Unravelling aspects of spatial and temporal distribution of *Verticillium dahliae* in olive, maple and ash trees and improvement of detection methods

Mojtaba Keykhasaber

Thesis committee

Promotors
Prof. Dr B.P.H.J. Thomma
Professor of Phytopathology
Wageningen University & Research

Prof. Dr P.J.G.M de Wit
Professor of Phytopathology
Wageningen University & Research

Co-promotor
Dr J.A. Hiemstra
Senior researcher, Wageningen Plant Research
Wageningen University & Research

Other members
Prof. Dr P.C. Struik, Wageningen University & Research
Dr A.J. Termorshuizen, Soil Cares Research, Wageningen
Dr J. Mercado Blanco, Institute for Sustainable Agriculture, Spain
Dr T.A. Wood, National Institute of Agricultural Botany (NIAB), England

This research was conducted under the auspices of the Graduate School Experimental Plant Sciences

Unravelling aspects of spatial and temporal distribution of *Verticillium dahliae* in olive, maple and ash trees and improvement of detection methods

Mojtaba Keykhasaber

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on 1st of March, 2017
at 1.30 p.m. in the Aula.

Mojtaba Keykhasaber
Unravelling aspects of spatial and temporal distribution of *Verticillium dahliae* in olive, maple and ash trees and improvement of detection methods, 163 pages.

PhD thesis, Wageningen University & Research, Wageningen, NL (2017)
With references, with summaries in English

ISBN: 978-94-6343-014-2
DOI: http://dx.doi.org/10.18174/396604

CONTENTS

Chapter 1

General introduction and outline of the thesis

During their lifetime, plants are exposed to a wealth of potential pathogens including viruses, bacteria, fungi, oomycetes, protozoa, and nematodes. These pathogens may differ with respect to their host range (ranging from a single species to whole genera or even a wide range of hosts belonging to various families of plants), the nature of infection (systemic or localized), the mode of perpetuation and spread (soil-, seed-, water- and air-borne, etc.), and the organ or tissue of the plant on which they can grow, which may even be age-dependent. As a result of the invasion of the pathogen and the development of disease, normal physiology and activities of the infected parts of the host plants may be compromised and tissues or organs may malfunction. Consequently, morphological and physiological changes may occur. Eventually, such changes may lead to death of plant parts or, ultimately, of the entire plant (Agrios, 2005; Horst, 2013; Smith et al., 1988).

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 (Agrios, 2005). 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 (Yadeta and Thomma, 2013).

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. (Bhat and Subbarao, 1999; Pegg and Brady, 2002; Smith et al., 1988; Fradin and Thomma, 2006). 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 (Inderbitzin et al., 2011). *Verticillium* is named after this verticillate

(=whorled) arrangement of the phialides on the conidiophore. The fungus forms microsclerotia, which are masses of melanized hyphae, as resting structures in dying plant tissue that are able to survive for many years in the soil (Barbara et al., 2003; Goud et al., 2003; Jabnoun-Khiareddine et al., 2010). The species can cause vascular wilt disease in at least 300 plant species, ranging from herbaceous annuals to woody perennials (Berlanger and Powelson, 2005; Klosterman et al., 2009). Control of Verticillium wilt is very difficult due to the long-term persistence of the pathogen in form of the microsclerotia in the field, the broad host range of the pathogen, and lack of curative measures once a plant is infected. Therefore, the use of practices to avoid spreading of the disease and to reduce soil inoculum levels, combined with resistant host plant cultivars if these are available, are the most effective measures to deal with Verticillium wilt disease (Barranco et al., 2010, Jiménez-Díaz et al., 1998; López-Escudero and Mercado-Blanco, 2011). To this end, detection and accurate diagnosis of the pathogen at cultivation sites prior to planting, as well as early during infection is essential. Conventional methods for identifying plant pathogens, for instance through interpretation of visual symptoms or isolation and culturing of the pathogen followed by determination, are often time-consuming, laborious and require expert knowledge. Over the last decade, advances in molecular methods have revolutionized detection and identification of fungal pathogens (Capote et al., 2012; McCartney et al., 2003; Tsui et al., 2011). Currently, PCR-based methods such as real-time PCR as well as array technology are increasingly used for rapid and sensitive detection and quantification of plant pathogens without the need for a culturing step (Lievens et al., 2003, 2005; 2006; Markakis et al., 2009; Mercado-Blanco et al., 2003; Schena et al., 2004a; Schena et al., 2004b). Improvement of the efficiency of such techniques, as well as the development of novel technologies to create a practical tool for a large-scale real-time disease monitoring under field conditions, will be valuable for early detection and monitoring of diseases and will facilitate decision making for proper management strategies to prevent the development and spread of diseases.

Verticillium wilt is quite intensively studied in relation to herbaceous hosts, whereas little is known about Verticillium wilts of trees, despite their economic and ecological impact. Worldwide, olive-growing regions and tree nurseries are affected by

this disease. In **Chapter 2**, the most important aspects of Verticillium wilt of woody hosts are reviewed, with emphasis on olive, ash and maple trees. The disease cycle, symptomatology, genetic diversity of the pathogen and defensive reactions of infected plants are discussed, as well as Verticillium wilt management in trees.

As discussed previously, detection and accurate identification of plant pathogens is one of the most important strategies for disease control. Early detection of pathogens in plant material to avoid the introduction of pathogens in non-infested growing areas is important. For that reason, the availability of fast, sensitive and accurate methods for detection and identification of pathogens is required. In the case of tree hosts, such methods particularly require attention for straightforward and non-destructive sampling methods. **Chapter 3** aims to design a straightforward and efficient sampling protocol for reliable detection and quantification of *V. dahliae* in olive trees. To this end, the suitability of twig and leaf samples for robust and reliable detection of *V. dahliae* in infected olive trees is tested. Furthermore the minimum number of samples required for reliable detection using real-time PCR is determined.

V. dahliae isolates that infect olive trees can be classified as defoliating (D) isolates that are highly virulent, and non-defoliating (ND) isolates that are generally less aggressive (Rodríguez-Jurado et al. 1993). The design of a novel, accurate method for accurate discrimination and sensitive detection of these *V. dahliae* isolates is described in **Chapter 4**. To this end, whole-genome sequences of various *V. dahliae* isolates were compared and primers that can discriminate the two groups of isolates were designed. Subsequently, the suitability of the primers to discriminate the two groups of isolates is validated.

The distribution patterns of D and ND isolates of *V. dahliae* in resistant and susceptible olive trees provides insight into the differences in their interactions. In **Chapter 5**, changes in quantities of D and ND *V. dahliae* in lower, middle and top parts of stems of susceptible and resistant cultivars of olive trees is assessed in a time course experiment. Furthermore, to characterize colonization in olive trees, GFP-labelled isolates are used for inoculation. To illustrate the growth and spread of the pathogen in infected

olive trees, epi-fluorescence and confocal laser microscopy were used to characterize pathogen progression.

Maple (*Acer* spp.) and ash (*Fraxinus* spp.) are popular shade trees that are affected by Verticillium wilt disease. Unlike infected maple trees, however, infected ash trees are able to recover from Verticillium wilt, even in cases of serious damage (Hiemstra and Harris 1998). Studying the progress of disease and proliferation and survival of *V. dahliae* in maple and ash trees provides useful information about differences in spatial and temporal distribution of *V. dahliae* colonization, as well as the recovery phenomenon. In **Chapter 6**, the progress of disease is monitored in inoculated maple and ash trees. Also changes in quantity of pathogen DNA is assessed in stem sections over time. Furthermore, the infection and changes in *V. dahliae* DNA quantities are analysed in xylem vessels of maple and ash over two consecutive years.

Finally, in **Chapter 7** the major results described in this thesis are discussed and a perspective on Verticillium wilt management in tree species is presented.

References

Agrios, G.N. (2005). Plant Pathology, 5th. Elsevier Academic Press, San Diego, California.

Barbara, D.J., Clewes, E. (2003). Plant pathogenic Verticillium species: how many of them are there?. Molecular Plant Pathology 4, 297-305.

Barranco, D., Fernandez-Escobar, R., Rallo, L. (2010). Olive Growing. Mundi Prensa/Australian Olive Association Ltd., Junta de Andalucía, 756 pp.

Berlanger, I., Powelson, M.L. (2005). Verticillium wilt. The Plant Health Instructor. DOI: 10.1094/PHI-I-PHI-I-2000-0801-01.

Bhat, R.G., Subbarao, K.V. (1999). Host range specificity in *Verticillium dahliae*. Phytopathology **89**, 1218-1285.

Capote, N., Pastrana, A.M., Aguado, A., Sánchez-Torres, P. (2012). Molecular tools for detection of plant pathogenic fungi and fungicide resistance. In: Cumagun C. J., 2012. Plant pathology: ISBN: 978-953-51-0489-6, InTech.

Fradin, E.F., Thomma, B.P.H.J. (2006). Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Molecular Plant Pathology 7, 71-86.

Goud, J.C., Termorshuizen, A.J., Gams, W. (2003). Morphology of *Verticillium dahliae* and *V. tricorpus* on semi-selective media used for the detection of *V. dahliae* in soil. Mycology Research **107**,822-830.

Hiemstra, J.A., Harris, D.C. (Eds.) (1998). A compendium of Verticillium wilts in tree species. Wageningen: Centre for Plant Breeding and Reproduction Research (CPRO-DLO)/West Malling: HRI-East Malling.

Horst, R.K. (2013). Westcott's plant disease handbook. Springer Netherlands: Dordrecht, DOI 10.1007/978-94-007-2141-8-8.

Inderbitzin, P., Bostock, R.M., Davis, R.M., Usami, T., Platt, H.W., Subbarao, K.V. (2011). Phylogenetics and Taxonomy of the Fungal Vascular Wilt Pathogen Verticillium with the Descriptions of Five New Species. PLoS ONE 6(12): e28341. doi:10.1371/journal. pone. 0028341.

Jabnoun-Khiareddine, H., Daami-Remadi, M., Barbara, D.J., El Mahjoub, M. (2010). Morphological variability within and among Verticillium species collected in Tunisia. Tunisian Journal of Plant Protection 5, 19-38.

Jiménez Díaz, R.M., Tjamos, E.C., Cirulli, M. (1998). Verticillium wilt of major tree hosts. Olive. Pages 13-16 in: A Compendium of Verticillium Wilt in Tree Species. J. A. Hiemstra and D. C. Harris, eds. Posen and Looijen, Wageningen, the Netherlands.

Klosterman, S.J., Atallah, Z.K., Vallad, G.E., Subbarao, K.V. (2009). Diversity, pathogenicity and management of *Verticillium* species. Annual Review of Phytopathology 47:39-62.

Lievens, B., Brouwer, M., Vanachter, A.C.R.C., Lévesque, C.A., Cammue, B.P.A., Thomma, B.P.H.J. (2003). Design and development of a DNA array for rapid detection and identification of multiple tomato vascular wilt pathogens. FEMS Microbiology Letters 223, 113-122.

Lievens, B., Thomma, B.P.H.J. (2005). Recent developments in pathogen detection arrays: Implications for fungal plant pathogens and use in practice. Phytopathology 95, 1374-1380.

Lievens, B., Claes, L., Vanachter, A.C., Cammue, B.P., Thomma, B.P.H.J. (2006). Detecting single nucleotide polymorphisms using DNA arrays for plant pathogen diagnosis. FEMS Microbiology Letters 255, 129-139.

López-Escudero, F.J., Mercado-Blanco, J. (2011). Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. Plant Soil **344**, 1-50.

Markakis, E.A., Tjamos, S.E., Antoniou, P.P., Paplomatas, E.J., Tjamos, E.C. (2009). Symptom development, pathogen isolation and real-time qPCR quantification as factors for evaluating the resistance of olive cultivars to Verticillium pathotypes. European Journal Plant Pathology 124, 603-611.

McCartney, H.A., Foster, S. J., Fraaije, B.A., Ward, E. (2003). Molecular diagnostics for fungal plant pathogens. Pest Management Science 59, 129-142.

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodríguez-Jurado, D., Jiménez-Díaz, R.M. (2003). Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction. Physiological and Molecular Plant Pathology 63, 91-105.

Pegg, G.F., Brady, B.L. (2002). Verticillium wilts. Wallingford, UK: CABI publishing.

Rodríguez-Jurado, D., Blanco-López, M.A., Rappoport, H.F., Jiménez-Díaz, R.M. (1993). Present status of Verticillium wilt of olive in Andalucía (southern Spain). European and Mediterranean Plant Protection Organization (EPPO) Bulletin 23, 513-516.

Schena, L., Nigro, F., Ippolito, A. (2004a). Real-time detection and quantification of soilborne fungal pathogens: The case of *Rosellinia necatrix*, *Phytophthora nicotianae*, *P. citrophthora* and *Verticillium dahliae*. Phytopathology Mediterranian 43, 273-280.

Schena, L., Nigro, F., Ippolito, A., Gallitelli, D. (2004b). Real-time quantitative PCR: A new technology to detect and study phytopathogenic and antagonistic fungi. European Journal of Plant Pathology 110, 893-908.

Smith, I.M., Dunez, J., Lelliott, R.A., Phillips, D.H., Archer, S.A. (1988). European handbook of plant diseases. Blackewell Scientific Publications, Oxford etc., 583 S.

Tsui, C.K.M., Woodhal, J., Chen, W., Lévesque, A., Lau, A., Schoen, C.D., Baschien, C., Najafzadeh, M.J., Sybren de hoog, G. (2011). Molecular techniques for pathogen identification and fungus detection in the environment. IMA Fungus 2(2), 177-189.

Yadeta, K.A., Thomma, B.P.H.J. (2013). The xylem as battleground for plant hosts and vascular wilt pathogens. Frontiers in plant science 4, 97.

Chapter 2

Verticillium wilt of woody plants with emphasis on olive and shade trees

Mojtaba Keykhasaber[a,b,c]

[a]Laboratory of Phytopathology, Wageningen University & Research, 6708 PB Wageningen, The Netherlands

[b]Wageningen Plant Research, Wageningen University & Research, P.O. Box 200, 6670 AE

[c]Department of Plant Biotechnology, Zabol University, Zabol, Iran

This chapter has been submitted for publication in European Journal of Plant Pathology.

Abstract

Olive plantations and tree nurseries are economically and ecologically important agricultural sectors. However, Verticillium wilt, caused by *Verticillium dahliae* Kleb., is a serious problem in olive-growing regions and in tree nurseries worldwide. In this review we describe common and differentiating aspects of Verticillium wilts in some of the main economically woody hosts. The establishment of new planting sites on infested soils, the use of infected plant material and the spread of highly virulent pathogen isolates are the main reasons of increasing problems with Verticillium wilt in tree cultivation. Therefore, protocols for quick and efficient screening of new planting sites as well as planting material for *V. dahliae* prior to cultivation is an important measure to control Verticillium wilt disease. Furthermore, screening for resistant genotypes that can be included in breeding programs to increase resistance to Verticillium wilt is an important strategy for future disease control. Collectively, these strategies are essential tools in an integrated disease management strategy to control Verticillium wilt in tree plantations and nurseries.

Introduction

Verticillium wilt caused by the soil-borne fungus *Verticillium dahliae* Kleb. is among the most widespread plant diseases worldwide (Pegg and Brady, 2002; Smith et al., 1988). Although no exact statistics exist on the number of species that are susceptible to Verticillium wilt, it is estimated that at least 300 (Berlanger and Powelson, 2005) to 400 (Klosterman et al., 2009) plant species, ranging from herbaceous annuals to woody perennials, are affected. All woody hosts that are susceptible to Verticillium wilt belong to the Dicotyledonaceae, whereas monocotyledonous trees and Gymnophytes are not affected (Hiemstra, 1998a; Sinclair and Lyon, 2005). Olive plantations in the Mediterranean Basin and tree nurseries in more temperate regions are the most important agricultural sectors that involve woody species affected by the disease (Goud et al., 2011; Hiemstra and Harris, 1998; Jiménez-Díaz et al., 2012). In this review we discuss about Verticillium wilt disease cycle, reactions of infected plants and recovery, Verticillium wilt in major tree hosts and symptoms, genetic diversity and detection of the pathogen, and management of Verticillium in the main woody hosts. In this review we will further

concentrate on olive as the most important fruiting species, and on ash and maple as the most important shade trees affected by Verticillium wilt.

Disease cycle of verticillium wilt of trees

The life cycle of *V. dahliae* consists of a parasitic part, in which the fungus lives in its host, and a non-parasitic part, in which it is dormant. For tree hosts the disease cycle of *V. dahliae* has been described in detail by Hiemstra (1998a) (Figure 1). During the non-parasitic phase in the soil, *V. dahliae* survives as microsclerotia, either as dispersed propagules or embedded within plant debris, mainly in the upper layer of the soil from where it can easily be spread by wind, rain or irrigation water, human and animal activities, and agricultural tools and machines (Pegg and Brady, 2002; Schnathorst, 1981; Wilhelm, 1950). Microsclerotia are very persistent and enable the pathogen to attack new plantings even after a long period without hosts being present (Wilhelm, 1955). The infection process of *V. dahliae* in woody plants is similar to that in herbaceous plants. Microsclerotia are stimulated to germinate by exudates from nearby growing roots (Schreiber and Green, 1963). *V. dahliae* begins its parasitic phase when hyphae from germinating microsclerotia penetrate roots of a susceptible host (Lockwood, 1977; Nelson, 1990; Schreiber and Green, 1963). Subsequently, hyphae grow inter- and intracellular within the root cortex to reach the xylem vessels and enter these (Prieto et al., 2009). Once inside the vessels, the fungus produces conidia that are carried with the flow of xylem fluid until they are trapped at vessel ends or protruding parts of vessel elements. Here they may germinate and the new hyphae penetrate into adjacent vessel elements (Beckman et al., 1976). At this step accumulation of *V. dahliae* hyphae, ultrastructural and chemical alterations resulting from defense reactions, and aggregates resulting from degradation of external material of the xylem vessel walls by fungal enzymatic activity may cause occlusion of *V. dahliae*-infected xylem vessels (Baídez et al., 2007; Hiemstra and Harris, 1998; Pegg and Brady, 2002). As a result, the water flow through the xylem is hampered and symptoms of water stress may develop. Wilting, defoliation and early senescence comprising chlorosis, necrosis, and stunting are the main symptoms of Verticillium wilt disease (Figure 2). Moreover, sparse foliage and branch

dieback may also occur (Berlanger and Powelson, 2005; Hiemstra, 1998a; Riffle and Peterson, 1989; Sinclair and Lyon, 2005). Plants with acute infections may start with symptoms on individual branches or on one side of the plant. This is often called "flagging", which can be diagnostic for Verticillium wilt disease. Furthermore, one or several branches may suddenly wilt and die and buds may fail to leaf out in spring (Douglas, 2008; Himelick, 1968; Piearce and Gibbs, 1981).

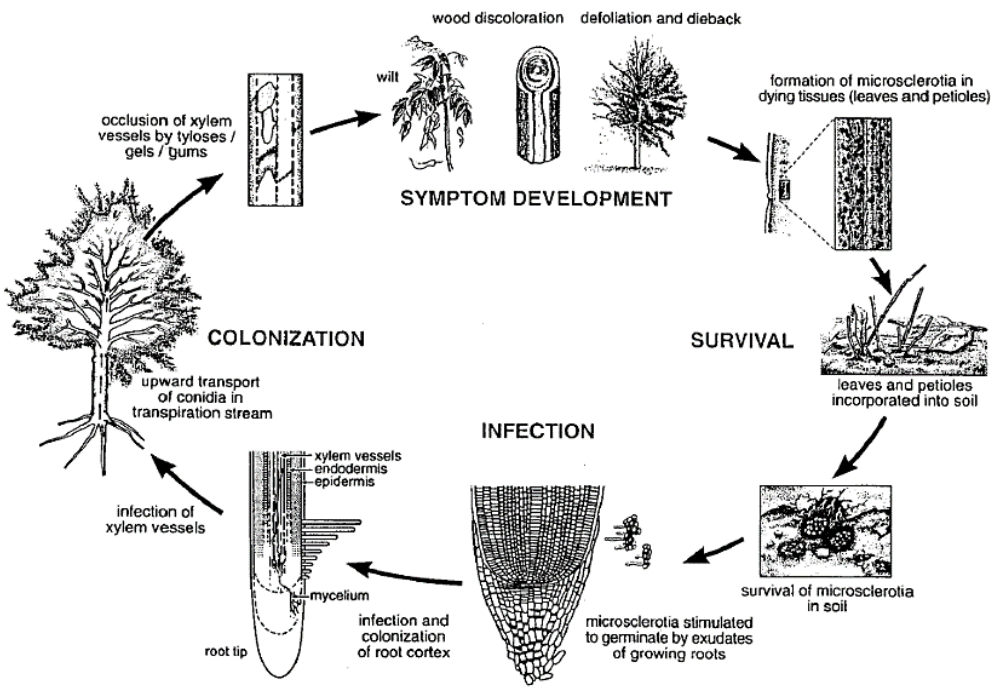


Figure 1. Disease cycle of *V. dahliae* in trees (Drawing by P.J. Kostense; reprinted with permission from Hiemstra and Harris, 1998).

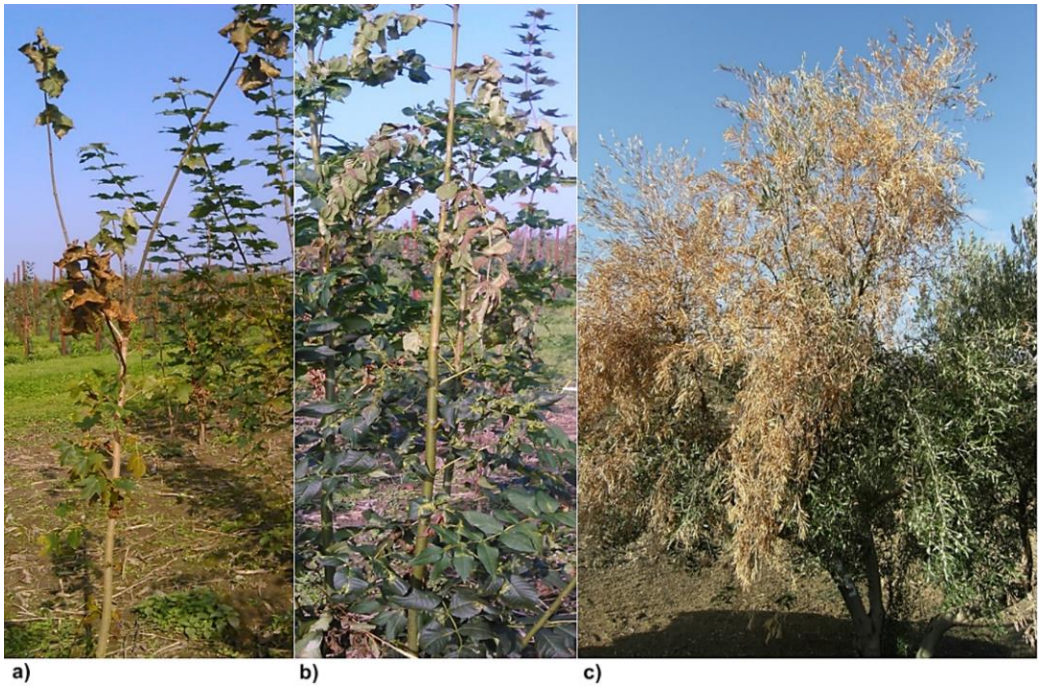


Figure 2. Wilting and leaf necrosis in maple, ash and olive trees affected by *Verticillium dahliae*. A) Wilting and desiccation of leaves in a young maple tree (photograph: M. Keykhasaber). B) Necrosis and wilting of leaves in a young ash tree (photograph: M. Keykhasaber). C) Partial dieback of shoots and branches in an olive tree (photograph: J.A. Hiemstra).

Finally, at late stages of the disease, microsclerotia are formed in dying tissues. The presence of *V. dahliae* in petioles of infected trees in the form of microsclerotia has been demonstrated for several tree species including *Acer* (Hiemstra 1997), *Liriodendron tulipifera* (Morehart and Melchior, 1982), olive (Prieto et al., 2009), and *Fraxinus* (Rijkers et al., 1992). Recently, formation of microsclerotia was also found inside peduncles and flowers of infected olive trees (Trapero et al., 2011). After incorporation of infected plant debris in the top soil layer and decomposition by the activity of soil-borne organisms, microsclerotia survive in the soil for prolonged times (years) and become available as inoculum for new infections (Hiemstra, 1997; Hiemstra and Harris, 1998; Morehart and Melchior, 1982; Rijkers et al., 1992; Tjamos and Botseas, 1987; Tjamos and Tsougriani, 1990; Townsend et al., 1990).

Reactions of the infected tree and recovery

In some cases trees infected by Verticillium wilt are able to recover from the disease (Hiemstra, 1998a). This phenomenon has been reported in olive as re-growth from existing crowns that suffer from limited dieback, or from the stem base after complete dieback (López-Escudero and Blanco-López, 2001; 2005; Levin et al., 2003; Markakis et al., 2009; Mercado-Blanco et al., 2001). Recovery has similarly been reported in *Catalpa bignonioides* as re-growth from the crown, in *Acer platanoides* with re-growth from stem base after extensive dieback (Goud et al., 2011), and in *Fraxinus excelsior* as re-growth without dieback of twigs (Hiemstra, 1998b). Differences in the severity of symptoms and in the percentage of recovery in tree species may be related to differences in the capacity to compartmentalize infected xylem. Compartmentalization was first proposed as a mechanism against spread of decay in trees by isolating the damaged tissues and replacing it by new functional tissues (Shigo and Marx, 1977). Later it was reported that this mechanism that causes changes in anatomy and chemistry of xylem cells also has an important role in protecting trees against colonization by vascular pathogens (Bonsen et al., 1985; Shigo, 1984; Tippett and Shigo, 1981; Manion, 2003; Smith, 2006).

The inherent structure of the xylem and the ability of trees to produce new layers of xylem also has a significant impact on the potential of recovery (Banfield, 1968; Emechebe et al., 1974; Sinclair et al., 1981; Tippett and Shigo, 1981). In ring-porous tree species (like robinia and ash) most of the water transport is in the most recent growth ring. This implicates that as long as these trees are able to produce new xylem vessels every year, they can substitute their blocked vessels with new ones, which enables complete recovery, often even without dieback of the crown. In tree species with a diffuse-porous structure of the xylem, such as maple, xylem vessels in each growth ring remain functional for several years. Hence loss of a major part of the water transport capacity in infected trees often cannot sufficiently be compensated by the wood in a new growth ring. Such trees therefore probably show much more dieback of the aerial parts and recovery starts by regrowth from healthy parts of the stem base or roots (Hiemstra and herris, 1998). Compared to the healthy plants, however, recovered plants have higher probability of becoming diseased again (Goud et al., 2011).

Expansion of *V. dahliae* in xylem vessels of infected plants triggers defense reactions, including ultrastructural and chemical alterations (Adams and Thomas, 1985; Hiemstra and herris, 1998; Pegg and Brady, 2002). In response to pathogen invasion, the cambium may form a barrier zone consisting of parenchymatous cells surrounding the infected tissues (Shigo, 1984). Host plants may also deposit coating materials (such as lipid-rich or fibrillar coatings) onto xylem vessel walls and into pit membranes (Robb et al., 1982; Street et al., 1986), and accumulate gums and form tyloses to prevent pathogen spread (Baídez et al., 2007).

Infected plants also secrete phytoalexins, terpenoid and phenolic substances that have antimicrobial activity during pre-vascular and vascular phases of infection (Daayf et al., 1997; Laouane et al., 2011; Mace et al., 1989; Mansfield, 2000; El-Zik, 1985; Rodríguez-Jurado et al., 1993; Ryan and Robards, 1998; Treutter, 2006). In olive trees infected with *V. dahliae* it was observed that the level of phenolic components such as quercetin, luteolin aglycons, rutin, oleuropein, luteolin-7-glucoside, tyrosol, p-coumaric acid and catechin increased in vascular tissues during infection and colonization (Baídez et al., 2007). Their antifungal activity against *V. dahliae* was substantiated by in vitro studies, suggesting they are involved in defense (Baídez et al., 2007).

Major tree hosts and symptoms

Olive (*Olea europaea* L.), a member of the *Oleaceae* family, is considered as one of the economically, socially and ecologically most important trees within olive producing countries. It originates from the Persian high plateau and coastal Syria, from where it was spread throughout the Mediterranean Basin, at first by the Greeks and Phoenicians, later by the Carthaginians, Romans, and Arabs. Later olive cultivation expanded to the Americas, South Africa, China, Japan and Australia (Blázquez-Martínez, 1996; Civantos, 2004). Verticillium wilt of olive was first reported from Italy (Ruggieri, 1946), and soon thereafter from various other regions, including California, European and Asian countries as well as Australia (López-Escudero and Mercado-Blanco, 2011; Navas-Cortes et al., 2008), and Argentina (Decampo et al., 1981). Initially, Verticillium wilt mostly occurred in olive groves that were established in fields that were previously used for cultivation of

crops that are susceptible to *V. dahliae*, especially cotton, or in groves established next to fields with susceptible crops (Jiménez-Díaz et al., 1998; 2012). Currently, Verticillium wilt is considered as the most important disease that threatens olive production, causing serious concern to growers, nursery companies and the olive-oil industry throughout the world (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al., 2012; Tsror, 2011). This is particularly relevant since most olive cultivars are susceptible to *V. dahliae* (Antoniou et al., 2001, 2008; Cirulli et al., 2008; López-Escudero et al., 2004; López-Escudero and Mercado-Blanco, 2011), although a number of relatively resistant genotypes have been identified in artificial inoculation assays (García-Ruiz et al., 2014; López-Escudero et al., 2004; Martos-Moreno et al., 2006) as well as in field experiments (López-Escudero and Mercado-Blanco, 2011; Trapero et al., 2013). However, most of the agronomically and economically relevant olive cultivars are susceptible or extremely susceptible to highly virulent strains of *V. dahliae* (López-Escudero and Mercado-Blanco, 2011).

In olive, two forms of Verticillium wilt have been distinguished: an acute form ('apoplexy') and a chronic form ('slow decline') (Blanco-López et al., 1984; Jiménez-Díaz 1998; Tosi and Zazzerini, 1998). The 'apoplexy' form, which mainly occurs in late winter and early spring, is characterized by rapid outbreaks involving severe wilting of main and secondary branches. Leaves first become chlorotic, and then turn light-brown and roll back towards the abaxial side while remaining attached to the branches. Ultimately, a rapid dieback of twigs, shoots and branches takes place, especially in young plants, which may result in death of the entire tree (Jiménez-Díaz, 1998; Jiménez-Díaz et al., 2012; López-Escudero and Blanco-López, 2001). The 'slow decline' syndrome is characterized by necrosis of inflorescences, chlorosis of leaves and heavy defoliation of green or dull green leaves. On infected plants, flowers mummify and remain attached to the shoots. The bark of affected shoots may become reddish-brown, and the inner vascular tissues show a dark-brown discoloration. These symptoms usually begin in spring and slowly progress to early summer (Jiménez-Díaz, 1998; Jiménez-Díaz et al., 2012; López-Escudero and Mercado-Blanco, 2011).

In addition to olive groves, Verticillium wilt is also a major problem in shade tree nurseries in more temperate regions, and can occur also in landscape plantings, especially at locations where susceptible field crops were grown previously (Hiemstra and Harris, 1998; Riffle and Peterson, 1989). Maples (*Acer* spp.) are popular trees for residential and commercial landscapes, but generally very susceptible to Verticillium wilt (Gleason and Hartman, 2001; Harris, 1998; Frank et al., 2012). Among the most frequently grown maple species, Norway maple (*A. platanoides*) is known as a highly susceptible species on which most of the investigations on Verticillium wilt of maple have been conducted (Harris, 1998; Townsend et al., 1990). This wilt can induce a range of symptoms in maple include leaf yellowing, curling, and wilting. Leaf scorch can also occur at leaf margins. Leaves on one side of the tree or on just an individual branch may suddenly wilt and die. Leaves are yellowish and smaller than normal. Also a dark olive-green discolouration develops in the sapwood that is more likely to be present in the larger branches than in the smaller twigs, and is more common near the bases of larger, symptomatic branches. Infected shoots may die back that leads to the death of branches, and possibly whole tree (Frank et al., 2012; Harris, 1998; Pscheidt and Ocamb, 2013a).

Ash (*Fraxinus* spp.), like olive a member of the *Oleaceae* family, is another widely cultivated genus with tree species that are well-known for their high quality timber and ornamental value. Several species in this genus, especially common ash (*F. excelsior*) which is the most widely distributed species in Europe (Fraxigen, 2005), may be severely affected by Verticillium wilt (Heffer and Regan, 1996; Hiemstra, 1998; 1998b; Worf et al,. 1994). Wilting and defoliation are the earliest symptoms of this disease on ash trees. Leaves may turn to a lighter greyish green colour or complete necrosis that can affect the entire crown or only part of it. Ash rarely produces the wilting and discoloration of sapwood common to other trees such as maple. However, some affected trees show a discoloration in the cambial zone, the wood or the pith of stems or branches. In summer after the first heat stress of the year upper branches may die back in a random or one-sided distribution on the tree (Hiemstra, 1998; Pscheidt and Ocamb, 2013b). In young trees, although death of affected trees may occur, most of the affected trees recover. Older trees, however, show more gradual disease progress and decline over a period of months or even

years. This disease may occur in all kinds of plantations: nurseries, roadside, amenity and recreational plantations as well as forest stands of ash (Hiemstra, 1995a; 1995b).

Apart from the above-mentioned major tree hosts, *V. dahliae* can attack fruit tree species including stone fruits, pistachio and cocoa, as well as other shade tree species including well known genera as *Catalpa*, *Tilia*, *Ulmus* and *Robinia* (Hiemstra, 1998a, Sinclair and Lyon, 2005). Wilt, leaf curling or dying, abnormal red or yellow colour of entire leaves, leaf scorch, defoliation, dieback and death, and sapwood discolouration are common symptoms in most of these woody hosts infected with Verticillium wilt (Hiemstra and harris, 1998; Sinclair et al., 2005; Stipes and Hansen, 2009). Eventually, particularly infected young trees may die slowly over a period of several years or suddenly within a few weeks (Adams et al., 2010; Douglas, 2008; Dykstra, 1997; Heimann and Worf, 1997).

Genetic diversity and detection of *V. dahliae*

Little information is available about variation of the virulence among *V. dahliae* isolates causing wilt in trees. An exception to this is the classification of isolates from olive as defoliating (D) and non-defoliating (ND) isolates (Rodríguez-Jurado et al., 1993). This dichotomy was first described by Schnathorst and Mathre (1966) for Verticillium infections on cotton (*Gossypium hirsutum* L.). Isolates belonging to the D pathotype are highly virulent and cause complete defoliation of affected plants, whereas isolates belonging to the ND pathotype are generally less aggressive and cause milder wilt symptoms that do not include defoliation (Schnathorst and Mathre, 1966). Interestingly, although isolates of both types cause defoliation in olive, isolates that belong to the D pathotype on cotton are also highly virulent on olive, while isolates that belong to the ND pathotype on cotton are also less virulent on olive (Rodríguez-Jurado et al., 1993; Schnathorst et al., 1971). However, despite the high virulence of isolates of the D pathotype on cotton and olive, different levels of virulence have been observed on other hosts. Moreover, on particular plant species D isolates can be highly virulent without inducing defoliation (Jiménez-Díaz et al., 2006; Korolev et al., 2008; Schnathorst and Mathre, 1966). So far, presence of the D pathotype has been reported in North and South

America, Europe, the Middle East, and Asia (Jiménez-Díaz et al., 2012). However, no information is available about the differential effects of these two types on other woody hosts.

Differentiation of *V. dahliae* pathotypes infecting cotton and olive from diverse regions has been conducted through the use of molecular markers (Mercado-Blanco et al., 2001; 2003; Pérez-Artés et al., 2000). Pérez-Artés et al., (2000) designed PCR primers specific for D and ND isolates of *V. dahliae*, based on sequences of pathotype-associated RAPD bands, and tested them on 67 *V. dahliae* isolates from cotton and olive collected from southern Spain, China, Italy and the USA. Subsequently, nested-PCR primers were designed and optimized for specific detection of D and ND pathotypes *in planta* and in soil samples (Mercado-Blanco et al., 2002; Pérez-Artés et al., 2005). However, although these primers have worked for several isolates tested worldwide, it was found that they do not produce the desired amplicon on all *V. dahliae* isolates (Collins et al., 2005) that can be explained by the high genetic variability that exists among *V. dahliae* isolates (De Jonge et al., 2012; 2013).

PCR based assays for detection of *V. dahliae* have been developed by several authors; for detection in soil (*e.g.* Pérez-Artés et al., 2005; DeBode and Van Poucke, 2011; Bilodeau et al., 2012) as well as for detection in plant samples (*e.g.* Schena et al., 2004; Karajeh and Masoud, 2006; Gayoso et al., 2007). So far, however, these protocols have not been developed into procedures for routine screening of planting stock or fields to be planted with crops susceptible to Verticillium wilt. If field soils are screened before planting this usually is done using laborious and time consuming wet or dry sieving and plating techniques (Hiemstra, 2015; Termorshuizen, 1998).

