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Developments in Detection Methods and Uncovering Resistant Agents Against *Verticillium dahliae* Imply for Effective Protection of Trees in Practice

Keykhasaber M*

Department of Plant Breeding and Biotechnology, University of Zabol, Zabol, Iran

Abstract

Verticillium wilt, caused by *Verticillium dahliae* Kleb., is a serious problem in tree nurseries and plantations worldwide. The best measure to control Verticillium wilt disease is using healthy planting material, and the deployment of resistant plants when *V. dahliae* is already present in the field. However, *V. dahliae* can thrive as an endophyte in plant hosts and asymptomatic infections may occur in recently infected plants that do not yet display symptoms. Therefore, reliable methods, such as PCR-based in planta detection methods, should be used for detection of the pathogen in plant material prior to planting to ensure use of healthy plant material and to avoid the introduction of pathogens in non-infested growing areas. In addition, in some trees recovery is enhanced by producing new vascular tissue, which allows novel vegetative growth of affected stems and branches. Studying the genes involve in recovery, and their impact on Verticillium-triggered changes in differentiation of cells from the cambium or even within existing tissues, may help to design strategies to stimulate recovery of susceptible trees. Identification of genetic sources of resistance is also an essential need for improving resistant trees aiming the effective control of Verticillium wilt in tree plantations. The discovery of candidate genes for disease resistance in trees based on genomics and transcriptomics, coupled with advancements in breeding technology, is expected to enable us to improve resistance particularly in commercially propagated valuable tree species such as olive in the future.

Keywords: PCR-based methods; Recovery; Resistant genes; Breeding

Introduction

Vascular wilts caused by xylem-colonizing pathogens are among the most devastating plant diseases worldwide. The microbial pathogens that cause these diseases are generally soil-borne and infect the plants through the roots. They traverse the cortex of the roots and enter the xylem vessels, after which they proliferate within the vessels, causing blockage of water and mineral flows that may result in wilting and death of the leaves, often followed by partial destruction or death of whole plants [1,2]. There are four fungal genera (*Ceratocystis, Ophiostoma, Verticillium, and Fusarium*), seven bacterial genera (*Clavibacter, Curtobacterium, Erwinia, Pantoea, Ralstonia, Xanthomonas, and Xylella*), and one oomycete genus (*Pythium*) that comprise the most important vascular wilt pathogens [3,4]. Verticillium wilt disease is one of the most common and destructive plant diseases worldwide and is most often caused by the soil-borne fungus *Verticillium dahliae* Kleb [5-8]. Up to today, no sexual stage has been observed for *V. dahliae*, but DNA evidence places the species within the class of Sodariomycetes in the phylum Ascomycota. Its vegetative mycelium is hyaline, septate, and multinucleate, while conidia are ovoid or ellipsoid and usually single-celled. They are borne on phialides, which are specialized hyphae produced in a whorl around each conidiophore, and each phialide carries a mass of conidia [9,10]. Verticillium is named after this verticillate (=whorled) arrangement of the phialides on the conidiophore. The species can cause vascular wilt disease in at least 300 plant species, ranging from herbaceous annuals to woody perennials [11-14]. Verticillium wilt disease is one of the major constraints for tree nurseries and plantations and causes substantial reduction in the production of orchards and high rates of tree mortality [15-20]. *V. dahliae* infection and colonization of woody hosts have been reviewed by [19]. In trees, *V. dahliae* begins its parasitic phase when microsclerotia in soil are stimulated to germinate by root exudates of nearby host roots. The resulting hyphae grow towards the roots of the host which they may penetrate inter- or intracellularly. Following the first penetration, hyphae grow inter- and intracellularly within the root cortex to reach and enter the xylem vessels. Next, conidiospores are produced within these vessels and the plant is colonized systemically by a combination of hyphal growth and conidiospores moving with the transpiration stream. The presence of the fungus and the responses of the plant ultimately cause widespread vascular dysfunctioning, leading to symptoms that comprise wilting, defoliation, necrosis and dieback. Infection and colonization of olive tree by *V. dahliae* has been studied by several research groups. Designing effective control strategies for this disease is difficult because of the long survival time of the pathogen in the form of microsclerotia in soil, broad host range of the pathogen that complicates crop rotation, and the absence of methods to cure infected trees and eradicate the pathogen from infested soils [21-24]. Several measures (such as employment of resistant cultivars or rootstocks, cultural practices to avoid spreading of the disease, disinfestation of *V. dahliae*-infested soil with fumigants, soil solarisation, green amendments or biological soil infestation. replacement of diseased trees with non-host plants, and use of biological control agents, including beneficial bacteria) have been suggested to control this disease [25]. However, as an important pre-planting measure, new plantations should not be established in or near fields with a known history of Verticillium infections. Arguably, the best measure to control Verticillium wilt disease is by planting on soils

