascular tissue of host plants.

(c) Suppressing host immunity

The ability to secrete effector proteins that can enter plant cells and manipulate host immunity is a key determinant of a successful plant pathogen (He *et al.*, 2020). In *V. dahliae*, the role of effectors in manipulating host immunity has been studied in detail (Fig. 3). To enable successful infection and proliferation in host plants, *V. dahliae* has evolved a range of effector proteins that inhibit host immune responses (Ding & Redkar, 2018), including inactivation of the salicylic acid (SA) pathway by isochorismate synthase VdIsc1 (Liu *et al.*, 2014), cellulose-binding module family 1-member (VdCBM1) (Gui *et al.*, 2017, 2018; Wang *et al.*, 2021b), small cysteine-rich protein VdSCP41 (Qin *et al.*, 2018), lysin motif

Table 1. Description of some secreted proteins involved in various biological processes of *Verticillium dahliae*.

Secreted protein	Function description	Cell wall degradation	Host immunity manipulation	Hormone homeostasis Interference	Cytotoxicity	Oxidative stress neutralization	Fungal nutrition	Morphological development	Microbiome manipulation	Others	Host molecular target	Reference
VdI8.5	Phytotoxic protein				✓							Palmer, Salecha & Lyon (2005)
VdH1	Class II hydrophobin gene							✓				Khimes & Dobinson (2006)
VdEg-1	Endoglucanase 1	✓						✓			Vel	Maruthachalam <i>et al.</i> (2011)
VdAve1	Avirulence gene of race 1		✓	✓								de Jonge <i>et al.</i> (2012); Snelders <i>et al.</i> (2020)
VdNLP1	Necrosis- and ethylene-inducing-like protein				✓			✓			GIPC sphingolipids	Zhou <i>et al.</i> (2012); Santhanam <i>et al.</i> (2013)
VdNLP2	Necrosis- and ethylene-inducing-like protein				✓						GIPC sphingolipids	Zhou <i>et al.</i> (2012); Santhanam <i>et al.</i> (2013)
VdSSP1	Cell wall degradation related protein	✓					✓					Liu <i>et al.</i> (2013)
VdISC1	Isochorismatase		✓	✓							Isochorismate	Liu <i>et al.</i> (2014)
VdPL3.1	Pectin lyase	✓										Chen <i>et al.</i> (2016)
VdPL3.3	Pectin lyase	✓										Chen <i>et al.</i> (2016)
VdZLysM	Chitin-binding lysin motif		✓					✓			Plant chitinases	Kombrink <i>et al.</i> (2017)
VdASP F2	Allergen Asp F2-like protein				✓			✓				Xie, Li & Yang (2017)
VdCBM1	Cellulose binding module 1	✓	✓	✓								Gui <i>et al.</i> (2017, 2018); Wang <i>et al.</i> (2020a)
VdCP1	Cerato-platanin protein 1	✓	✓	✓							Plant chitinases	Zhang <i>et al.</i> (2017a)
VdEG1	GH12 domain-containing protein	✓	✓	✓								Gui <i>et al.</i> (2017)
VdEG3	GH12 domain-containing protein	✓	✓	✓								Gui <i>et al.</i> (2017)
VdSCP7	Small cysteine-rich protein	✓	✓	✓								Zhang <i>et al.</i> (2017b)
VdPEL1	Pectate lyase	✓	✓	✓								Yang <i>et al.</i> (2018)
VdPL1	Polysaccharide lyase	✓	✓	✓								Zhang <i>et al.</i> (2018)
VdCUT11	Cutinase	✓	✓	✓								Gui <i>et al.</i> (2018)
PevD1	Alt a 1 family protein	✓	✓	✓							GhPR5, ORE1	Zhang <i>et al.</i> (2021); Liang <i>et al.</i> (2018)
VdSCP41	Small cysteine-rich protein	✓	✓	✓							CBP60g and SARD1	Qin <i>et al.</i> (2018)
VdOCH1	Alpha-1,6-mannosyltransferase						✓	✓				Zhang <i>et al.</i> (2019b)
VdPDA1	Polysaccharide deacetylase	✓	✓	✓							Chitin oligomer	Gao <i>et al.</i> (2019)
VdSSEP1	Secretory Ser protease 1	✓	✓	✓						✓	Plant chitinases	Han <i>et al.</i> (2019)
VdSCP27	Small cysteine-rich protein	✓	✓	✓								Wang <i>et al.</i> (2020a)
VdSCP113	Small cysteine-rich protein	✓	✓	✓								Wang <i>et al.</i> (2020a)
VdSCP126	Small cysteine-rich protein	✓	✓	✓								Wang <i>et al.</i> (2020a)
VdSOD3	Superoxide dismutase				✓							Tian <i>et al.</i> (2020)
VdAMP2	Antimicrobial effector				✓							Snelders <i>et al.</i> (2020)
VdSOD5	Superoxide dismutase				✓							Tian <i>et al.</i> (2021a)
VdSOD1	Cu/Zn superoxide dismutase				✓							Tian <i>et al.</i> (2021b)

Table 1. (Cont.)