Another way to characterize genetic diversity in fungi is to classify individual isolates in vegetative compatibility groups. According to the ability of individual fungal strains to undergo hyphal anastomosis and form stable heterokaryons they can be classified into vegetative compatibility groups (VCGs), such that compatible isolates are placed in the same VCG group (Joaquim and Rowe, 1990; Katan, 2000; Leslie, 1993). *V. dahliae* isolates have been classified into six VCGs (VCG1 through VCG6). VCG1, VCG2 and VCG4 were further divided into subgroups A and B based on the frequency

and vigour of complementation (Chandelier et al., 2003; Chen, 1994; Dervis et al., 2010; Goud and Termorshuizen, 2002; Jiménez-Díaz et al., 2006; Jiménez-Díaz et al., 2011; Jiménez-Díaz et al., 2012; Korolev et al., 2000; 2001; 2008; López-Escudero and Mercado-Blanco, 2011). So far, vast numbers of *V. dahliae* isolates from maple, ash, olive and some other woody hosts in USA and Europe have been analysed through complementation tests and classified into VCG1A, VCG1B, VCG2A, VCG2B and VCG4B (Chandelier et al., 2003; Chen, 1994; Hiemstra and Rataj-Guranowska, 2000; Jiménez-Díaz et al., 2011; Jiménez-Díaz et al., 2012; Neubauer et al., 2009). Recently, however, Papaioannou and Typas (2015) showed that although VCGs may be helpful in characterising different isolates, they are genetically not completely isolated.

Management of the disease

Control of Verticillium wilt is very difficult due to the characteristics of the pathogen and the nature of the infection, especially the long survival time of microsclerotia in soil, the long lifetime of a tree with continuous exposure to inoculum present in the soil, and the absence of methods to cure infected trees are important factors. Consequently, the use of an integrated strategy is the best way to deal with this disease. This includes the employment of resistant cultivars or rootstocks, cultural practices (i.e., avoid intercropping with *V. dahliae* susceptible crops; minimise cultivation practices that damage the roots; avoid contaminated equipment; and avoid irrigation that may disseminate the pathogen) to avoid spreading of the disease, and measures (i.e., disinfestation of *V. dahliae*-infested soil with fumigants, soil solarisation) to avoid build-up of soil inoculum and to reduce soil inoculum levels wherever possible (Barranco et al., 2010, Jiménez-Díaz et al., 1998; López-Escudero and Mercado-Blanco, 2011; Tjamos and Jiménez-Díaz, 1998). Green amendments or biological soil infestation would be also a promising method for control of Verticillium wilt in tree nurseries, but it is a costly method and rely on soil quality (Blok et al., 2000; Hiemstra et al., 2013). Moreover, accurate quantification of inoculum in soil would provide valuable information for disease prediction, since density of inoculum in soil is correlated with final disease incidence values (López-Escudero and Blanco-López, 2007). Replacement of diseased trees with

non-host plants might also be an environmentally friendly management solution to control Verticillium wilt. Studies on replacement of dead or severely diseased olive trees with apple trees revealed that this would be an appropriate approach in an integrated disease management to control Verticillium wilt disease (Karajeh and Owais, 2012). Use of biological control agents, including beneficial bacteria is of the other practices to manage Verticillium wilts (Prieto et al., 2009). However, the use of resistant cultivars and the screening of new planting sites and planting stock for infection by *V. dahliae* are the most efficient tools for control of Verticillium wilt of trees (López-Escudero and Mercado-Blanco, 2011; Tjamos and Jiménez-Díaz, 1998).

Conclusion

Over the last decades, spreading of Verticillium Wilt of olive was associated with the establishment of new olive orchards on infested soils, the use of infected plant material, and the spread of highly virulent pathogen isolates. Therefore, improving the resistance of cultivars, as well as protocols for fast and reliable detection of *V. dahliae* in planting stocks and at planting sites are of the highest importance for establishing an integrated disease management strategy. PCR-based methods for sensitive and accurate detection and discrimination of *V. dahliae* isolates allow for the rapid and reliable assessment of soil contaminations and plant infections by *V. dahliae*. Presently used methods, however, are not efficient enough and need to be improved for use in standard screening protocols. Furthermore, revealing the genetics and molecular background of resistance mechanisms, and of the recovery phenomenon, may provide essential information that can be used in breeding programs to increase the natural resistance of trees against Verticillium wilt. Collectively, these strategies are essential tools in an integrated disease management strategy to control Verticillium wilt in tree plantations and nurseries.

Acknowledgments

Work on Verticillium wilts of trees at Applied Plant Research of Wageningen UR was supported financially by a scholarship of the ministry of science and technology of Iran to MK.

References

Adams, G., Thomas, C. (1985). Verticillium wilt of shade trees and woody ornamentals. Extension Bulletin E-1860.

Adams, S., Miller, F., Taylor, D., Belshaw, C. (2010). Plant health care report. Scouting Report of the Morton Arboretum, Issue 2009. 02.

Antoniou, P.P., Tjamos, E.C., Kaltsis, J., Tjamos, S.E. (2001). Resistance evaluation to *Verticillium dahliae* in young olive cultivars or in root stocks of established olive orchards. Proceedings of the 8th International Verticillium Symposium, 2001. Córdoba, Spain, p 31.

Antoniou, P.P., Markakis, E.A., Tjamos, S.E., Paplomatas, E.J., Tjamos, E.C. (2008). Novel methodologies in screening and selecting olive varieties and root-stocks for resistance to *Verticillium dahliae*. European Journal of Plant Pathology, 110, 79-85.

Baídez, A.G., Gomez, P., Del Rio, J.A., Ortuno, A. (2007). Dysfunctionality of the xylem in *Olea europaea* L. plants associated with the infection process by *Verticillium dahliae* Kleb. Role of phenolic compounds in plant defense mechanism. Journal of Agricultural and Food Chemistry, 55, 3373-3377.

Banfield, W.M. (1968). Dutch elm disease recurrence and recovery in American elm. Phytopathologische Zeitschrift, 62, 21-60.

Barranco, D., Fernandez-Escobar, R., Rallo, L. (2010). Olive Growing. MundiPrensa/Australian Olive Association Ltd., Junta de Andalucía, 756 pp.

Beckman, C.H., Vandermolen, G.E., Mueller, A.C. (1976). Vascular structure and distribution of vascular pathogens in cotton. Physiology of Plant Pathology, 9, 87-94.

Bell, A.A. (1994). Mechanisms of disease resistance in Gossypium species and variation in *Verticillium dahliae*. In G. A. Constable, and N. D. Forrester (Eds.), Proceedings of world cotton research conference. Melbourne, Australia: CSIRO pp. 225-235.

Berlanger, I., Powelson, M.L. (2005). Verticillium wilt. *The Plant Health Instructor*. DOI: 10.1094/PHI-I-PHI-I-2000-0801-01.

Bilodeau, G.J.; Koike, S.T.; Uribe, P.; Martin, F.N. (2012). Development of an assay for rapid detection and quantification of *Verticillium dahliae* in soil. Phytopathology, 102, 331-343.

Blanco-López, M.A., Jiménez-Díaz, R.M., Caballero, J.M. (1984). Symptomatology, incidence and distribution of Verticillium wilt of olive trees in Andalucía. Phytopathology Mediterranean, 23, 1-8.

Blázquez-Martínez, J.M. (1996). Evolution and history, p. 17–54. In: International Olive Oil Council (IOOC) (eds.). World Olive Encyclopedia. Madrid, Spain.

Blok, W.J., Lamers, J.G., Termorshuizen, A.J., Bollen, G.J. (2000). Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. Phytopathology 90, 253-259.

Bonsen, K.J.M., Scheffer, R.J., Elgersma, D.M. (1985). Barrier zone formation as a resistance mechanism of elms to Dutch elm disease. International Association of Wood Anatomists Bulletin n.s., 6, 71-77.

Chandelier, A., Laurent, F., Dantinne, D., Mariage, L., Etienne, M., Cavelier, M. (2003). Genetic and molecular characterization of *Verticillium dahliae* isolates from woody ornamentals in Belgian nurseries. European Journal of Plant Pathology, 109, 943-952.

Chen, W. (1994). Vegetative compatibility groups of *Verticillium dahliae* from ornamental woody plants. Phytopathology, 84, 214-219.

Cirulli, M., Colella, C., D'Arnico, M., Amenduni, M., Bubici, G. (2008). Comparison of screening methods for the evaluation of olive resistance to *Verticillium dahliae* Kleb. Journal of Plant Pathology, 90, 7-14.

Collins, A., Mercado-Blanco, J., Jiménez-Díaz, R.M., Olivares, C., Clewes, E., Barbara, D.J. (2005). Correlation of molecular markers and biological properties in *Verticillium dahlia*e and the possible origins of some isolates. Plant Pathology, 54, 549-557.

Daayf, F., Nicole, M., Boher, B., Pando, A., Geiger, J.P. (1997). Early vascular defense reactions of cotton roots infected with defoliating mutant strain of *Verticillium dahliae*. European Journal of Plant Pathology, 103, 125-136.

DeBode, J.; Van Poucke, K. (2011). Detection of multiple Verticillium species in soil using density flotation and real-time polymerase chain reaction. Plant Disease, 95, 1571-1580.

De Jonge, R., Peter van Esse, H., Maruthachalam, K., Bolton, M.D., Santhanam, P., Keykha Saber, M., Zhang, Z., Usami, T., Lievens, B., Subbarao, K.V., Thomma, B.P.H.J. (2012). Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. Proceedings of the National Academy of Sciences of the United States of America, 109(13):5110-5115.

De Jonge, R., Bolton, M.D., Kombrink, A., van den Berg, G.C.M., Yadeta, K. A., Thomma, B.P.H.J. (2013). Extensive chromosomal reshuffling drives evolution of virulence in an asexual pathogen. Genome Research 23(8):1271-1282.

Dervis, S., Mercado-Blanco, J., Erten, L., Valverde-Corredor, A., Perez-Artes, E. (2010). Verticillium wilt of olive in Turkey: a survey on disease importance, pathogen diversity and susceptibility of relevant olive cultivars. European Journal of Plant Pathology, 127, 287-301.

Docampo, D.M., Vázquez, A.A., Laguna, I.G. (1981). *Verticillium dahliae* Kleb. causal de la paralisis del olivo en el area olivera centro oeste Argentina. Rev Invest Agropecuarias XVI (2):197-204 (in Spanish).

Douglas, S.M. (2008). Verticillium wilt of ornamental trees and shrubs. The Connecticut Agricultural Experiment Station.

Dykstra, M.D. (1997). Verticillium wilt of maple. Pest Diagnostic Clinic, Laboratory Services Division, University of Guelph, California: 4.

El-Zik, K.M. (1985). Integrated control of Verticillium wilt of cotton. Plant Disease, 69, 1025-1032.

Emechebe, A.M., Leakey, C.L.A., Banage, W.B. (1974). Verticillium wilt of cacao in Uganda: wilt induction by mechanical vessel blockage and mode of recovery of diseased plants. The East African Agricultural and Forestry Journal, 39, 337-343.

Frank, S.D., Klingeman, W.E., White, S.A., Fulcher, A.F. (2012). Reduced risk insecticides to control scale insects and protect natural enemies in the production and maintenance of urban landscape plants. Published by the southern nursery IPM working group, Knoxville, TN; in cooperation with the southern region IPM center. Chapter 10, ISBN: 978-0-9854998-1-5.

Fraxigen (2005). Ash species in Europe: biological characteristics and practical guidelines for sustainable use. Oxford Forestry Institute, University of Oxford, UK. 128 pp.

García-Ruiz, G.M., Trapero, C., Del Río, C., López-Escudero, F.J. (2014). Evaluation of Resistance of Spanish Olive Cultivars to *Verticillium dahliae* in Inoculations Conducted in Greenhouse. Phytoparasitica, 42, 205-212.

Gayoso, C.; Martínez de Illárduya, O.; Pomar, F.; Merino de Cáceres, F. (2007). Assessment of real-time PCR as a method for determining the presence of *Verticillium* dahliae in different Solanaceae cultivars. European Journal of Plant Pathology, 118, 199-209.

Gleason, M., Hartman, J. (2001). Chapter 60, Maple diseases, In: Diseases of woody ornamentals and trees in nurseries, APS Press. P. 236-241.

Goud, J.K.C., Termorshuizen, A.J., van Bruggen, A.H.C. (2011). Verticillium wilt in nursery trees: damage thresholds, spatial and temporal aspects. European Journal of Plant Pathology, 131, 451-465.

Goud, J.K.C., Termorshuizen, A.J. (2002). Pathogenicity and virulence of the two Dutch VCGs of *Verticillium dahliae* to woody ornamentals. European Journal of Plant Pathology, 108, 771-782.

Harris, D.C. (1998). Maple. In: Hiemstra JA, Harris DC, 1998. A compendium of Verticillium wilts in trees: 35-36.

Heffer, V.J., Regan, R.P. (1996). First report of Verticillium wilt caused by *Verticillium dahlia*e of ash trees in Pacific Northwest nurseries. Plant Disease, 80, 342.

Heimann, M.F., Worf, G.L. (1997). Maple and other trees disorder: Verticillium wilt. University of Wisconsin-Extension, Cooperative Extension, 608-262-8076.

Hiemstra, J.A. (1995a). Recovery of Verticillium-infected ash trees. Phytoparasitica, 23, 64-65.

Hiemstra, J.A. (1995b). Verticillium wilt of *Fraxinus excelsior*. PhD Thesis, Wageningen Agricultural University, The Netherlands.

Hiemstra, J.A. (1997). Petioles from infected trees spread *Verticillium dahliae*. 7th. International Verticillium Symposium, Athens. Book of abstracts, p 47.

Hiemstra, J.A. (1998a). Some general features of Verticillium wilts in trees. In: Hiemstra JA, Harris DC, 1998. A compendium of Verticillium wilts in trees: 5-11.

Hiemstra, J.A. (1998b). Ash. In: Hiemstra JA, Harris DC, 1998. A compendium of Verticillium wilts in trees: 35-36.

Hiemstra, J.A. (2015). Der schnelle Nachweis von Verticillium. Jahrbuch der Baumpflege 2014: 108-120.

Hiemstra, J.A., Harris, D.C. (Eds.) (1998). A compendium of Verticillium wilts in tree species. Wageningen: Centre for Plant Breeding and Reproduction Research (CPRO-DLO)/West Malling: HRI-East Malling.

Hiemstra, J.A., Rataj-Guranowska, M. (2000). Vegetative compatibility groups in *Verticillium dahliae* in the Netherlands. In: Tjamos EC, Rowe RC, Heale JB and Fravel DR (eds) Advances in Verticillium: Research and Disease Management (pp 100–102) APS Press, St. Paul.

Hiemstra, J.A., Korthals, G.W., Visser, J.H.M., Dalfsen, P., van, Sluis, B.J., van der, Smits, A.P. (2013). Control of Verticillium in tree nurseries through biological soil disinfestation. 11th International Verticillium Symposium, Georg-August-Universität Göttingen, Germany, 5-8 May 2013. - Göttingen: DPG Spectrum Phytomedizin, 2013 - ISBN 9783941261129 - p. 62 - 62.

Himelick, E.B. (1968). Verticillium wilt of trees and shrubs. Illinois Natural History Survey, Bl-1968, Botany and Plant Pathology Section, Urbana, Illinois. 2 pp.

Jiménez-Díaz, R.M, Cirulli, M., Bubici, G., Jiménez-Gasco, M., Antoniou, P.P., Tjamos, E.C. (2012). Verticillium wilt a major threat to olive production: Current status and future prospects for its management. Plant Disease, 96(3), 304-329.

Jiménez-Díaz, R.M., Mercado-Blanco, J., Olivares-García, C., ColladoRomero, M., Bejarano-Alcázar, J., Rodríguez-Jurado, D., Giménez-Jaime, A., García-Jiménez, J., Armengol, J. (2006). Genetic and virulence diversity in *Verticillium dahliae* populations infecting artichoke in eastern-central Spain. Phytopathology, 96, 288-298.

Jiménez-Díaz, R.M., Olivares-García, C., Landa, B.B., Jiménez-Gasco, M.M., Navas-Cortés, J.A. (2011). Region-wide analysis of genetic diversity in *Verticillium dahliae* populations infecting olive in southern Spain and agricultural factors influencing the distribution and prevalence of vegetative compatibility groups and pathotypes. Phytopathology, 101, 304-315.

Jiménez-Díaz, R.M., Tjamos, E.C., Cirulli, M. (1998). Verticillium wilt of major tree hosts. In: Hiemstra JA, Harris DC, 1998. A compendium of Verticillium wilts in trees: 13-16.

Joaquim, T.R., Rowe, R.C. (1990). Reassessment of vegetative compatibility relationships among strains of *Verticillium dahliae* using nitrate-nonutilizing mutants. Phytopathology, 80, 1160-1166.

Karajeh, M.R.; Masoud, S.A. (2006). Molecular detection of *Verticillium dahliae* Kleb. in asymptomatic olive trees. Journal of Phytopathology, 154, 496-499.

Karajeh M.R., Owais S.J. (2012). Reaction of selected apple cultivars to wilt pathogen *Verticillium dahliae*. Plant Protection Science 48: 99-104.

Katan, T. (2000). Vegetative compatibility in populations of Verticillium: an overview. In: Tjamos E.C., Rowe R., Heale J.B., Fravel D.R. (eds.) Advances in Verticillium: research and disease management. Proceeding of 7th International Verticillium Symposium. The American Phytopathological Society, St. Paul, MN, pp 69–86.

Klosterman, S.J., Atallah, Z.K., Vallad, G.E., Subbarao, K.V. (2009). Diversity, pathogenicity and management of *Verticillium* species. Annual Review of Phytopathology, 47, 39-62.

Korolev, N., Katan, J., Katan, T. (2000). Vegetative compatibility groups of *Verticillium dahliae* in Israel: Their distribution and association with pathogenicity. Phytopathology, 90, 529-536.

Korolev, N., Pérez-Artés, E., Bejarano-Alcázar, J., Rodríguez-Jurado, D., Katan, J., Katan, T., Jiménez-Díaz, R.M. (2001). Comparative study of genetic diversity and

pathogenicity among populations of *Verticillium dahliae* from cotton in Spain and Israel. European Journal Plant Pathology, 107, 443-456.

Korolev, N., Pérez-Artés, E., Mercado-Blanco, J., Bejarano-Alcázar, J., Rodríguez-Jurado, D., Jiménez-Díaz, R.M., Katan, T., Katan, J. (2008). Vegetative compatibility of cotton defoliating *Verticillium dahliae* in Israel and its pathogenicity to various hosts. European Journal Plant Pathology, 122, 603-617.

Ladux, J.L., Jotayan, L., Otero, M.L., González Vera, C., Ortiz, J. (2014). Incidence of *Verticillium dahliae* in traditional orchards of the olive 'Arauco' in northwest Argentina (La Rioja). Acta Horticulturae, 1057, 127-132.

Laouane, H., Lazrek, H.B., Sedra, M.H. (2011). Synthesis and toxicity evaluation of cinnamyl acetate: a new phytotoxin produced by a strain of *Verticillium dahliae* pathogenic on olive tree. International Journal of Agriculture and Biology, 13, 444-446.

Leslie, J.F. (1993). Fungal vegetative compatibility. Annual Review of Phytopathology, 31, 127-150.

Levin, A.G., Lavee, S., Tsror, (Lahkim) L. (2003). Epidemiology and effects of Verticillium wilt on yield of olive trees (cvs. Barnea and Souri) irrigated with saline water in Israel. Phytoparasitica, 31, 333-343.

Lockwood, J.L. (1977). Fungistasis in soils. Biological Reviews, 52, 1-43.

López-Escudero, F.J., Blanco-López, M.A. (2001). Effect of a single or double soil solarisation to control Verticillium wilt in established olive orchards in Spain. Plant Disease, 85, 489-496.

López-Escudero, F.J., Blanco-López, M.A. (2005). Recovery of young olive trees from *Verticillium dahliae*. European Journal Plant Pathology, 113, 367-375.

López-Escudero, F.J., Del Río, C., Caballero, J.M., Blanco-López, M.A. (2004). Evaluation of olive cultivars for resistance to *Verticillium dahliae*. European Journal Plant Pathology, 110, 79-85.

López-Escudero, F.J., Mercado-Blanco, J. (2011). Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. Plant Soil, 344, 1-50.

Mace, M.E., Stipanovic, R.D., Bell, A.A. (1989). Histochemical localization of desoxyhemigossypol a phytoalexin in *Verticillium dahlia*e infected cotton stems. New Phytologist, 111, 229-232.

Manion, P.D. (2003). Evolution of concepts in forest pathology. Phytopathology, 93, 1052-1055.

Mansfield, J.W. (2000). Antimicrobial compounds and resistance: the role of phytoalexins and phytoanticipins. In A Slusarenko, R Fraser, L van Loon, eds, Mechanisms of Resistance to Plant Diseases. Kluwer Academic Publishers, Dordrecht, the Netherlands, pp 325 - 370.

Markakis, E.A., Tjamos, S.E., Antoniou, P.P., Paplomatas, E.J., Tjamos, E.C. (2009). Symptom development, pathogen isolation and real-time qPCR quantification as factors for evaluating the resistance of olive cultivars to Verticillium pathotypes. European Journal Plant Pathology, 124, 603-611.

Martos-Moreno, C., López-Escudero, F.J., Blanco-López, M.A. (2006). Resistance of olive cultivars to the defoliating pathotype of *Verticillium dahliae*. HortScience, 41, 1313-1316.

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodríguez-Jurado, D., Jiménez-Díaz, R.M. (2003). Quantitative monitoring of colonization of olive genotypes by *Verticillium dahlia* pathotypes with real-time polymerase chain reaction. Physiological and Molecular Plant Pathology, 63, 91-105.

Mercado-Blanco, J., Rodríguez-Jurado, D., Perez-Artes, E., Jiménez-Díaz, R.M. (2001). Detection of the nondefoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR. Plant Pathology, 50, 609-19.

Mercado-Blanco, J., Rodríguez-Jurado, D., Pérez-Artés, E., Jiménez Díaz, R.M. (2002). Detection of the defoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR. European Journal Plant Pathology, 108, 1-13.

Morehart, A.L., Melchior, G.L. (1982). Influence of water stress on Verticillium wilt of yellow-poplar. Canadian Journal of Botany, 60, 201-209.

Navas-Cortes, J.A., Landa, B.B., Mercado-Blanco, J., Trapero-Casas, J.L., Rodríguez-Jurado, D., Jiménez-Díaz, R.M. (2008). Spatiotemporal analysis of spread of infections by *Verticillium dahliae* pathotypes within a high tree density olive orchard in southern Spain. Phytopathology, 98, 167-180.

Nelson, E.B. (1990). Exudate molecules initiating fungal responses to seeds and roots. Plant and Soil, 129, 61-73.

Neubauer, C., Vogel, C., Heitmann, B. (2009). Morphology, Vegetative Compatibility and Pathogenicity of *Verticillium dahliae* isolates from Woody Ornamentals in Germany. Journal of Plant Diseases and Protection, 116, 109-114.

Papaioannou, I. A., Typas, M.A. (2015). Barrage formation is independent from heterokaryon incompatibility in *Verticillium dahliae.* European Journal Plant Pathology, 141, 71-82.

Pegg, G.F., Brady, B.L. (2002). Verticillium wilts. Wallingford, UK: CABI publishing.

Pérez-Artés E., Garcia-Pedrajas M., Bejarano-Alcazar D., Jime-nez-Díaz R.M., (2000). Differentiation of cotton-defoliating and nondefoliating pathotypes of *Verticillium dahliae* by RAPD and specific PCR analyses. European Journal Plant Pathology, 106, 507-517.

Pérez-Artés, E., Mercado-Blanco, J., Ruz-Carrillo, A.R., Rodríguez-Jurado, D., Jiménez-Díaz, R.M. (2005). Detection of the defoliating and nondefoliating pathotypes of *Verticillium dahliae* in artificial and natural soils by nested PCR. Plant Soil, 268, 349-356.

Piearce, G.D., Gibbs, J.N. (1981). Verticillium wilt of trees and shrubs. Arboricultural leaflet 9. Dept. of the Environment, Forestry Commission, HMSO, London. 8 pp.

Prieto, P., Navarro-Raya, C., Valverde-Corredor, A., Amyotte, S.G., Dobinson, K.F., Mercado-Blanco, J. (2009). Colonization process of olive tissues by *Verticillium dahliae* and its *in planta* interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7. Microbial Biotechnology, 2(4), 499-511.

Pscheidt, J.W., Ocamb, C.M. (2013a). Maple (*Acer* spp.)-Verticillium Wilt. Pacific Northwest Plant Disease Management Handbook.

Pscheidt, J.W., Ocamb, C.M. (2013b). Ash (*Fraxinus* spp.)-Verticillium Wilt. Pacific Northwest Plant Disease Management Handbook.

Riffle, J.W., Peterson, G.W. (1989). Diseases of trees in the Great Plains. Gun. Tech. Rep. RM-129. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Forest and Range Experiment Station; 1986. 149 p.

Rijkers, A.J.M., Hiemstra, J.A., Bollen, G.J. (1992). Formation of microsclerotia of *Verticillium dahliae* in petioles of infected ash trees. Netherlands Journal of Plant Pathology, 98, 261-264.

Robb, J., Smith, A., Busch, L. (1982). Wilts caused by *Verticillium* species. A cytological survey of vascular alterations in leaves. Canadian Journal of Botany, 60, 825-837.

Rodríguez-Jurado, D., Blanco-López, M.A., Rappoport, H.F., Jiménez-Díaz, R.M. (1993). Present status of Verticillium wilt of olive in Andalucía (southern Spain). European and Mediterranean Plant Protection Organization (EPPO) Bulletin, 23, 513-516.

Ruggieri, G. (1946). Una nuova malatia dell'olivo. L'Italia Agricola, 83, 369-372.

Ryan, D., Robards, K. (1998). Phenolic compounds in olives. Analyst, 123, 31-44.

Schena, L., Nigro, F., Ippolito A. (2004). Real-time PCR detection and quantification of soilborne fungal pathogens: the case of *Rosellinia necatrix*, *Phytophthora nicotianae*, *P. citrophthora*, and *Verticillium dahliae*. Phytopathology Mediterranean, 43, 273-380.

Schnathorst, W.C. (1981). Life cycle and epidemiology of Verticillium. In Fungal Wilt Diseases of Plants (Mace, M.E., Bell, A.A. and Beckman, C.H., eds), New York: Academic Press. pp. 81–111.

Schnathorst, W.C., Mathre, D.E. (1966). Host range and differentiation of a severe form of *Verticillium albo-atrum* in cotton. Phytopathology, 56, 1155-1161.

Schnathorst, W.C., Sibbett, G.S. (1971). The relation of strains of *Verticillium albo-atrum* to severity of Verticillium wilt in *Gossypium hirsutum* and *Olea europaea* in California. Plant Disease Reports, 9, 780-782.

Schreiber, L.R., Green, R.J. (1963). Effect of root exudates on germination of conidia and microsclerotia of *Verticillium albo-atrum* inhibited by the soil fungistatic principle. Phytopathology, 53, 260-264.

Shigo, A.L. (1984). Compartmentalization: A conceptual framework for understanding how tree grow and defend themselves. Annual Review of Phytopathology, 22, 189-214.

Shigo, A.L., Marx, H.G. (1977). Compartmentalization of decay in trees. Agricultural Information Bulletin USDA Forest Service. No.405.73pp.

Sinclair, W.A., Lyon, H.H. (2005). Diseases of trees and shrubs. 2nd ed. Ithaca, NY: Cornell University Press. 659 p.

Sinclair, W.A., Smith, K.L., Larsen, A.O. (1981). Verticillium wilt of maples: symptoms related to movement of the pathogen in stems. Ethiology 71:340-345.

Smith, K.T. (2006). Compartmentalization today. Arboricultural Journal, 9, 173-18.

Smith, I.M., Dunez, J., Lelliott, R.A., Phillips, D.H., Archer, S.A. (1988). European handbook of plant diseases. Blackewell Scientific Publications, Oxford etc., 583 S.

Stipes, R.J., Hansen, M.A. (2009). Verticillium wilt of shade trees. Virginia Polytechnic Institute and State University, publication 450-619.

Street, P.F.S., Robb, J., Ellis, B.E. (1986). Secretion of vascular coating components by xylem parenchyma cells of tomatoes infected with *Verticilliurn albo-atrum*. Protoplasma, 132, 1-11.

Termorshuizen, A.J. (1998). Quantification of *Verticillium dahliae* in soil. In: Hiemstra JA, Harris DC, 1998. A compendium of Verticillium wilts in trees: 47.

Tippett, J.T., Shigo, A.L. (1981). Barrier zone formation: a mechanism of tree defense against vascular pathogens. International Association of Wood Anatomists Bulletin n.s. 2, 163-168.

Tjamos, E.C., Botseas, D. (1987). Occurrence of *Verticillium dahliae* in leaves of Verticillium-wilted olive trees. Canadian Journal of Plant Pathology, 9, 86.

Tjamos, E.C.; Jiménez-Díaz, R.M. (1998). Management of disease. In: Hiemstra JA, Harris DC, 1998. A compendium of Verticillium wilts in trees: 55-57.

Tjamos, E.C., Tsougriani, H. (1990). Formation of *Verticillium dahliae* microsclerotia in partially disintegrated leaves of Verticillium affected olive trees. 5th International Verticillium Symposium, Book of Abstracts, Leningrad, Soviet Union, p 20.

Tosi, L., Zazzerini, A. (1998). An epidemiological study on Verticillium wilt of olive in Central Italy. Olivae, 71, 50-55. (In Spanish).

Townsend, A.M., Schreiber, L.R., Hall, T.J., Bentz, S.E. (1990). Variation in response of Norway maple cultivars to *Verticillium dahliae*. Plant Disease, 74, 44-46.

Trapero, C., Roca, L. F., Alcantara, E., Lopez-Escudero, F. J. (2011). Colonization of olive Inflorescences by *Verticillium dahliae* and its Significance for Pathogen Spread. Journal of Phytopathology, 159, 638-640.

Trapero, C., Serrano, N., Arquero, O., Del Río, C., Trapero, A., López-Escudero, F.J. (2013). Field resistance to Verticillium wilt in selected olive cultivars grown in two naturally infested soils. Plant Disease, 97, 668-674.

Treutter, D. (2006). Significance of flavonoids in plant resistance: a review. Environmental Chemistry Letters, 4, 147-157.

Tsror, L. (2011). Epidemiology and control of Verticillium Wilt on Olive. Israel Journal of Plant Sciences, 59, 59-69.

Wilhelm, S. (1950). Vertical distribution of *Verticillium albo-atrum* in soils. Phytopathology, 40, 776-777.

Wilhelm, S. (1955). Longevity of Verticillium wilt fungus in the laboratory and field. Phytopathology, 45, 180-181.

Worf, G.L., Spear, R., Heimann, S.M.F. (1994). Verticillium induced scorch and chlorosis in ash. Journal of Environmental Horticulture, 12, 124-130.

Chapter 3

Reliable detection of unevenly distributed *Verticillium dahliae* in diseased olive trees

M. Keykhasaber[a,b,c], K.T.K. Pham[b], B.P.H.J. Thomma[a], J.A. Hiemstra [b*]

[a] Laboratory of Phytopathology, Wageningen University & research, 6708 PB Wageningen, The Netherlands

[b]Wageningen Plant Research, Wageningen University & Research, P.O. Box 200, 6670 AE

[c] Department of Plant Biotechnology, Zabol University, Zabol, Iran

This chapter has been published in Plant Pathology.

Abstract

Verticillium wilt caused by *Verticillium dahliae* Kleb. is one of the most threatening diseases of olive worldwide. For pre-planting and post-planting control of Verticillium wilt in olive trees, availability of a rapid, reliable and non-destructive method for detection of *V. dahliae* is essential. For such a method, suitable and easily performed sampling and efficient processing of samples for extraction of DNA are necessary. In this study, we assessed the suitability of young twig and leaf samples of olive trees that are easy to collect and extract DNA for the detection of *V. dahliae* in routine procedures. The lower and top parts of twigs as well as leaves from infected olive trees were screened for *V. dahliae* infection and distribution using real-time PCR. We observed that the biomass of *V. dahliae* detected in individual twigs was highly variable, but there was no significant difference between mean quantities of *V. dahliae* DNA detected in top and lower parts of twigs. It was furthermore demonstrated that analysis of combined samples containing DNA extracted from five twigs of an infected tree accurately detected the presence of the pathogen. Similarly, testing combined samples of 5-10 leaves enabled reliable detection of the pathogen in an infected tree. The development of this assay provides for reliable detection of *V. dahliae* in infected olive trees that can aid in management decisions for the implementation of integrated disease management.

Introduction

Olive (*Olea europaea* L.) is one of the most ancient cultivated plant species and has a huge economic and social importance in olive-producing countries worldwide (Blázquez-Martínez, 1996; Civantos, 2004). Verticillium wilt caused by the fungus *V. dahliae* Kleb. is a major disease of this tree crop, causing serious concern to growers, nursery companies and the olive-oil industry (Jiménez-Díaz et al., 2012; López-Escudero and Mercado-Blanco, 2011). The outbreak of this disease was first reported in Italy in 1946 and soon thereafter in California, European and Asian countries and Australia (López-Escudero and Mercado-Blanco, 2011), and most recently in Argentina (Ladux et al., 2014). Currently, Verticillium wilt is considered one of the most important disease of olive with most olive

cultivars being susceptible to *V. dahliae* (Jiménez-Díaz et al., 2012; López-Escudero and Mercado-Blanco, 2011).

V. dahliae is a soil-borne fungus that can survive as microsclerotia in the soil or on plant debris for prolonged periods of time (Pegg and Brady, 2002). The presence of root exudates from nearby growing roots of host plants stimulates germination of microsclerotia which gives rise to the formation of infective hyphae. The fungus penetrates the roots of the host plants, and once inside the root of a susceptible plant the fungus grows into the xylem vessels where it produces conidia and spreads upward by a combination of passive movement of conidia with the transpiration stream and active growth of hyphae into neighbouring xylem vessels. Collectively, this enables rapid colonization of the above-ground parts of tree hosts by the fungus (Hiemstra, 1998; Pegg and Brady, 2002). As a result of the presence of the fungus and the defence reactions of the infected plant, such as secretions of gums and gels into xylem vessels as well as the formation of tyloses, xylem vessels may be occluded. This reduces water transport capacity, triggering the typical wilt symptoms, but also defoliation and dieback of shoots or even death of complete trees (Baídez et al., 2007; Hiemstra, 1998; Pegg and Brady, 2002).

Control of Verticillium wilt of olive is difficult, particularly due to the long survival time of microsclerotia in soil, the long lifetime of olive trees with continuous exposure to inoculum present in the soil, the broad range of hosts which enhances the pathogen survival capacity, and the absence of methods to cure infected trees (López-Escudero and Mercado-Blanco, 2011; Tjamos, 1993; Tjamos and Jiménez Díaz, 1998). Therefore, the best way of controlling Verticillium wilt in olive is implementation of an integrated disease management strategy. Using pathogen-free plant material, especially when planting in areas free of *V. dahliae*, and swift identification of diseased trees aiming to minimise damage by preventing an outbreak occurring or stopping Verticillium wilt from spreading further are essential elements in such a strategy (Barranco et al., 2010; Hiemstra, 2015; López-Escudero and Mercado-Blanco, 2011). To this end, a rapid, reliable and preferably non-destructive method for detection of *V. dahliae*, particularly in symptomless infected plants, is highly desired.

Traditional methods for detection of fungi from diseased plants involve plating of small subsamples of infected plant material onto agar plates and identification by visual inspection of the resulting colonies. Such plating assay procedures are laborious and time-consuming, and therefore not suitable for routine testing of large numbers of samples. Detection of *V. dahliae* using plating assays takes at least 7-10 days (Termorshuizen, 1998). However, real-time PCR technology has allowed the design of fast and sensitive methods for detection and quantification of DNA of pathogens. The use of real-time PCR for detection and quantification of *V. dahliae* in samples from diseased olive trees has been reported by several authors (e.g. Ceccherini et al., 2013; Gramaje et al., 2013; Lievens et al., 2006; Markakis et al., 2009 Mercado-Blanco et al., 2003).