***Corresponding author:** Keykhasaber M, Department of Plant Breeding and Biotechnology, University of Zabol, Zabol, Iran, Tel: 98-54-31232080-2; E-mail: mkeikhasaber@uoz.ac.ir

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without Verticillium and preventing introduction of the pathogen into fields by using healthy planting material, and also the deployment of resistant plants when *V. dahliae* is already present [26-30].

Literature Review: Use of Healthy Planting Material

Endophytic colonization of *V. dahliae*

Selection of planting material only based on (the lack of) visible symptoms is not reliable, since asymptomatic infections have been reported to occur in several host plants [31-36]. *V. dahliae* could be detected when samples from trunks and branches of asymptomatic infected olive trees were subjected to amplification by PCR using *V. dahliae*-specific ITS primers [37]. Moreover, nested-PCR analysis and plating assays have shown that seeds harvested from asymptomatic olive trees can transmit the pathogen to seedlings [38]. This may be explained by the fact that *V. dahliae* can colonize plant species strictly as an endophyte without inducing any visible symptoms of disease [39,40]. Currently, endophytic colonization of *V. dahliae* has been reported mainly from monocotoledonous plant species, such as barley, oat and wheat. However, also numerous weeds, including dicotelydonous ones such as common blackberry (*Rubus allegheniensis* Porter ex L. H. Bailey), nettle (*Urtica* spp.), Pennsylvania smartweed *(Polygonum pennsylvanicum* L.), lamb's quarters (*Chenopodium album*), common purslane (*Portulaca oleraceae*), and black nightshade (*Solanum nigrum*) are known as symptomless hosts of *Verticillium* spp. [41-45]. Thus, the fact that *V. dahliae* can thrive as an endophyte in plant hosts has the important implication that asymptomatic plants may serve as a reservoir of inoculum and may potentially initiate epidemics of Verticillium wilt disease.

Latent period and asymptomatic infection

Asymptomatic infections may also occur in recently infected plants that do not yet display symptoms; a phenomenon that is also known as the latent period (Figure 1). Depending on host and pathogen genotypes as well as environmental conditions, this period can last for longer or shorter periods. Upon artificial inoculation, pathogen DNA can be detected in symptomless olive plants at much earlier time points than when the first Verticillium wilt symptoms appear [46-50]. Thus, considering that latency is a phenomenon that is associated with Verticillium infections, reliable methods should be used for detection of the pathogen in plant material prior to planting to ensure use of healthy plant material and to avoid the introduction of pathogens in non-infested growing areas.