Secreted protein	Function description	Cell wall degradation	Host immunity manipulation	Hormone homeostasis Interference	Cytotoxicity	Oxidative stress neutralization	Fungal nutrition	Morphological development	Microbiome manipulation	Others	Host molecular target	Reference
Av2	Avirulence gene of race 2		√								V2	Chavarró-Carrero <i>et al.</i> (2021)
VdEIX3	Ethylene-inducing xylanase	√	√	√							EIX2	Yin <i>et al.</i> (2021)
VdXyn4	Xylanase	√	√	√	√						PUB25/26	Wang <i>et al.</i> (2021a)
VDAL	Asp f2-like protein		√									Ma <i>et al.</i> (2021)
VdAMP3	Antimicrobial effector								√			Snelders <i>et al.</i> (2021)
VdR3c	Avirulence gene of race 3		√									Wang <i>et al.</i> (2021b)
VdRTX1	Ribonuclease		√							√		Yin <i>et al.</i> (2022)

CBP60g, calmodulin binding protein 60 family member g; EIX2, leucine-rich repeat receptor-like protein; GIPC, glycosylinositol phosphorylceramide; ORE1, A senescence-associated NAC transcription factor; PR5, pathogenesis-related protein 5 (PR5)-like protein; PUB25/26, plant U-box 25/26; SARD1, systemic acquired resistance deficient 1; Ve1, leucine-rich repeat receptor-like protein.

(LysM) protein Vd2LysM (Kombrink *et al.*, 2017), secretory serine protease 1 (VdSSEP1) (Han *et al.*, 2019) and polysaccharide deacetylase (VdPDA1) (Gao *et al.*, 2019) (Fig. 3; Table 1). For instance, VdPDA1 deacetylates chitin oligomers; since the *N*-acetyl group contributes to host LysM-containing receptor recognition for ligand-triggered immunity, the action of VdPDA1 renders the host susceptible to *V. dahliae* by evading host surveillance through inactivation of a chitin-triggered host immune response (Gao *et al.*, 2019). Together, these effectors mainly function in the early stages of infection and employ a variety of strategies to overcome immunity to facilitate pathogen colonization in roots.

(d) Fungal nutrition

Fungi secrete many plant CWDEs such as cellulase, hemicellulase, and pectin-degrading enzymes that act on plant tissues to produce monomeric and/or small polymeric sugars that are subsequently transported into the fungal cell *via* membrane-bound transporters (Glass *et al.*, 2013). In this way, *V. dahliae* obtains nutrition for proliferation in the vascular tissue. Many secreted carbohydrate-active enzymes from *V. dahliae* catalyse the release of nutrients as the degradation products of cell walls (Klosterman *et al.*, 2011; Xiong, Wang & Tian, 2015; Chen *et al.*, 2016) (Table 1, Fig. 3), some of which function in the generation of energy and acetyl-CoA during nutrient starvation (Chu *et al.*, 2015; Xiong, Wang & Tian, 2015). The *V. dahliae* alpha-1,6-mannosyltransferase (VdOCH1) plays a crucial role in *N*-linked oligosaccharide glycoprotein metabolism (Zhang *et al.*, 2019b). The abundant pectate lyases secreted by *V. dahliae* are thought to have key roles in its adaptation to its vascular niche (Klosterman *et al.*, 2011).

(e) Microbiome manipulation

Although pathogen effectors are typically considered to act exclusively through direct host immunity manipulation, plant pathogens also utilize effectors to target the host microbiota in order to facilitate niche colonization (Rovenich, Boshoven & Thomma, 2014; Snelders *et al.*, 2018). Recent work (Snelders *et al.*, 2020, 2021) suggests that *V. dahliae* exploits secreted proteins to manipulate the host microbiome through its antibacterial or antifungal properties, to promote infection. The *V. dahliae* avirulence effector (VdAve1) displays antimicrobial activity and facilitates colonization of tomato and cotton through the selective manipulation of the host microbiota in the roots, as well as in the xylem by suppressing antagonistic bacteria. The antimicrobial protein VdAMP2, is exclusively expressed by *V. dahliae* when in the soil and exerts antibacterial activity that contributes to niche establishment (Snelders *et al.*, 2020). Thus, VdAMP2 and VdAve1 likely have complementary functions for optimal soil and host colonization. Another ancient antimicrobial protein (VdAMP3) is specifically expressed to ward off fungal niche competitors during resting structure formation in senescing host mesophyll tissues (Snelders *et al.*, 2021), allowing *V. dahliae* to tailor the expression of microbiome-manipulating effectors based on the