For a robust and reliable detection of the pathogen in practical disease management using PCR techniques, suitable and easily performed sampling and efficient processing of samples for extraction of DNA are required. Arguably, young twigs and leaves are the most appropriate parts for sampling, due to the ease of collection and suitability for DNA extraction; and the non-destructive character of such a sampling method. However, it is currently unknown whether *V. dahliae* will always be present in these tissues in every infected tree. Moreover, as a result of the way in which infected trees are colonized, i.e. through conidia transported by the sapstream in the xylem, the resulting distribution of *V. dahliae* may be discontinue and parts of symptomatic trees may remain free of the fungus. Thus, information on the distribution of *V. dahliae* within these parts of infected olive trees may help to design an appropriate and efficient sampling method for reliable detection of the pathogen. Therefore, we investigated the distribution of *V. dahliae* in young twigs and leaves of naturally infected olive trees by real-time quantification of *V. dahliae* DNA, aiming to assess the suitability of twig and leaf samples to be used in routine procedures to screen for *V. dahliae* infection. Additionally, we investigated the possibility of combining individual twig or leaf subsamples in one analysis per tree for reliable detection of *V. dahliae* DNA using real-time PCR. The final aim was to design an easy and efficient sampling protocol for reliable detection of *V. dahliae* in olive trees that can provide information for diagnosis-based strategies to manage Verticillium wilt in olive production.

Materials and methods

Plant material and sampling. This study was carried out with samples from olive trees (*Olea europaea* L.) from five olive cultivars (table 1) showing symptoms of Verticillium wilt. Samples were collected in Spain, Portugal and Greece in different seasons of the years 2012 and 2013. In total 58 trees were sampled, organized in 6 groups according to the region and date of sampling (Table 1).

Table 1. Overview of the olive trees used in this study.

Sample group[a]	number of trees evaluated	trees positive[b] for *V. dahliae*	% samples positive for *V. dahliae* after PCR testing	% samples positive for *V. dahliae* after standard plating	Cultivar	Sampling date	Country
0	14	11	52	12	Picual	April (2012)	Spain
1	10	10	100	n.d.[c]	Cobrançosa, Galega	February (2013)	Portugal
2	5	5	100	n.d.	Picual	April (2013)	Spain
3	6	6	100	0	Picual	September (2012)	Spain
4	14	5	36	n.d.	Picual	August (2013)	Spain
5	9	9	100	88	Konservolia, Magaritikes	October (2012)	Greece

[a] Samples collected by the last author as part of the work in the EU-Vertigeen project (www.vertigeen.eu).

[b] After PCR testing of 5 individual twigs per tree (groups 1-5), and 2-6 twigs per tree (group 0) (detection limit 0.001 ng *V. dahliae* DNA).

[c] No data (not tested).

From each tree, 5-10 twigs (about 50-60 cm. in length) with leaves attached to them were collected from symptomatic branches in the middle region of the crown at different sides of the trees (Figure 1A). Samples were collected in labelled plastic bags (1 sample per bag) kept out of the sun during the field work and shipped to the laboratory in the

Netherlands by courier. Temperature recorders included in one of the shipments (Table 1, group 4) showed that temperatures during transport did not exceed 32 ºC and were below 30 ºC for most of the time. At the laboratory the samples were stored in a cold room (4 ºC) until processing, usually within 4-6 weeks from collection.

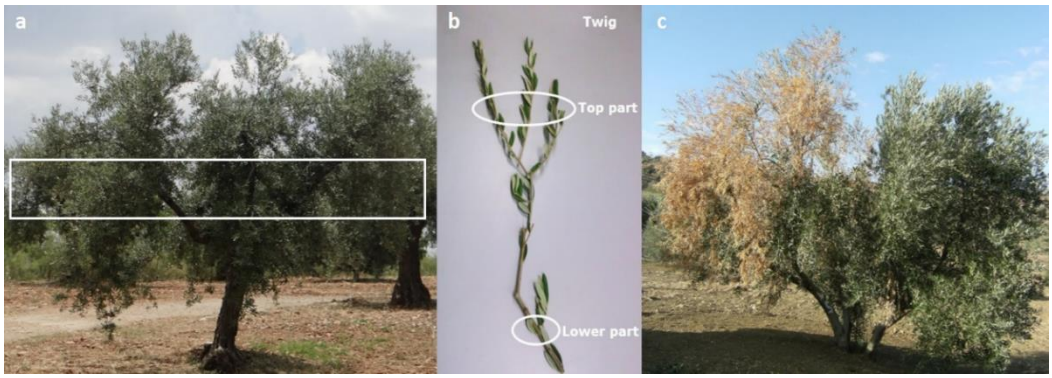


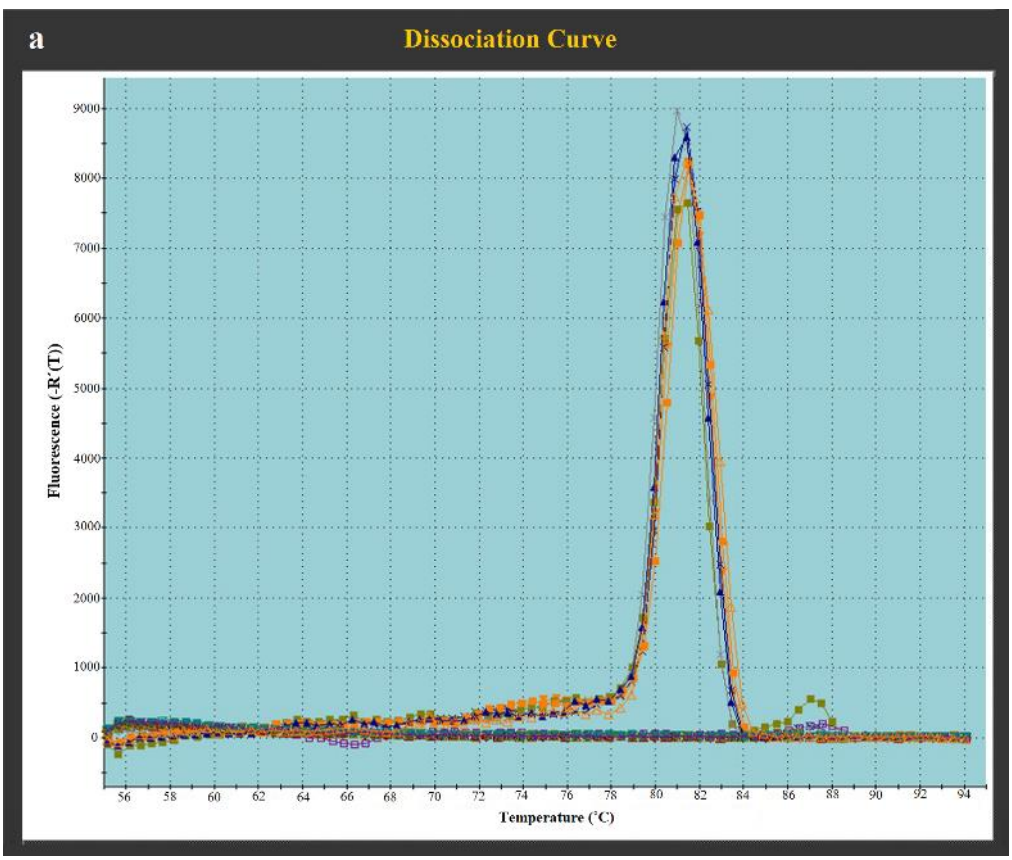
Figure 1. Sampling of olive trees. A) Adult olive tree as a representative of the sampled trees, the rectangle indicates the part of the crown that was sampled. From this part of the tree, 5 twigs were collected from different sides of the tree. B) Picture of a representative twig. The ovals indicate the lower (about 50 cm from top) and top (about 5 cm from top) parts of the twig that were used for subsequent analyses. C) Adult olive tree with serious symptoms in only part of the crown whereas other parts of the crown remain free of symptoms.

DNA isolation. For DNA isolation from twigs, 300-400 mg of woody parts from top (about 5 cm from the top) and lower parts (about 50 cm from the top) of the twigs were used (Figure 1B). First 10 cm long pieces of the top and lower part of a twig were washed under running tap water for 1-2 minutes and then dried for a few minutes on sterile paper. Then, the bark was removed under sterile conditions and small (about 2-5 mm) pieces of xylem tissue were taken by using a sterilized (70% alcohol) scalpel and put in a 2 ml tube containing 1 ml of lysis buffer AP1 of the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and 4-5 stainless steel beads (3.2 mm diameter, BioSpec, US/Canada). Next, the tubes were incubated for 15-30 min at 65˚C and then shaken in a Retsch® mixer mill (MM 400) for 15 minutes at 30 Hz. After centrifugation at 10,000 rpm for 5 minutes, 400 µl of suspension was used for total genomic DNA extraction using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was quantified using a BioPhotometer (Eppendorf AG, Hamburg, Germany) and concentrations were equalized by adding elution buffer or DNase-free water. For DNA

isolation from leaves, samples were ground in liquid nitrogen to a fine powder using a mortar and pestle (Dellaporta et al. 1983). Next, about 100-150 mg of powder was transferred to a 2 ml tube with 1 ml of lysis buffer AP1 of the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) for DNA extraction according to the manufacturer's instructions.

Quantification assay. Real-time PCR using a *V. dahliae*-specific primer pair designed using the internal transcribed spacer (ITS) region (Van Doorn et al., 2009) (VerDITSF: 5'-CCGGTCCATCAGTCTCTCTG-3', VerDITSRk: 5'-CACACTACATATCGCGTTTCG-3') was performed to quantify the amount of *V. dahliae* DNA. These primers in previous work were shown to be highly specific to *V. dahliae* as they only reacted with *V. dahliae* isolates but neither with other *Verticillium* species (*albo-atrum*, *longisporum*, *tricorpus*) nor with a series of common plant pathogenic or soil inhabiting fungi (Van Doorn et al., 2009; Hiemstra et al. 2013). Amplification was carried out in a 25 µl final reaction volume containing: 1.5 µl of DNA extract, 10 nM of each primer, 12.5 µl of SYBR Green Supermix (2X) and sterile nuclease-free water to reach the appropriate volume. Each run included a positive control containing pure *V. dahliae* (isolate V117; supplied from the collection of the Laboratory of Plant Pathology, Department of Agronomy, University of Córdoba) DNA, a negative control containing nuclease-free water instead of DNA, as well as a negative control containing DNA isolated from leaves collected from a healthy ornamental olive tree in the Netherlands. At least two simultaneous replicates were carried out for each sample. All real-time PCR reactions were performed in a Max 3000P^{TM} STRATAGENE real-time PCR machine. The real-time PCR program consisted of an initial step of denaturation for 10 min at 95˚C, followed by 45 cycles of 15 sec at 95˚C, 40 sec at 62˚C, and 40 sec at 72˚C. A melting curve program was also run for which measurements were made at 0.5˚C temperature increments every 10 s within a range of 56-94˚C to determine signals from specific and non-specific products. Based on the melting profiles obtained from preliminary runs the reading step for fluorescence emission was set at T_m = 81˚C for all runs (figure 2a). To determine the quantity of *V. dahliae*, a standard curve was generated by plotting the logarithm of known DNA concentrations of a ten-fold dilution series of

DNA (10 ng/µl) of *V. dahliae* isolate V117, against the threshold cycle (Ct) obtained in the real-time PCR assays. A Ct of 36 (0.001 ng of DNA according to the standard curve) was considered to be the threshold value in the standard curve suitable for quantification (Figure 2b). Primers specific for the plant cytochrome oxidase (*COX*) gene (Weller et al., 2000) were used to quantify the amount of plant DNA for calculation of the relative quantity of *V. dahliae* DNA in the tested samples based on the quantity of *V. dahliae* DNA (ng) in 100 ng total DNA (including pathogen and plant DNA) isolated from infected plant materials. Mean values were compared using T-test analysis and the Fisher protected LSD at P=0.05.



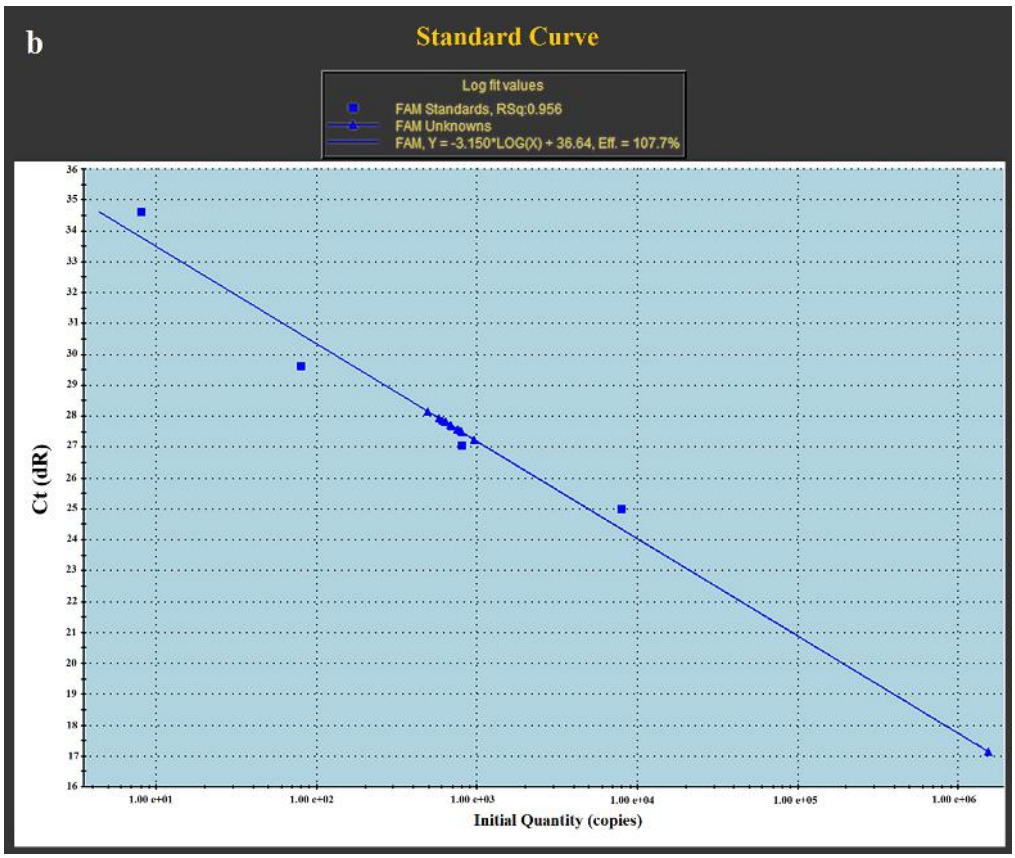


Figure 2. Melting and standard curves obtained from real-time PCR assays. In melting curve (a), measurements were made at 0.5˚C temperature increments every 10 s within a range of 56-94˚C to determine signals from specific and non-specific products. Based on the melting profiles obtained from preliminary runs the reading step for fluorescence emission was set at $T_m = 81$˚C for all runs. Standard curve (b) obtained by plotting the logarithm of known DNA concentrations of a ten-fold dilution series of DNA (10 ng/µl) of *V. dahliae* isolate V117 against the Ct values obtained from real-time quantitative PCR assays. This curve served to calculate the amount of *V. dahliae* DNA in total genomic DNA samples extracted from infected olive tissues.

Plating assay. To isolate *V. dahliae*, stem samples of 10 cm were first washed under running tap water. After drying, the bark was peeled off and chips from xylem sheets of the two most recent growing years were taken and disinfected in 10% chloramine-T hydrate 98% for 1 minute. Afterwards, wood chips were washed with sterile water for 30 seconds and dried on Whatman filter paper. Chips then were placed onto PDA plates that

were incubated at 24ºC in the dark. After 7 days the number of wood chips yielding *V. dahliae* colonies was counted.

Results

***Efficiency of real time PCR in comparison to plating assay*.** In a preliminary experiment 60 shoot samples collected from 1 healthy and 13 diseased trees varying in disease severity (Table 1, group 0) were examined for presence of *V. dahliae* using both real-time PCR and plating assays. By plating assay *V. dahliae* was detected in only 7 samples (12%), all from trees with severe disease symptoms, whereas by real-time PCR the pathogen was detected in 31 samples (52 %) including samples from trees with moderate of even little symptoms of Verticillium wilt (Table 2). The efficiency of reactions based on the slope of standard curve (-3.150) was 107.7% (i.e., calculated from the equation in Figure 2b), indicating the high-efficiency of the real-time PCR assay in our study. Samples from a healthy tree were negative with both methods. These results indicate that the PCR method we used has a higher sensitivity for detection of *V. dahliae* in olive shoot samples than the standard plating assay, especially in trees showing only little or moderate symptoms. However, the percentage of Vd-positive samples per tree varied strongly, from 17% (1 out of 6) till 100% (6 out of 6) suggesting that for a reliable result several shoot samples per tree should be analysed.

Table 2. Detection of *V. dahliae* in shoot samples from diseased olive trees with varying disease severity (table 1, group 0) by means of real-time PCR and standard plating assays.

Disease class	**trees**	**Vd-pos after plating**[a]	**Vd-pos after real time PCR**[a]
Healthy	1	0 (0/3)	0 (0/3)
Slight symptoms	2	0 (0/5)	1 (1/5)
Moderate	3	0 (0/18)	3 (2/18)
Severe symptoms	8	5 (7/34)	7 (28/34)
total	**14**	**5 (7/60)**	**11 (31/60)**

[a] First figure is number of Vd-positive trees; between brackets the number of Vd-positive samples and the total number of samples that was examined.

Identification of infected trees. All twigs (five per tree) collected from 44 trees in five sampling groups (Table 1, groups 1-5) were tested individually with real-time PCR through analysis of one sub-sample per twig containing fragments from different parts of that twig (Table 1, groups 1-5). Results from this analysis confirmed the presence of *V. dahliae* in 35 out of the 44 sampled trees (i.e. the fungus was detected in at least one sample per tree). This again was substantially more than the number of infected trees detected by standard plating assays that were performed in parallel for part of the trees; out of the 15 sampled tree from groups 3 and 5 that were positive for *V. dahliae* after PCR testing only from 8 trees *V. dahliae* was recovered by the plating assay (Table 1). The trees in which *V. dahliae* was not detected, likely because of absence of *V. dahliae* in the sampled twigs or because its amount was lower than the threshold value (0.001 ng of DNA) were discarded from further research.

Presence of V. dahliae in individual twigs. To investigate the distribution of *V. dahliae* in twigs of the infected trees identified in the first step, 55 twigs from 11 infected trees (2, 2, 1, 5 and 1 tree(s) respectively from the groups 1-5 in Table 1) were analysed for *V. dahliae* colonization. To this end, top (about 5 cm from the top) and lower parts (about 50 cm from the top) of five twigs per tree were analyzed separately with real-time PCR. At least two technical repeats were run for each DNA sample. The analysis of mean values of the real-time PCR results for individual samples revealed that *V. dahliae* quantities were highly variable between and within shoots. Out of the 110 samples that were examined, 28 individual samples (25%) yielded no *V. dahliae* DNA and many samples yielded very low levels of *V. dahliae* DNA, whereas in 14 out of 110 samples *V. dahliae* DNA levels over 5% up to almost 50% of total DNA were detected (Figure 3). Moreover, although many of the differences between samples from top and lower parts of a twig were statistically significant, there was no consistent pattern of one of the two of sample types (top or lower part of twigs) containing higher amounts of *V. dahliae* DNA.

Detection of V. dahliae in combined twig samples. Because of the high variation in the amount of *V. dahliae* DNA detected in individual twig samples of infected trees, combined samples from five twigs per tree were processed and used for *V. dahliae*

detection. To this end, for all 35 infected trees, DNA samples isolated from five individual twigs per tree were mixed in equal amounts, separately for the top and lower parts, and used for quantification of *V. dahliae* DNA. In this manner, *V. dahliae* DNA was detected in all trees, both in the combined samples from the lower and from the top parts.

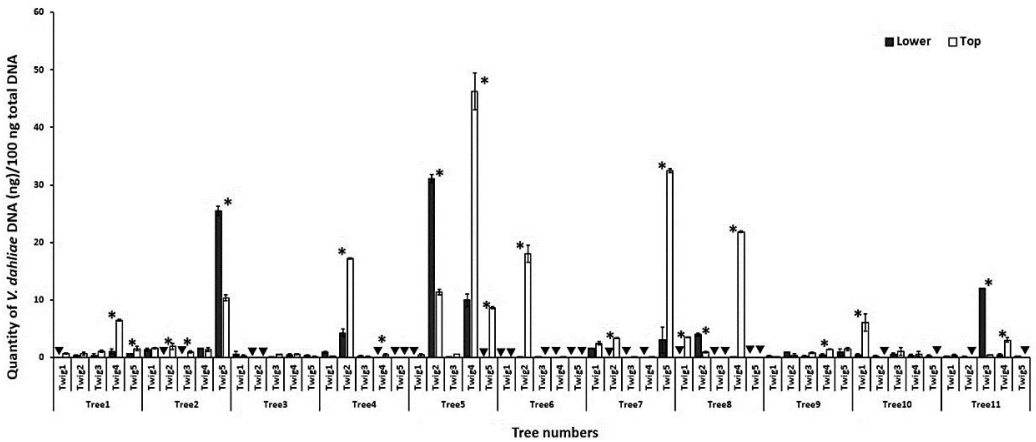


Figure 3. Mean quantities of *V. dahliae* DNA (ng) in 100 ng of total DNA extracted from individual twigs. From each tree, the top (about 5 cm from top) and lower (about 50 cm from top) parts of five individual twigs were analysed separately. A triangle (▼) indicates that *V. dahliae* DNA was not detected (threshold value 0.001 ng of DNA according to the standard curve). Each bar is the mean value of at least two replications for each DNA sample. Error bars show standard deviation. An asterisk indicates trees for which significantly different quantities of *V. dahliae* DNA were detected in lower and top parts (P=0.05).

Again, although for some of the trees the differences between amounts detected in top and lower parts were significant, no consistent pattern of top or lower parts yielding more *V. dahliae* DNA was detected and in most of the trees no significant differences were observed in the mean quantities of *V. dahliae* DNA detected in top and lower parts of twigs (P=0.05) (Figure 4). These results show that twig samples can be used for detection of *V. dahliae* infected olive trees, and that analysis of mixed DNA samples from top or lower parts of five twigs per tree gives reliable results whereas the results from individual twigs are much more variable. In our study testing samples from 35 infected trees for presence of *V. dahliae*, analysis of just one sample per tree (top or bottom part of a twig) gave 25% negative results, combined top and bottom parts from one twig per tree gave 10% negative results and analysis of combined top or bottom parts from 5 different twigs

per tree gave 0% negative results. We subsequently investigated if *V. dahliae* detection could be made more time efficient by combining samples of five twigs per tree prior to DNA extraction. To this end, 25 twigs sampled from five infected trees were used, and after combining subsamples taken from top or lower parts of five twigs per tree, total DNA was isolated. Then, all DNA samples were analysed using real-time PCR for detection of *V. dahliae* DNA. Interestingly, *V. dahliae* DNA was detected in all DNA samples from combined twigs (Figure 5) and the amounts of *V. dahliae* detected were comparable to mean quantities of 5 individual twigs (figure 4). Similar to the results of individual twigs statistical analysis did not show significant differences between results for samples from top and lower parts of shoots for most of the samples (P=0.05) (Figure 5). Hence, detection of *V. dahliae* after extraction of DNA from combined samples of five twigs would be as robust as detection in mixed DNA samples extracted from five individual twigs, and significantly reduces the amount of samples that need to be processed and extracted in order to reliably detect *V. dahliae* in infected olive trees.

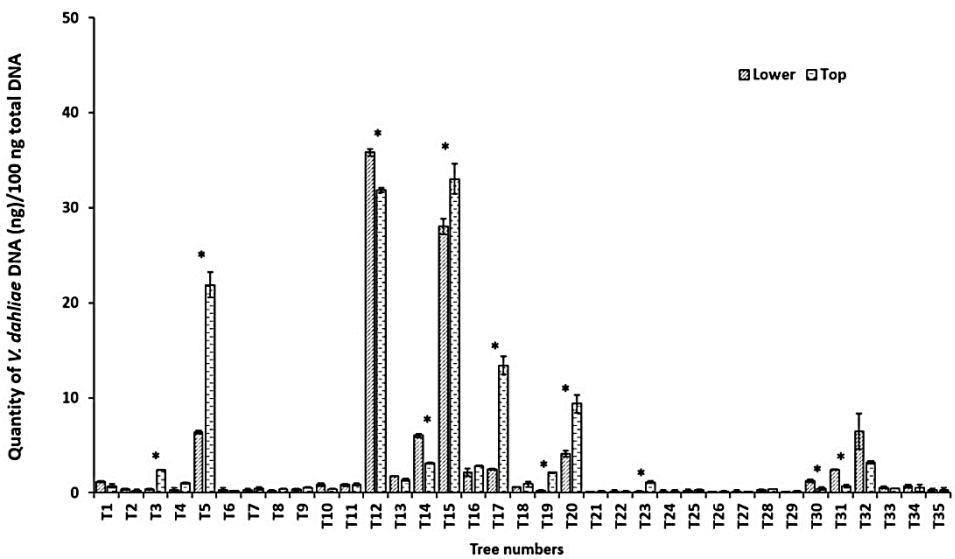


Figure 4. Mean quantities of *V. dahliae* DNA (ng) in 100 ng of mixed DNA samples from lower (about 50 cm from top) and top (about 5 cm from top) parts of twigs (Figure 1) as detected after mixing equal amounts of DNA isolated separately from five twigs per tree. Top and lower parts of twig samples of 35 trees were separately analysed. Each bar is the mean value of two replications for each DNA sample with standard deviation. Means of the results of top and lower parts were compared for each tree separately, based on T-test analysis. An asterisk indicates trees for which significantly different quantities of *V. dahliae* DNA were detected in lower and top parts (P=0.05).

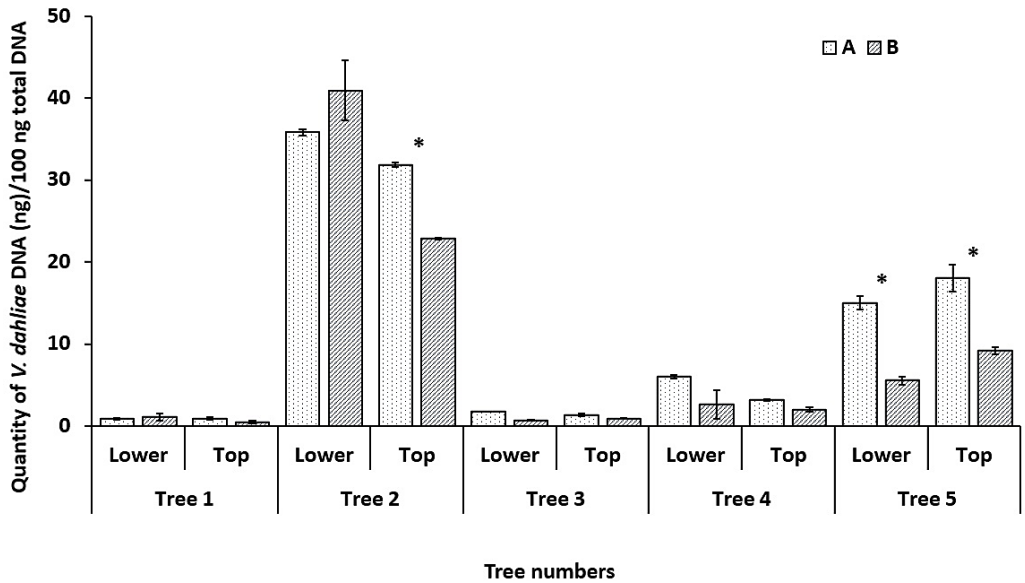


Figure 5. Quantities of *V. dahliae* DNA detected in 100 ng of mixed DNA samples extracted from top (about 5 cm from top) and lower (about 50 cm from top) parts of five individual twigs (A bars), and in 100 ng DNA extracted from combined samples of five twigs (B bars) (Figure 1). Each bar is the mean value of at least three replications for each DNA sample. Error bars show standard deviation. Mean quantities of *V. dahliae* DNA in mixed DNA samples extracted from five individual twigs and from combined samples of five twigs were compared, based on T-test analysis. An asterisk indicates cases where the quantities of *V. dahliae* DNA detected by the two approaches were significantly different (P=0.05).

Detection of V. dahliae in leaves. To examine the use of leaves of infected olive trees as a substrate for reliable detection of *V. dahliae,* 40 individual leaves randomly collected from symptomatic twigs of four infected olive trees (10 leaves from each tree) were tested. DNA was extracted from all individual leaves and analyzed with real-time PCR. At least two repeats were run for each DNA sample. Although *V. dahliae* DNA was detected in most of the samples, analysis of mean values of real-time PCR results showed that, like we observed for individual twigs, quantities of *V. dahliae* appeared to be highly variable between individual leaves of the same tree (Figure 6). Therefore, we assessed the suitability of combined samples from sets of 5, 10, 15, 20 or 25 leaves, randomly collected from symptomatic twigs of three infected trees, for detection of *V. dahliae* DNA. For each tree we tested at least three replications of each size set of leaves. For each DNA sample

extracted from each set of leaves two repeats were run in real-time PCR assays. *V. dahliae* DNA was readily detected in all sets of leaves. Also mean quantities of *V. dahliae* DNA detected after repeated testing of different sets of leaves from the same tree showed less variation than the variation that was observed after testing individual leaves of the same tree. This indicates that testing of combined samples of leaves is appropriate for fast and reliable detection of *V. dahliae* in infected trees. Furthermore, statistical analysis did not show significant differences between different set sizes that were tested for the same tree (Figure 7). Notably, as the combination of 5 or 10 leaves already accurately betrays fungal presence, samples composed of 5-10 leaves collected from symptomatic branches are considered sufficient for detection of *V. dahliae* in infected olive trees.

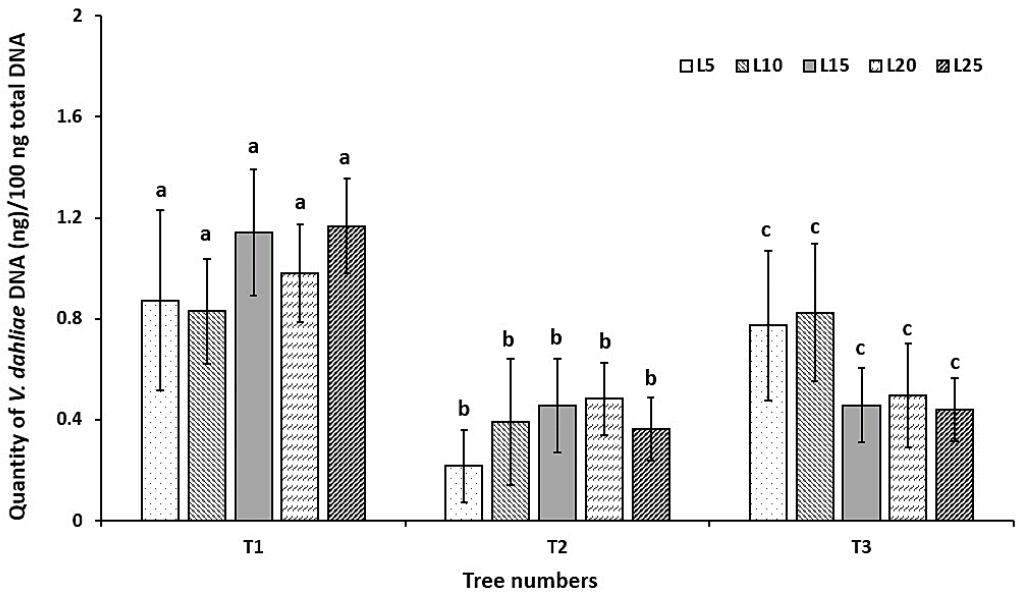


Figure 6. Quantities of *V. dahliae* DNA (ng) in 100 ng of total DNA extracted from individual leaves that were randomly sampled from symptomatic branches of four infected trees. From each tree 10 leaves were assayed separately. Triangles (▼) indicate that the *V. dahliae* DNA failed to be detected. Each bar is the mean value of at least two replications. Error bars show standard deviation.

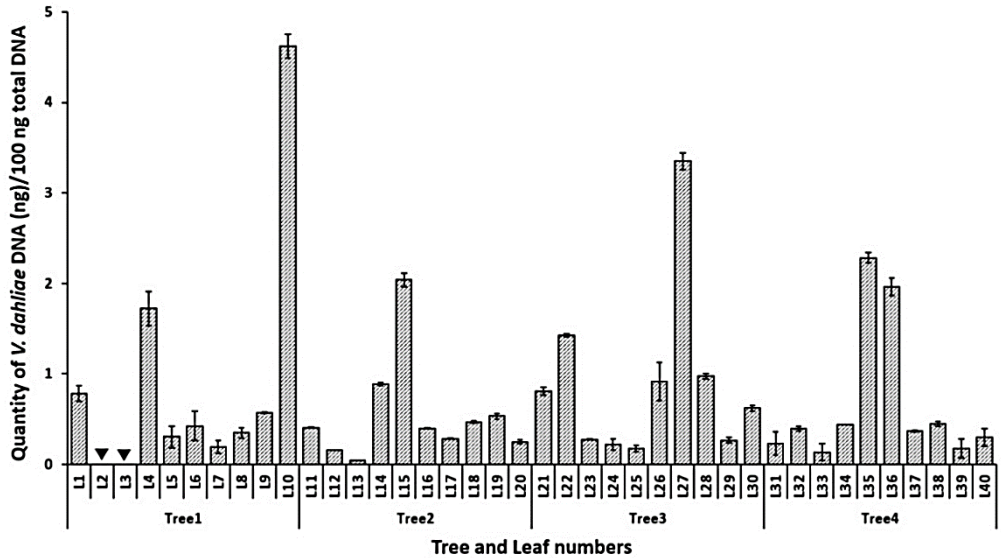


Figure 7. Quantities of *V. dahliae* DNA (ng) in 100 ng of total DNA extracted from sets consisting of 5, 10, 15, 20 or 25 leaves from infected olive trees. Leaves were randomly collected from symptomatic branches of three infected trees. Each bar is the mean value of at least 3 independent real-time PCR assays for different sets of leaves. The same letters above bars indicate that there were no significant differences between different sets of leaves, according to the Fisher protected LSD test (P=0.05). Error bars show standard deviation.

Discussion

The main goal of this study was to provide information on distribution of *V. dahliae* within diseased olive trees needed for designing efficient non-destructive sampling protocols for reliable detection and quantification of *V. dahliae* using PCR technology. We focussed on sampling of young twigs and leaves because these samples can be collected relatively easy and collecting them is not destructive to the tree. Indeed, in our experiments *V. dahliae* DNA could be successfully detected and quantified both in twig and leaf samples collected from naturally infected trees in olive orchards in different periods and in different regions. The real-time PCR with ITS-based primers (Van Doorn et al., 2009) that we used also appeared to be much more sensitive than the standard plating method as the percentage of samples positive for *V. dahliae* was much higher (Table 1) which is in agreement with the results of Morera et al. (2005) and Karajeh and Masoud (2006) who also reported that detection using PCR technology is much more reliable for detecting infected olive trees than the traditional plating method.

Analysis of twig samples collected from different sides of the crown of infected olive trees, resulted in highly variable quantities of *V. dahliae* DNA detected even within twigs, implying a non-uniform distribution of the fungus within infected parts of diseased olive trees. In the past, several authors have reported a non-continuous distribution of *V. dahliae* in infected olive trees (e.g. Vigouroux, 1975; Wilhelm and Taylor, 1965). In our results, however, the number of negative samples was very low. This may be attributed to the real-time PCR method being much more sensitive than the plating method used by those authors. Also in our results the relative amount of *V. dahliae* DNA in most of the samples is low indicating limited fungal growth within the tissues of the host. This is in accordance with a colonisation process that supposedly is based on the fungus mainly being transported by conidia that passively move with the sap stream until they are trapped at vessel ends or other obstructions within the xylem vessels of the host where they may germinate, start hyphal growth and form new conidia (Hiemstra, 1998). The rather high relative amounts of *V. dahliae* DNA detected in some samples may refer to such sites with relatively profuse hyphal growth. Furthermore, resistance mechanisms of the host plant that pose widespread local occlusions of xylem vessels may restrict uniform spread of the pathogen (Hiemstra, 1998; Pegg and Brady, 2002). In naturally occurring infections it is very likely that the uneven distribution of *V. dahliae* in soil results in just a part of the root system being infected. Because olive trees are highly sectored with direct vascular connection of specific roots and shoots (Lavee, 1996) infection through one major root may lead to a tree with serious symptoms in just a part of the crown whereas other parts of the crown remain free of symptoms (Levin et al., 2003). This explains the common phenomenon that diseased olive trees show some branches with severe symptoms of Verticillium wilt whereas other parts of the tree may be completely free from symptoms (Figure 1). This, however, did not affect our results as samples always were collected from symptomatic parts of the trees. Our results demonstrate that in these parts *V. dahliae* clearly is present in a more or less continuous pattern, although the relative amount of the fungus may be varying strongly and incidentally the pathogen may not be detected at all.