Timely testing of plant material for *V. dahliae* infection

PCR-based methods such as real-time PCR are increasingly used for rapid and sensitive detection and quantification of *V. dahliae* in artificially inoculated as well as in naturally infected trees [51,52]. In artificially inoculated trees, detection of the pathogen early after inoculation generally works well, owing to the high inoculum concentration that is generally used to promote consistency of disease incidence in pathogenicity tests [53]. However, the amount of fungal inoculum in asymptomatic infected plants, as likely occurs in natural infections in tree nurseries as well, combined with the non-uniform distribution of the fungus within the tree [54,55], complicates robust and reliable early detection of the pathogen in natural infections. Several studies have been conducted to improve PCR-based methods for early in planta detection and quantification of *V. dahliae* in symptomatic and asymptomatic tissues that carry low amounts of pathogen DNA. Loop-mediated isothermal amplification (LAMP) is a method that recently has been developed as a highly sensitive and specific isothermal PCR-based method that can be used for effective diagnostic assays. Moreover, the sampling strategy may have a major influence. It was demonstrated that the testing mixed samples instead of individual samples improves the robustness of detection methods. Thus, exploitation of these PCR-based *in planta* detection methods, in combination with sampling strategies facilitates robust testing of planting material for *V. dahliae* presence, aiming to provide pathogen-free planting material for establishing new plantations [56,57].

Recovery: A natural phenomenon to overcome verticillium infection

In several tree species such as almond, peach, apricot, ash, catalpa, pistachio, cocoa, avocado, and olive it has been observed that Verticillium wilt symptoms of infected trees may be reduced in a next growing year [58-60]. Also, it was observed that, despite the fast occurrence of disease symptoms in ash trees in the year of inoculation, a high portion of diseased ash trees were recovered from Verticillium wilt symptoms in the year after inoculation [61]. Interestingly, analysis of the distribution of the pathogen in the year after inoculation showed that new xylem sheaths in recovered ash trees were not infected by *V. dahliae*, whereas new xylem sheaths of both maple and symptomatic ash trees were infected. This implies that occurrence of recovery in ash trees is associated with impeding new infections. It also has been observed that olive trees that have recovered from a single inoculation will not express wilt symptoms again, unless new infections occur [62]. Sources of new infections, however, may be either internal (i.e. previously infected xylem sheets) or external (i.e. contracted from the environment). Infested soil is the major external source of new infections in the field. Therefore, practices that reduce inoculum sources in the soil and prevent new infections have an impact on the occurrence and persistence of natural recovery [63-65]. In this context, soil treatments such as soil solarization, soil fumigation, and organic or biological amendments that reduce the inoculum density of *V. dahliae* in the soil around the tree and therefore reduce the number of new invasions of rootlets not only prevent new disease but also stimulate recovery from disease [66-70].

Compartmentalization facilitates recovery

As noted above, in trees infected xylem sheets may provide an internal source of inoculum for infections of new vessel elements in the next year showed that pathogen DNA can be isolated from the xylem of two successive years in diseased maple trees, while in recovered ash trees pathogen DNA could be isolated only from old vessels and not from newly formed vessels in the wood after inoculation. In this experiment, plants received a single inoculation. This indicates that new xylem sheets in maple trees were infected by spreading of the pathogen from old vessels, while in recovered ash trees the ability of *V. dahliae* to invade adjacent vascular bundles was impaired. Thus, mechanisms that hinder spread of the pathogen from old vessels to the new vessels or other parts of infected trees can stimulate recovery of infected trees. Compartmentalization is a boundary-setting process that is activated following fungal vascular invasion and tends to limit the spread of infection and the loss of normal functioning of sapwood [71]. The principle of the compartmentalization lies in the establishment of four types of "walls". While wall 1 restricts pathogen movement longitudinally, wall 2 consists of the growth ring boundary and restricts pathogen movement centripetally, and wall 3 limits the tangential movement of pathogen and is associated with ray parenchyma. Wall 4 is the strongest and referred to as the parenchymatous "barrier zone", produced by cambial activity, and separates the tissue present at the time of infection from new, uninfected tissue. Studies on clones

of *Populus deltoides* Bartr. (eastern cottonwood) and *Liquidambar barstyraciflua* L. (sweetgum) have shown that different clones vary in their compartmentalization ability, suggesting that this phenomenon is under genetic control, and making it possible to screen species for genotypes that display superior compartmentalization traits [72-75].