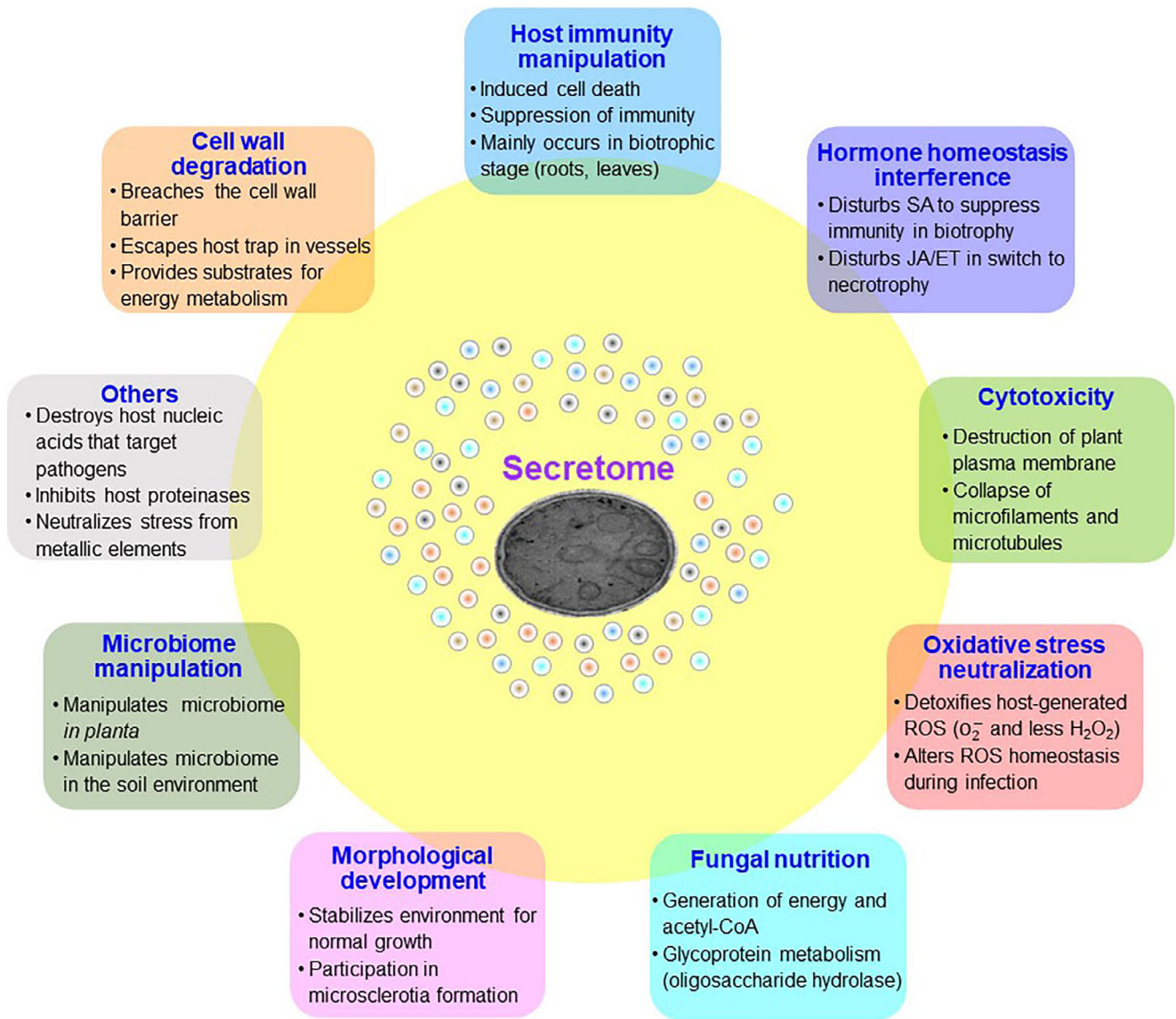


Fig. 1. Versatile biological functions of the secretome in *Verticillium dahliae*. Acetyl-coA, acetyl co-enzyme A; ET, ethylene; JA, jasmonic acid; ROS, reactive oxygen species; SA, salicylic acid.

microbiota that it encounters during different stages of the disease cycle. This promotes *V. dahliae* colonization and proliferation in host plants to accelerate symptom development.

(2) Xylem wall collapse and deposition of polysaccharides

Compared to many fungi, the genome of *V. dahliae* encodes a significant expansion of gene families involved in plant cell wall degradation, and specific members of this repertoire may be essential for adaptation to its vascular niche (Klosterman *et al.*, 2011). For example, among plant cell wall polysaccharides, pectin has the highest structural and functional complexity (Mohnen, 2008; Harholt, 2010), with four major structural classes: homogalacturonan (HG), rhamnogalacturonan I (RG-I),

xylogalacturonan (XG), and rhamnogalacturonan II (RG-II). Enzymes required for efficient degradation of pectin (Martens-Uzunova & Schaap, 2009) include polygalacturonases and rhamnogalacturonases (glycoside hydrolases family 28, GH28), polysaccharide lyases (pectin and pectate lyases, PL1, PL2, and PL3), and rhamnogalacturonan lyases (PL4), as well as pectin methylesterases (carbohydrate esterase family 8, CE8), pectin acetyl esterases (CE12 and CE13), and rhamnogalacturonan acetyl esterases (CE12). Among all sequenced fungal genomes, *V. dahliae* has one of the most complete repertoires of polysaccharide lyases and associated glycoside hydrolases for the breakdown of complex pectin (Klosterman *et al.*, 2011). Experimental evidence indicates that the *V. dahliae* polysaccharide lyase VdPL1 (Zhang *et al.*, 2018), pectate lyase VdPEL1 (Yang *et al.*, 2018), pectin lyases VdPL3.1 and VdPL3.3

(Chen *et al.*, 2016), and the pathotype-specific secretory protein VdSSP1 (Liu *et al.*, 2013) are all involved in virulence and pectin degradation. Since pectin is enmeshed with other polysaccharides to form plant cell walls, the result is that incompletely degraded and intermediate polysaccharides contribute directly to vascular occlusion (Kubicek, Starr & Glass, 2014). In addition, in response to infections, pectin gels are usually released into the xylem (Rioux *et al.*, 1998; Clériveret *et al.*, 2000). Similarly, starch hydrolysis also contributes to vessel occlusion related to wilt symptoms in olive (*Olea europaea* L.) stems of susceptible cultivars infected by *V. dahliae* (Trapero *et al.*, 2018). Thus, while degradation of the xylem walls and pit membranes by *V. dahliae* CWDEs supplies nutrients for pathogen proliferation in the low-nutrient confines of the xylem, it also causes xylem collapse and blockage by deposition of insufficiently degraded polysaccharides.