Because of the varying amount of the pathogen within infected parts of diseased olive trees, analysis of samples collected from different twigs in the diseased part of a tree will

increase reliability of the screening for presence of *V. dahliae* as has been suggested before by Karajeh and Masoud (2006) and Levin et al. (2003). This is confirmed by our results as for all infected trees analysis of a mixed sample from five twigs resulted in detection of *V. dahliae* whereas part of the twig samples were negative when tested individually. Moreover, results demonstrated that testing mixed samples from different twigs in one go, i.e. DNA extraction and PCR analysis on one mixed sample with small subsamples from different twigs, gives positive results for all infected trees that were tested. From an economical point of view, testing mixed DNA samples instead of DNA samples from individual twigs is highly favourable as it reduces the costs of quantification assays by reducing the number of tests needed for reliable analysis of individual trees. This is particularly important in large-scale diagnostic experiments.

Several studies have demonstrated the presence of *V. dahliae* in petioles of infected olive trees (Prieto et al., 2009; Tjamos and Botseas, 1987). The present results, however, represent the first systematic testing of large sets of leaves as a good approach with practical advantages. Comparable to the results using twig samples, high variation was observed in relative quantities of *V. dahliae* DNA detected in individual leaves. This again may be explained by the fact that distribution of the pathogen throughout the vascular system of naturally infected trees is not uniform. Therefore, like twigs, the level of infection would be highly variable between leaves. To overcome the variation caused by testing individual leaves, DNA from several sets of combined leaves was tested and *V. dahliae* DNA was detected in all sets (5, 10, 15, 20, and 25) of leaves with less variation in quantities of *V. dahliae* DNA between replications of each set in the same tree. The maxima for the relative amount of *V. dahliae* DNA detected in leave samples were substantially lower than for twig samples. This probably can be explained by the type of samples. As long as its host is still alive *V. dahliae* is confined to the xylem vessels. The part that was analysed from twig samples only consists of xylem tissues (i.e. vessels, fibres and some parenchyma cells, with part of the tissue being not alive (vessels and part of fibres), i.e. without DNA, whereas in leaf samples parts of the whole leaf were used, i.e. veins and leaf lamina. The latter sample therefore contains relatively more living plant cells and most probably a higher percentage of plant DNA.

In conclusion, to minimise damage of Verticillium wilt on olive trees and to stop the pathogen from spreading further, early detection of the pathogen as part of an integrated control strategy is necessary (Hiemstra, 2015; López et al., 2003). For a robust and reliable detection of the pathogen as part of such a strategy non-destructive and easily performed sampling is required (López et al., 2003). Due to the distribution of the pathogen within the tree detection using single samples is unreliable. Data presented in this study demonstrate that testing of one combined sample comprising subsamples from at least 5 twigs from different sides of the tree, or 5-10 leaves randomly collected from symptomatic twigs can reliably detect the pathogen. With this valuable information for the design of easy and efficient sampling protocols for detection of *V. dahliae* in olive trees as part of integrated management strategies for the control of Verticillium wilt in olive is provided. Our results demonstrate that the present procedure works well for reliable and efficient detection of *V. dahliae* in diseased olive trees. Because our study only addressed symptomatic trees, its robustness in early detection of the pathogen in symptomless trees, whether tolerant (i.e. infected without symptoms) or recently infected (i.e. not yet showing symptoms) remains to be studied in more detail.

Acknowledgments

The work of MK at the Applied Plant Research organization of Wageningen University and Research Center (WUR-PPO) was supported by a scholarship of the ministry of science and technology of Iran. Samples and information about the trees were obtained through cooperation within the EU-Vertigeen project (www.vertigeen.eu). The work of JAH in the EU-Vertigeen project was co-funded by the European Commission under the seventh framework programme for research and technological development of the European Union (Fp7-sme-2011-2-286140). We thank the local participants in the Vertigeen project for their help in collecting the samples. We also thank Suzanne J. Breeuwsma (WUR-PPO) for valuable technical support in analysing the samples. Finally we thank dr. F. J. López-Escudero, Departamento de Agronomía, Universidad de Córdoba, who kindly supplied the V117 isolate of *V. dahliae*.

References

Baídez, A.G., Gomez, P., Del Rio, J.A., Ortuno, A. (2007). Dysfunctionality of the xylem in *Olea europaea* L. plants associated with the infection process by *Verticillium dahliae* Kleb. Role of phenolic compounds in plant defense mechanism. Journal of Agricultural and Food Chemistry 55, 3373-3377.

Barranco, D., Fernandez-Escobar, R., Rallo, L. (2010). *Olive Growing*. MundiPrensa/Australian Olive Association Ltd., Junta de Andalucía, 756 pp.

Blázquez-Martínez, J.M. (1996). Evolution and history. *In: World Olive Encyclopaedia, International Olive Oil Council*, 1996. P. 17-58.

Ceccherini, M.T., Luchi, N., Pantani, O.L., Ascher, J., Capretti, P., Pietramellara, G. (2013). Upward movement of *Verticillium dahliae* from soil to olive plants detected by qPCR. World Journal of Microbiology and Biotechnology 29(10), 1961-7.

Civantos, L. (2004). Olive growing in the world and in Spain, p. 17–35. *In: D. Barranco, R. Fernández - Escobar, and L. Rallo (eds.)*, 2010. Olive Growing. RIRDC, Australia.

Dellaporta, S.L., Wood, J., Hicks, J.B. (1983). A Plant DNA Minipreparation: Version II. Plant Molecular Biology Reporter 1, 19-21.

García-Cabello, S., Pérez-Rodríguez, M., Blanco-López, M.A., López-Escudero, F.J. (2012). Distribution of *Verticillium dahliae* through watering systems in widely irrigated olive growing areas in Andalucía (southern Spain). European Journal of Plant Pathology 133, 877-885.

Gramaje, D., Pérez-Serrano, V., Montes-Borrego, M., Navas-Cortés, J.A., Jiménez-Díaz, R.M., Landa, B.B. (2013). A comparison of real-time PCR protocols for the quantitative monitoring of asymptomatic olive infections by *Verticillium dahliae* pathotypes. Phytopathology 103, 1058-1068.

Hiemstra, J.A. (1998). Some general features of *Verticullium* wilt in trees. In: Hiemstra and Harris 1998. *A compendium of Verticillium wilts in tree species.* Wageningen: Centre for Plant Breeding and Reproduction Research (CPRO-DLO)/West Malling: HRI-East Malling, 5-11.

Hiemstra, J.A. (2015). Control of Verticillium wilt in olive; guide of best practices. EU-Vertigeen project (FP7-SME-2011-2-286140).
[http://www.vertigeen.eu/files_media/1429516669_vertigeen_deliverable_6_13_guide_of_best_practices_final.pdf]

Hiemstra, J.A., Peters, J., Duran, C., Gonzalez, I., Keykha Saber, M., Breeuwsma, S., Pham, K., Van Doorn, J. (2013). Optimizing detection of *Verticillium dahliae* in

olive tree samples. *Proceedings of the 11th International Verticillium Symposium – Göttingen*, 2013. DPG Verlag, Braunschweig. P. 130.

Jiménez-Díaz, R.M., Cirulli, M., Bubici, G., Jiménez-Gasco, M.D., Antoniou, P.P., Tjamos, E.C. (2012). Verticillium wilt, a major threat to olive production: current status and future prospects for its management. Plant Disease 96, 304-329.

Karajeh, M.R., Masoud, S.A. (2006). Molecular detection of *Verticillium dahliae* Kleb. in asymptomatic olive trees. Journal of Phytopathology 154, 496-499.

Ladux, J.L., Jotayan, L., Otero, M.L., González Vera, C., Ortiz, J. (2014). Incidence of *Verticillium dahliae* in traditional orchards of the olive 'Arauco' in northwest Argentina (La Rioja). Acta Horticulturae 1057, 127-132.

Lavee, S. (1996). Biology and physiology of the olive. *In: World olive encyclopaedia. International Olive Oil Council*, Madrid, 59-110.

Levin, A.G., Lavee, S., Tsror, L. (2003). Epidemiology of *Verticillium dahliae* on olive (Picual) and its effect on yield under saline conditions. Plant Pathology 52, 212-218.

Lievens, B., Brouwer, M., Vanachter, A.C.R.C., Cammue, B.P.A., Thomma, B.P.H.J. (2006). Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples. Plant Science 171, 155-165.

López MM, Bertolini E, Olmos A, Caruso P, Gorris MT, Llop P, Penyalver R, Cambra M, 2003. Innovative tools for detection of plant pathogenic viruses and bacteria. International Microbiology 6(4), 233-243.

López-Escudero, F.J., Mercado-Blanco, J. (2011). Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. Plant Soil 344, 1- 50.

Markakis, E.A., Tjamos, S.E., Antoniou, P.P., Paplomatas, E.J., Tjamos, E.C. (2009). Symptom development, pathogen isolation and real-time qPCR quantification as factors for evaluating the resistance of olive cultivars to Verticillium pathotypes. European Journal of Plant Pathology 124, 603-611.

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodriguez-Jurado, D., Jimenez-Diaz, R.M. (2003). Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction. Physiological and Molecular Plant Pathology 63, 91-105.

Morera, B., Páez, J.I., Vega, J.M., Montes, F. (2005). Comparación de métodos de diagnóstico de *Verticillium dahliae* en olivo: aislamiento en medio de cultivo y PCR. Boletín de sanidad vegetal. Plagas 31, 267-275.

Pegg, G.F., Brady, B.L. (2002). Verticillium wilts. Wallingford, UK: CABI publishing.

Prieto, P., Navarro-Raya, C., Valverde-Corredor, A., Amyotte, S.G., Dobinson, K.F., Mercado-Blanco, J. (2009). Colonization process of olive tissues by *Verticillium dahliae* and its in planta interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7. Microbial Biotechnology 2(4), 499-511.

Termorshuizen, A.J. (1998). Disease diagnosis and identification of the pathogen. In: Hiemstra JA, Harris DC, 1998. *A compendium of Verticillium wilts in tree species*. Wageningen: Centre for Plant Breeding and Reproduction Research (CPRO-DLO)/West Malling: HRI-East Malling, 41-43.

Tjamos, E.C., Botseas, D. (1987). Occurrence of *Verticillium dahliae* in leaves of Verticillium wilted olive trees. Canadian Journal of Plant Pathology 9, 86.

Tjamos, E.C. (1993). Prospects and strategies in controlling Verticillium wilt of olive. Bull. OEPP/EPPO Bull. 23, 505-512.

Tjamos, E.C., Jiménez-Díaz, R.M. (1998). Management of disease. In: Hiemstra JA, Harris DC, 1998. *A compendium of Verticillium wilts in tree species*. Wageningen: Centre for Plant Breeding and Reproduction Research (CPRO-DLO)/West Malling: HRI-East Malling, 55-57.

Van Doorn, J., Pham, K.T.K., Hiemstra, J.A. (2009). Molecular detection of *Verticillium dahliae* in soil. *Abstract book of the 10th International Verticillium Symposium*, 2009. Corfu Island, Hellas, p. 94.

Vigouroux, A. (1975). *Verticillium dahliae*, Agentd'un dépérissement de l'olivier en France. Annual Phytopathology 7, 37-44 (in French).

Weller, S.A., Elphinstone, J.G., Smith, N.C., Boonham, N., Stead, D.E. (2000). Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Applied Environmental Microbiology 66, 2853-2858.

Wilhelm, S., Taylor, J.B. (1965). Control of Verticillium wilt of olive through natural recovery and resistance. Phytopathology 55, 310-316.

Chapter 4

A robust method for discriminating defoliating and the non-defoliating pathotypes of *V. dahliae*

Mojtaba Keykhasaber[a,b,c], Luigi Faino[a], Grardy C. M. van den Berg[a], Jelle A. Hiemstra[b], Bart P.H.J. Thomma[a]

[a]Laboratory of Phytopathology, Wageningen University & Research, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

[b]Wageningen Plant Research, Wageningen University & Research, P.O. Box 200, 6670 AE

[c] Department of Plant Biotechnology, Zabol University, Zabol, Iran

Abstract

Verticillium dahliae isolates infecting olive and cotton are traditionally classified into highly virulent defoliating (D) and mildly virulent non-defoliating (ND) pathotypes. Previously, a PCR-based method was developed for detection and discrimination of D and ND isolates of *V. dahliae*, but it later became clear that this method failed to assign particular *V. dahliae* isolates to either pathotype. In the present study, comparative population genomics was employed to identify similarities within and differences between pathotypes to find genomic regions that could reliably be associated to D and ND isolates of *V. dahliae*, aiming to design robust markers for discrimination of these pathotypes. Comparison of eight recently sequenced genomes of *V dahliae* isolates, as well as the genomes of *V. dahliae* isolates JR2 and VdLs17, revealed that isolates belonging to the D and the ND pathotype are monophyletic and developed as two distinct *V. dahliae* lineages. Our analysis resulted in the identification of a particular deletion that is observed in all sequenced ND isolates but not in D isolates. Thus, a set of primers was designed across the deletion that was able to generate differentially sized amplicons for isolates belong to the different pathotypes of *V. dahliae*. Subsequently, a nested PCR assay was developed that allowed detection of very low quantities of pathogen DNA *in planta* and discrimination of D and ND *V. dahliae* isolates.

Introduction

Verticillium dahliae Kleb. is a soil-borne fungus that causes Verticillium wilt worldwide in various plant species, ranging from herbaceous annuals to woody perennials (Berlanger and Powelson, 2005; Fradin and Thomma, 2006; Klosterman et al., 2009; Pegg and Brady, 2002; Smith et al., 1988). Verticillium symptoms comprise wilting, chlorosis, stunting, necrosis, vein clearing and defoliation, in advanced stages of the disease especially in woody hosts often leading to dieback or death of plants. Despite the name of the disease, wilting symptoms do not generally occur and symptom display is highly variable between hosts; there are no unique symptoms that belong to all plants infected by this fungus (Fradin and Thomma, 2006). Based on the symptoms that are inflicted, on some host species, particularly on cotton (*Gossypium hirsutum* L.) and olive (*Olea europaea* L.), *V. dahliae* isolates are traditionally classified into a highly virulent defoliating (D) and a

mildly virulent non-defoliating (ND) pathotype (Schnathorst and Mathre, 1966). This typing was first described for Verticillium isolates infecting cotton according to their differential level of virulence and the ability to cause defoliation. On cotton, the D pathotype comprises isolates that are highly virulent and causes complete defoliation of infected plants, whereas the ND pathotype comprises isolates that are moderately virulent and only cause mild wilt without defoliation (Schnathorst and Mathre, 1966). Interestingly, isolates that belong to the D pathotype on cotton are also highly aggressive on olive, whereas isolates of the ND pathotype on cotton are less aggressive (Rodríguez-Jurado et al., 1993; Schnathorst et al., 1971).

Despite the fact that the strict correlation with the occurrence of defoliation symptoms as occurs on cotton is not observed on olive plants (Rodríguez-Jurado et al., 1993), assignment of isolates to the D or ND pathotype is still meaningful as it directly correlates with fungal aggressiveness and damage caused by the disease. Moreover, differentiation between D and ND pathotype is important in order to predict the severity of disease, and decide on appropriate disease management strategies to take (López-Escudero and Mercado-Blanco, 2011; Hiemstra, 2015). More specifically, isolates belonging to the D pathotype cause much more severe disease symptoms in olive than isolates belonging to the ND pathotype (Bell, 1994; Schnathorst and Mathre, 1966). Initially, whereas the ND pathotype of *V. dahliae* occurred ubiquitously, the D pathotype only occurred in particular sites in the Americas (Mathre et al., 1966; Schnathorst, 1966), China (Zhengjun et al., 1998) and Spain (Bejarano-Alcázar et al., 1996). However, since then the D pathotype has also spread significantly (Jiménez-Díaz et al., 2012).

Conventional methods for discrimination of pathotypes involve virulence tests on cotton or olive that are time-consuming, laborious and often require expert knowledge. Therefore, more rapid, sensitive and accurate tools such as DNA-based methodologies are desired to assign *V. dahliae* isolates to the correct pathotype. To this end, a variety of PCR-based methods has been developed (Aljawasim and Vincelli, 2015; Carder et al., 1994; Mercado-Blanco et al., 2001, 2002, 2003; Pérez-Artés et al., 2000). Differentiation of D and ND isolates of *V. dahliae* became possible by using primer pair DB19/DB22 (Carder et al., 1994) that resulted in generating specific polymorphic DNA bands of 539

or 523 bp for the D and ND pathotype, respectively. However, these primers occasionally produced unspecific bands for D isolates (Collins et al., 2005; Mercado-Blanco et al., 2003). Therefore, a new primer (espdef01) was designed that, in combination with the DB19 primer, allowed for specific amplification of a 334 bp marker for D isolates (Mercado-Blanco et al., 2003). Later it became clear that this 334 bp amplicon is generated not only for D isolates but also for particular ND isolates (Collins et al., 2005, Jiménez-Díaz et al., 2006). Finally, a PCR-method based on sequences of pathotype-associated RAPD bands, was developed for detection and discrimination of *V. dahliae* isolates (Pérez-Artés et al., 2000). This method allowed for the detection and differentiation of several D and ND isolates of *V. dahliae* collected worldwide (Pérez-Artés et al., 2000). Subsequently, nested PCR primers were designed to increase the sensitivity of the assay to allow for detection of D and ND pathotypes *in planta* and in soil samples (Mercado-Blanco et al., 2002; 2001 Pérez-Artés et al., 2005). Unfortunately, however, it later became clear that this method also does not work for all *V. dahliae* isolates, as isolates were encountered for which this PCR method did not result in any amplification, and thus the PCR was not able to assign the isolate to the correct pathotype (Collins et al., 2005).

Comparative population genomics is a powerful tool to query for similarities and differences between isolates that belong to the same species (De Jonge et al., 2012). Previously, we have used comparative population genomics in combination with transcriptome sequencing to identify the Ave1 effector molecule that is secreted by *V. dahliae* race 1 isolates that are recognized by the Ve1 immune receptor of resistant tomato plants. The comparative genomics strategy was based on the comparison of the genomes of race 1 isolates with race 2 isolates that defy recognition (de Jonge et al., 2012). Appreciating the success of this approach, the aim of the present study was to compare whole-genome sequences of *V. dahliae* isolates that belong to the ND and the D pathotype to design an improved marker that can discriminate these isolates.

Materials and methods

V. dahliae isolates and plant materials. Initially, in this study 18 *V. dahliae* isolates with a known pathotype were used for testing the primers used in this study (Table 1). Of these 18 isolates, eight were previously determined to belong to the ND pathotype whereas ten isolates were determined to belong to the D pathotype by means of biological pathotyping on cotton or olive (Bejarano-Alcázar et al., 1995; 1996; Joaquim and Rowe, 1991; Jiménez-Díaz 2008; Mace et al., 1990; Xu et al., 2012). Additionally, ten uncharacterized isolates recovered from naturally infected olive trees in two commercial olive plantations in central Greece were used. Finally, to set up an *in planta* detection assay, eight twig samples from naturally infected olive trees (cv Picual) from southern Spain, were used. The Greek Isolates and Spanish samples were obtained through cooperation within the EU-Vertigeen project (FP-SME-2011-286140; www.vertigeen.eu).

Table 1. Source, original host, geographic origin and pathotype of the *V. dahliae* isolates used in this study.

Isolate	Host	Origin	Provider of the isolate	Pathotype	PCR[#]	Sequenced[*]
V4	Olive	Spain	dr. F. J. Lopez-Escudero, department of agronomy, university of Cordoba	ND	X	X
BP2	Cotton	China	dr Baolong Zhang, Jiangsu Academy of Science, China	ND	X	X[**]
B1-3	Olive	Spain	dr. F. J. Lopez-Escudero, department of agronomy, university of Cordoba	ND	X	
B4-10	Olive	Spain	dr. F. J. Lopez-Escudero, department of agronomy, university of Cordoba	ND	X	
B4-12	Olive	Spain	dr. F. J. Lopez-Escudero, department of agronomy, university of Cordoba	ND	X	
V-096	Olive	Spain	dr. F. J. Lopez-Escudero, department of agronomy, university of Cordoba	ND	X	
V117	Olive	Spain	dr. F. J. Lopez-Escudero, department of agronomy, university of Cordoba	D	X	X
T9	Cotton	USA	dr Baolong Zhang, Jiangsu Academy of Science, China	D	X	X
F4-A20	Olive	Spain	dr. F. J. Lopez-Escudero, department of agronomy, university of Cordoba	D	X	
F4-A83	Olive	Spain	dr. F. J. Lopez-Escudero, department of agronomy, university of Cordoba	D	X	
V-054	Olive	Spain	dr. F. J. Lopez-Escudero, department of agronomy, university of Cordoba	D	X	
V-071	Olive	Spain	dr. F. J. Lopez-Escudero, department of agronomy, university of Cordoba	D	X	
F3-A23	Olive	Spain	dr. F. J. Lopez-Escudero, department of agronomy, university of Cordoba	D	X	
V991	Cotton	China	dr Baolong Zhang, Jiangsu Academy of Science, China	D	X	X[**]
V-76/463	Cotton	Mexico	dr Volker Lipka Department or plant cell biology, Gottingen	D	X	
1cd3_2	Cotton	China	dr Baolong Zhang, Jiangsu Academy of Science, China	ND		X

1HN_1	Cotton	China	dr Baolong Zhang, Jiangsu Academy of Science, China	ND		X
4TM6_15	Cotton	China	dr Baolong Zhang, Jiangsu Academy of Science, China	D		X
VD-270	Olive	Greece	EU-Vertigeen project (FP-SME-2011-286140; www.vertigeen.eu)	-	X	-
VD-272	Olive	Greece	EU-Vertigeen project (FP-SME-2011-286140; www.vertigeen.eu)	-	X	-
VD-273	Olive	Greece	EU-Vertigeen project (FP-SME-2011-286140; www.vertigeen.eu)	-	X	-
VD-274	Olive	Greece	EU-Vertigeen project (FP-SME-2011-286140; www.vertigeen.eu)	-	X	-
VD-276	Olive	Greece	EU-Vertigeen project (FP-SME-2011-286140; www.vertigeen.eu)	-	X	-
VD-277	Olive	Greece	EU-Vertigeen project (FP-SME-2011-286140; www.vertigeen.eu)	-	X	-
VD-280	Olive	Greece	EU-Vertigeen project (FP-SME-2011-286140; www.vertigeen.eu)	-	X	-
VD-287	Olive	Greece	EU-Vertigeen project (FP-SME-2011-286140; www.vertigeen.eu)	-	X	-
VD-291	Olive	Greece	EU-Vertigeen project (FP-SME-2011-286140; www.vertigeen.eu)	-	X	-
VD-294	Olive	Greece	EU-Vertigeen project (FP-SME-2011-286140; www.vertigeen.eu)	-	X	-

* paired-end sequencing using 500 bp insert size library
** mate-pair sequencing using 5000 bp and paired-end sequencing using 500 bp insert size library
# isolates used for testing the primers used in this study

Genome assemblies and comparative analysis. *V. dahliae* isolates V991 and BP2 were paired-end sequenced using two libraries (500 bp and 5000 bp insert size, 100 bp read length) while six additional isolates (Table 1) were paired-end sequenced using one library only (500 bp, 100 bp read length) with Illumina sequencing. About 1 Gb of data was produced from each library. The genomes of V991 and BP2 were assembled using the A5 pipeline (Tritt et al., 2012). The reads of the additional six isolates were mapped onto the V991 and the BP2 genomes using Bowtie2 software with default settings (Langmead et al., 2009). The mapping results were used in combination with BED tools software (Quinlan and Hall, 2010) using default settings to retrieve coverage plots and to identify polymorphic regions between the genomes of the ND and D isolates. A phylogenetic tree was constructed using RealPhy software (Bertels et al., 2014) using the genome of *V. dahliae* isolate JR2 as a reference (Faino et al., 2015). Primer pairs were designed to amplify the polymorphic regions.

Polymerase chain reaction (PCR). All PCR reactions were performed using a PTC-200 Thermal Cycler (MJ Research, Watertown, MA, USA), and primers used in this study are listed in Table 2. Further to the primers designed in this study we also used the previously

described D1-D2 (Pérez-Artés et al. 2000) and NDr-NDf (Mercado-Blanco et al. 2001) primers to discriminate D and ND isolates of *V. dahliae*, respectively. PCR reactions with D1-D2 and NDr-NDf primers were performed as previously described (Pérez-Artés et al., 2000; Mercado-Blanco et al., 2001). For the new primers designed in this study (Table 2), the temperature profile consisted of an initial denaturation step for 4 minutes at 94˚C, followed by 40 cycles of 45 seconds at 94˚C, 30 seconds at 56˚C, and 60 seconds at 72˚C. Subsequently, a final extension step was performed for 4 minutes at 72˚C. Also nested PCR assays were performed using this temperature profile in both primary and secondary rounds of the PCR. Primers specific for the plant cytochrome oxidase (*COX*) gene (Weller et al. 2000) were used as a control.

Table 2. Primers used in this study for detection of D and ND isolates of *V. dahliae* by PCR.

Primer	Sequence	Amplicon size (bp)	
		ND	D
Vdf1	5´-AGGCTGACTGGGAGAGTTGA-3´	423	694
Vdr1	5´- GGCCCTCAAGGTCAAACTCT-3´		
Vdf2	5´- GGCCCTCAAGGTCAAACTCT-3´	297	568
Vdr2	5´- GACCAGTATCACGGCAGGTT -3´		
D1	5´- CATGTTGCTCTGTTGACTGG -3´	-	548
D2	5´- GACACGGTATCTTTGCTGAA -3´		
NDf	5´- ATCAGGGGATACTGGTACGAGA-3´	1410	-
NDr	5´- GAGTATTGCCGATAAGAACATG-3´		

Results

Attempted differentiation of D and ND isolates of V. dahliae. Initially, the previously described ND- and D-specific primers (NDf-NDr and D1-D2, respectively; Pérez-Artés et al. 2000) were tested on 6 isolates belonging to the D pathotype and 9 isolates belonging to the ND pathotype of *V. dahliae*, collected from different parts of the world (Table 1). The PCR assay revealed that the NDf-NDr primers adequately recognized all tested ND isolates (Figure 1A). In contrast, however, whereas the D1-D2 primers accurately recognized most of the D isolates, no amplicons were observed for the T9 and V76 isolates that belong to the D pathotype (Puhalla and Hummel, 1983; Pérez-Artés et al. 2000) (Figure 1B). Thus, we confirm that the currently available markers to discriminate ND

and D isolates are not accurate and cannot reliably assign every *V. dahliae* isolate to its corresponding pathotype.

Identification of specific primers for D and ND pathotypes using sequencing data. In order to determine why the previously described ND- and D-specific primers (Pérez-Artés et al. 2000) are not consistently able to discriminate isolates that belong to the D and ND pathotypes, we aimed to investigate the genomic basis of the PCR assay. First we generated a reference genome assembly for an isolate belonging to the D pathotype and the ND pathotype, respectively. To this end, the genomes of *V. dahliae* isolates V991 (D) and BP2 (ND) were sequenced by paired-end sequencing of two libraries (500 bp and 5000 bp insert size, 100 bp read length) for each isolate. The subsequent genome assembly produced ~650 contigs for each of the isolates, with an N50 of ~35 kb and ~48 kb for V991 and BP2, respectively (Table 3).

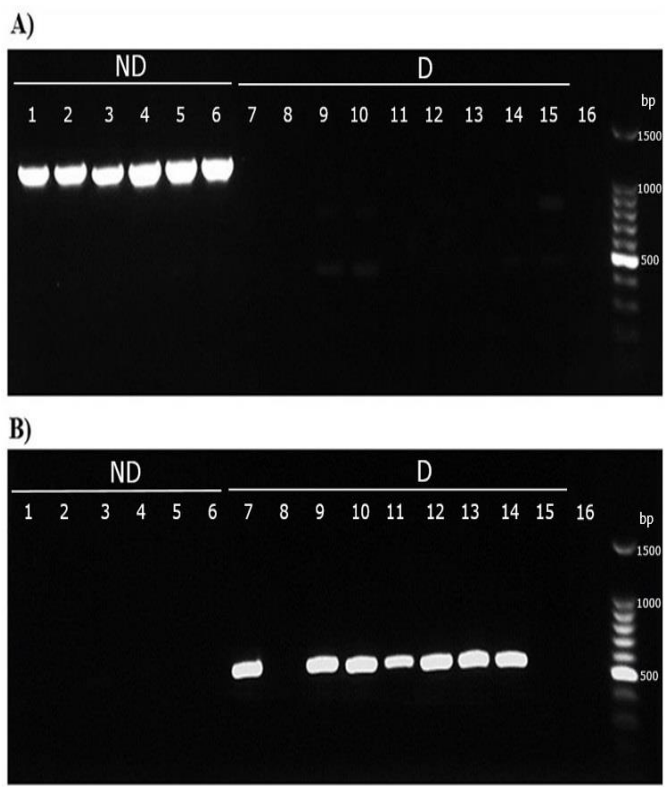


Figure 1. PCR products obtained with primer pair NDr-NDf to identify *V. dahliae* isolates belonging to the ND pathotype (A), and primer pair D1-D2 to identify isolates belonging to the D (defoliating) pathotype (B). Lanes 1-6: *V. dahliae* ND isolates: V4 (1); Bp2 (2); B1-3 (3); B4-10 (4); B4-12 (5); V-096 (6). Lanes 7-17: *V. dahliae* D isolates: V117 (7); T9 (8); F4-A20 (9); F20-A83 (10); V-054 (11); V-071 (12); F3-A23 (13); V-991 (14); V-76 (15). Amplification reactions with NDr-NDf primers yielded a 1410 bp band for all tested ND isolates and no band for D isolates. Amplification reactions with D1-D2 primers yielded a 548 bp band for D isolates except for T9 (lane 8) and V-76 (lane 15). D1-D2 primers did not amplify any fragment for ND isolates. Lane 16: DNase-free water as negative control.

Table 3. Assembly statistics of the genome sequences of *V. dahliae* isolates V991 and BP2

Isolate	# contigs	Largest contig (kb)	N50 (kb)	# N's per 100 kb
V991	1,991	1,430,085	354,213	1,524.20
BP2	1,675	2,190,310	483,968	1,353.53

Additionally, the genome sequences of six additional *V. dahliae* isolates were determined by paired-end Illumina sequencing of a single library (500 bp, 100 bp read length), three of which belonged to the D pathotype and three to the ND pathotype (Table 1; Table 3). A phylogenetic analysis was conducted using the eight sequenced genomes as well as the genomes of *V. dahliae* isolates JR2 and VdLs17 that were previously sequenced (Faino et al. 2015). The analysis showed that isolates belonging to the D and the ND pathotype cluster in different clades, suggesting that the two pathotypes developed as two distinct *V. dahliae* lineages (Figure 2).

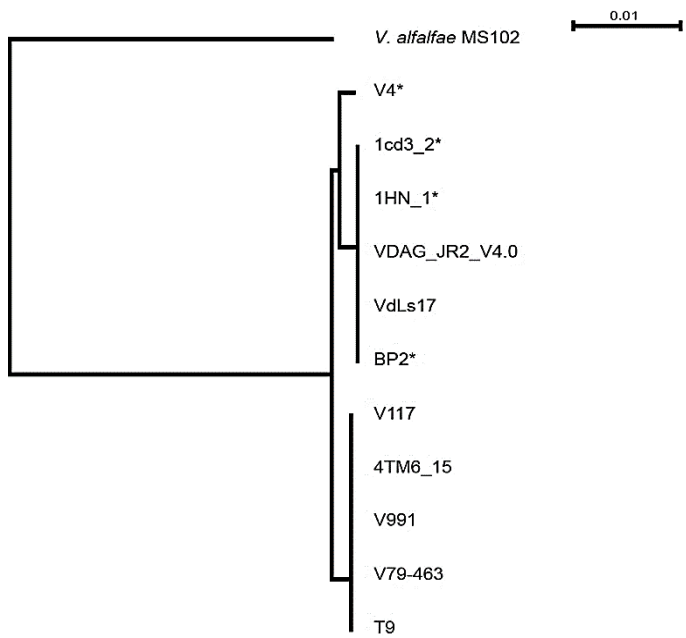


Figure 2. Isolates belonging to the defoliating and to the non-defoliating pathotype are monophyletic in the *Verticillium dahliae* population. Phylogenetic relationship between eleven sequenced *V. dahliae* isolates was inferred using RealPhy (Bertels et al. 2014). The *V. dahliae* isolate JR2 (VDAG_JR2_V4.0) was used as reference isolate while the *V. alfalfa* MS 102 was used as root of the tree.

Next, the genomes of V991 and BP2 were used to determine the annealing sites of the D and ND primers, respectively. While the target sequence of the ND primer set was found in the BP2 genome, it was absent from the V991 genome (Figure 3A). Similarly, the target sequence of the D primer set was identified in the V991 genome, while it was absent from the BP2 genome (Figure 3B). Subsequently, the reads from the three additionally sequenced D and ND isolates (Table 1) were mapped to the genome assemblies of BP2 and V991. Like for BP2, the additional ND isolates carried the target region of the ND primers, while the D isolates did not (Figure 3A). Similarly, the D isolates 4TM61, V991 and V117 were found to carry the target region of the D primers, while the ND isolates did not (Figure 3B). Importantly, however, the T9 isolate that belongs to the D pathotype did not carry the target region of the D primers (Figure 3B). Thus we conclude that the D primers have apparently been developed on a genomic region that is not ubiquitously present in all D isolates. Subsequently, we queried the sequencing data to identify genomic regions that could reliably be associated to isolates either belonging to the D or the ND pathotype and that could be used to design an improved identification assay. This time, however, we aimed for a polymorphic marker that would be detected in all isolates, rather than separate markers for the different pathotypes. To this end, the genome assembly of the D pathotype V991 was queried as a reference, and used in a comparative genomic analysis with the other 7 genomes. This resulted in the identification of a deletion on scaffold V911_N.5.1 which is observed in all sequenced ND isolates (Figure 4). A set of primers was designed across the deletion (Vdf1-Vdr1; Table 2; Figure 5), such that for all *V. dahliae* isolates an amplicon will be obtained. However, the amplicon that will be obtained for an isolate that belongs to the ND pathotype (423 bp) will be significantly shorter than the amplicon that will be obtained for an isolate that belongs to the D pathotype (694 bp) (Figure 4; Figure 5).

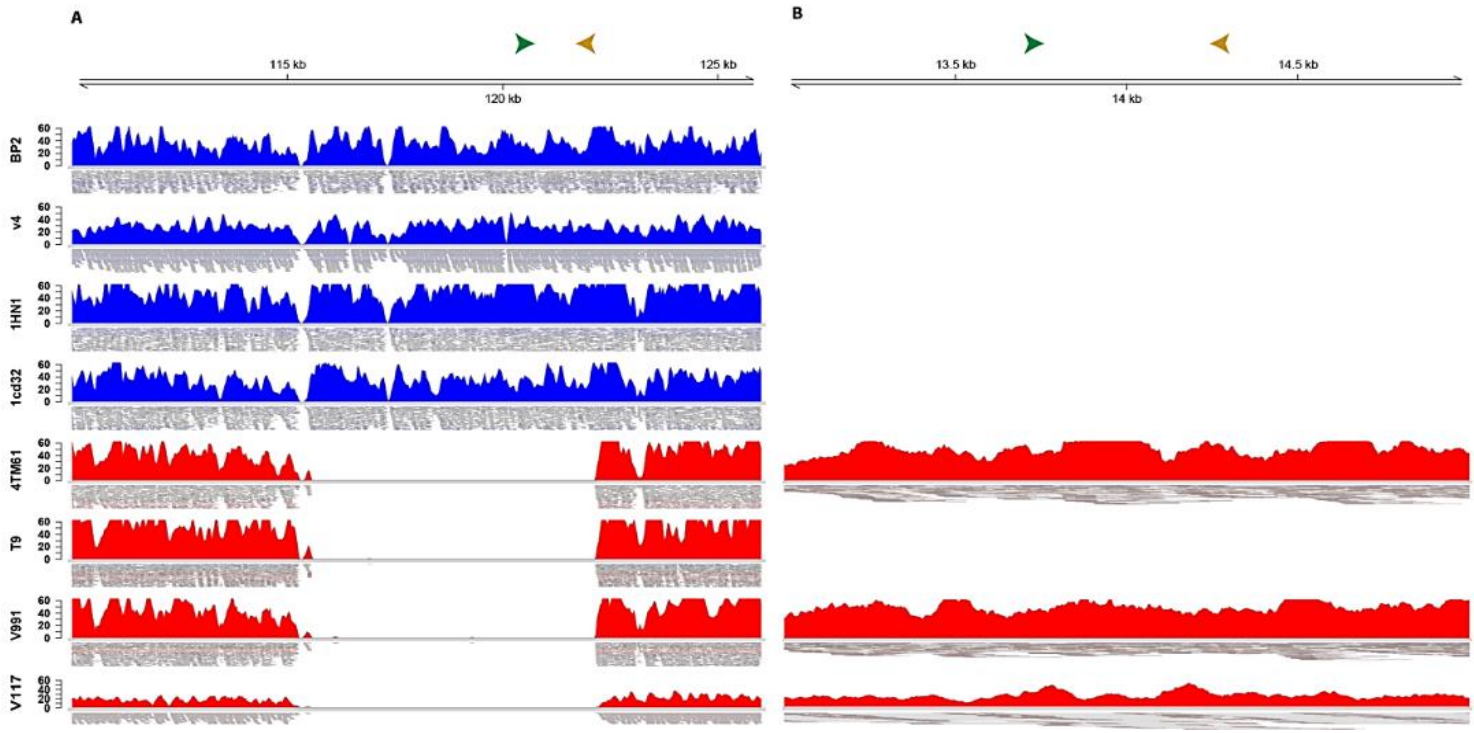


Figure 3. Genomic target region of the primer pairs used to discriminate isolates belonging to the ND (in blue) and D (in red) pathotypes, respectively. On the left the names of the isolates used for the genomic comparison are indicated. The region targeted by the ND and D primers were compared between isolates using the target BP2 ND region (A) and target V991 D region (B) as a reference, respectively. On top is indicated the size of the target region and the arrows indicate the annealing sites of the forward (green arrow) and reverse (yellow arrow) primers for both regions. In grey is represented the reads that map to the target region in the different isolates, while the depth of the coverage is indicated in colour.