Restoration of vascular tissue enhances recovery

Recovery is also enhanced by producing new vascular tissue, which allows novel vegetative growth of affected stems and branches [76]. In trees in temperate climate zones every year a new zone of xylem elements (growth ring) is formed if the cambium survives. This enables recovery of infected trees through replacement of the infected vascular tissue. In annual plant species diseased plants at least two different strategies in response to invasion of vascular pathogens to produce new xylem vessels have been reported: 1) trans differentiation which is defined as the conversion of one cell type into another with a different function. 2) vascular hyperplasia which is generally defined as an induced increase in cell number as a result of infection [77-80]. In vascular diseases, infection may induce transdifferentiation of bundle sheath cells to novel, functional xylem vessels, or may increase xylem cells within the vascular bundle as a result of prolonged or renewed activity of the vascular cambium. Seven putative NAC (for NAM, ATAF1/2, and CUC2) transcription factors have been identified in the *Arabidopsis thaliana*, which are involved in transdifferentiation and fall into the subfamily of VND (Vascular related NAC Domain) [81-85]. Within this subfamily, VND6 and VND7 seem to have specific roles on Verticillium-triggered transdifferentiation of bundle sheath cells, with VND6 regulating metaxylem (xylem tissue that consists of rigid thick-walled cells and occurs in parts of the plant that have finished growing) formation, and VND7 inducing protoxylem (the first-formed xylem tissue, consisting of extensible thin-walled cells thickened with rings or spirals of lignin) development [86]. It would be very interesting to see if similar mechanisms do occur in tree species resulting in increased numbers of vascular elements being formed after vascular infection. Interestingly, homologs of NAC domain protein genes (*PtVNS/PtrWND*) have been identified in poplar (*Populus trichocarpa*) and their role in differentiation of the xylem vessel element has been demonstrated [87,88]. Thus, studying the distribution of these genes or their homologs in other trees, and their impact on Verticillium-triggered changes in differentiation of cells from the cambium or even within existing tissues, may help to design strategies to stimulate recovery of susceptible trees [89,90].

Exploiting resistance sources to control verticillium wilt

Genetic resistance is the most preferred strategy to control Verticillium wilt diseases because of its potentially effective and environmentally-friendly nature [91-95]. Several experiments have been carried out to identify Verticillium wilt resistance in various tree species, such as maple, pistachio, and olive [96-100]. Cultivars that have been introduced as resistant show reduction in disease progression when they are inoculated with *V. dahliae*, while can still be colonized by the pathogen as the pathogen could be isolated from inoculated trees. This suggests that resistance in these cultivars is partial and despite the efficacy in reduction of disease symptoms, such plants may serve as a reservoir of inoculum and contribute to spread of the pathogen. Furthermore, when these cultivars are used as rootstock, the pathogen may grow through the rootstock and cause significant disease when it reaches the susceptible scion. Therefore, identification of genetic sources of resistance is an essential need for improving resistant trees aiming the effective control of Verticillium wilt in tree plantations.

Genetic resistance against Verticillium wilt diseases has been reported in several crop species, such as alfalfa (*Medicago sativa*), cotton (*Gossypium hirsutum*), potato (*Solanum tuberosum*), strawberry (*Fragaria vesca*), sunflower (*Helianthus annuus*), and tomato (*Solanum*