(3) Induction of host response that aggravate vascular occlusion

While secretion of an array of CWDEs is required for breaching cell wall barriers and nutrient acquisition, *V. dahliae* also must overcome host defence responses. It achieves this with a combination of secreted effectors and interference with host hormone signalling, both of which contribute to vascular occlusion and wilt symptoms. Among plant hormones, SA, jasmonic acid (JA) and ethylene (ET) are all significant in the hormonal regulation of plant immune responses (Shigenaga & Argueso, 2016). SA has been implicated in the protection of plant tissues during the initial biotrophic phase of *V. dahliae* infection, while JA is more prominent following the establishment of *V. dahliae* in the xylem and its switch to a necrotrophic lifestyle (Dhar *et al.*, 2020). ET activates defence responses to limit spread of the pathogen during the early stages of *V. dahliae* infection; but during subsequent colonization and the switch to the necrotrophic phase, ET can facilitate establishment of the pathogen and accelerate the progression of *Verticillium* wilt (Dhar *et al.*, 2020). The *V. dahliae* secretome interferes with the intricate and delicate balance of phytohormones during *V. dahliae*–plant interactions, regulating the immune responses in a synergistic or antagonistic manner that contributes to *Verticillium* wilt progression (Dhar *et al.*, 2020). Manipulation of SA–JA signalling has been demonstrated for several known effectors in *V. dahliae*, including four VdSCPs (Wang *et al.*, 2012; Wang *et al.*, 2020a), the cutinase VdCUT11 (Gui *et al.*, 2018), the necrosis- and ethylene-inducing-like protein VdNLP (Wang *et al.*, 2004), the endoglucanases VdEG1 and VdEG3 (Gui *et al.*, 2017), VdSCP7 (Zhang *et al.*, 2017a,b) and the xylanase VdXyn4 (Wang *et al.*, 2021a,b) (Fig. 3). Additionally, VdNLP involved in ET signalling aids in the establishment of the pathogen and accelerates the progression of *Verticillium* wilt and death of the host (Wang *et al.*, 2004; Dhar *et al.*, 2020), and PevD1 induces ET biosynthesis by directly binding to the senescence-associated NAC transcription factor ORE1 (Zhang *et al.*, 2021).

In response to *V. dahliae* infection and colonization, the plant may mount a rapid defence including deposition of

coating materials such as suberin on vascular cell walls, forming a barrier to fungal penetration and horizontal spread (Robb, Powell & Street, 1989). At this stage, infection can result in vessel occlusion by gums, gels, tyloses and other deposited resins secreted by the neighbouring parenchyma cells (Robb *et al.*, 1979; Benhamou, 1995). In addition, plant hormone levels can be manipulated by effectors secreted by pathogens, influencing plant responses to the pathogen, including vascular occlusion. During plant–pathogen interactions, many phytohormones regulate the formation of coating materials on vascular cell walls, including ET, which is involved in lignin and suberin biosynthesis, and tyloses formation, while abscisic acid coordinates suberin deposition, JA contributes to lignin biosynthesis and tyloses formation, and SA can abolish the induction of tyloses by JA (Sun *et al.*, 2007; Leśniewska *et al.*, 2017; Hu *et al.*, 2018; Liu *et al.*, 2020; Wang *et al.*, 2020b). Some of these regulatory mechanisms have been inferred in *V. dahliae*–plant interactions (Hu *et al.*, 2019; Dhar *et al.*, 2020). In *V. longisporum* infections, *Arabidopsis thaliana* plants can generate xylem *de novo* by transdifferentiation of the chloroplast-containing bundle sheath cells into functional xylem elements, enhancing water transport, storage capacity, and drought tolerance (Reusche *et al.*, 2012). Whether a similar mechanism exists during *V. dahliae*–plant interactions with a role in relieving plant wilt symptoms requires further investigation. Together, the secreted effectors from *V. dahliae* cause host immune responses and disturb the phytohormone balance contributing to vascular occlusion and the induction of *Verticillium* wilt symptoms (Fig. 2).

(4) Toxicity

A cytotoxic function for all identified secreted proteins is difficult to demonstrate explicitly because a similar plant necrosis phenotype accompanies immunity- and cytotoxin-induced necrosis. While some studies have shown that crude *Verticillium* culture extracts cause microfilaments and microtubules to collapse in plant cells (Zhao *et al.*, 2020), it remains unclear whether it is toxic activity that directly causes necrosis. A prominent example of a fungal protein with cytotoxic activity is NLP purified from the culture filtrates of *Fusarium oxysporum* (Bailey, James & James, 1997). A NLP member identified from the mycelia of a *V. dahliae* strain pathogenic to cotton displayed the ability to cause foliar necrosis in various plant species (Wang *et al.*, 2004). With the advent of genomics, seven additional members of the NLP family have been identified from the *V. dahliae* genome, two of which cause cellular necrosis (Zhou *et al.*, 2012; Santhanam *et al.*, 2013). The proposed mechanism of NLP-mediated cell lysis is that glycosylinositol phosphorylceramide (GIPC) sphingolipids act as NLP receptors to form complexes with terminal monomeric hexose moieties of GIPCs that insert into the plant plasma membrane (Ottmann *et al.*, 2009; Lenarčič *et al.*, 2017) (Fig. 3). Additionally, a member of the xylanase family, VdXyn4 induces vein necrosis and plant wilting during advanced stages of infection, but not immunity-triggered cell death, suggesting that it is a