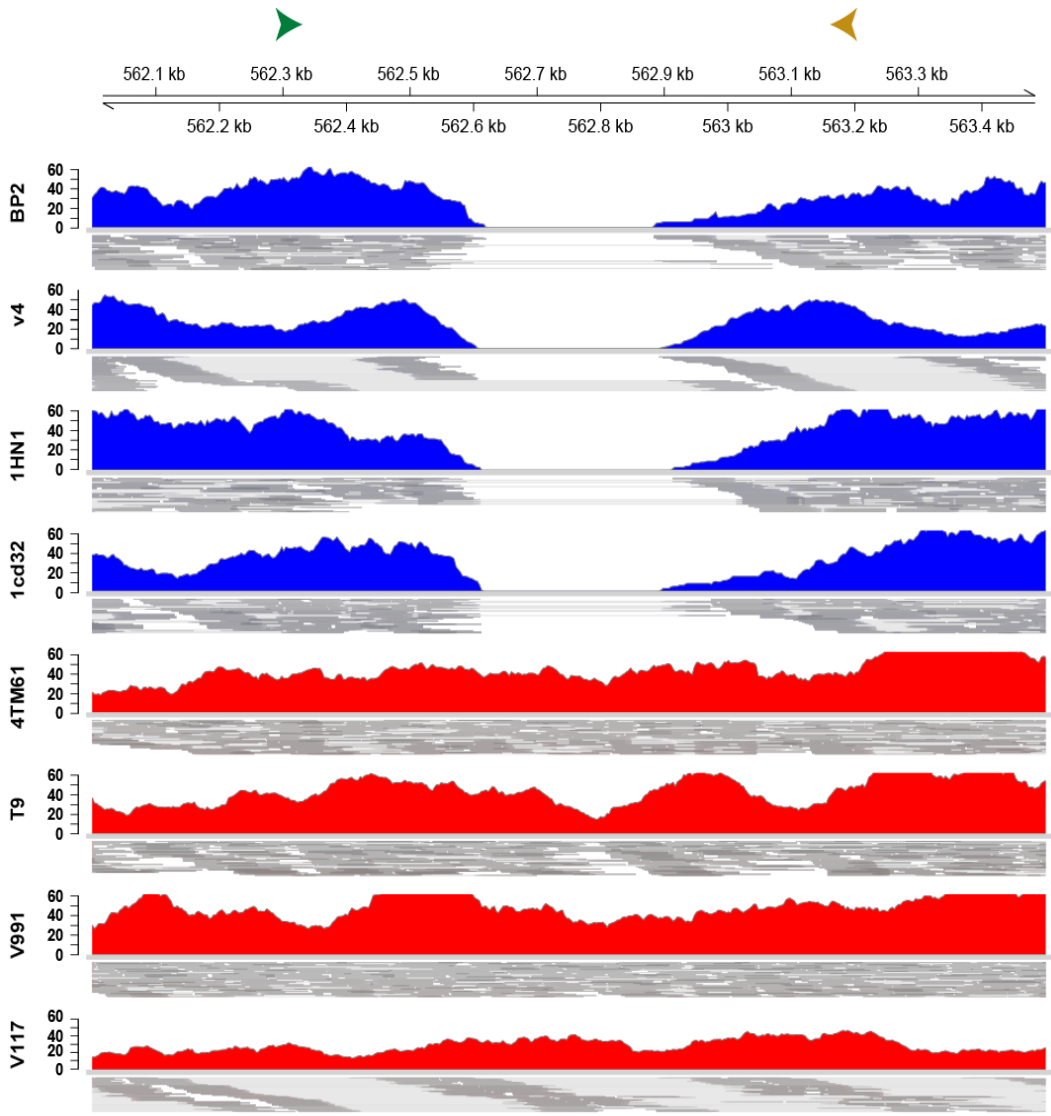


Figure 4. Genomic target region of the primer pairs developed to discriminate isolates belonging to the ND (in blue) and D (in red) pathotypes, respectively. On the left the names of the isolates used for the genomic comparison are indicated. The targeted region was compared between isolates using the target V991 regions as reference. On top is indicated the size of the target region and the arrows indicate the annealing sites of the forward (green arrow) and reverse (yellow arrow) primers for the region. In grey is represented the reads that map to the target region in the different isolates, while the depth of the coverage is indicated in colour.

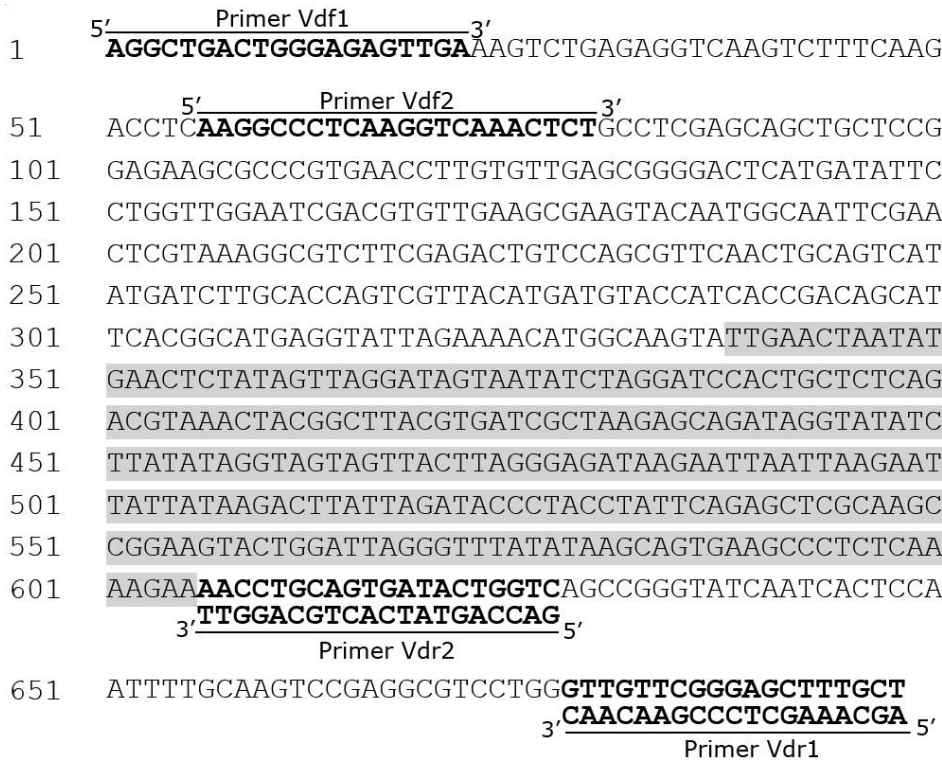


Figure 5. Nucleotide sequence of the target region and the position of the newly designed primer pairs Vdf1-Vdr1 and Vdf2-Vdr2 to discriminate isolates belonging to the ND or D pathotype, respectively. The area highlighted in grey indicates the deletion of 271 bp on scaffold V911_N.5.1 which occurs in all sequenced ND isolates.

Robust differentiation of D and ND isolates of V. dahliae. The newly designed primer pair Vdf1-Vdr1 (Table 2; Figure 5) was evaluated by testing on all D and ND isolates of *V. dahliae* used in this study (Table 1). As expected, amplicons were obtained for all tested *V. dahliae* isolates, including T9 and V76. Whereas the ND isolates yielded a 423 bp amplicon, the D isolates yielded a 694 bp amplicon (Figure 6). Thus, whereas the previously designed assay failed to assign the T9 and V76 isolates to the D pathotype, our newly designed assay correctly assigned them to the D pathotype by yielding a 694 bp amplicon. (Figure 6).

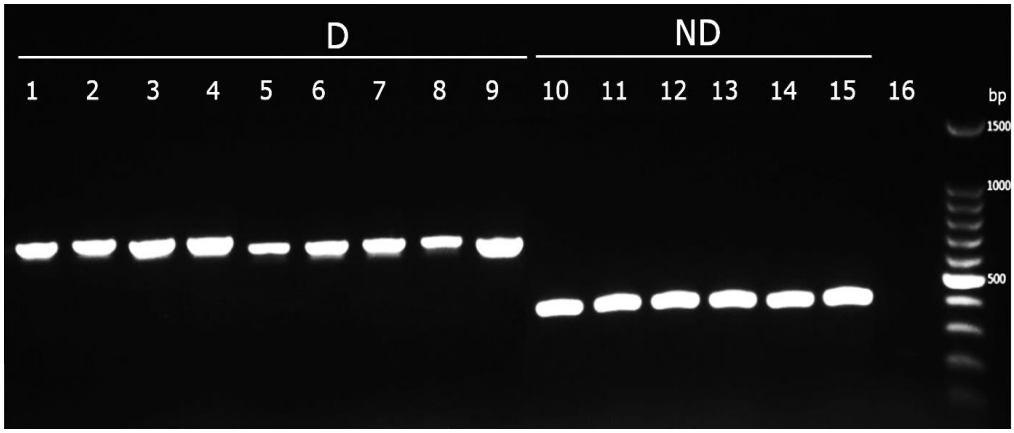


Figure 6. Detection of D (defoliating) and ND (non-defoliating) *V. dahliae* isolates with Vdf1-Vdr2 primers designed in this study. Lanes 1-9: *V. dahliae* D isolates:V117 (1); T9 (2); F4-A20 (3); F20-A83 (4); V-054 (5); V-071 (6); F3-A23 (7); V-991 (8); V-76 (9). Lanes 10-15: *V. dahliae* ND isolates: V4 (10); Bp2 (11); B1-3 (12); B4-10 (13); B4-12 (14); V-096 (15). Amplification reactions yielded a 694 bp band for all tested D isolates, and a 423 bp band for all tested ND isolates. Lane 16: negative control reaction with no DNA template.

The novel assay as well as the previously designed assay were further used to characterize ten *V. dahliae* isolates that were recovered from naturally infected olive trees in central Greece (Table 1). Remarkably, whereas the D1-D2 as well as the NDf-NDr primers did not produce an amplicon for any of these isolates (Figure 7A, 7B), the Vdf1-Vdr1 primers produced a 694 bp amplicon for all of them, revealing that they belong to the D pathotype (Figure 7C).

In planta detection of D and ND isolates of V. dahliae using nested PCR. The Vdf1-Vdr1 primers were used in PCR assays aimed at *in planta* detection of D and ND isolates of *V. dahliae*. To this end, eight samples from naturally infected olive trees were used. Infection of these trees was confirmed by real-time PCR, using a *V. dahliae*-specific primer pair designed on the internal transcribed spacer (ITS) region (van Doorn et al., 2009), through analysis of sub- samples containing fragments from different parts of twigs, and the fungus was detected at least in one sample per infected tree. For *V. dahliae* DNA detection by the newly designed assay, mixed DNA samples composed out of five twigs per tree were tested. However, PCR with the Vdf1-Vdr1 primers did not lead to a

visible amplification product, presumably due to a low amount of *V. dahliae* DNA in the total DNA that was isolated from the infected plant material (Figure 8A). Therefore, a nested-PCR procedure was designed to increase the sensitivity and improve detection of D and ND isolates *in planta*. To that end, Vdf2 and Vdr2 primers were designed (Figure 5) that were used for the second round of PCR. To this end, a 25 μl PCR reaction was performed with 1.5 μl of 20x diluted PCR mixture that resulted from the first round of PCR as template. Interestingly, the second round of PCR yielded amplicons for all tested samples, showing that the nested PCR assay was able to detect the *V. dahliae* infection (Figure 8B). Moreover, the assay yielded a 568 bp fragment for six of the samples, pointing towards infection with the D pathotype, and a 297 bp fragment for two of the samples, indicating infection with the ND pathotype of *V. dahliae* (Figure 8B).

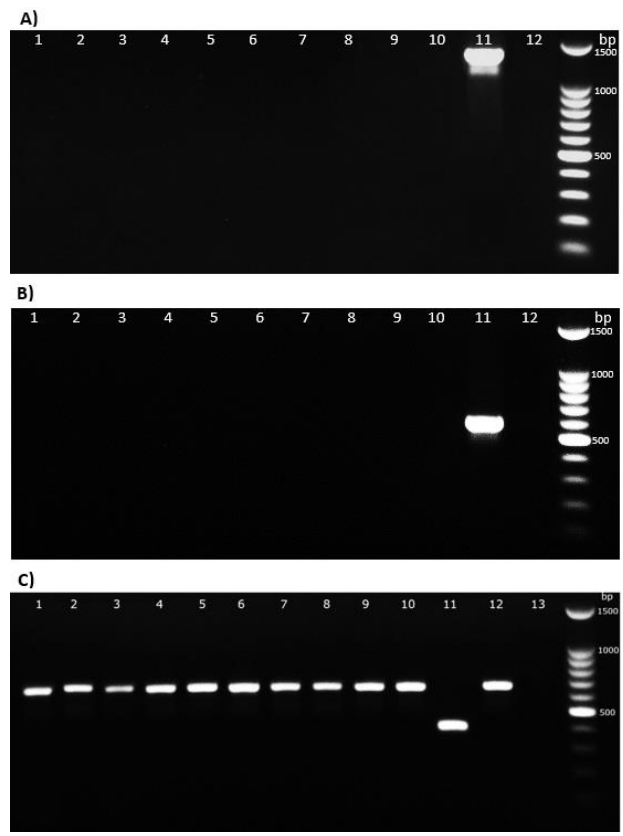


Figure 7. PCR results obtained with novel primers as well as the previously designed primers using DNA from 10 *V. dahliae* isolates recovered from naturally infected olive trees in central Greece. (A) PCR results obtained with NDf-NDr primers (Lanes 1-10). Lane 11 correspond to amplifications using genomic DNA from BP2 used as control for ND pathotype. Lane 12: negative control reaction with no DNA template. (B) PCR results obtained with D1-D2 primers (Lanes 1-10). Lane 11 correspond to amplifications using genomic DNA from VT9 used as control for D pathotype. Lane 12: negative control reaction with no DNA template. (C) PCR results obtained with Vdf1-Vdr2 primers (Lanes 1-10). Lanes 11 and 12 correspond to amplifications using genomic DNA from Bp2 and VT9 isolates used as controls for the ND and D pathotype, respectively. Lane 13: negative control reaction with no DNA template.

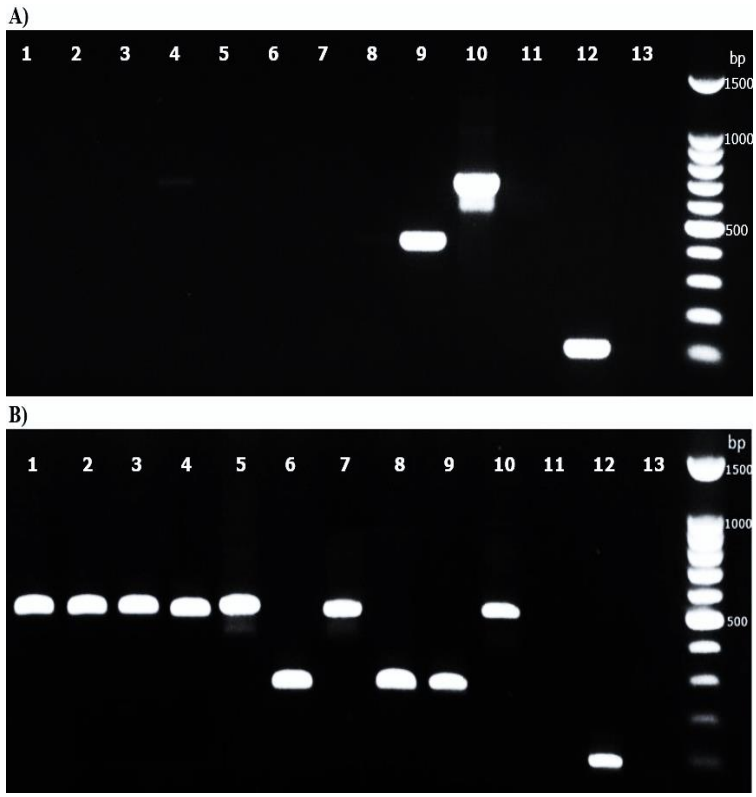


Figure 8. Nested PCR results obtained for total DNA samples extracted from twigs of infected olive trees. A) The first round of PCR carried out with Vdf1-Vdr1 primers. No amplification was detected after the first PCR round (Lanes 1-8). B) The second round of PCR was carried out using 1.5 µl of 20x diluted solution of mixture after the first round of PCR as template, using primer pair Vdf2-Vdr2. PCR reactions in the second round of PCR produced a 297 bp fragment for two of the samples (lanes 6 and 8), indicating infection with the ND pathotype of *V. dahliae*. PCR reactions in the second round of PCR produced a 568 bp fragment for six of the samples (lanes 1-5 and 7), indicating infection with the D pathotype of *V. dahliae*. Lanes 9 and 10 correspond to amplifications using genomic DNA from Bp2 and VT9 isolates used as controls for the ND and D pathotype, respectively, whereas lane 11 contains genomic DNA from non-infected olive; primers specific for the cytochrome oxidase (*COX*) gene were used as control for presence of plant DNA in sample from non-infected olive (lane 12). Lane 13: negative control reaction with no DNA template.

Discussion

V. dahliae isolates belonging to the D pathotype cause considerably more severe disease symptoms in olive and cotton than isolates belonging to the ND pathotype (Bell, 1994; Schnathorst and Mathre, 1966). Discrimination between D and ND pathotypes of *V. dahliae* isolates will provide information for assessment of disease risk as well as for

designing a more efficient disease control strategy through risk assessment (López-Escudero and Mercado-Blanco, 2011; Hiemstra, 2015). This is particularly important because of the alarming spread of highly virulent isolates of the D pathotype worldwide (Jiménez-Díaz et al., 2012). The D- and ND-specific primers that were previously designed based on pathotype-associated RAPD bands (Mercado-Blanco et al., 2001, 2002; Pérez-Artés et al., 2000) allowed for the discrimination of many D and ND isolates collected worldwide. Nevertheless, they failed amplification for particular *V. dahliae* isolates (Collins et al., 2005). This failure was confirmed in our study where the D-specific primers did not amplify a fragment for the D isolates T9 and V76. In our study, comparative population genomics revealed that the D-specific primers were developed on a genomic region that is not ubiquitously present in all D isolates, and that was indeed lacking in the isolates T9 and V76 (Figure 3B). Previously, Pérez-Artés et al. (2000) reported amplification of a D-specific band from the T9 isolate while we did not obtain an amplicon with the same primers. However, based on our findings, the T9 isolate indeed belongs to the D pathotype, but does not carry the target region of the D-specific primers.

The observation that particular genomic regions are not shared among all strains fits with the extensive intra- and inter-chromosomal rearrangements that have been observed among strains of *V. dahliae* (de Jonge et al., 2013; Seidl et al., 2014; Faino et al., 2015). This genomic plasticity is correlated with the appearance of lineage-specific regions that are unique to, or shared only by a subset of, *V. dahliae* isolates and that are implicated in aggressiveness (de Jonge et al., 2013; Seidl et al., 2015; Faino et al., 2015). Comparative genomics is a powerful tool to identify genomic similarities and differences for isolates within, and between, species, and to identify sequence differences that may be responsible for phenotypic differences among pathogen strains (Hu et al., 2011; De Jonge et al., 2012). Previously, through high-throughput population genome sequencing and comparative genomics it was found that a single locus (named *Ave1*) that is shared only by race 1 strains and encodes an effector that contributes to fungal virulence, is recognized by the tomato immune receptor Ve1 (de Jonge et al., 2013). In the present study, we similarly compared genomes of multiple D and ND strains of *V. dahliae* to find differences between these two pathotypes, aiming to design a marker for robust discrimination. This analysis

led to identification of a region absent in all sequenced ND isolates, while present from all D isolates. Then, a new set of primers was designed across this region to detect and discriminate all D and ND isolates. It is worth mentioning that this new method only uses one pair of primers to identify both pathotypes, whereas for previously described assays, two independent primers sets (one for each pathotype) were used in parallel (Mercado-Blanco et al., 2002; 2001; Pérez-Artés et al., 2000). Thus, our new method is time and cost efficient, and also more accurate as the whole typing is based on a single locus. Ideally, discrimination of pathotypes should be based on markers that are designed on the genetic element that is responsible for the phenotypic difference. Arguably, the difference in aggressiveness of the D and ND pathotypes is based on (a) lineage-specific effector(s) shared among D isolates. Although we illustrated that the region that is targeted by the current assay is lineage-specific, it is not the only genomic region that is differential among strains of the ND and the D pathotype. Thus, a causal relationship with aggressiveness needs to be further studied to find if there is (are) effector(s) encoded in this region.

Primers that we designed in this study also enabled characterization of 10 *V. dahliae* isolates recovered from infected olive trees from central Greece that in preliminary tests did not react with the available D/ND primers. Interestingly, the results of the PCR assay revealed that all these isolates belong to D pathotype. Thus far, only ND isolates were reported to occur in olive infections in Greece, although presence of the D pathotype had occasionally been reported in cotton fields (Elena and Paplomatas, 1998; 2001). Our assay identified infections of olive trees with the D pathotype for the first time in this region. Future surveys are required to assess the distribution of the D pathotype in central Greece.

For effective management of Verticillium wilt in olive, as well as in other field crops, efficient and reliable procedures for testing of planting material and of new planting sites for infection by *V. dahliae* are essential. In addition characterisation of the pathotype of available *V. dahliae* populations is needed. As the specific primers developed in this study enable detection and characterisation of the pathogen in one go they represent a major step towards the design of such procedures.

Acknowledgment

We thank dr. F. J. Lopez-Escudero (Department of Agronomy, University of Córdoba), who kindly supplied the isolate of *V. dahliae.*

References

Aljawasim, B., Vincelli, P. (2015). Evaluation of polymerase chain reaction (PCR)-based methods for rapid, accurate detection and monitoring of *Verticillium dahliae* in woody hosts by real-time PCR. Plant Disease 99, 866-873.

Bejarano-Alcázar, J., Blanco-López, M.A., Melero-Vara, J.M., Jiménez-Díaz, R.M. (1996). Etiology, importance, and distribution of Verticillium wilt of cotton in southern Spain. Plant Disease 80, 1233-1238.

Bejarano-Alcázar, J., Melero-Vara, J.M., Blanco-López, M.A., Jiménez-Díaz, R.M. (1995). Influence of inoculum density of defoliating and non-defoliating pathotypes of *Verticillium dahliae* on epidemics of Verticillium wilt of cotton in southern Spain. Phytopathology 85, 1474-1481.

Bell, A.A. (1994). Mechanisms of disease resistance in Gossypium species and variation in *Verticillium dahliae*. In G. A. Constable, and N. D. Forrester (Eds.), Proceedings of world cotton research conference. Melbourne, Australia: CSIRO pp. 225-235.

Berlanger, I., Powelson, M.L. (2005). Verticillium wilt. The Plant Health Instructor. DOI: 10.1094/PHI-I-PHI-I-2000-0801-01.

Bertels, F., Silander, O.K., Pachkov, M., Rainey, P.B., Van Nimwegen, E. (2014). Automated reconstruction of whole-genome phylogenies from short-sequence reads. Molecular Biology and Evolution 31, 1077-1088.

Carder, J.H., Morton, A., Tabrett, A.M., and Barbara, D.J. (1994). Detection and differentiation by PCR of subspecific groups within two Verticillium species causing vascular wilts in herbaceous hosts. Pages 91-97 in: Modern Assays for Plant Pathogenic Fungi. A. Schots, F. M. Dewey, and R. Oliver, eds. CAB International, Wallingford, UK.

Collins, A., Mercado-Blanco, J., Jiménez-Díaz, R. M., Olivares, C., Clewes, E., Barbara, D. J. (2005). Correlation of molecular markers and biological properties in *Verticillium dahlia*e and the possible origins of some isolates. Plant Pathology 54, 549-557.

De Jonge, R., Peter van Esse, H., Maruthachalam, K., Bolton, M.D., Santhanam, P., Keykha Saber, M., Zhang, Z., Usami, T., Lievens, B., Subbarao, K.V., Thomma, B.P.H.J. (2012). Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. Proceedings of the National Academy of Sciences of the United States of America 109(13), 5110-5115.

De Jonge, R., Bolton, M.D., Kombrink, A., van den Berg, G.C.M., Yadeta, K. A., Thomma, B.P.H.J. (2013). Extensive chromosomal reshuffling drives evolution of virulence in an asexual pathogen. Genome Research 23(8), 1271-1282.

Elena, K., Paplomatas, E.J. (1998). Vegetative compatibility groups within *Verticillium dahliae* isolates from different hosts in Greece. Plant Pathology 47, 635-640.

Elena, K., Paplomatas, E.J. (2001). The defoliating strain of *Verticillium dahliae* on cotton: First report for Greece. Phytopathology Mediterranean 40, 70.

Faino, L., Seidl, M.F., Datema, E., van den Berg, G.C.M, Janssen, A., Wittenberg, A.H.J., Thomma, B.P.H.J. (2015). Single-molecule real-time sequencing combined with optical mapping yields completely finished fungal genome. mBio 6(4):e00936-15. doi:10.1128/mBio.00936-15.

Fradin, E.F., and Thomma, B.P.H.J. (2006). Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Molecular Plant Pathology 7, 71-86.

Hiemstra, J.A. (2015). Control of Verticillium wilt in olive; guide of best practices. EU-Vertigeen project (FP7-SME-2011-2-286140).
[http://www.vertigeen.eu/files_media/1429516669_vertigeen_deliverable_6_13_guide_of_best_practices_final.pdf]

Hu, B., Xie, G., Lo, C., Starkenburg, S.R, Chain, P.S.G. (2011). Pathogen comparative genomics in the next-generation sequencing era: genome alignments, pangenomics and metagenomics. Briefings in Functional Genomics 10, 322-333.

Joaquim, T. R., and Rowe, R. C. (1991). Vegetative compatibility and virulence of strains of *Verticillium dahliae* from soil and potato plants. Phytopathology 81, 552-558.

Jiménez-Díaz, R. M., Mercado-Blanco, J., Olivares-García, C., ColladoRomero, M., Bejarano-Alcázar, J., Rodríguez-Jurado, D., Giménez-Jaime, A., García-Jiménez,J., and Armengol, J. (2006). Genetic and virulence diversity in *Verticillium dahliae* populations infecting artichoke in eastern central Spain. Phytopathology 96, 288-298.

Jiménez-Díaz, R.M., Rodríguez Jurado, D., Landa del Castillo, B.B., Trapero Casas, J.L., Navas Cortés, J.A. (2008). Dispersión de la Verticilos is a través de las hojas de olivos infectadas por el patotipo defoliante. Vida Rural 265, 40-44.

Jiménez-Díaz, R.M, Cirulli, M., Bubici, G., Jiménez-Gasco, M., Antoniou, P.P., Tjamos, E.C. (2012). Verticillium wilt a major threat to olive production: Current status and future prospects for its management. Plant Disease 96(3), 304-329.

Klosterman, S.J., Atallah, Z.K., Vallad, G.E., Subbarao, K.V. (2009). Diversity, pathogenicity and management of *Verticillium* species. Annual Review of Phytopathology 47, 39-62.

Langmead, B., Trapnell, C., Pop, M., Salzberg, S.L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biology 10(3), R25.

López-Escudero, F.J., Mercado-Blanco, J. (2011). Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. Plant Soil 344, 1-50.

Mace, M.E., Stipanovic, R.D., Bell, A.A. (1990). Relation between sensitivity to terpenoid phytoalexins and virulence to cotton of *Verticillium dahliae* strains. Pesticide Biochemistry and Physiology 36 (1), 79-82.

Mathre, D., Erwin, D.C., Paulus, A.O., Ravenscroft, A.V. (1966). Comparison of the virulence of isolates of Verticillium albo-atrum from several of the cotton-growing regions in the United States, Mexico and Peru. Plant Disease Report 50, 930-933.

Mercado-Blanco, J., Rodríguez-Jurado, D., Perez-Artes, E., Jiménez-Díaz, R.M. (2001). Detection of the nondefoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR. Plant Pathology 50, 609-19.

Mercado-Blanco, J., Rodríguez-Jurado, D., Pérez-Artés, E., Jiménez Díaz, R.M. (2002). Detection of the defoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR. European Journal Plant Pathology 108, 1-13.

Mercado-Blanco, J., Rodríguez-Jurado, D., Parrilla-Araujo, S., Jiménez-Díaz, R.M. (2003). Simultaneous detection of the defoliating and nondefoliating *Verticillium dahliae* pathotypes in infected olive plants by duplex, nested polymerase chain reaction. Plant Disease 87, 1487-1494.

Pegg, G.F., Brady, B.L. (2002). Verticillium wilts. Wallingford, UK: CABI publishing.

Pérez-Artés, E., Garcia-Pedrajas M., Bejarano-Alcázar D., Jime-nez-Díaz J.R.M. (2000). Differentiation of cotton-defoliating and nondefoliating pathotypes of *Verticillium dahliae* by RAPD and specific PCR analyses. European Journal Plant Pathology 106, 507-517.

Pérez-Artés, E., Mercado-Blanco, J., Ruz-Carrillo, A.R., Rodríguez-Jurado, D., Jiménez-Díaz, R.M. (2005). Detection of the defoliating and nondefoliating pathotypes of *Verticillium dahliae* in artificial and natural soils by nested PCR. Plant Soil 268, 349-356.

Puhalla, J.E., Hummel, M. (1983). Vegetative compatibility groups within *Verticillium dahliae*. Phytopathology 73, 1305-1308.

Quinlan, A.R., Hall, I.M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 26, 841-842.

Rodríguez-Jurado, D., Blanco-López, M.A., Rappoport, H.F., Jiménez-Díaz, R.M. (1993). Present status of Verticillium wilt of olive in Andalucía (southern Spain). European and Mediterranean Plant Protection Organization (EPPO) Bulletin 23, 513-516.

Seidl, M. F., Faino, L., Shi-Kunne, X., van den Berg, G. C., Bolton, M. D., and Thomma, B. P. (2015). The Genome of the Saprophytic Fungus *Verticillium tricorpus* Reveals a Complex Effector Repertoire Resembling That of Its Pathogenic Relatives. Molecular Plant Microbe Interactions 28(3), 362-373.

Seidl, M.F., Thomma, B.P.H.J. (2014). Sex or no sex: evolutionary adaptation occurs regardless. Bioessays 36, 335–345.

Schnathorst, W.C., Mathre, D.E. (1966). Host range and differentiation of a severe form of *Verticillium albo-atrum* in cotton. Phytopathology 56, 1155-1161.

Schnathorst, W.C., Sibbett, G.S. (1971). The relation of strains of *Verticillium albo-atrum* to severity of Verticillium wilt in *Gossypium hirsutum* and *Olea europaea* in California. Plant Disease Reporter 9, 780-782.

Smith, I.M., Dunez, J., Lelliott, R.A., Phillips, D.H., Archer, S.A. (1988). European handbook of plant diseases. Blackewell Scientific Publications, Oxford etc., 583 S.

Tritt, A., Eisen, J.A., Facciotti, M.T., Darling, A.E. (2012). An Integrated Pipeline for de Novo Assembly of Microbial Genomes. PLoS ONE 7(9): e42304.

Van Doorn, J., Pham, K. T. K., Hiemstra, J. A., (2009). Molecular detection of *Verticillium dahliae* in soil. 10th International Verticillium Symposium, abstract book p. 94.

Weller, S. A., J. G. Elphinstone, N. C. Smith, N. Boonham, and D. E. Stead., (2000). Detection of Ralstonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Applied Environmental Microbiology 66, 2853-2858.

Xu, F., Yang, L., Zhang, J., Guo, X., Zhang, X., Li, G. (2012). Prevalence of the defoliating pathotype of Verticillium dahliae on cotton in central China and virulence on selected cotton cultivars. Journal of Phytopathology 160, 369-376.

Zhengjun, X., Achar, P. N., and Gu, B. (1998). Vegetative compatibility grouping of Verticillium dahliae from cotton in mainland China. European Journal of Plant Pathology 104, 871-876.

Chapter 5

Distribution of defoliating and non-defoliating *V. dahliae* isolates in root-inoculated olive trees that display differential levels of susceptibility

M. Keykhasaber[a,b,e], G.M. Garcia-Ruiz[c], M. Pérez-Rodríguez[d], B.P.H.J. Thomma[a], J.A. Hiemstra[b]

[a] Laboratory of Phytopathology, Wageningen University & Research, 6708 PB Wageningen, The Netherlands

[b] Wageningen Plant Research, Wageningen University & Research, P.O. Box 200, 6670 AE Zetten, The Netherlands

[c] IFAPA Center Alameda del Obispo, 14080 Córdoba, Spain

[d] Department of Agronomy, Cordoba University, Campus of Rabanales, 14071 Córdoba, Spain

[e] Department of Plant Biotechnology, Zabol University, Zabol, Iran

This chapter has been submitted for publication in Plant Pathology

Abstract

Verticillium wilt, caused by *Verticillium dahliae* Kleb., is the main disease of olive (*Olea europaea* L.) in most producer countries. In this study the distribution of a defoliating (V117) and a non-defoliating (V4) isolate of *V. dahliae* in young root-dip inoculated plants of a susceptible (Picual) and a partially resistant (Frantoio) cultivar of olive was studied by using real-time PCR. The infection and colonization processes of *V. dahliae* in these cultivars was also studied through microscopy by using GFP-labelled isolates of both *V. dahliae* isolates. *V. dahliae* was detected by real-time PCR in the lower, middle and top parts of inoculated plants in all treatment groups at only one week after inoculation and several weeks before symptom development. During the whole experiment an uneven distribution of pathogen DNA was observed along the main stem of the inoculated plants, with the highest levels being found in the lower part of the stems in all treatments. Microscopical analysis of inoculated plants illustrated that *V. dahliae* in the stem of inoculated plants is limited to the xylem during the phase of symptom development. With real-time PCR, we also observed lower amounts of both *V. dahliae* isolates in 'Frantoio' associated with significant lower disease severity when compared with 'Picual' for which higher amounts of *V. dahliae* were observed associated with higher disease severity. In each cultivar isolate V117 caused higher disease severity than isolate V4, and the amount of *V. dahliae* detected in V117-inoculated plants was higher than in V4-inoculated plants. Thus, both olive genotype and pathogen pathotype contribute to the observed differences in *V. dahliae* colonization of olive trees.

Introduction

Olive (*Olea europaea* L.) is one of the most ancient cultivated plants and the only species within the *Oleaceae* family with edible fruit. Nowadays, olive is a crop with a huge economic, social and ecological importance especially within the Mediterranean Basin (Blázquez-Martínez 1996; Civantos 2004). However, in most of the producer countries this crop is affected by Verticillium wilt, causing serious concern to growers, nursery companies and the olive-oil industry (Jiménez-Díaz et al. 2012; López-Escudero and Mercado-Blanco 2011). Verticillium wilt of olive is caused by *Verticillium dahliae* Kleb., and disease incidence and symptom severity strongly depend on the virulence of the

infecting *V. dahliae* isolate, being classified as belonging to the defoliating (D) or non-defoliating (ND) pathotype (Jiménez-Díaz et al. 2011; López-Escudero et al. 2004, 2007; Rodríguez-Jurado et al. 1993). The D isolates causes infections that can be deadly to olive trees, whereas ND isolates usually do not produce severe disease symptoms and plants may even recover from the disease (Jiménez-Díaz et al. 1998; Tjamos et al. 1991).

V. dahliae infection and colonization of woody hosts have been reviewed by Hiemstra (1998). In trees, including olive, *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 by *V. dahliae* has been studied by several research groups (e.g. Baídez et al. 2007; Prieto et al. 2009). However, the amount of information about differences in distribution of *V. dahliae* D and ND isolates in susceptible and (partially) resistant plants is limited.

The purpose of this study was: (I) to study the distribution of D and ND *V. dahliae* isolates in young root-inoculated olive cultivars with different levels of susceptibility, using real-time PCR, and (II) to study the infection and colonization processes of *V. dahliae* in these trees by using GFP-labelled isolates in a microscopical analysis.