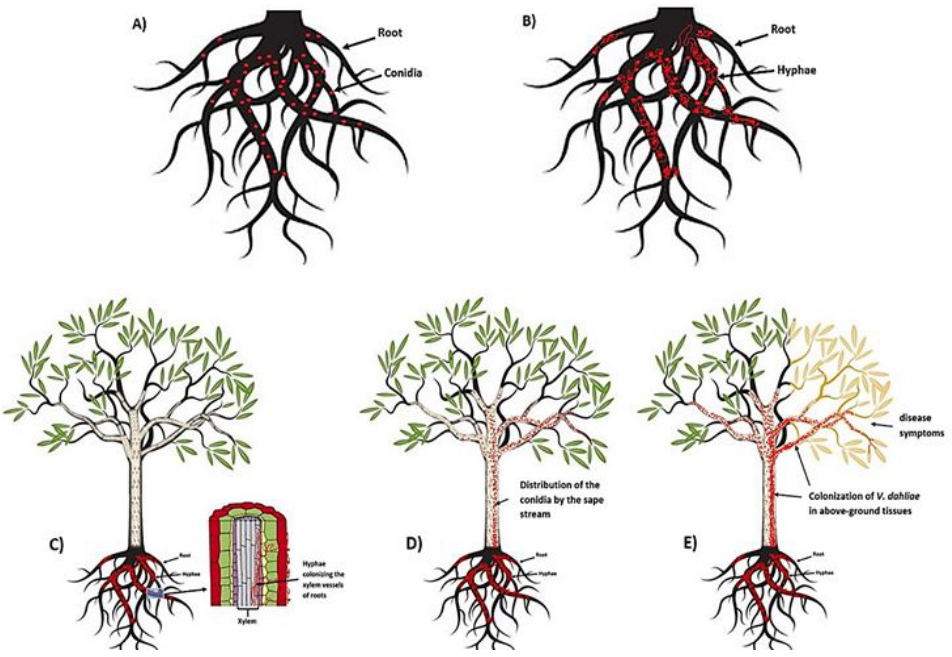

Figure 1: (A-E) Progression of *V. dahliae* and disease symptoms in olive upon artificial inoculation. **A)** Attachment of conidiospores (red spots) to the root surface. **B)** Dense hyphal colonization of the root system. **C)** Hyphae colonizing the xylem vessels of roots. **D)** Distribution of conidia throughout the tree upon transport by the transpiration stream of the host. *V. dahliae* is detectable in all above-ground tissues (main stem, branches, twigs and leaves). **E)** Colonization of *V. dahliae* in above-ground tissues leads to display of extensive disease symptoms in parts of the infected tree.

lycopersicum) [101-105]. Nevertheless, for many other crops and tree species, genetic resistant is not readily available [106,107]. The *Ve* locus in tomato is the only cloned and functionally characterized locus in terms of plant resistance against Verticillium wilt. This locus contains two genes, *Ve1* and *Ve2,* encoding extracellular leucine-rich repeat receptor-like proteins (eLRR-RLPs). However, of these genes only *Ve1* provides resistance against race 1 isolates of *V. dahliae* and *V. albo-atrum* via recognition of Ave1 effector, which was identified only in race 1 isolates [108-110]. Intriguingly, phylogenetic analysis showed that homologues of *Ve1* are widely distributed in plants. So far, several *Ve1* homologous genes that confer race-specific resistance against *V. dahliae* have been reported such as *SlVe1* from *Solanum lycopersicoides*, *StVe1* from *S. tuberosum*, *StVe* and *StoVe1* from *S. torvum*, *mVe1* from *Mentha longifolia*, and *Vr1* from *Lactuca sativa*. Recently, the *Ve1*-like genes *GbVe1* and *Gbvdr5* were cloned from island cotton, which is resistant to Verticillium wilt. Transgenic expression of these genes in susceptible *Arabidopsis* and upland cotton induced significant resistance to both D and ND isolates of *V. dahliae*. Moreover, the *Ve1*-like gene *VvVe* was recently cloned from *Vitis vinifera.* Overexpression of *VvVe* in transgenic *Nicotiana benthamiana* conferred resistance to the V991 isolate (D pathotype) of *V. dahliae* [111-115]. Recently, Gómez-Lama Cabanás et al. conducted a transcriptomic analysis to identify systemic defense responses induced/repressed in aerial tissues of the tolerant olive cultivar (Frantoio) upon root colonization by *V. dahliae*. They reported transcription factor *GRAS1* and disease resistance-responsive protein (*DRR2*) could be further evaluated as markers of the tolerance level to *V. dahliae.* However, genes conferring resistance to *V. dahliae* D and ND isolates have not been reported from tree hosts thus far [116-125].