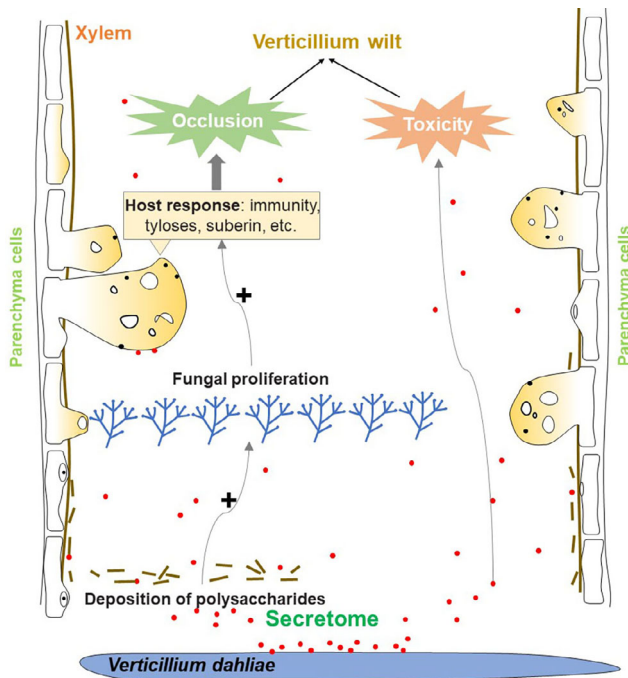


Fig. 2. The secretome of *Verticillium dahliae* causes vascular occlusion which results in Verticillium wilt. Its secretome contributes to vascular occlusion by promoting fungal proliferation and polysaccharide deposition, and by inducing host responses including tyloses formation and suberin production. The secretome also contributes to Verticillium wilt through cytotoxicity.

candidate toxicity factor (Wang *et al.*, 2021a) (Fig. 3). Thus, constituents of the *V. dahliae* secretome may induce foliar wilting directly *via* toxicity, even if the definition of toxicity is narrow in this context.

III. HOW THE SECRETOME COMPROMISES THE HOST TO CAUSE VASCULAR OCCLUSION AND VERTICILLIUM WILT

The pathogenic status of *V. dahliae* is dynamic during the different stages of host infection (Fradin & Thomma, 2006), and the secretome plays a critical role in Verticillium wilt disease progression by both supplying toxins and contributing to vascular occlusion to cause irreversible and lethal plant wilting (Fig. 4).

(1) Initial conflict zone in roots

Verticillium dahliae initially attacks plants through the root tip or through lateral roots by developing an infectious structure known as hyphopodium (Zhao, Zhou & Guo, 2016), which assists in establishing biotrophy (Fradin & Thomma, 2006). The hyphopodium develops a septin ring while piercing the

root epidermis and cortical cells, that acts as a functional fungus–host penetration interface for the delivery of secreted fungal proteins (Zhou, Zhao & Guo, 2017). Subsequently, the secretome overcomes host defence responses by manipulating host immunity, interfering in hormone homeostasis, neutralization of oxidative stress, and cytotoxicity. The secreted CWDEs breach the plant cell walls to allow further penetration and proliferation (Fradin & Thomma, 2006). Even with these specialized tools, only a few invading hyphae successfully traverse into the vessel cells (Zhao *et al.*, 2014). In addition, several CWDEs are involved in manipulating host immunity and altering hormone homeostasis (Table 1). Prior to penetration of plant roots, the rhizosphere environment is also crucial for *V. dahliae* survival and its eventual invasion. Here, effector proteins can suppress the growth of antagonistic bacteria (Snelders *et al.*, 2020, 2021). Thus, ‘toxic’ activities of the *V. dahliae* secretome are critical for successful penetration, infection, and xylem colonization (Fig. 4).

(2) The vascular battlefield

Once *V. dahliae* crosses the endodermis and reaches the xylem (generally 2–4 days), the hyphae begin to branch, develop terminal conidia and proliferate in the xylem (Fradin & Thomma, 2006). At this stage, the pathogen channels energy into vegetative growth resulting in hyphal extension and conidia formation in the xylem (Zhao *et al.*, 2014). During this stage, *V. dahliae* uses its extensive range of CWDEs to survive in the barren environment of the xylem (Klosterman *et al.*, 2011), and also secretes effectors with toxicity functions, for example VdXyn4 acts as a toxin during the colonization of vessels by destroying the host vascular system (Wang *et al.*, 2021a). Concurrently, the host plants respond to the extensive sporulation and proliferation in the xylem by initiating rapid defence responses (Benhamou, 1995; Heinz *et al.*, 1998; Pegg & Brady, 2002). In resistant hosts, colonization and proliferation of the pathogen are hindered by its compartmentalization in the vascular tissue. In susceptible hosts, the pathogen escapes these host traps by counteracting host defences *via* secreted effectors and hyphal growth that progresses through the occluded xylem (Fradin & Thomma, 2006). In this process, *V. dahliae* effectors also disturb the xylem microbiome (Snelders *et al.*, 2020, 2021), further accelerating fungal proliferation and initiating a resistance response, that in turn leads to further xylem occlusion. Therefore, in the battle over control of the plant xylem, *V. dahliae* deploys its secretome to aid mycelial proliferation, resulting in release of incompletely degraded polysaccharides from the plant cell walls and induction of biomacromolecules from a host response, all of which contribute to vascular occlusion and the appearance of wilt symptoms (Fig. 4).