Material and methods

Plant and fungal material. Nine-month-old plants (70-100 cm height) of the olive cultivars 'Picual' (susceptible to isolates that belong to the D and ND pathotype), and 'Frantoio' (partially resistant to isolates that belong to the D pathotype and resistant to those of the ND pathotype) (López-Escudero et al. 2004), were provided by F. J. López-Escudero (Department of Agronomy, University of Córdoba, Spain) from a nursery in Spain and used in two experiments:

Experiment 1. In this experiment 100 plants of each of the two cultivars were used. While 45 plants of each cultivar were inoculated with *V. dahliae* isolate V117 (D), 45 plants were inoculated with *V. dahliae* isolate V4 (ND) and ten plants were root-dipped in sterile water before planting and used as controls. While ten plants of each treatment were kept to monitor disease development, the other plants were used for sampling at regular intervals after the inoculation. The two *V. dahliae* isolates were supplied from the collection of the Laboratory of Plant Pathology, Department of Agronomy, University of Córdoba. Inoculum was prepared by adding small fragments from a potato dextrose agar (PDA) culture to liquid Czapek-Dox medium in Erlenmeyer flasks that were put in a shaker at 100 rpm at room temperature in the dark for about seven days to allow conidiospores to be produced. After filtration over cheese cloth, the conidiospore suspension was centrifuged to remove growth medium, and the pellet was resuspended in sterile water. Then, the concentration of conidia was determined and the suspension was diluted to a concentration of 10^7 conidia/ml. For inoculation, the root system of healthy plants was dipped in the conidiospore suspension for 20 minutes (López-Escudero et al. 2004). Control trees were root-dipped in sterile water. After inoculation, the plants were potted in a coco peat mix in 2 L containers and placed for 15 weeks in a 64 m^2 greenhouse on tables according to a complete randomized design at 70% relative humidity and a temperature of 18±3°C at night and 22±3°C during daytime, with a light/dark regime of 16/8 hours. The plants received 100 Wm^{-2} supplemental lighting (Agro SON-T, 400 W lamps) when the sunlight influx intensity was less than 150 Wm^{-2}.

Experiment II. In this experiment, 80 of each of the two cultivars were used. While 35 plants of each cultivar were root-dip inoculated with a GFP-labelled isolates of the *V. dahliae* isolate V117 (defoliating), 35 plants were inoculated with GFP-labelled *V. dahliae* isolate V4 (non-defoliating) and 10 control trees were root-dipped in sterile water before planting. GFP-labelled strains of V4 and V117 were generated by means of *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Santhanam, 2012). The inoculum was prepared and the inoculation was conducted as described for experiment 1. Inoculated plants were grown for 13 weeks in the same greenhouse and under the same conditions as the plants in experiment 1.

Disease assessment. Disease severity was monitored by rating disease symptoms on 10 trees per treatment at 2-week intervals on a severity scale from 0 to 4, based on the percentage (estimated by visual inspection) of plant tissue affected by chlorosis, leaf and shoot necrosis or defoliation (0 = plant without symptoms of Verticillium wilt; 1 = 1% to 33% of the plant affected; 2 = 34% to 66% of the plant affected; 3 = 67% to 99% of the plant affected; 4 = dead plant) (López-Escudero et al. 2004).

DNA isolation. To quantify the amount of *V. dahliae* DNA, total genomic DNA was extracted from stem samples from lower, middle and top parts of the stems of inoculated plants. Each time disease symptoms were recorded, also five plants per treatment were sampled. Stem fragments of about 10 cm length were washed under running tap water for 1-2 minutes, dried with cleaning paper, and left to dry for a few minutes on cleaning paper. Then, the bark was removed under sterile conditions and small (2-5 mm) pieces of woody tissue (300-400 mg) were taken by using a sterilized scalpel (flamed after submergence in 70% ethanol) and transferred to a 2 mL tube containing 1 mL of lysis buffer AP1 of the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and 4-5 stainless steel beads (3.2 mm diameter, BioSpec, Bartlesville, US/Canada). Next, the tubes were incubated for 15-30 min at 65˚C and then shaken in a Retsch® mixer mill (MM 400, Retsch, Haan, Germany) for 15 minutes at 30 Hz. After centrifugation at 10,000 rpm for 5 minutes, 400 µL of the suspension was used for total genomic DNA extraction using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA amounts were quantified using a BioPhotometer (Eppendorf AG, Hamburg, Germany) and concentrations were equalized by adding elution buffer or DNase-free water.

Quantification assay. Real-time PCR assays were performed using a *V. dahliae*-specific primer pair designed on the internal transcribed spacer (ITS) region (van Doorn et al., 2009) (VerDITSF: 5'-CCGGTCCATCAGTCTCTCTG-3', VerDITSRk: 5'-CACACTACATATCGCGTTTCG-3') and a primer pair for the plant cytochrome oxidase (*COX*) gene (Weller et al. 2000) to quantify the amount of *V. dahliae* and plant DNA, respectively. All real-time PCR reactions were performed in a STRATAGENE Max 3000P™ real-time PCR machine (Agilent Technologies, Santa Clara, United States).

The real-time PCR program consisted of an initial step of denaturation for 10 min at 95˚C, followed by 45 cycles of 15 sec at 95˚C, 40 sec at 62˚C, and 40 sec at 72˚C. A melting curve program was also run for which measurements were made at 0.5˚C temperature increments every 10 s within a range of 60-100˚C. Amplification was carried out in a 25 µl final reaction volume containing 1.5 µl of DNA extract, 10 nM of each primer, 12.5 µl of SYBR Green Supermix (2X) and sterile nuclease-free water to reach the appropriate volume. Each run also included a negative control containing nuclease-free water instead of DNA, as well as a negative control containing DNA isolated from disease free olive leaves. Two or three simultaneous replicates (technical repeats) were carried out for each sample. The quantities of *V. dahliae* and plant DNA were determined using a standard curve by plotting the logarithm of a ten-fold dilution series prepared from 10 ng/µl DNA suspension of *V. dahliae* isolate V117, and a ten-fold dilution series prepared from 10 ng/µl plant (olive) DNA suspension, respectively, against the threshold cycle (Ct) obtained in the real-time PCR assays. The relative quantity of *V. dahliae* DNA in the tested samples was calculated based on the quantity of *V. dahliae* DNA (ng) in 100 ng total DNA (i.e. including pathogen and plant DNA as quantified by simultaneously conducting plant-specific real-time PCR and pathogen-specific real-time PCR) isolated from inoculated plant tissues.

***Data analysis*.** Based on disease severities assessed on 10 trees for each treatment at different time points, the area under the disease progress curve percentage (AUDPCP) was calculated with the formula of Campbell and Madden (1990):

$$\text{AUDPCP} = \left[(t/2) * \left(S_2 + 2 * S_3 + \ldots + S_i\right)/4 * n\right] * 100$$

(t = days between observations; S = mean severity; Si = final mean severity; 4 = maximum disease rating; n = number of observations).

Also, based on the relative quantities of pathogen DNA obtained from 5 trees for each treatment at different time points, the area under the quantity progress curve percentage (AUQPCP) was calculated with the modified formula of Campbell and Madden (1990):

$$\text{AUQPCP} = \left[(t/2) * \left(S_2 + 2 * S_3 + ... + S_i\right)/n\right] * 100$$

(t = days between observations; S = relative quantity; Si = final relative quantity; 1= maximum relative quantity; n = number of testing time points).

Data obtained from disease assessment (AUDPCP) and quantification of pathogen DNA in stems of inoculated trees (AUQPCP) were subjected to analysis of variance (ANOVA) for a complete randomized design, using the SPSS program. Mean values of AUDPCP as well as mean values of AUQPCP were compared using the Fisher protected LSD at *P=0.05*.

Results

Progression of Verticillium wilt in inoculated olive plants. Initially, 45 olive plants that belong to the 'Frantoio' and 'Picual' cultivars were inoculated with V117 and V4 isolates of *V. dahliae* that are representatives of the D and the ND pathotype, respectively, while ten plants from each cultivar were used as non-inoculated controls. To monitor disease development, ten plants of each combination of olive cultivar and *V. dahliae* isolate ('Frantoio'/V117, 'Frantoio'/V4, 'Picual'/V117, 'Picual'/V4) were used for assessment of disease severity at 2-week intervals. At each time point, disease severity on ten trees per treatment was assessed by visually estimating the percentage of plant tissue affected by chlorosis, leaf and shoot necrosis or defoliation, and rated on 0 (plant without symptoms), 1 (1% to 33% of the plant affected), 2 (34% to 66% of the plant affected), 3 (67% to 99% of the plant affected), and 4 (dead plant) severity scales. The first obvious symptoms on 'Picual' plants inoculated with the V117 and V4 isolates were recorded at 7 weeks post inoculation (wpi), while the first symptoms on 'Frantoio' plants were observed only at 9 wpi. From 9 wpi onwards, disease severity slightly increased in all four combinations of olive cultivar and *V. dahliae* isolate. At the end of the experiment at 15 wpi, the highest disease severity was observed in 'Picual' plants inoculated with isolate V117, whereas the lowest disease severity occurred in 'Frantoio' plants inoculated with isolate V4 (Figure 1A). Based on the disease severities assessed at different time points, the area under the disease progress curve percentage (AUDPCP) was calculated for each

combination. Statistical analysis of AUDPCP data showed that for both isolates the severity of disease in 'Picual' plants was significantly higher than the disease severity in 'Frantoio' plants, confirming that 'Picual' is more susceptible to Verticillium wilt than 'Frantoio'. Furthermore, analysis of the AUDPCP also revealed that in both cultivars the disease severity caused by isolate V117 was significantly higher (P=0.05) than the disease severity caused by isolate V4 (Figure 1B).

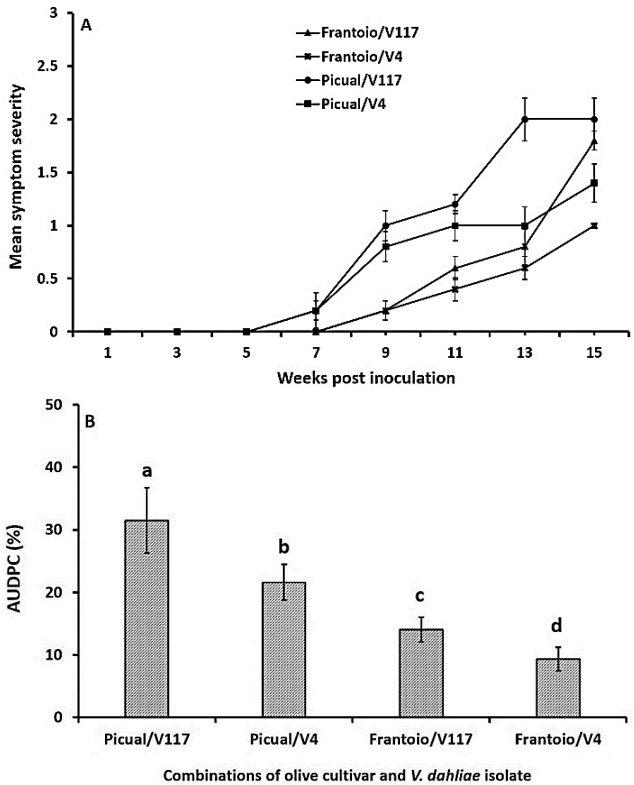
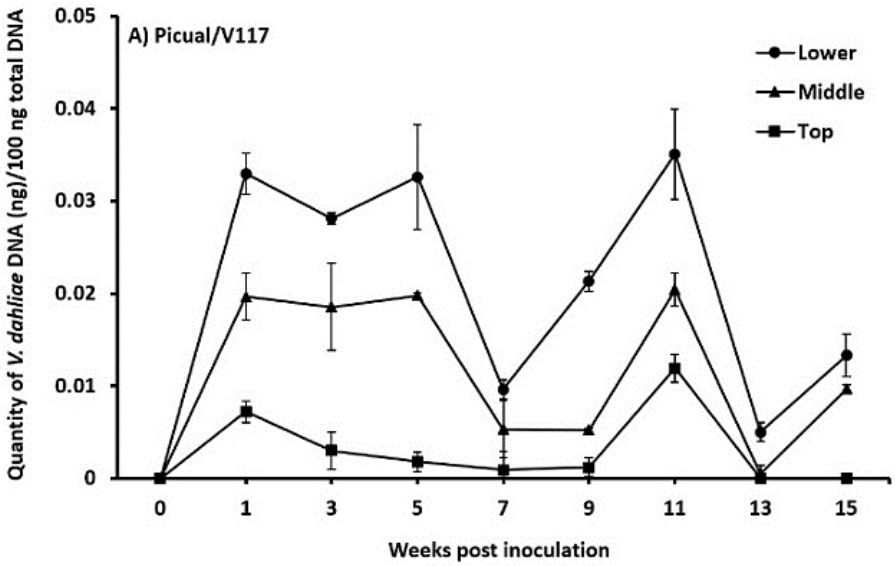
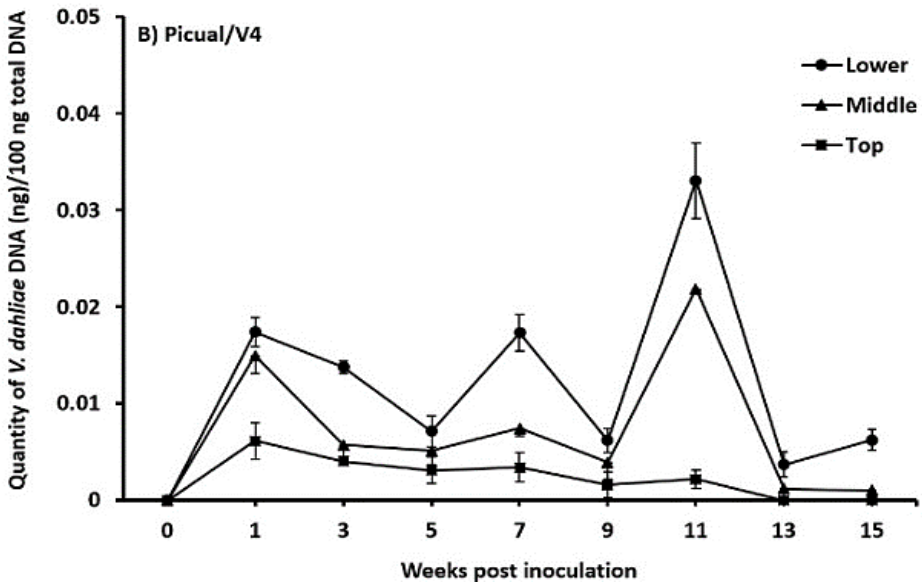
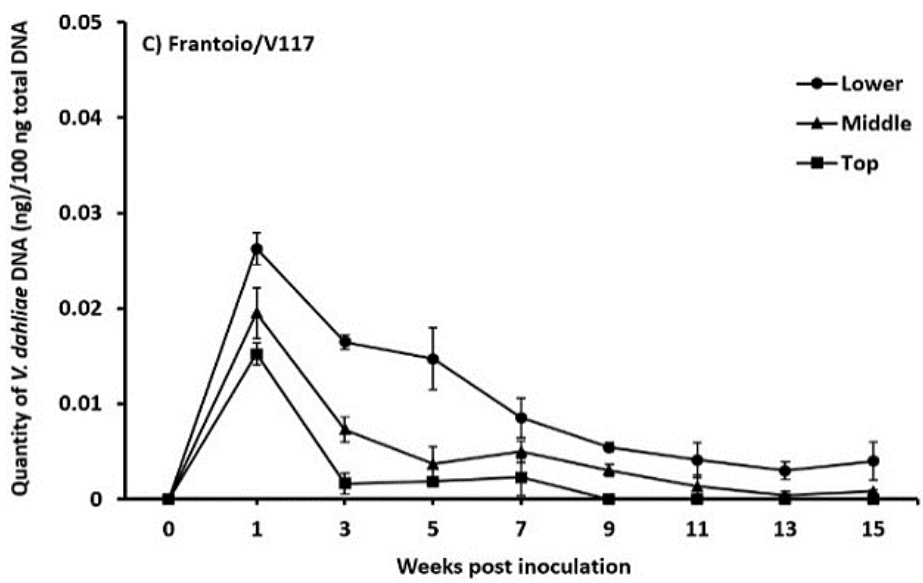


Figure 1. Progression of Verticillium wilt disease in young 'Frantoio' and 'Picual' olive plants that were root-dip inoculated with 10^7 conidia mL^{-1} of defoliating (V117) and nondefoliating (V4) *V. dahliae* isolates. (A) Verticillium wilt disease progression curves. Severity of symptoms was assessed on a 0-4 rating scale (0 = plant without symptoms of Verticillium wilt; 1 = 1% to 33% of the plant affected; 2 = 34% to 66% of the plant affected; 3 = 67% to 99% of the plant affected; 4 = dead plant). Each plotted point is the mean value of 10 plants. (B) Area under the disease progress curve percentage (AUDPCP) for 'Frantoio' and 'Picual' plants inoculated with V117 and V4 isolates of *V. dahliae*. The AUDPCP was calculated by the trapezoidal integration method of Campbell and Madden (1990). Each bar represents the mean value of 10 trees with error bars showing standard deviation. Mean values of all treatment were compared based on the T-test analysis and different letters above bars indicate significant differences between mean values (P=0.05).

Quantification of V. dahliae DNA in inoculated plants. Distribution of V117 and V4 isolates of *V. dahliae* in inoculated 'Frantoio' and 'Picual' cultivars of olive in experiment 1 was studied by real-time PCR quantification of *V. dahliae* DNA in samples collected from lower, middle and top parts of five infected plants at different intervals after inoculation. At each time point, mean relative quantities of *V. dahliae* DNA (means of five plants per time point) were assessed for each part separately for all four treatments and plotted against sampling time points (Figure 2). Already at one week after inoculation, i.e. well before the onset of symptoms, *V. dahliae* DNA was detected in all parts of the inoculated plants for all treatments. The amount of *V. dahliae* V4 and V117 DNA detected in 'Picual' plants fluctuated strongly during the 15 weeks after inoculation, reaching a peak of 0.033 ng/100 ng total DNA in the Picual/V4 combination and 0.035 ng/100 ng total DNA in the Picual/V117 combination in samples from the lower part at 11 wpi. In both Picual/V4 and Picual/V117 combinations the amount of *V. dahliae* bottomed at 13 wpi (Figure 2A, 2B). In contrast, in 'Frantoio' plants the amount of *V. dahliae* V4 and V117 continuously decreased from the first week after inoculation onwards (Figure 2C, 2D). Overall, the maximum amounts of DNA of both isolates were detected in the lower parts of inoculated trees of both cultivars, while at any given time point the quantities detected in the top parts were generally lower than those in the lower parts with quantities in the middle parts being intermediate (Figure 2).




B) Picual/V4
Lower
Middle
Top
Quantity of V. dahliae DNA (ng)/100 ng total DNA
0.05
0.04
0.03
0.02
0.01
0
0
1
3
5
7
9
11
13
15
Weeks post inoculation


C) Frantoio/V117
Lower
Middle
Top
Quantity of V. dahliae DNA (ng)/100 ng total DNA
0.05
0.04
0.03
0.02
0.01
0
0
1
3
5
7
9
11
13
15
Weeks post inoculation

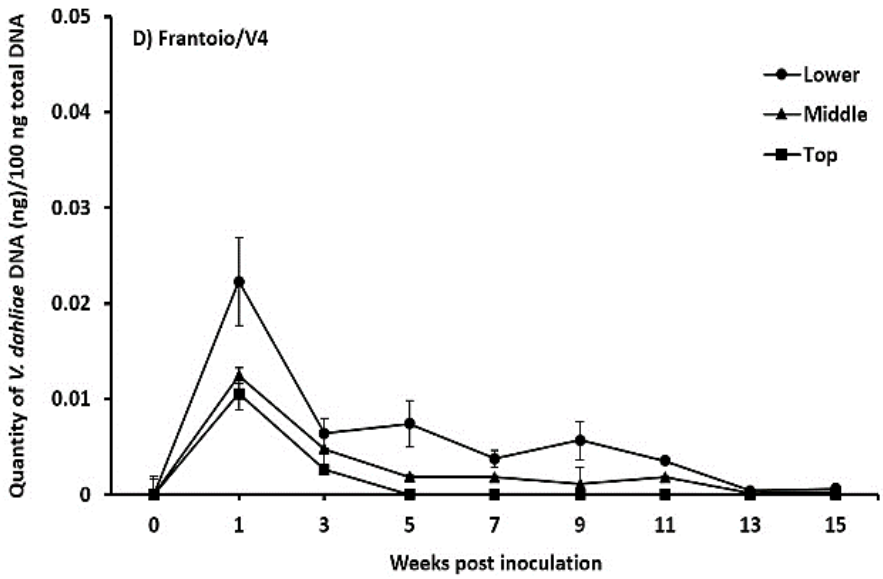


Figure 2. Amount of *V. dahliae* DNA (ng) in 100 ng total DNA extracted from lower, middle and top parts of 'Frantoio' and 'Picual' olive trees inoculated with *V. dahliae* isolate V117 (A, C) and V4 (B, D). Quantification assays were run at nine time points during 15 weeks after inoculation. Each value is the mean of five replications (trees). Error bars show standard deviations.

At the end of the experiment (15 wpi), the area under quantity progress curve percentage (AUQPCP) of *V. dahliae* DNA was calculated for each part separately for all four treatments. Analysis revealed that there were significant differences between different parts of the inoculated olive trees (P=0.05) (Figure 3). Also, mean quantities of *V. dahliae* DNA in trees of each treatment group were calculated at each time point by averaging the amounts detected in different parts (lower, middle and top) of five trees at each time point. The AUQPCP analysis of mean values showed that quantities of both isolates detected in 'Frantoio' plants were significantly lower than quantities in 'Picual' plants, while the amount of V117 in both cultivars was significantly higher than the amount of V4 (P=0.05) (Figure 4). These results were consistent with the results of the AUDPCP analysis in which V117 caused significantly more disease symptoms than V4 in both cultivars, and the severity of disease in 'Picual' was significantly higher than that in 'Frantoio' (P=0.05).

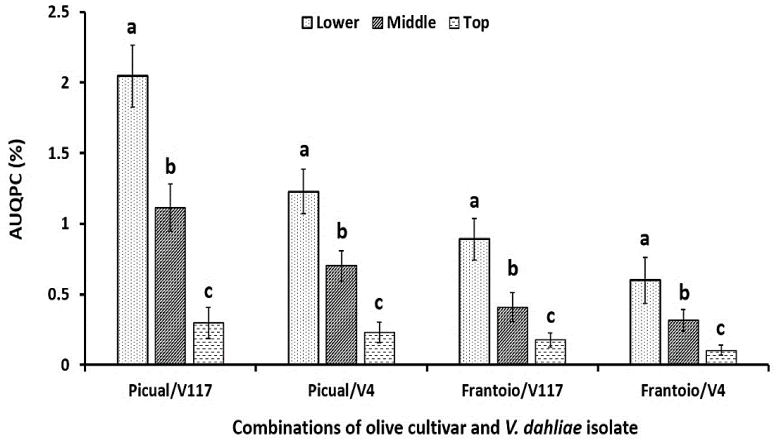


Figure 3. Area under the quantity progress curve percentage (AUQPCP) of the amount of *V. dahliae* DNA (ng) in 100 ng of total DNA extracted from lower, middle and top parts of 'Frantoio' and 'Picual' olive trees after inoculation with V117 and V4 isolates of *V. dahliae*. The AUQPCP values were calculated by the trapezoidal integration method of Campbell and Madden (1990) of the data of figure 2. Error bars show standard deviations. Means of the results of lower, middle and top parts were compared for each treatment ('Frantoio'/V117, 'Frantoio'/V4, 'Picual'/V117 and 'Picual'/V4) separately, based on Fisher protected LSD at P=0.05. Different letters above bars in each treatment group indicates significantly different quantities of *V. dahliae* DNA detected in lower, middle and top parts of plants within each combination group.

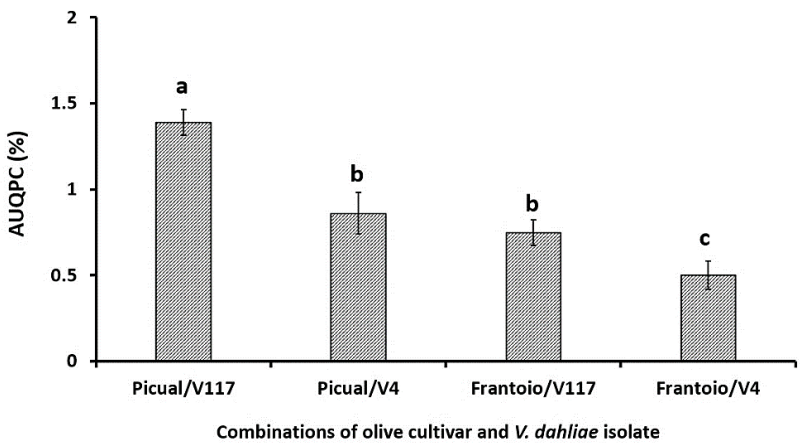


Figure 4. Area under the quantity progress curve percentage (AUQPCP) for mean values of *V. dahliae* DNA quantities in Frantoio/V117, Frantoio/V4, Picual/V117 and Picual/V4 combinations. Mean quantities of *V. dahliae* DNA were obtained by averaging the amounts detected in different parts (lower, middle and top) of tested trees (five trees per time point). The AUQPCP values were calculated by the trapezoidal integration method of Campbell and Madden (1990). Different letters above the bars show significant differences between mean values (T-test, P=0.05), while error bars indicate standard deviations.

Infection of olive trees with GFP-expressing isolates. To study the process of infection and colonisation by V117 and V4 isolates of *V. dahliae* in 'Frantoio' and 'Picual' olive cultivars, we used GFP-labelled isolates of *V. dahliae* V4 and V117 for inoculation of 'Picual' and 'Frantoio' plants. To this end, GFP-expressing transgenes were generated by means of *Agrobacterium tumefaciens*-mediated transformation (ATMT) of *V. dahliae* isolates V117 and V4, and each transgene was used for inoculation of 35 plants from each 'Picual' and 'Frantoio' cultivar. Ten plants from each cultivar were used as controls. To monitor disease development, ten plants of each combination of olive cultivar and GFP-expressing *V. dahliae* transgene were used for assessment of disease severity at 2-week intervals, in the same way as we did for plants inoculated with wild-type V117 and V4. As expected, the disease development as observed in plants inoculated with GFP-labelled transgenes of *V. dahliae* V117 and V4 was similar to that observed in plants inoculated with *V. dahliae* V117 and V4 (data not shown).

The amounts of the GFP-labelled isolates of *V. dahliae* in the stem of inoculated trees was quantified by real-time PCR at different time points (two weeks intervals). Total DNA was isolated from mixed subsamples taken from three different parts of the stem and used for quantification of *V. dahliae* DNA. At each time point, five plants from each treatment were sampled and mean relative quantities of *V. dahliae* DNA (means of five plants) were plotted against sampling time points (Figure 5A). Results were similar to those of experiment 1. One week after inoculation, *V. dahliae* DNA could be detected in the stem of trees from all four treatments with more *V. dahliae* DNA detected in 'Picual' plants than in 'Frantoio' plants. Like in experiment 1, in Frantoio plants a gradual decline of the amount of *V. dahliae* DNA was observed, whereas in Picual the amount of *V. dahliae* DNA was more variable with a decline at the end of the period of observation. The AUQPCP analysis of mean values showed, similar to experiment 1, that quantities of *V. dahliae* DNA in 'Picual' plants were significantly higher than quantities in 'Frantoio' plants for both GFP-labelled isolates, and the quantities of GFP-V117 were significantly different from quantities of GFP-V4 in both cultivars (P=0.05) (Figure 5B).

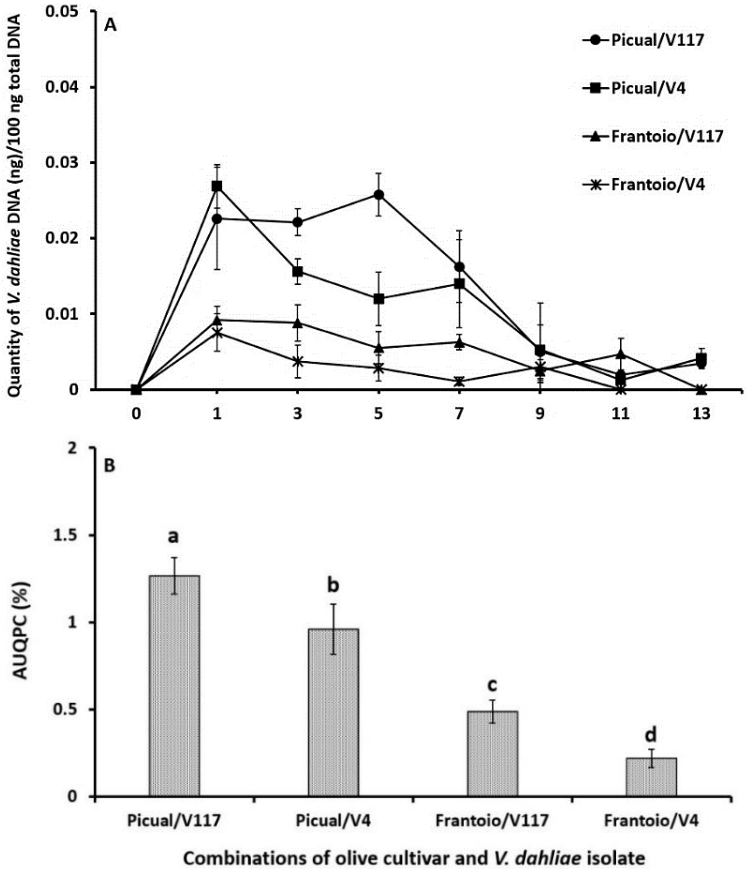


Figure 5. Mean quantity of *V. dahliae* DNA (ng) in 100 ng total DNA extracted from 'Frantoio' and 'Picual' olive trees inoculated with GFP-V117 and GFP-V4 transgenes. (A) Progress curve of the mean quantities of *V. dahliae* DNA in different treatments. Mean quantities of *V. dahliae* DNA in tested trees were obtained by averaging the amounts detected in different parts (lower, middle and top). Quantification assays were run at eight time points during 13 weeks after inoculation. Each value is the mean of five replications (trees) per time point. Error bars show standard deviations. (B) Area under the quantity progress curve percentage (AUQPCP) of the mean quantities of *V. dahliae* DNA in the different treatment groups. The AUQPCP values were calculated by the trapezoidal integration method of Campbell and Madden (1990). Different letters above the bars show significant differences between mean values (T-test, P=0.05) and error bars show standard deviations.

To illustrate in which part of the vascular system of olive trees, and in what form, *V. dahliae* grows and spreads, samples from root and stem of olive trees inoculated with GFP-expressing isolates were analysed with epi-fluorescence and confocal laser microscopy. Two to four dpi, germinating conidia were frequently observed on the surface

of the roots (Figure 6A, 6B, 6C). At this time point, also formation of more complex networks of hyphae were observed locally on the epidermis of the roots (Figure 6D, 6E, 6F). Intriguingly, before penetration and colonization of the cortical tissues, inflated structures that are recognized as "hyphopodia" and that are thought to be functionally analogous to appressoria (Vallad et al., 2008, Reusche et al., 2014; Zhao et al., 2016) were observed at the penetration site (Figure 6G). One week after inoculation, the surface of the roots was locally intensively colonized by hyphae at seemingly random positions along the roots (Figure 7A, 7B).

Simultaneously, hyphae were observed growing intra- and intercellularly within the root cortex and the central cylinder to colonize the vascular system of the root (Figure 7C, 7D, 7E). Two weeks after inoculation, germinating conidia were observed in xylem vessels of middle parts of the inoculated trees (Figure 8A, 8B, 8C, 8D), while formation of hyphae was observed in lower parts (Figure 8E, 8F, 8G). Notably, colonization in the stem was restricted to the xylem vessels (Figure 8E1-8E3). These observations were similar for GFP-V4 and GFP-V117 transgenes in 'Frantoio' and 'Picual' olive cultivars.

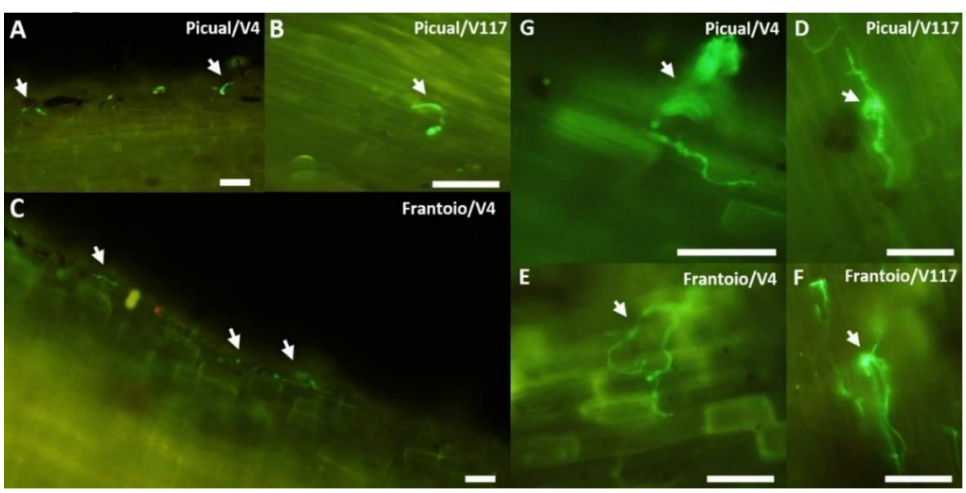


Figure 6. Colonization of the surface of olive roots by GFP-labelled *V. dahliae* at 48 h after inoculation as observed with epi-fluorescence microscopy (A, B and C). Conidia on the root surface of plants from different treatments (D, E, F and G). Growth of *V. dahliae* hyphae on the root surface (G). Arrows indicate appressorium-like structures at penetration sites. Scale bar = 50 µm.

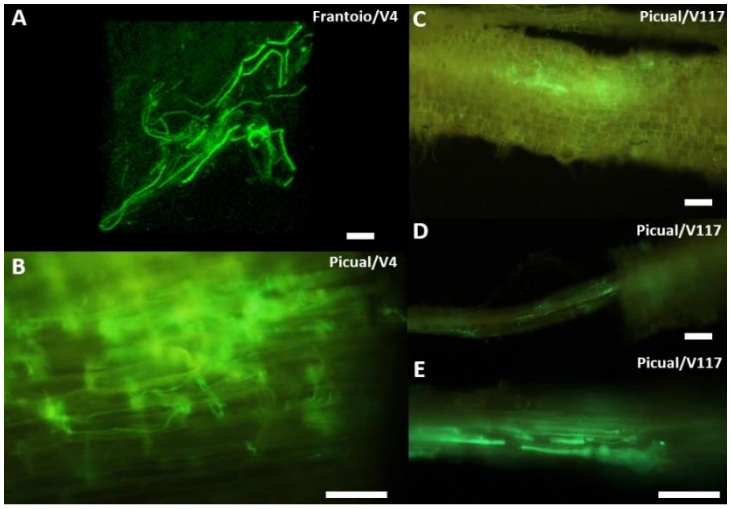


Figure 7. Presence of GFP-labelled *V. dahliae* in cortex and vessels of olive roots at one week after inoculation. GFP-labelled *V. dahliae* in the root cortex (A, B and C). Colonization of GFP-labelled *V. dahliae* in root vessels (D, E). Images were captured using confocal laser microscopy (A) or epi-fluorescence microscopy (B, C, D and E). Scale bar=50 μm.

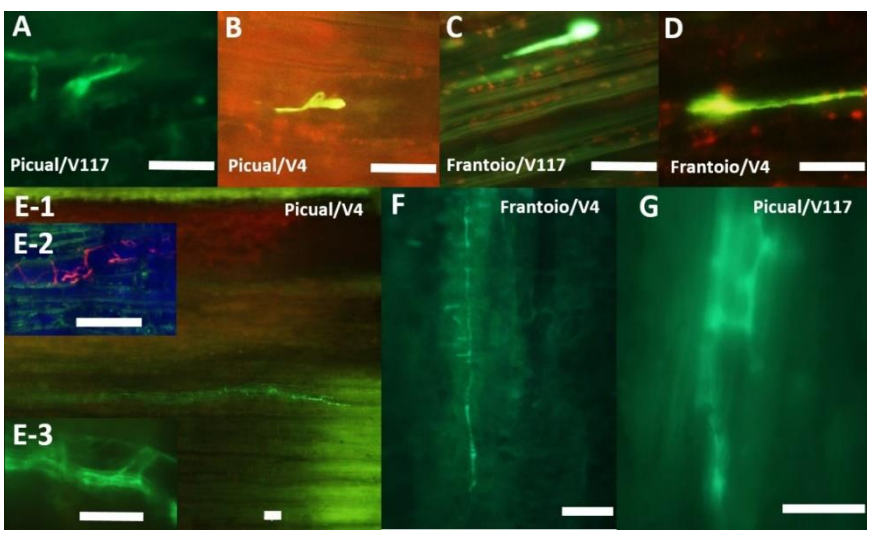


Figure 8. Presence of GFP-labelled *V. dahliae* in xylem vessels of the stem of olive plants at 2 weeks after root-dip inoculation. Germinating conidia in xylem vessels of middle parts of infected olive trees (A, B, C, D). Hyphae of GFP-labelled *V. dahliae* in xylem vessels of lower parts of infected olive trees (E, F and G). Images were captured using epi-fluorescence microscopy (A, B, C, D, E1, E2, F, and G) and confocal laser microscopy (E2). Scale bar = 50 μm.