Discussion and Conclusion

Putative resistant cultivars may be identified by screening genotypes preserved in germplasm banks, or by screening wild relatives or progenies generated in breeding programs. Several screenings of commercial olive cultivars and wild olive germplasm have been carried out to identify sources of resistance to Verticillium wilt [126]. Although olive genotypes that display some degree of resistance to *V. dahliae* have been found, most of the commercial olive cultivars are still susceptible or extremely susceptible to Verticillium wilt. Thus, the development of breeding programs may act as an important approach to generate resistant cultivars that also have desirable agronomic traits. Breeding for resistance typically includes:

1) Identification of genotypes that carry a useful disease resistance trait, even if this is combined with less desirable other traits.

2) Crossing of a susceptible preferred cultivar with the resistance source.

3) Testing of the progeny of the cross for reduced disease susceptibility.

4) Selection of disease-resistant individuals and crossing back to the recurrent parent.

This process is repeated for as many back crosses as needed to obtain a line as identical as possible to the recurrent parent with the addition of the gene of interest. Especially in perennial species this is a long term approach that takes many years, often even decades.

Diversity in plant genetic resources is the basis for selection and for plant improvement in breeding programs [127]. In the absence of enough diversity, mutagenesis followed by screening for enhanced resistance is a means to identify novel resistance traits. Through the years, mutagenesis has played a significant role in plant breeding programs by producing a vast amount of genetic diversity in crops and tree species. Several technologies have been developed for random mutation, e.g., radiation (gamma and X-ray), chemical mutagens such as ethyl methanesulfonate and sodium azide and methylnitrosourea, T-DNA- or transposon-based activation tagging. Besides, *in vitro* culture techniques are particularly relevant for mutagenesis as large populations of cells can be treated and screened before being regenerated into complete plants. Among the different *in vitro* methods, however, somatic embryogenesis is the most useful tool for the selection and multiplication of mutants as somatic embryos usually originate from single cells. Furthermore, a few subcultures can be performed in a short time to increase the mutagenized population for selection. Therefore, combination of mutagenesis and *in vitro* culture techniques can generate an appropriate genetic diversity to be used in breeding programs for improvement of resistant cultivars.

To evaluate the resistance level of genotypes that are developed in a breeding program, they should be challenged with the pathogen. Reported that olive cultivars that are highly resistant to isolates that belong to the ND pathotype may be highly susceptible to isolates that belong to the D pathotype. This indicates that resistance in trees is only active against isolates of the species, and not to others, equivalent to the occurrence of a race-structure that is frequently observed with the deployment of resistance genes. As isolates of *V. dahliae* are mostly considered host-adapted rather than host-specific, i.e. are more virulent to the host from which they were isolated it is important to include isolates representing differential virulence in programs for evaluating host resistance to *V. dahlia.*

Advances in genetic transformation technology through use of selected strains of *Agrobacterium tumefaciens* and subsequent regeneration via somatic embryogenesis have provided new possibilities for the biotechnological improvement of resistance in tree species. However, for this strategy understanding host-pathogen interactions and molecular characterization of the genes and proteins that are responsible for resistance is essential. In tomato, genetic analysis has shown that the *Ve1*-mediated resistance signaling pathway requires the EDS1 (Enhanced Disease Susceptibility 1), NDR1 (Non-race-specific Disease Resistance 1), BAK1 (BRI1-Associated Kinase 1), MEK2 (MKK2, MAP kinase 2), and SOBIR1 (LRR-RLK Suppressor of BIR1-1) proteins. Also, it has been reported that GhNDR1 and GhMKK2 are required for resistance mediated by the *GbVe1* and *Gbvdr5* genes in cotton. In tree hosts, however, many aspects of defense responses remain unknown and require investigation. With recent genomic and transcriptomics advances we are now better equipped to begin unraveling the mechanisms underlying plant-pathogen interactions in woody hosts. The discovery of candidate genes for disease resistance in trees based on genomics and transcriptomics, coupled with advancements in breeding technology, is expected to enable us to improve resistance particularly in commercially propagated olive and other valuable tree species in the future.

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