(3) Final victory in the shoot

The successful proliferation of *V. dahliae* in the xylem results in two damaging effects: vascular occlusion blocking water and nutrient transport; and rapid pathogen transmission to

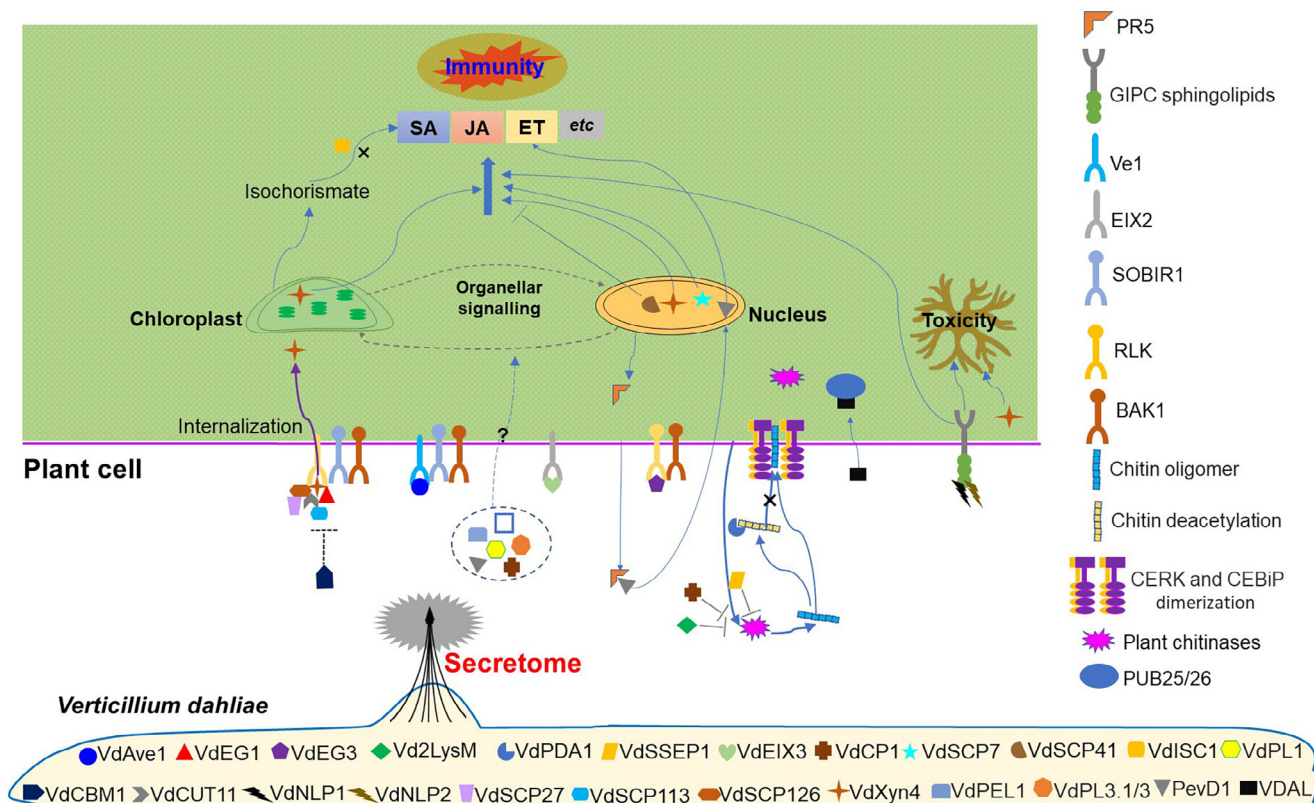


Fig. 3. *Verticillium dahliae* employs its secretome to manipulate host immunity and interfere with hormone homeostasis. See Table 1 for *V. dahliae* protein names. VdAve1 is recognized as an avirulence determinant by tomato plants that carry the corresponding Ve1 immune receptor. VdEG1 and VdEG3 are glycoside hydrolase 12 proteins that trigger immunity dependent on the LRR-RLPs/SOBIR1/BAK1 and LRR-RLKs/BAK1 complexes, respectively. Vd2LysM binds long- or short-chain chitin oligomers and prevents degradation of chitin by plant chitinase. VdPDA1 directs deacetylation of chitin oligomers and inhibits perception by host LysM-containing receptors, thus avoiding ligand-triggered immunity. VdSSEP1 hydrolyses cotton Chi28 directly, inhibiting the production of chitin oligomers. VdEIX3 exhibits immunity-inducing activity in *Nicotiana benthamiana*, recognized by the leucine-rich repeat receptor-like protein NbEIX2. VdCP1 protects the *V. dahliae* cell wall from chitinase degradation. VdSCP7 targets the host nucleus to modulate plant immunity. VdSCP41 targets the plant-specific transcription factors CBP60g and SARD1 to modulate immunity. VdISC1 disrupts the plant salicylate metabolism pathway by suppressing the transformation from isochlorismate to salicylic acid. VdPL1 plays a virulence function during infection of cotton. VdCBM1 suppresses VdEG1-, VdEG3-, VdSCP27-, VdSCP113-, VdSCP126-, VdCUT11- and VdXyn4-induced cell death and some PAMPs-triggered immunity in *N. benthamiana*. VdCUT11 induces plant defence responses in *N. benthamiana* in a BAK1- and SOBIR1-dependent manner. VdNLP1 and VdNLP2 are GIPC sphingolipids that act as NLP toxin receptors; NLPs form complexes with terminal monomeric hexose moieties of GIPCs and insert into the plant plasma membrane, causing cell lysis. VdSCP27, VdSCP113 and VdSCP126 induce defence responses in *N. benthamiana* in a BAK1- and SOBIR1-dependent manner. VdXyn4 plays a cytotoxic function and induces a necrosis phenotype in *N. benthamiana*, depending on simultaneous localization to the nuclei and chloroplasts in a BAK1- and SOBIR1-dependent manner. VdPEL1 exhibits pectin hydrolytic activity and induces cell death in plants. VdPL3.1/3 have virulence functions during infection of cotton. PevD1 induces ethylene biosynthesis by directly binding to ORE1. VDAL protects transcription factor MYB6 from degradation by interacting with the E3 ligases PUB25 and PUB26 to enhance *Verticillium* wilt resistance. BAK1, LRR-RLK BRI1-associated kinase-1; CBP60g, calmodulin binding protein 60 family member g; CEBiP, chitin-elicitor binding protein; CERK, receptor chitin elicitor receptor kinase; Chi28, chitinase 28; EIX2, leucine-rich repeat receptor-like protein; ET, ethylene; GIPC, glycosylinositol phosphorylceramide; JA, jasmonic acid; LRR, leucine-rich repeat; LysM, lysin motif; MYB6, MYB domain protein 6; NLP, necrosis- and ethylene-inducing-like protein; ORE1, A senescence-associated NAC transcription factor; PAMP, pathogen-associated molecular pattern; PR5, pathogenesis-related protein 5 (PR5)-like protein; PUB25/26, plant U-box 25/26; RLK, receptor-like kinase; RLP, receptor-like protein; SA, salicylic acid; SARD1, systemic acquired resistance deficient 1; SOBIR1, LRR-RLK suppressor of BIR1-1; Ve1, leucine-rich repeat receptor-like protein.

the leaves *via* the vascular plumbing (Pegg & Brady, 2002; Fradin & Thomma, 2006). The pathogen can easily degrade the cell walls of leaf tissues since these are mainly coated with aliphatic biopolymers composed of cutin rather than suberin

(Schreiber, 2010). To infect foliar cells, secreted proteins are released that manipulate host immunity and break hormone homeostasis (Liu *et al.*, 2014; Gui *et al.*, 2017; Gao *et al.*, 2019). For example, *V. dahliae* induces leaf senescence and wilting by