Discussion

Verticillium wilt disease incidence and severity of symptoms in olive trees are strongly dependent on the virulence of the infecting *V. dahliae* isolate, the pathotype (D or ND) to which the isolate belongs (Jiménez-Díaz et al. 2011; López-Escudero et al. 2004, 2007; Rodríguez-Jurado et al. 1993), and on the level of resistance of the host tree (Mercado-Blanco et al. 2003). More knowledge of the dynamics of pathogen distribution in infected plants may help to elucidate the reason for differences in the extent and severity of disease caused by these two pathotypes in resistant and susceptible olive genotypes. Therefore, the objective of this study was to investigate the distribution of a defoliating (D) and a non-defoliating (ND) isolate of *V. dahliae* (V117 and V4 respectively) in relation to disease progression in a susceptible (Picual) and a partially resistant cultivar (Frantoio) of olive.

As expected, disease severity caused by isolate V117 was significantly higher than that caused by isolate V4 in both cultivars, and disease severity in 'Frantoio' plants was less than in 'Picual' plants for both isolates, confirming that isolate V117 is more virulent than isolate V4, and 'Picual' is more susceptible to Verticillium wilt than 'Frantoio'. Colonization of the above ground parts of infected olive plants arguably occurs by means of conidiospores that are transported with the xylem sap stream, the pathogen occurs in the upper parts already at one week post inoculation. Conceivably, this is too fast to be achieved by hyphal growth only, as the maximum growth rate of *V. dahliae* hyphae is about 8 mm/day (ElSharawy, 2015; Rampersad, 2010). This conclusion is further underpinned by the observation of conidia and germinating conidia and the absence of hyphal growth in xylem vessels in samples from the middle part of inoculated stems at the second week after inoculation. By this time, profuse hyphal growth was only observed on the root surface and in the cortex of infected roots, and some hyphae were detected in vessels in samples from the lower stem parts. Moreover, in the stem samples the fungus could only be detected in the xylem, indicating that *V. dahliae* in the stem of infected plants is limited to the vascular system of the plant during symptom development.

The highest levels of *V. dahliae* DNA within the stem of infected plants (before the onset of symptoms) were detected already at one week after inoculation with symptoms

developing only from week seven (Picual) or week nine (Frantoio) onward. Street and Cooper (1984) indicated that vessel occlusion is the primary cause of water stress and wilting in Verticillium wilt. This occlusion might be caused by physical blockage of the plant's xylem by the pathogen itself or by host defence responses that are aimed at vessel plugging. Data from our study show that symptoms are the result of reactions by the plant to the presence of the pathogen, rather than to the presence of the pathogen itself (e.g. through clogging of vessels). This hypothesis is supported by the low levels of *V. dahliae* DNA that were detected, i.e. up to 0.035 % of the total DNA extracted for isolate V117 in 'Picual' plants (0.035 ng in 100 ng total DNA) at the most (Fig. 2). In this respect, it should be taken into account that many plant cells in the sample volume are dead (i.e. the xylem vessels) and therefore do not contain DNA. This leads to the conclusion that despite the occurrence of severe symptoms, only limited growth of the fungus within the plant stems occurs during the period of symptom development. This conclusion was further substantiated by the microscopical analysis that only rarely revealed *V. dahliae* presence, and if so usually to a limited extent.

Interestingly, real-time PCR analysis revealed that initial *V. dahliae* DNA levels detected at one week post inoculation were rather similar for all treatments, while striking differences developed in the weeks after. In 'Picual' plants the quantity of *V. dahliae* DNA fluctuated for both isolates and remained at a high level until 13 weeks post inoculation, whereas in 'Frantoio' plants the quantities of both pathotypes gradually decreased till the end of the experiment. Notably, in each cultivar the higher amount of pathogen biomass was observed in the interaction with the V117 isolate. Apparently in the susceptible cultivar (Picual), *V. dahliae* is able to overcome the defense reactions of the host, as several peaks in the amount of *V. dahliae* DNA were observed in infected plants of this cultivar. However, in the 'Frantoio' the defense mechanisms of the host are apparently more effective as in this cultivar the quantity of *V. dahliae* DNA gradually decreases. Despite the differences in disease development and pathogen biomass as measured by real-time PCR, we did not observe visible difference in the infection and colonization process of the GFP-labelled isolates of *V. dahliae* V117 and V4 in the 'Frantoio' and 'Picual' trees. This illustrates that quantitative nature of GFP-visualisation of colonisation

in this study is rather poor, because microscopical analysis concerns only localized sites and only provides a quantitative rather than a quantitative view.

The highest amounts of *V. dahliae* DNA were always detected in the lowest parts of the inoculated plants. Also the distribution patterns of the two isolates in the lower, middle and top parts of the tested olive cultivars showed that differences in symptom severity correlated to higher amounts of the pathogen in the lower and middle parts of the trees. Actually, colonization of the pathogen in top parts of the stem of inoculated plants was not significantly different between treatments. Resistance mechanisms of woody plants against a vascular pathogen like *V. dahliae* include physical and biochemical components, serving to seal off infected sites and immobilize the pathogen (Hiemstra 1998). For olive, several components including production of tyloses extruding from the paravascular parenchyma into the vessel elements, aggregates, and polysaccharide-type materials have been described (Baídez et al. 2007; Rodríguez-Jurado et al. 1993). In both olive cultivars these defense mechanisms are not sufficient to prevent the fungus from colonizing the upper parts of the stem of inoculated plants. Moreover, the interaction between the plant and the fungus results in significant differences in disease intensity that can be related to differences in the quantity of *V. dahliae* in the lower and middle parts of the stems. Thus, for a better understanding of differences in susceptibility of olive cultivars to Verticillium wilt, it would be very interesting to investigate the presence and activity of different proteins and secondary metabolites in the xylem sap that contribute directly or indirectly to plant defense, especially in the xylem of the upper part of the root system and the lower part of the stem of olive cultivars that display differential levels of susceptibility.

Acknowledgment

M. Keykhasaber is supported by a scholarship of the Ministry of Science and Technology of Iran. We sincerely thank F. J. Lopez-Escudero, Departamento de Agronomía, Universidad de Córdoba, for supplying the V117 and V4 isolates of *V. dahliae* and the olive plants used in this study. We also thank K.T.K. Pham for very valuable technical support and Dr. N.C.A. de Ruijter, Manager of Wageningen Light Microscopy Center (WLMC), for the support during microscopical imaging and analysis.

References

Baídez, A.G., Gomez, P., Del Rio, J.A., Ortuno, A. (2007). Dysfunctionality of the xylem in Olea europaea L. plants associated with the infection process by *Verticillium dahliae* Kleb. Role of phenolic compounds in plant defense mechanism. Journal of Agricultural and Food Chemistry 55, 3373-3377.

Blázquez-Martínez, J.M. (1996). Evolution and history. In: World Olive Encyclopaedia, International Olive Oil Council (1996), pages 17-58.

Campbell, C.L., Madden, L.V. (1990). Introduction to Plant Disease Epidemiology. New York: John Wiley and Sons.

Chen, P., Lee, B., Robb, J. (2004). Tolerance to a non-host isolate of *Verticillium dahliae* in tomato. Physiology and Molecular Plant Pathology 64, 283-291.

Civantos, L. (2004). Olive growing in the world and in Spain, p. 17–35. In: D. Barranco, R. Ferna´ndez- Escobar, and L. Rallo (eds.), (2010). Olive Growing. RIRDC, Australia.

Gold J., Robb J. (1995). The role of the coating response in Craigella tomatoes infected with *Verticillium dahliae*, races 1 and 2. Physiology and Molecular Plant Pathology 47, 141-57.

Heinz, R., Lee, S.W., Saparno, A., Nazar, R.N., Robb, J. (1998). Cyclical systemic colonization in *Verticillium*-infected tomato. Physiological and Molecular Plant Pathology 52, 385-96.

Hiemstra, J.A. (1998). Some general features of Verticillium wilts in trees. Pages 5-11 in: Hiemstra, J.A., Harris, D.C. (Eds.) (1998). A compendium of Verticillium wilts in tree species. Wageningen: CPRO-DLO/West Malling: HRI-East Malling. 80 pp.

Hu, X., Nazar, R.N., Robb, J. (1993). Quantification of Verticillium biomass in wilt disease development. Physiological and Molecular Plant Pathology 42, 23-36.

Jiménez-Díaz, R.M., Tjamos, E.C., Cirulli, M. (1998). Verticillium wilt of major tree hosts. In: Hiemstra JA, Harris DC, 1998. A compendium of Verticillium wilts in trees: 13-16.

Jiménez-Díaz, R.M., Olivares-García, C., Landa, B.B., Jiménez-Gasco, M.M., Navas-Cortés, J.A., (2011). Region-wide analysis of genetic diversity in *Verticillium dahliae* populations infecting olive in southern Spain and agricultural factors influencing the distribution and prevalence of vegetative compatibility groups and pathotypes. Phytopathology 101, 304-315.

Jiménez-Díaz, R.M., Cirulli, M., Bubici, G., Jiménez-Gasco, M.D., Antoniou, P.P., Tjamos, E.C., (2012). Verticillium wilt, a major threat to olive production: current status and future prospects for its management. Plant Disease 96, 304-329.

López-Escudero, F.J., Del Río, C., Caballero, J.M., Blanco-López, M.A. (2004). Evaluation of Olive Cultivars for Resistance to *Verticillium dahliae*. European Journal of Plant Pathology 110, 79-85.

López-Escudero, F.J., Blanco-López, M.A., Del Río Rincón, C., Caballero Reig, J.M. (2007). Response of olive cultivars to stem puncture inoculation with a defoliating pathotype of *Verticillium dahliae*. HortScience 42, 294-298.

López-Escudero, F.J., Mercado-Blanco, J. (2011). Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. Plant Soil 344, 1-50.

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodriguez-Jurado, D., Jimenez-Diaz, R.M., (2003). Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction. Physiological and Molecular Plant Pathology 63, 91-105.

Prieto, P., Navarro-Raya, C., Valverde-Corredor, A., Amyotte, S.G., Dobinson, K.F., Mercado-Blanco, J. (2009). Colonization process of olive tissues by *Verticillium dahliae* and its in planta interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7. Microbial Biotechnology 2(4), 499-511.

Reusche M, Truskina J, Thole K, Nagel L, Rindfleisch S, et al. (2014) Infections with the vascular pathogens and *Verticillium dahliae* induce distinct disease symptoms and differentially affect drought stress tolerance of *Arabidopsis thaliana*. Environmental and Experimental Botany 108: 23-37.

Rodríguez-Jurado, D., Blanco-López, M.A., Rappoport, H.F., Jiménez-Díaz, R.M. (1993). Present status of Verticillium wilt of olive in Andalucía (southern Spain). EPPO Bulletin 23, 513-516.

Santhanam, P. (2012). Random insertional mutagenesis in fungal genomes to identify virulence factors. Methods in molecular biology 835, 509-517.

Tjamos, E.C., Biris, D.A., Paplomatas, E.J., (1991). Recovery of olive trees with Verticillium wilt after individual application of soil solarisation in established olive orchards. Plant Disease 75, 557–562.

Vallad, G.E., Subbarao, K.V. (2008). Colonization of resistant and susceptible lettuce cultivars by a green fluorescent protein-tagged isolate of *Verticillium dahliae*. Phytopathology 98, 871-885.

Van Doorn, J., Pham, K.T.K., Hiemstra, J.A. (2009). Molecular detection of *Verticillium dahliae* in soil. Abstract book of the 10th International Verticillium Symposium, (2009). Corfu Island, Hellas, p. 94.

Weller, S.A., Elphinstone, J.G., Smtih, N.C., Boonham, N., Stead, D.E. (2000). Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Applied and Environmental Microbiology 66, 2853-2858.

Zhao Y-L, Zhou T-T, Guo H-S (2016). Hyphopodium-Specific VdNoxB/VdPls1-Dependent ROS-Ca2+ Signaling Is Required for Plant Infection by *Verticillium dahliae*. PLoS Pathog 12(7): e1005793. doi:10.1371/journal.ppat.1005793.

Chapter 6

Distribution and persistence of *V. dahliae* in the xylem of Norway maple and European ash trees

Mojtaba Keykhasaber[a,b,c], Bart P.H.J. Thomma[a] , Jelle A. Hiemstra[b]

[a]Laboratory of Phytopathology, Wageningen University & Research, 6708 PB Wageningen, The Netherlands

[b]Wageningen Plant Research, Wageningen University & Research, P.O. Box 200, 6670 AE

[c]Department of Plant Biotechnology, Zabol University, Zabol, Iran

This chapter is (provisionally) accepted for publication in European Journal of Plant Pathology.

Abstract

V. dahliae colonizes the xylem vessels of susceptible host plants. Hence it can be expected that the distribution of the fungus as well as disease progress will be influenced by the anatomy of the xylem of that host. Here, we studied the spatial and temporal distribution of *V. dahliae* in relation to recovery from disease symptoms in young European ash (*Fraxinus excelsior*) trees and Norway maple (*Acer platanoides*) that differ in their vascular anatomy. Quantifying the amount of *V. dahliae* DNA at different heights in the stem of inoculated trees at different time points after inoculation showed that in the year of inoculation the speed of colonization of these two species by *V. dahliae* was highly similar. Nevertheless, in the year after inoculation disease incidence and also quantities of *V. dahliae* detected in maple trees were significantly higher than in ash trees, suggesting that the xylem of ash trees is much less supportive for growth and survival of *V. dahliae* than that of maple trees. Moreover in this second year *V. dahliae* could not be detected at all in the wood of ash trees that had recovered from disease whereas it easily could be detected in the wood of diseased ash and maple trees. Furthermore, despite the presence of a layer of parenchyma cells between growth rings in ash trees, in symptomatic ash trees *V. dahliae* was present in the xylem of the new growth ring. We observed that *V. dahliae* can move downward from the point of inoculation into the root collar. This may provide a way for infection of new growth rings by circumventing the physical barriers within the stem xylem.

Introduction

Verticillium wilt caused by the soil-borne fungus *Verticillium dahliae* Kleb., is a major disease worldwide that not only affects herbaceous annuals but also woody perennials (Pegg and Brady, 2002; Smith et al., 1988). Shade tree nurseries and landscape plantings, especially in areas where field crops suffered from Verticillium wilt in the past, are agricultural settings that are confronted with this disease (Hiemstra and Harris, 1998; Riffle and Peterson, 1989). Maples (*Acer* spp.) are probably the best known shade trees that are susceptible to Verticillium wilt (Gleason and Hartman, 2001; Harris, 1998; Townsend et al., 1990). *A. platanoides* (Norway maple) is an important shade tree species

in Europe that is known to be highly susceptible to *V. dahliae* (Harris, 1998; Piearce and Gibbs, 1981). Ash trees (*Fraxinus* spp.) are widely cultivated because of their high-quality timber (strong but elastic) and their ornamental value. Several species in this genus, and especially *F. excelsior*, are also highly susceptible to Verticillium wilt in nurseries as well as after being planted out in the landscape (Heffer and Regan, 1996; Hiemstra, 1998; Worf et al., 1994).

The disease cycle of *V. dahliae* on tree hosts has been described in detail by Hiemstra (1998). Microsclerotia (resting structures) of *V. dahliae* in the soil are stimulated to germinate by exudates from nearby growing roots. Hyphae from germinating microsclerotia grow towards the roots, penetrate the root surface (Lockwood, 1977; Nelson, 1990; Schreiber and Green, 1963) and grow inter- and intracellularly through the root cortex to reach the xylem vessels (Prieto et al., 2009). Once inside the vessels, the fungus starts to produce conidia that are spread throughout the infected tree with the flow of xylem fluid. At vessel ends or against protruding parts of vessel elements, conidia are trapped and may germinate. The new hyphae penetrate into adjacent vessel elements, produce conidia and the process starts all over again, finally leading to systemic colonization of upper parts of infected plants (Baídez et al., 2007; Rodríguez-Jurado, 1993).

Fungal propagules and host defense products may block xylem vessels in infected plants. As a result, the water flow throughout the xylem is hampered and symptoms of water stress develop. Consequently, wilting, desiccation and defoliation are among the early symptoms of Verticillium wilt disease in trees. If the plugged vessels are not replaced in time by novel ones, dieback of shoots, branches or even the whole tree may follow. Not all tree species show dieback to the same extent. Whereas ash is able to recover completely from Verticillium wilt (Hiemstra, 1995b), serious dieback is generally observed in maple trees (Harris, 1998; Piearce and Gibbs, 1981).

The capability of tree species to recover from vascular infections has been related to the inherent structure of their xylem and the ability to produce new layers of healthy xylem tissue around diseased xylem (Banfield, 1968; Emechebe et al., 1974; Sinclair et al., 1987; Tippett and Shigo, 1981). Ash is a ring-porous tree with most of the water transport taking

place in the xylem vessels of the most recent growth ring and a marginal parenchyma sheath between successive growth rings that may act as a barrier zone, effectively separating the latewood vessels of one growth ring from the earlywood vessels of the next growth ring (Braun, 1970; Grosser, 1977; Schweingruber, 1990). This implies that as long as infected ash trees are able to produce new uninfected xylem vessels every year they can substitute their blocked vessels, which enables complete recovery, often even without dieback of the crown. In maple, which is a diffuse-porous tree, xylem vessels remain functional for several years in each growth ring. Therefore, loss of a major part of the water transport capacity in infected trees often cannot sufficiently be compensated by new growth rings. Consequently, these trees show much more dieback of the aerial parts and recovery, if it occurs, starts by regrowth from healthy parts of the stem base or roots (Hiemstra, 1998).

De novo xylem formation as a mechanism to recover from Verticillium wilt implies the requirement to protect new xylem vessels from infection by the fungus that is already present in the tree. Compartmentalization resulting from the inherent structure of the wood, in combination with changes in anatomy and chemistry of xylem after infection, has been suggested to play an important role in protecting trees against colonization by vascular pathogens (Bonsen et al., 1985; Manion, 2003; Shigo, 1984; Tippett and Shigo, 1981; Smith, 2006). However, although recovery from Verticillium wilt has been described not only for ash, but also for other tree species including almond and peach (Ciccarese et al., 1990), apricot (Taylor and Flentje, 1968; Vigouroux and Castelain, 1969), pistachio (Paplomatas and Elena, 1998), cocoa (Emechebe et al., 1974), avocado (Latorre and Allende, 1983), and olive (López-Escudero and Blanco-López, 2005), there is little information about the fate of the fungus in infected trees in the years following the initial infection.

In this research we investigated (1) if the spread of *V. dahliae* is different in two tree species that differ in vascular anatomy, (2) if *V. dahliae* is still present in the xylem of a tree host at one year after infection, and (3) if recovery is correlated to containment of the pathogen in the xylem of the year of infection. To this end, we studied the spatial and temporal distribution of *V. dahliae* as well as recovery in Norway maple and European

ash trees. We monitored disease progression and quantified the amount of pathogen that is present at different heights in the stem of inoculated trees during the year of inoculation and in the subsequent year. In that second year we also investigated the presence of the pathogen in the newly formed ring of stem xylem.

Materials and methods

Plant and fungal material. Two-year-old seedlings of Norway maple (*Acer platanoides*) (79 trees) and ash (*Fraxinus excelsior*) (74 trees) were stem-inoculated on August (8th) 2013 with *V. dahliae* isolate Vd1 that originates from maple (collection of Applied Plant Research (PPO), Wageningen University and Research Center). The inoculum was prepared by adding small fragments from a potato dextrose agar (PDA) culture to liquid Czapek-Dox medium in Erlenmeyer flasks. The flasks were put in a shaker at 100 rpm at room temperature in the dark for about seven days to allow conidiospores to be produced. After filtration over cheese cloth, the conidiospore suspension was centrifuged to remove growth medium, and the pellet was resuspended in sterile water. Then, the concentration of conidia was determined and diluted to 10^6 conidia/ml. This conidiospore suspension was used for inoculation of healthy ash and maple trees. To this end, a horizontal incision of a few millimetres deep was made through the bark of the stem into the xylem with a snap-off cutter, around 30 cm above the soil level. Next, 1-2 drops of 50-100 µl of the conidial suspension were put on the cutter blade with a disposable transfer pipette, with the blade still inside the incision (Figure 1). Within a few seconds the conidial suspension was drawn into the stem as a result of the low pressure potential within the xylem vessels. Additionally, 35 maple trees and 27 ash trees were not inoculated to be used as controls. From each species, 40 inoculated trees were kept for assessment of disease progression, and the remainder of the inoculated trees (39 maple trees and 34 ash trees) were used for quantification of the pathogen.

Figure 1. Illustration of stem-inoculation of a maple seedling. With a snap-off cutter a horizontal incision of about 5 mm deep is made through the bark into the xylem of the stem. With the knife still inside the incision, 1-2 drops of 50-100 µl of a conidiospore suspension were put on the knife with a disposable transfer pipette. The conidiospore suspension is drawn into the stem within a few seconds as a result of the negative pressure potential within the xylem vessels.

Sampling. To monitor the upward and downward distribution of *V. dahliae* from the point of inoculation within the stem of inoculated trees, and also to examine changes in *V. dahliae* biomass in infected trees over time, 10 cm samples were taken at different heights from the stem of 5 individual trees at different time points: 0 days past inoculation (dpi; i.e. about an hour after inoculation), 10 dpi, 24 dpi and 60 dpi; as well as 8 months past inoculation (mpi), 11 mpi and 14 mpi. As the ash trees were shorter than the maple trees, we tested eight samples from ash trees (P1 = 5 cm below the soil level, P2 = 5 cm above the soil level, P3 = 5 cm below the inoculation point, and P4 to P8 = 5, 10, 20, 40 and 60 cm above the inoculation point, respectively) and ten samples from maple trees (P1-P8 as for ash trees and P9 and P10 at 80 and 100 cm above the inoculation point, respectively) (Figure 2). These samples were used for quantification of *V. dahliae* DNA and for reisolation of the pathogen through plating.

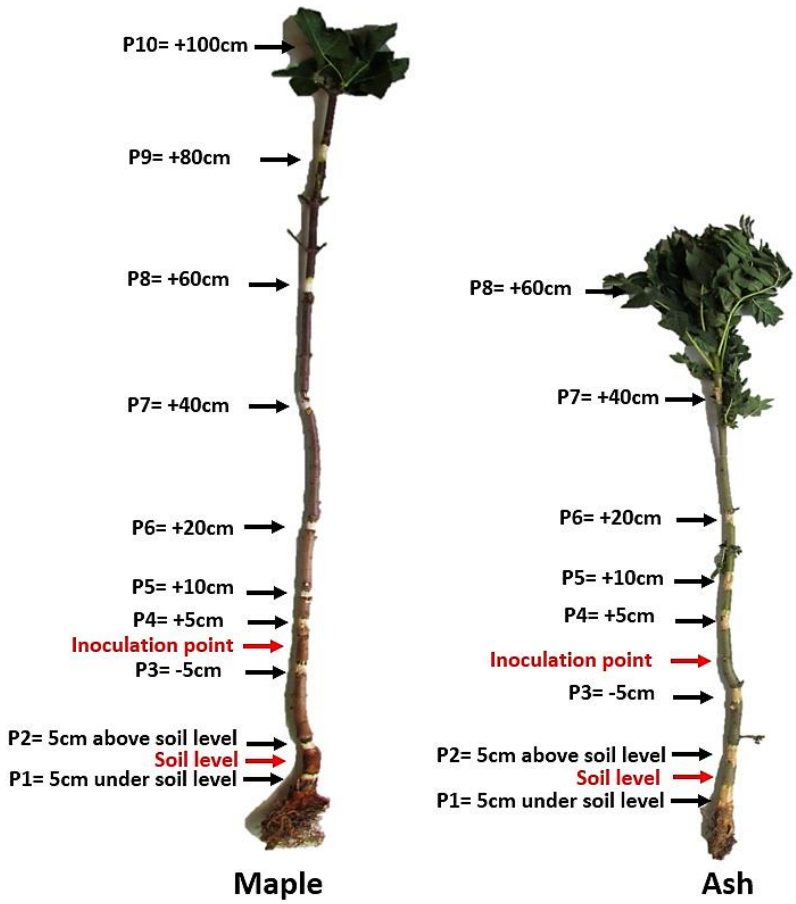


Figure 2. Locations in the stem of inoculated trees that were sampled for detection and quantification of *V. dahliae* DNA shown on an uprooted maple (left) and ash (right) tree that was photographed with most leaves removed before taking samples. Positions of the soil level and inoculation point are indicated.

DNA isolation. For DNA isolation the stem samples were first washed under running tap water for 1-2 minutes, dried with cleaning paper and left to dry for a few minutes on cleaning paper. Then, the bark was removed under sterile conditions and small (2-5 mm) pieces of woody tissue (300-400 mg) were taken by using a sterilized scalpel (flamed after submergence in 70% alcohol) and transferred to a 2 ml tube containing 1 ml of lysis buffer AP1 of the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and 4-5 stainless steel beads (3.2 mm diameter, BioSpec, Bartlesville, US/Canada). Next, the tubes were incubated for 15-30 min at 65˚C and then shaken in a Retsch® mixer mill (MM 400, Retsch, Haan, Germany) for 15 minutes at 30 Hz. After centrifugation at 10,000 rpm for

5 minutes, 400 µl of the suspension was used for total genomic DNA extraction using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA amounts were quantified using a BioPhotometer (Eppendorf AG, Hamburg, Germany) and concentrations were equalized by adding elution buffer or DNase-free water.

DNA quantification. Real-time PCR assays were performed using a *V. dahliae*-specific primer pair designed on the internal transcribed spacer (ITS) region (van Doorn et al., 2009) (VerDITSF: 5'-CCGGTCCATCAGTCTCTCTG-3', VerDITSRk: 5'-CACACTACATATCGCGTTTCG-3') and a primer pair for the plant cytochrome oxidase (*COX*) gene (Weller et al. 2000) to quantify the amount of *V. dahliae* DNA and plant DNA, respectively. All real-time PCR reactions were performed in a STRATAGENE Max 3000P^{TM} real-time PCR machine (Agilent Technologies, Santa Clara, United States). The real-time PCR program consisted of an initial step of denaturation for 10 min at 95˚C, followed by 45 cycles of 15 sec at 95˚C, 40 sec at 62˚C, and 40 sec at 72˚C. The quantities of *V. dahliae* and plant DNA were determined using a standard curve by plotting the logarithm of a ten-fold dilution series prepared from 10 ng/µl DNA suspension of *V. dahliae* isolate V117 (supplied by F. J. Lopez-Escudero of the Laboratory of Plant Pathology, Department of Agronomy, University of Córdoba, Spain), and a ten-fold dilution series prepared from 10 ng/µl plant (maple/ash) DNA suspension, respectively, against the threshold cycle (Ct) obtained in the real-time PCR assays. The relative quantity of *V. dahliae* DNA in the tested samples was calculated based on the quantity of *V. dahliae* DNA (ng) in 100 ng total DNA (i.e. including pathogen and plant DNA as quantified by simultaneously conducting plant-specific real-time PCR and pathogen-specific real-time PCR) isolated from inoculated plant tissues.

Disease assessment. To monitor disease progress, 40 inoculated trees were selected randomly from the group of inoculated trees and severity of disease symptoms on these trees was recorded at the day of inoculation (0 dpi = days post inoculation) and at the end of the growing season in the year of inoculation (60 dpi) and, after the dormant period in the winter from 2013-2014, at 11 mpi (mid-season; mpi = months post inoculation) and

14 mpi (end of the growing season) in the year after inoculation. Disease symptoms of each tree were rated on a scale from 0 to 4 based on the percentage of plant tissue affected by chlorosis, leaf and shoot necrosis or dieback (0 = no symptoms; 1 = slight (<30%) foliar symptoms; 2 = severe foliar symptoms (>30%) with or without slight (<10%) dieback of top or shoot tips; 3 = severe dieback of top or shoot tips (>10%); 4 = dead plant) (scale modified from Hiemstra, 1995a).

Plating assay. To re-isolate *V. dahliae*, stem samples of 10 cm were first washed under running tap water. After drying, the bark was peeled off and chips from xylem sheets of the two most recent growing years were taken and disinfected in 10% chloramine-T hydrate 98% for 1 minute. Afterwards, wood chips were washed with sterile water for 30 seconds and dried on Whatman filter paper. Chips then were placed onto PDA plates and incubated at 24ºC in dark for 7 days.

Results

Disease incidence. In this study, 79 maple trees and 74 ash trees were stem-inoculated with a *V. dahliae* conidiospore suspension to investigate the disease progression and distribution of the pathogen. Notably, in naturally infected trees this is difficult to investigate because every year new upward surges of the pathogen from infected roots are possible, as well as new infections from the soil. However, in stem-inoculated trees the infection essentially is a one-time event which makes it possible to investigate differences between tree species in their capacity to limit spread of the pathogen in the year of infection as well as in their capacity to contain the pathogen effectively and prevent it from spreading into newly formed tissues in the next year. To monitor disease progression, the severity of disease symptoms on 40 inoculated trees of each of the two species were recorded in a time course (0 dpi, 60 dpi, 11 mpi, 14 mpi). Two months after inoculation (i.e. at the end of the growing season) disease symptoms had developed in both species, although the percentage of diseased trees varied strongly. At this time point, 75% of the inoculated ash trees showed symptoms of Verticillium wilt, with 55% of the trees showing severe symptoms (Table 1A), whereas only 17.5% of the inoculated maple trees showed disease symptoms, with 5% displaying severe symptoms (Table 1B).

Interestingly, early in the following growing season (11 mpi), the disease incidence in ash trees was decreased strongly, with 70% of the trees being devoid of disease symptoms, whereas disease incidence in maple was strongly increased with only 35% of the trees remaining symptomless. During that second season, incidence and severity of disease increased again in both species, with ash being notably less affected than maple. At the end of the second growing season (14 mpi) still 37.5% of the ash trees remained symptomless and 40% showed only slight leaf symptoms. In contrast, the disease index for maple trees was strongly increased by that time, resulting in 80% of the maple trees showing symptoms, including 30% dead trees. These data illustrate the potential of ash to recover from Verticillium wilt disease, despite the fast occurrence of disease symptoms in the first year. At the end of the year after inoculation, the percentage of seriously affected trees was much lower than at the end of the year of inoculation. In contrast, disease in maple trees developed much slower in the year of inoculation, but showed a strong increase in the second year (Figure 3).

Upward movement of V. dahliae. To investigate upward movement of *V. dahliae* within the stem of maple and ash trees, different heights of the inoculated stems were analysed in a time course by real-time PCR for presence of the pathogen. The results of the real-time PCR analysis of samples collected at different heights of the inoculated stems showed that in ash trees *V. dahliae* was already present at P4, P5 and P6 (i.e. 5, 10 and 20 cm above the inoculation point) at the day of inoculation, at P7 (i.e. 40 cm above the inoculation point) at 10 days after inoculation, at P8 (i.e. 60 cm above the point of inoculation) at 24 days after inoculation, and at P9 (i.e. in the top of the stem, 80 cm above the point of inoculation) at only 60 days after inoculation (Figure 4A). At the day of inoculation, *V. dahliae* was detected at P4 and P5 in maple, while at 10 days after inoculation the fungus was also detected at P6, P7 and P8. At 24 days after inoculation *V. dahliae* DNA was detected at P9 and at 60 dpi the fungus was detected at P10 (i.e. in the top of the stem) (Figure 4B). These results show that the speed of *V. dahliae* colonization in the inoculated ash and maple trees does not really differ between the two species.

Table 1. Number (#) and percentage (%) of diseased trees of ash (A) and maple (B) at different time points after inoculation with *V. dahliae.*

A. Ash (*Fraxinus excelsior*)

Disease Index (DI)[1]	2013				2014			
	0 dpi[2] (9-Aug.)		60 dpi (14-Oct.)		11 mpi[3] (14-July)		14 mpi (23-Sep.)	
	#	%	#	%	#	%	#	%
0	40	100	10	25	28	70	15	37.5
1	0	0	8	20	7	17.5	16	40
2	0	0	22	55	2	5	4	10
3	0	0	0	0	1	2.5	2	5
4	0	0	0	0	2	5	3	7.5

B. Maple (*Acer platanoides*)

Disease Index (DI)	2013				2014			
	0 dpi (9-Aug.)		60 dpi (14-Oct.)		11 mpi (14-July)		14 mpi (23-Sep.)	
	#	%	#	%	#	%	#	%
0	40	100	33	82.5	14	35	8	20
1	0	0	5	12.5	9	22.5	7	17.5
2	0	0	2	5	4	10	8	20
3	0	0	0	0	6	15	5	12.5
4	0	0	0	0	7	17.5	12	30

[1] Disease symptoms of each tree were rated on a scale from 0 to 4 based on the percentage of plant tissue affected by chlorosis, leaf and shoot necrosis or dieback (0 = no symptoms; 1 = slight (<30%) foliar symptoms; 2 = severe foliar symptoms (>30%) with or without slight (<10%) dieback of top or shoot tips; 3 = severe dieback of top or shoot tips (>10%); 4 = dead plant).

[2] dpi = days post inoculation.

[3] mpi = months post inoculation.

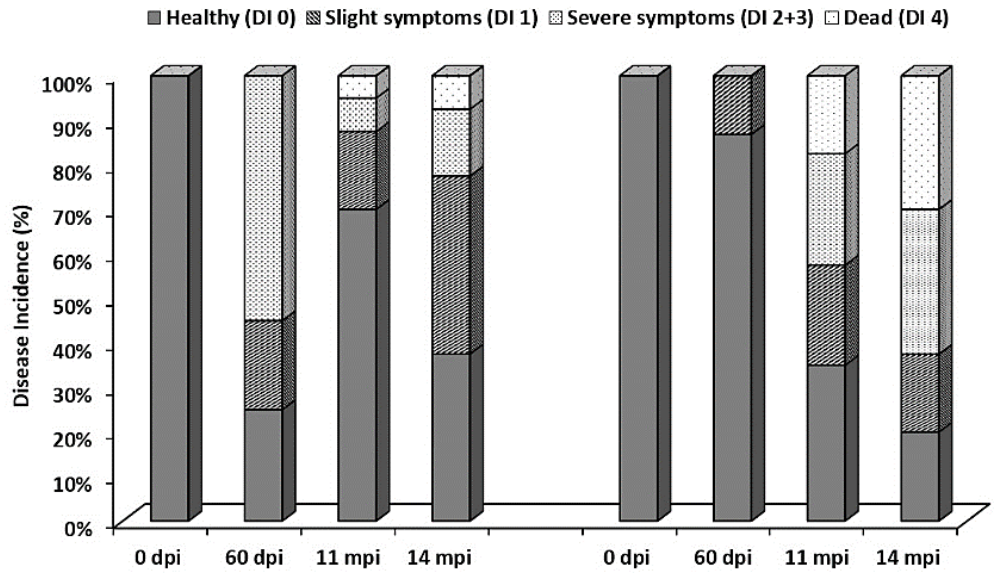
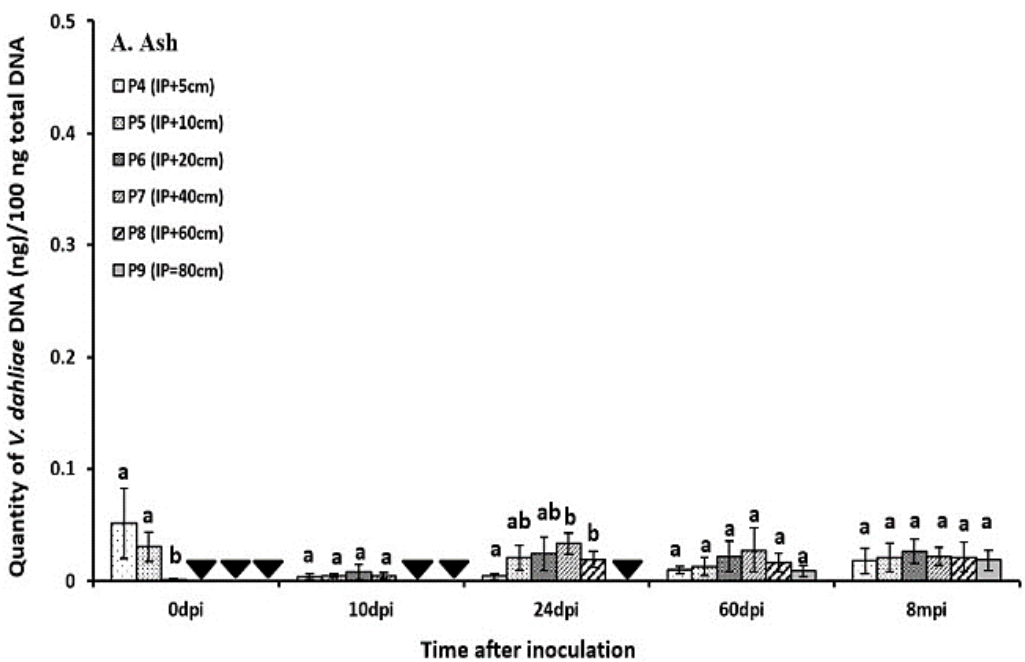


Figure 3. Disease incidence in ash (left) and maple (right) at different time points. Disease index (DI) categories: trees without symptoms (DI 0), with slight symptoms (DI 1), severe symptoms (DI 2+3) and dead trees (DI 4). Dpi = days post inoculation, mpi = months post inoculation.