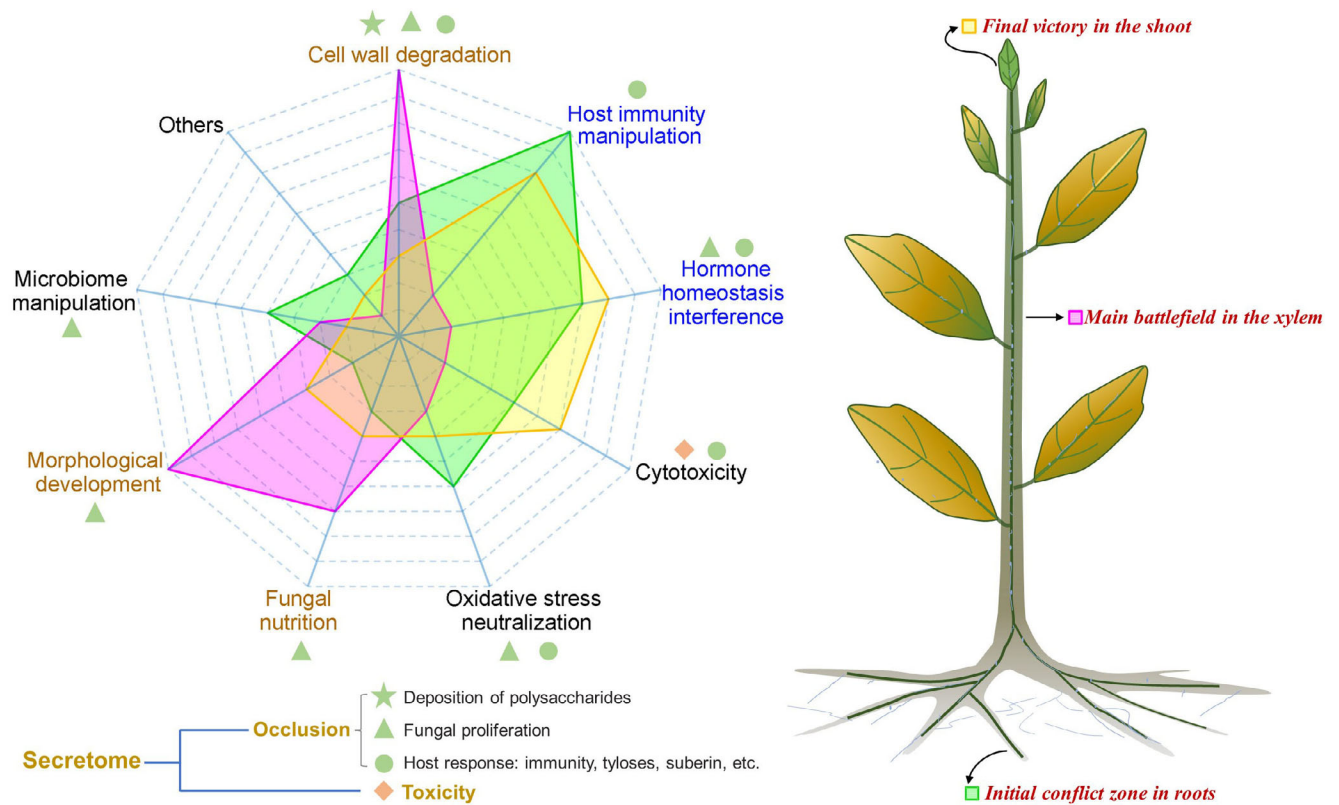


Fig. 4. Intensity model of biological functions of the secretome at different stages of *Verticillium* wilt infection. Left: the main contributions of different functions of the secretome in polysaccharide deposition, fungal proliferation, host response and toxicity. Cell wall degradation, morphological development and fungal nutrition predation (shown in brown font) represent the main effects of the secretome in vessels; and host immunity manipulation and hormone interference (shown in blue font) represent the main effects of the secretome in roots and leaves. The green region shows the biological functions of the secretome that operate during the initial stages of the colonization, the pink region shows those operating when the fungus is present in the xylem, and the yellow region is for the final stage of infection when the pathogen has reached the leaves (see plant image on right).

secreting the alt a1 family protein PevD1 to regulate the ORE1–ACS6 (1-aminocyclopropane-1-carboxylate synthase) cascade and enhance ET production (Zhang *et al.*, 2021). In addition, cytotoxicity also plays a critical role in inducing cell necrosis, as VdNLPs are constitutively expressed during later stages of infection (Santhanam *et al.*, 2013), accelerating disease symptoms and the production of microsclerotia to begin a new disease cycle. *V. dahliae* effectors contribute to microsclerotia formation in senescing mesophyll tissue by warding off other fungal niche competitors (Snelders *et al.*, 2020, 2021).

IV. CONCLUSIONS

(1) *Verticillium* wilt caused by *V. dahliae* is a notorious vascular wilt disease that leads to chlorosis, stunting and wilting. Two notable hypotheses exist regarding the mechanisms underlying *Verticillium* wilt symptom development: vessel occlusion and direct toxicity through secreted toxins (Fradin & Thomma, 2006; Báidez *et al.*, 2007). Vascular occlusion occurs as a result of the biomass of the pathogen

present in the plant xylem, in addition to defensive tylose or colloid production by the plant (Báidez *et al.*, 2007). The toxin hypothesis suggests that the pathogen delivers complex compounds into its host, including large polymers of polysaccharides, protein-lipopolysaccharides, glycoproteins, enzymes, etc., that induce foliar wilting *via* cytotoxicity (Fradin & Thomma, 2006; de Sain & Rep, 2015).

(2) Similar to other plant pathogens, the secretome of *V. dahliae* has evolved to alter host structure and physiology, facilitating colonization. Specifically, components of the secretome of *V. dahliae* degrade plant cell walls, manipulate host immunity, exert cytotoxicity, neutralize host oxidative stresses, compete with its host for nutrients, and alter host endophytic associations and the rhizosphere microbiome, finally contributing to *Verticillium* wilt.

(3) Except for some *V. dahliae* secretome members with direct cytotoxicity, *V. dahliae* mainly employs its secretome to support proliferation in the plant xylem. These activities contribute to xylem wall collapse and the deposition of polysaccharides, and induction of host plant responses that promote vascular occlusion, finally compromising the host by causing total vascular occlusion. Thus, the *V. dahliae* secretome manipulates plant

defences to contribute to vascular occlusion simultaneously with its toxicity effects to cause wilt symptoms.

(4) There are several key phases in the *V. dahliae*–plant interaction in which the weapons available in the secretome play critical roles in the induction of Verticillium wilt symptoms. In the initial infection zone in the roots, these effector activities specifically counter plant defences, neutralize oxidative stress, manipulate host immunity, and interfere with hormone homeostasis. Subsequently, in the vascular battlefield, the secretome aids mycelial proliferation, the release of incompletely degraded polysaccharides from plant cell walls and the induction of biomacromolecules from an active host defence response, all of which contribute to vascular occlusion. Final victory in shoot occurs as the toxic functions of the secretome manipulate immunity and hormone homeostasis to aggravate wilt symptoms further.

(5) Description of the functions of the secretory proteins in detail will allow their exact roles in vascular occlusion and toxicity to be clarified. Unravelling the bridge that links the *V. dahliae* secretome to the historical vascular occlusion and toxin hypotheses will shed new light into novel means to control this and related plant vascular pathogens.

V. ACKNOWLEDGEMENTS

We apologize in advance to all investigators whose research could not be cited due to space limitations. We extend special thanks to Drs Nikhil Dhar and Peter Henry for thoughtful pre-submission comments. This work was supported by grants from the National Key Research and Development Program of China (2018YFE0112500), the Fundamental Research Funds for Central Non-profit Scientific Institution in CAAS (Y2021XK22), and the Elite Youth Program CAAS to J.-Y.C.

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(Received 16 November 2021; revised 12 April 2022; accepted 14 April 2022; published online 27 April 2022)