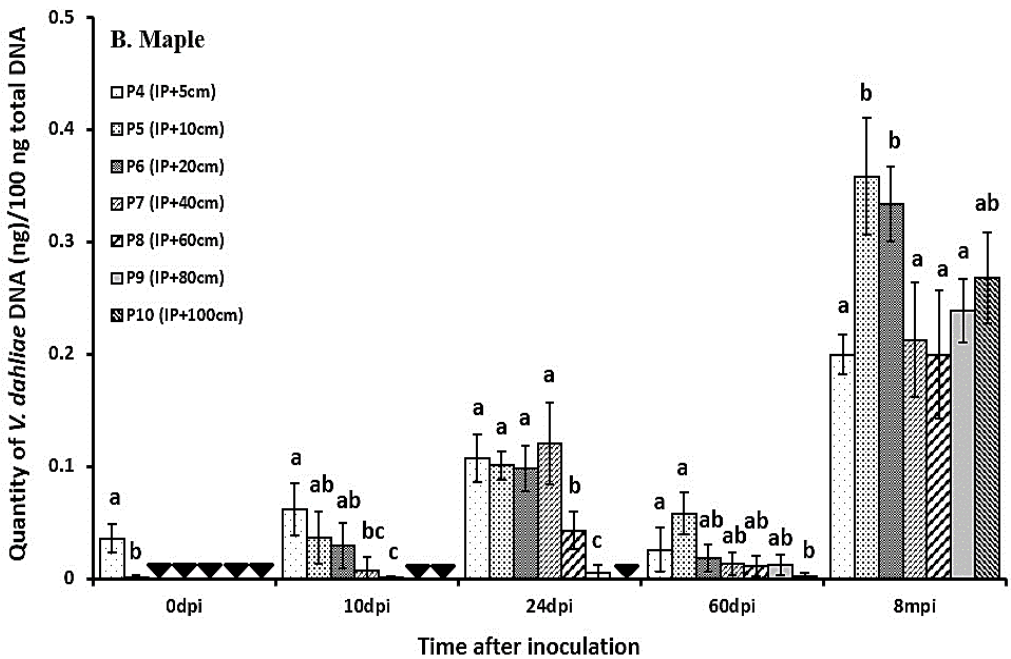


Figure 4. Amount of *V. dahliae* DNA detected at different heights above the inoculation point (IP) in the stem of inoculated ash (A) and maple trees (B). Assessments were conducted at 0, 10, 24, 60 dpi and 8 mpi. Each bar is the mean value of *V. dahliae* DNA quantities detected at corresponding stem positions in 5 trees. Error bars show standard errors. An inverted solid triangle (▼) indicates that *V. dahliae* DNA was not detected (threshold value 0.001 ng of DNA according to the standard curve). Significant differences in quantities of *V. dahliae* DNA detected in different stem positions at each time point have been indicated by different letters on top of the bars (P=0.05).

Downward movement of V. dahliae. The potential of downward movement of *V. dahliae* after stem inoculation was studied by analysis of stem samples at three points below the inoculation point (P1 = 5 cm under soil level, P2 = 5 cm above the soil level and P3 = 5 cm below the inoculation point) taken at 0 dpi, 10 dpi, 24 dpi, 60 dpi in the year of inoculation; and at 8 mpi in the year after inoculation (Figure 5). Directly after inoculation, high amounts of *V. dahliae* DNA were detected in P3 samples of both species, while *V. dahliae* DNA could not be detected in P2 and P1 samples. At 10 dpi, *V. dahliae* DNA was detected also at P2 in both species, while at 24 days after inoculation *V. dahliae* DNA was detected at all three sites below the inoculation point, which evidences downward spread of the pathogen in the year of inoculation. Analysis of P1, P2, and P3 samples taken at 8 mpi (i.e. in the next growing year) showed that *V. dahliae* was still present at these three

sites in both species. Although perhaps unexpected, these findings show that *V. dahliae* is able to move downward in the xylem against the direction of the water flow.

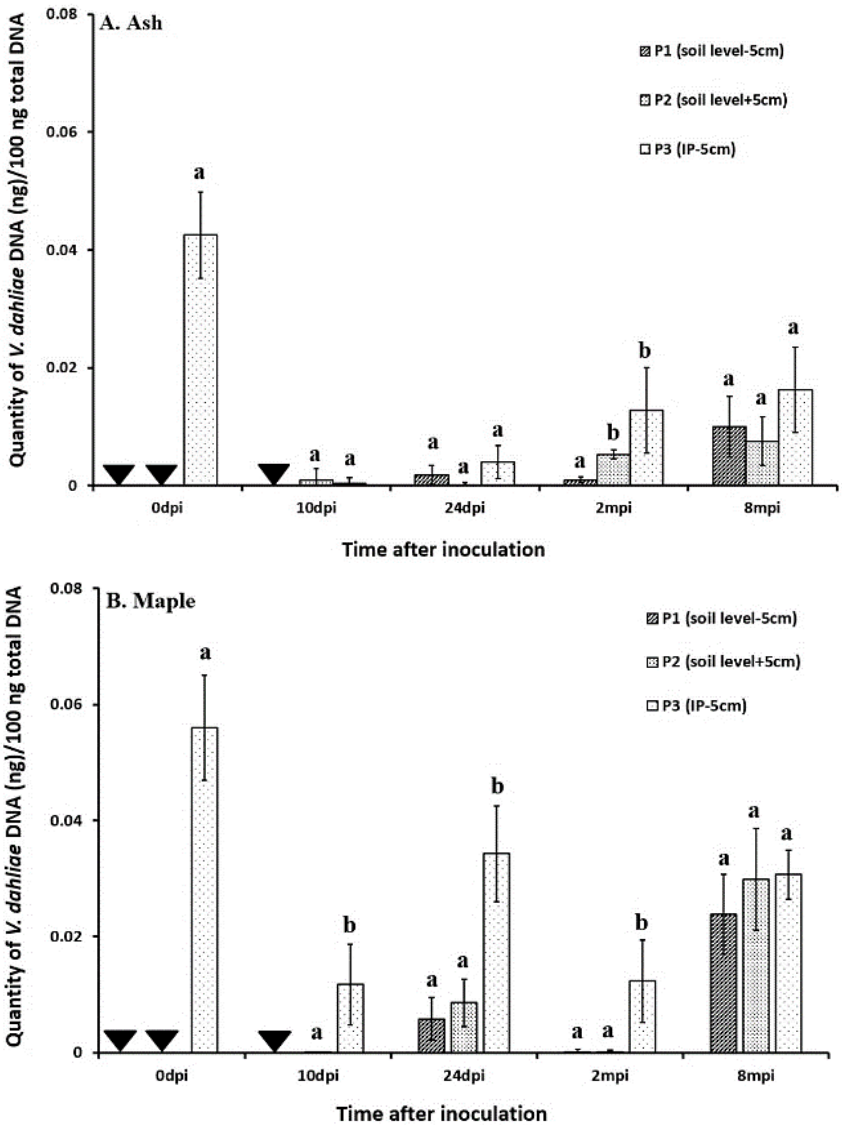


Figure 5. Amount of *V. dahliae* DNA detected at three points below the inoculation point (IP) in the stem of inoculated ash (A) and maple trees (B) at different time points (dpi=days post inoculation, mpi = months post inoculation). Each bar represent the mean of samples from 5 individual trees. Error bars show standard deviations. An inverted solid triangle (▼) indicates that *V. dahliae* DNA was not detected (threshold value 0.001 ng of DNA according to the standard curve). Significant differences in quantities of *V. dahliae* DNA detected in different stems positions at each time point have been indicated by different letters added on top of the bars ($P = 0.05$).

Changes in biomass of V. dahliae in infected maple and ash trees over time. To investigate if there is a difference in proliferation of *V. dahliae* in inoculated ash and maple trees, we examined the *V. dahliae* biomass within the infected trees over time. To this end, at each time point we measured the *V. dahliae* DNA quantities at different heights in the stems. The mean values of *V. dahliae* DNA quantities detected at the corresponding positions in five trees tested at each time point and standard error of the means for all tested points were calculated (Table 2).

Table 2. Quantities of *V. dahliae* DNA (ng) in 100 ng total DNA (including pathogen and plant DNA) detected in samples collected at different positions in the stem of inoculated ash (A) and maple trees (B) at different time points[1].

A. Ash		Ash						
Position in stem	(cm)[2]	0 dpi[6]	10 dpi	24 dpi	60 dpi	8 mpi[7]	11 mpi	14 mpi
P12	+140	X[3]	x	x	x	x	x	x
P11	+120	x	x	x	x	x	x	x
P10	+100	x	x	x	x	x	x	x
P9	+80	x	x	x	0.0090±0.0050^{a}	0.0185±0.0041^{a}	x	x
P8	+60	ND[4]	ND	0.0190±0.0075^{b}	0.0161±0.0081^{a}	0.0211±0.0070^{a}	x	x
P7	+40	ND	0.0048±0.0017^{a}	0.0333±0.0093^{b}	0.0274±0.0135^{a}	0.0215±0.0081^{a}	0.0658±0.0122^{c}	0.0131±0.0021^{b}
P6	+20	0.0002±0.0001^{b}	0.0077±0.0047^{a}	0.0242±0.0149^{ab}	0.0217±0.0094^{a}	0.0204±0.0109^{a}	0.0074±0.0024^{b}	0.0077±0.0010^{ab}
P5	+10	0.0304±0.0134^{a}	0.0045±0.0020^{a}	0.0205±0.0112^{ab}	0.0126±0.0044^{a}	0.0209±0.0125^{a}	0.0061±0.0007^{b}	0.0090±0.0040^{ab}
P4	+5	0.0516±0.0211^{a}	0.0039±0.0018^{a}	0.0040±0.0027^{a}	0.0099±0.0031^{a}	0.0177±0.0102^{a}	0.0002±0.0001^{a}	0.00240.0002^{a}
IP								
P3	-5	0.0425±0.0073^{a}	0.0004±0.0001^{b}	0.0039±0.0018^{a}	0.0128±0.0072^{a}	0.0163±0.0071^{a}	x	x
P2	+5	ND	0.0009±0.0002^{b}	0.0002±0.0001^{a}	0.0053±0.0007^{b}	0.0075±0.0041^{a}	0.0002±0.0001^{a}	0.0018±0.0003^{a}
SL								
P1	-5	ND	ND	0.0018±0.0006^{a}	0.0010±0.0004^{b}	0.0099±0.0051^{a}	x	x
		0.0312±0.0120^{Avr5}	0.0037±0.0017^{Avr*}	0.0133±0.0081^{Avr}	0.0134±0.0085^{Avr}	0.0171±0.0051^{Avr}	0.0159±0.0101^{Avr}	0.0068±0.0040^{Avr*}

B. Maple		Maple						
Position in stem	(cm)	0 dpi	10 dpi	24 dpi	60 dpi	8 mpi	11 mpi	14 mpi
P12	+140	x	x	x	x	0.2355±0.0266^{a}	x	x
P11	+120	x	x	x	x	0.3473±0.0316^{b}	x	x
P10	+100	ND	ND	ND	0.0026±0.0022^{b}	0.2676±0.0404^{ab}	x	x
P9	+80	ND	ND	0.0055±0.0007^{c}	0.0123±0.0092^{ab}	0.2386±0.0286^{a}	x	x
P8	+60	ND	0.0010±0.0001^{c}	0.0431±0.0167^{b}	0.0109±0.0091^{ab}	0.1995±0.0568^{a}	x	x
P7	+40	ND	0.0073±0.0011^{bc}	0.1205±0.0362^{a}	0.0134±0.0101^{ab}	0.2124±0.0509^{a}	0.1521±0.0214^{b}	0.2508±0.0395^{a}
P6	+20	ND	0.0296±0.0203^{ab}	0.0983±0.0204^{a}	0.0187±0.0012^{ab}	0.3334±0.0330^{b}	0.2152±0.0239^{b}	0.2233±0.0257^{a}
P5	+10	0.0009±0.0002^{b}	0.0367±0.0235^{ab}	0.1010±0.0125^{a}	0.0579±0.0186^{ab}	0.3579±0.0520^{b}	0.1971±0.0230^{b}	0.2676±0.0231^{a}
P4	+5	0.0360±0.0130^{a}	0.0616±0.0231^{a}	0.1073±0.0216^{a}	0.0259±0.0193^{a}	0.1995±0.0179^{a}	0.1403±0.0024^{b}	0.2068±0.0229^{a}
IP								
P3	-5	0.0560±00.0090^{a}	0.0118±0.0069^{ab}	0.0343±0.0082^{b}	0.0123±0.0071^{ab}	0.0307±0.0041^{d}	x	x
P2	+5	ND	ND	0.0086±0.0041^{c}	0.0002±0.0001^{c}	0.0299±0.0087^{d}	0.0144±0.0001^{a}	0.1589±0.0199^{a}
SL								
P1	-5	ND	ND	0.0059±0.0037^{c}	0.0003±0.0001^{c}	0.0238±0.0067^{d}	x	x
		0.0310±0.0128^{Avr}	0.0247±0.0123^{Avr}	0.0583±0.0281^{Avr}	0.0193±0.0107^{Avr}	0.2353±0.0736^{Avr*}	0.1438±0.048^{Avr*}	0.2215±0.0422^{Avr*}

[1]Values represent means ± standard error for five trees at each time point. Significant differences in quantities of *V. dahliae* DNA detected in different stems positions at each time point have been indicated by different letters added to the values ($P = 0.05$).
[2] Distances (cm) from the inoculation point (IP) (P3-P12) and from the soil level (SL) (P1 and P2).
[3] X = points that did not exist in tested trees.
[4] ND = indicates positions in which *V. dahliae* was not detected.

[5] Avr = averages of the *V. dahliae* DNA quantities as detected at *V. dahliae*-positive positions at each time point. Mean values of *V. dahliae* DNA quantities that are indicated with asterisks in table A are significantly different (P=0.05) from the mean values of *V. dahliae* DNA quantities as calculated for other time points in ash trees. Mean values of *V. dahliae* DNA quantities that are indicated with asterisks in table B are significantly different (P=0.05) from the mean values of *V. dahliae* DNA quantities as calculated for other time points in maple trees. In each table, mean values of *V. dahliae* DNA quantities that are indicated with asterisks are not significantly differing from each other.
[6] dpi = days post inoculation.
[7] mpi = months post inoculation.

In ash trees, from 10 dpi onward, the quantities of *V. dahliae* DNA detected at different heights in the stem were more or less at the same level with the differences between the quantities detected at different levels in the stem generally statistically not being significant (P=0.05) (Table 2A). In contrast, *V. dahliae* DNA quantities detected in maple varied much stronger with in the year of inoculation the quantities detected at higher points (P8 at 10 dpi, P8 and P9 at 24 dpi, and P10 at 60 dpi) being significantly (P=0.05) lower than the quantities detected at points closer to the inoculation site. However, in the year after inoculation (8 mpi), the amount of *V. dahliae* DNA in top part (P10) did not differ significantly from lower parts (P=0.05) (Table 2B). Comparison of the mean *V. dahliae* DNA quantities in ash and maple trees at different time points, as determined by averaging the amounts detected at different heights in the stem of the examined trees, revealed that in the year of inoculation there was no significant difference between maple and ash trees at each of the time points tested, except at 24 dpi (Figure 6; see also Table 2A and B, last lines). At this time point, the mean quantity of *V. dahliae* DNA in maple trees was significantly higher than the mean quantity of *V. dahliae* DNA in ash trees (P=0.05). However, from 8 mpi (start of the growing season in the year after inoculation) onward, the amounts of *V. dahliae* DNA in the stem of maple trees showed a significant increase when compared with the quantities detected at 0, 10, 24 and 60 dpi (in the year of inoculation), while quantities of *V. dahliae* DNA in the stem of ash trees did not show any increase (P=0.05) (Table 2). Notably, from 8 mpi onward, quantities of *V. dahliae* DNA in ash trees were significantly lower than in maple trees (P=0.05) (Figure 6). These data show that the ash xylem is less supportive than the maple xylem for the growth of *V. dahliae*.

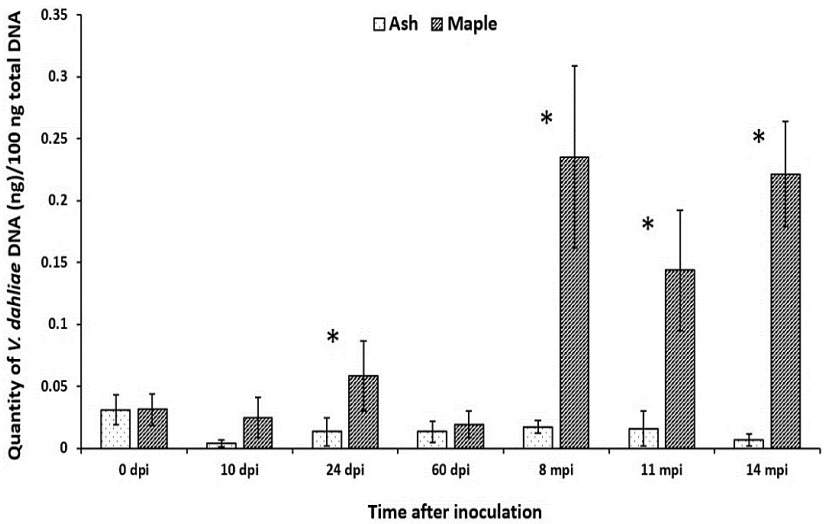


Figure 6. Comparison between mean relative quantities of *V. dahliae* DNA detected in maple and ash trees at different time points after inoculation. Each bar is the mean value of *V. dahliae* DNA quantities as detected at different heights in the stem of five examined trees (see Table 2). Asterisks indicate significant differences in quantities of *V. dahliae* DNA detected in inoculated maple and ash trees at that time point (P=0.05). Dpi = days post inoculation, mpi = months post inoculation.

Presence of V. dahliae upon de novo xylem formation. To investigate movement of *V. dahliae* from the xylem of the growth ring of the year of inoculation into newly formed xylem vessels of the next year's growth ring, we analysed subsamples from the xylem of two successive years in both species by plating assays using samples collected at 11 mpi and 14 mpi. Four maple trees at each time point, as well as four ash trees at 11 mpi and six ash trees at 14 mpi, were examined. Two of the tested ash trees at each of the time points were recovered trees, meaning that they showed clear disease symptoms in the year of inoculation but became symptomless in the year after inoculation (11 mpi/14 mpi). At each time point, one non-inoculated tree from each species was used as control. Interestingly, *V. dahliae* was not found in symptomless ash trees based on plating assays, while it was recovered from most samples from old as well as new growth rings of symptomatic ash trees at both time points (11 mpi and 14 mpi) (Table 3A). In maple, *V. dahliae* was recovered from old and new growth rings of most of the tested trees at both time points, whereas the pathogen could not be recovered from two tested trees (tree 3 at 11 mpi and tree 1 at 14 mpi) (Table 3B).

Table 3. Results of isolation of *V. dahliae* from new and old xylem of both recovered and symptomatic ash trees (A) and from symptomatic maple trees (B) in the year after inoculation (11 mpi, 14 mpi).

A. Ash	11 mpi[5]										14 mpi													
	Recovered trees				Symptomatic trees				Control		Recovered trees				Symptomatic trees								Control	
Tested trees	Tree 1 (49)[1]		Tree 2 (26)		Tree 3 (94)		Tree 4 (16)		Tree 5 (3)		Tree 1 (19)		Tree 2 (67)		Tree 3 (95)		Tree 4 (100)		Tree 5 (17)		Tree 6 (46)		Tree 7 (81)	
Growth ring	Old[2]	New[3]	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New
Number of chips placed on agar	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15
Number of Vd-Positive chips	-[4]	-	-	-	6	2	1	1	-	-	-	-	-	-	1	-	-	-	7	2	2	2	-	-

B. Maple	11 mpi										14 mpi									
Tested trees	Tree 1 (193)		Tree 2 (131)		Tree 3 (190)		Tree 4 (154)		Tree 5 (168) Control		Tree 1 (102)		Tree 2 (148)		Tree 3 (145)		Tree 4 (132)		Tree 5 (187) Control	
Growth ring	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New
Number of chips placed on agar	10	20	10	20	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15
Number of Vd-Positive chips	1	10	4	14	-	-	-	15	-	-	-	-	1	14	-	13	10	6	-	-

[1] Between brackets are the identification numbers of the trees from which the chips originated.
[2] Old = growth ring of the year of inoculation.
[3] New = growth ring of the year after inoculation.
[4] - = *V. dahliae* was not recovered.
[5] mpi = months post inoculation.

Presence of *V. dahliae* in the xylem of the growth ring of the year of inoculation as well as in that of the next year of ash (both recovered and symptomatic trees) and maple trees was further studied by real-time PCR at two time points in the year after inoculation (11 mpi and 14 mpi). To this end, xylem subsamples from both growth rings, separated by using scalpel and forceps under a binocular at three points above the inoculation point (P4, P6, P8 in maple trees, and P4, P5, P6 in ash trees) were examined. In this assessment, 75% (27 out of 36) of the subsamples tested from six symptomatic ash trees at 11 mpi and 14 mpi contained *V. dahliae* DNA in the vessels of both years (Table 3A). In contrast, from four symptomless ash trees at 11 mpi and 14 mpi *V. dahliae* DNA was detected only in 25% (3 out of 12) of the tested subsamples, and always in the xylem from the year of inoculation and never in the xylem of the new growth ring (Table 4A). In maple trees, *V. dahliae* DNA was detected in over 80% of all tested subsamples from new and old vessels of tested trees (Table 4B). Notably, *V. dahliae* was not detected in negative control samples from ash and maple trees when tested by real-time PCR and plating assays.

Table 4. Quantities of *V. dahliae* DNA (ng) in 100 ng of total DNA (including pathogen and plant DNA) as detected in successive growth rings of ash (A) and maple (B) trees in the year after inoculation (11 mpi, 14 mpi) by real-time PCR. In each species samples from three different positions in the stem (P4, P5 and P6 in ash and P4, P6 and P8 in maple trees) were examined.

A. Ash		11 mpi[5]					14 mpi						
		Recovered		Symptomatic		Control	Recovered		Symptomatic				Control
Position in stem	Growth ring	Tree 1 (49)[3]	Tree 2 (26)	Tree 3 (94)	Tree 4 (16)	Tree 5 (3)	Tree 1 (19)	Tree 2 (67)	Tree 3 (95)	Tree 4 (100)	Tree 5 (17)	Tree 6 (46)	Tree 7 (81)
P4	New[1]	ND[4]	ND	0.0243	0.0106	ND	ND	ND	ND	0.0013	ND	ND	ND
	Old[2]	ND	ND	0.0285	0.0224	ND	0.0029	ND	0.0099	0.0091	0.0136	0.0081	ND
P5	New	ND	ND	ND	0.0019	ND	ND	ND	ND	ND	0.0020	0.0026	ND
	Old	ND	0.0015	0.0199	0.0216	ND	ND	ND	0.0372	0.0019	0.0068	0.0098	ND
P6	New	ND	ND	ND	0.0035	ND	ND	ND	0.0018	ND	ND	ND	ND
	Old	0.0050	ND	ND	0.0235	ND	ND	ND	0.0006	0.0058	0.0177	0.0232	ND

B. Maple		11 mpi					14 mpi				
Position in stem	Growth ring	Tree 1 (193)	Tree 2 (131)	Tree 3 (190)	Tree 4 (154)	Tree 5 (168) Control	Tree 1 (102)	Tree 2 (148)	Tree 3 (145)	Tree 4 (132)	Tree 5 (187) Control
P4	New	ND	ND	ND	0.2555	ND	ND	0.5568	0.0213	0.5605	ND
	Old	ND	ND	0.0086	0.3316	ND	0.0093	0.0168	0.1929	0.2969	ND
P6	New	ND	0.3641	0.0005	0.0514	ND	ND	0.7419	0.2209	0.5661	ND
	Old	0.2935	0.5135	0.0113	0.1876	ND	0.0059	0.0916	0.2875	0.2273	ND
P8	New	0.1148	0.5956	ND	0.0624	ND	0.0068	0.7965	0.2433	0.2428	ND
	Old	0.3225	0.2415	0.0168	0.0094	ND	ND	0.0147	0.3845	0.0981	ND

[1] Old = growth ring of the year of inoculation.
[2] New = growth ring of the year after inoculation.
[3] Between brackets are the number of the tree from which the chips originated.
[4] ND = indicates positions in which *V. dahliae* was not detected.
[5] mpi = months past inoculation.

Discussion

Little is known about differences in the pattern of *V. dahliae* distribution in the stems of infected tree species that differ in anatomy of the xylem. As vascular pathogens like *V. dahliae* colonize their hosts through the xylem vessels, it can be expected that the speed and extent of colonization after a localized infection will be influenced by the xylem anatomy of that host. Also, some tree species such as olive, cherry, apricot, peach, cacao, catalpa, and ash are able to recover from Verticillium wilt; a capability in which the anatomy of the xylem is supposed to play an important role (Banfield, 1968; Emechebe et al., 1974; Hiemstra and Harris, 1998; Sinclair et al., 1987; Tippett and Shigo, 1981). However, the fate of *V. dahliae* in recovered trees in the years following the initial infection has been unrevealed so far. This study investigated the spatial and temporal distribution of *V. dahliae* in relation to disease progression and recovery in stem-inoculated maple and ash trees, two species that differ strongly in vascular anatomy with maple having a diffuse porous xylem anatomy whereas ash has a ring porous xylem anatomy (Schweingruber et al., 2013). The main difference between these two types of xylem anatomy is that in ring porous species the xylem vessels that are formed early in the growth season have a much larger diameter (~ 2.5 to 3.5 times) than the vessels formed later in the season, whereas in diffuse porous species the diameter of the xylem vessels is more or less the same regardless of the position in the ring (Cochard and Tyree 1990; Core et al., 1979). Despite these innate differences in the anatomy of their xylem, the speed of *V. dahliae* colonization in the inoculated ash and maple trees did not really differ between the two species. This may be due to the inoculations being carried out relatively late in the season and the cut into the stem being only few millimeters deep into the xylem. As a result, the conidia were likely introduced mainly in the vessels of the outer part of the growth ring; in the case of ash in the smaller sized latewood vessels. Additionally, the

plants of both species were rather young, when shoot vessel dimensions are usually smaller than in mature stems (Zimmermann, 1983). The latter aspect may also explain the decrease in speed of colonization towards the top of the maple plants.

Directly after inoculation the pathogen was detected up to 10 cm both upward and downward from the inoculation site in inoculated stems of both species. As there was no time for hyphal growth, this must be the result only of the conidial suspension being drawn into the severed vessels as a result of the low pressure potential within those vessels. In the first ten days after inoculation, the fungus moved at least 30 cm upward in ash and even 50 cm in maple, corresponding to 3-5 cm per day. From day 10 on the speed of colonization in maple is decreasing (Table 2), but is still well over the maximum growth rate of *V. dahliae* hyphae of about 8 mm/day (ElSharawy, 2015; Rampersad, 2010). These results confirm the important role of conidiospore transport upward with the sap stream in the xylem of infected trees.

Hiemstra and Harris (1998) reported that ash trees are able to recover from Verticillium wilt, whereas maple trees usually show progressive dieback of the aerial parts. Our observations confirm this earlier observation. Notably, the difference in disease incidence in maple and ash trees correlated with a difference in quantities of *V. dahliae* DNA detected in these two species in the year after inoculation (Figure 6). Moreover, in the year after inoculation we were not able to detect or recover the pathogen in new xylem sheaths and only rarely in old xylem sheaths of recovered ash trees (Tables 3A, 4A). Similar results were observed for olive trees infected with *V. dahlia*, where reduction in symptoms was associated with a decrease in *V. dahliae* DNA in newly developed asymptomatic shoots (Markakis et al., 2009; Mercado-Blanco et al., 2003). Therefore, it appears that recovery correlates with the inactivation of the fungus in the xylem and impeding new infections (Hiemstra, 1995a, b; Rodríguez-Jurado, 1993; Sinclair et al., 1981; Talboys, 1968; Wilhelm and Taylor, 1965). It has been reported that *V. dahliae* can become inactivated by high air temperature or other non-favourable environmental conditions in the field (Wilhelm and Taylor, 1965; Taylor and Flentje, 1968) or by antimicrobial phenolic components that are produced by the host (Baídez et al. 2007; Markakis et al. 2010). Based on our data it can be ruled out that the remission of symptoms

in ash is caused by unfavorable environmental factors for the pathogen because the maple trees in the same field showed a steady increase of symptoms over the same two year period in which a number of the diseased ash trees recovered. Thus, recovery of the ash trees must be the result of inherent characteristics of (the xylem of) this species.

In the present study, *V. dahliae* DNA could be detected in both successive xylem sheaths of maple and symptomatic ash trees. Moreover, the pathogen could also be re-isolated from both xylem sheaths. This demonstrates that the fungus can still be present and alive in the xylem of a tree one year after infection. For ash it also shows that, despite the presence of a layer of parenchyma cells between two growth rings which is supposed to restrict penetration of pathogens from the xylem of one growth ring to the xylem of the growth ring of next year (Braun, 1970), infection of the new xylem layer did occur. One explanation is that *V. dahliae* is able to penetrate through this layer of parenchyma. Another explanation, however, could be that infection of xylem of the new growth year in infected ash trees occurs from the root area where the percentage of vessels per unit area is much higher than in the stem and branch wood (Banfield, 1968). To this end, downward movement of the pathogen within infected xylem vessels toward roots would be required. Surprisingly, in this study we observed that *V. dahliae* indeed spreads downwards in the stem of both species and with considerable speed (Table 2), as pathogen DNA was detected at 5 cm under the soil level of the main stem of stem-inoculated ash trees at 24 days after inoculation. Consequently, it is a well possible that infection of new xylem vessels of ash trees occurred from the root area.

Summarizing, it can be concluded that differences in the xylem anatomy of ash and maple did not significantly affect the speed and extent of the upward spread of the pathogen in stem-inoculated trees. Furthermore, despite the presence of a layer of marginal parenchyma cells between the growth rings in ash trees, infection of the new xylem layer did occur in the year after inoculation. Nevertheless, this transition to the new growth ring was not observed in recovered ash trees, while in recovered ash trees proliferation of the pathogen is also impeded suggesting that the ash xylem is much less supportive for the growth of *V. dahliae* than the maple xylem. However, further studies are necessary to uncover the mechanisms responsible for the reduction of the presence of

the pathogen in recovered trees. We also observed a fast downward movement of the pathogen from the point of inoculation into the root collar. This may provide a way for infection of the xylem of the new growth ring by circumventing the mechanical barriers in the stem xylem. Moreover, in addition to the inoculum from infected leaves falling from diseased ash trees (Rijkers et al., 1992), it may provide new inoculum (from infected roots) for contamination of the soil in case of cultivating infected plants in non-infested soil.

Acknowledgments

Work on Verticillium wilts of trees at Applied Plant Research organization (PPO) of Wageningen University has been supported financially by a scholarship of the Ministry of Science and Technology of Iran. We also thank K.T.K. Pham, Gloria M. García-Ruiz and Mario Pérez-Rodríguez for very valuable technical support in the lab.

References

Baídez, A.G., Gomez, P., Del Rio, J.A., Ortuno, A. (2007). Dysfunctionality of the xylem in Olea europaea L. plants associated with the infection process by *Verticillium dahliae* Kleb. Role of phenolic compounds in plant defense mechanism. Journal of Agricultural and Food Chemistry 55, 3373-3377.

Banfield, W.M. (1968). Dutch elm disease recurrence and recovery in American elm. Journal of Phytopathology 62, 21-60.

Bonsen, K.J.M., Scheffer, R.J., Elgersma, D.M. (1985). Barrier zone formation as a resistance mechanism of elms to Dutch elm disease. IAW ABull, n.s., 6, 71-77.

Braun, H.J. (1970). Funktionelle Histologie der sekundären Sprossachse. I. Das Holz. Gebrüder Borntraeger, Berlin - Stuttgart. 190 pp.

Ciccarese, F., Frisullo, S., Cirulli, M. (1990). Natural disease recovery from Verticillium wilt in peach. Page6 in : Proceedings of the 5th International Verticillium Symposium, 25th-30th June 1990, Leningrad, USSR, 11 3pp.

Cochard, H., Tyree, M.T. (1990). Xylem dysfunction in Quercus: vessel sizes, tyloses, cavitation and seasonal changes in embolism. Tree Physiology 6, 393-407.

Core, H.A., Côté, W.A., Day, A.C. (1979). Wood structure and identification. 2nd ed. Syracusa University Press. 182 pp.

ElSharawy, A.A., Hu, X., Yang, J. (2015). Trade-Offs between Growth Rate, Sporulation and Pathogenicity in *Verticillium dahliae*. Journal of Agricultural Science 7 (7), 35-41; doi:10.5539/jas.v7n7p35.

Emechebe, A.M., Leakey, C.L.A., Banage, W.B. (1974). Verticillium wilt of cacao in Uganda: wilt induction by mechanical vessel blockage and mode of recovery of diseased plants. The East African Agricultural and Forestry Journal 39, 337-343.

Gleason, M., Hartman, J. (2001). Chapter 60, Maple Diseases, In: Diseases of Woody Ornamentals and Trees in Nurseries, APS Press P. 236-241.

Grosser, D. (1977). Die Hölzer Mitteleuropas. Ein mikrophotographischer Lehratlas. Springer-Verlag, Berlin etc. 208 pp.

Harris, D.C. (1998). Maple. In: Hiemstra JA, Harris DC, 1998. A compendium of Verticillium wilts in trees: 35-36.

Heffer, V.J., Regan, R.P. (1996). First report of Verticillium wilt caused by *Verticillium dahliae* of ash trees in Pacific Northwest nurseries. Plant Disease 80, 342.

Hiemstra, J.A. (1995a). Verticillium wilt of *Fraxinus excelsior*. PhD Thesis, Wageningen Agricultural University, The Netherlands, xvi + 213 pp. ISBN 90-5485-360-3.

Hiemstra, J.A. (1995b). Recovery of verticillium-infected ash trees. Phytoparasitica 23, 64-65.

Hiemstra, J.A. (1998). Some general features of Verticillium wilts in trees. In: Hiemstra JA, Harris DC, 1998. A compendium of Verticillium wilts in trees: 5-11.

Hiemstra, J.A., Harris, D.C. (Eds.) (1998). A compendium of Verticillium wilts in tree species. Wageningen: CPRO-DLO/West Malling: HRI-East Malling.

López-Escudero, F.J., Blanco-López, M.A. (2005). Recovery of young olive trees from *Verticillium dahliae*. European Journal of Plant Pathology 113, 365-375.

Latorre, B.A., Allende, T. (1983). Occurrence and incidence of Verticillium wilt of Chilean avocado groves. Plant Disease 67, 445-447.

Lockwood, J.L. (1977). Fungistasis in soils. Biological Reviews 52, 1-43.

Manion, P.D. (2003). Evolution of concepts in forest pathology. Phytopathology 93, 1052-1055.

Markakis, E.A., Tjamos, S.E., Antoniou, P.P., Paplomatas, E.J., Tjamos, E.C. (2009). Symptom development, pathogen isolation and real-time qPCR quantification as factors for evaluating the resistance of olive cultivars to Verticillium pathotypes. European Journal Plant Pathology 124, 603-611.

Markakis, E.A., Tjamos, S.E., Antoniou, P.P., Roussos, P.A., Paplomatas, E.J., and Tjamos, E.C. (2010). Phenolic responses of resistant and susceptible olive cultivars induced by defoliating and nondefoliating *Verticillium dahlia*e pathotypes. Plant Disease 94:1156-1162.

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodríguez-Jurado, D., Jiménez-Díaz, R.M. (2003). Quantitative monitoring of colonization of olive genotypes by *Verticillium dahlia* pathotypes with real-time polymerase chain reaction. Physiological and Molecular Plant Pathology 63, 91-105.

Nelson, E.B. (1990). Exudate molecules initiating fungal responses to seeds and roots. Plant and Soil, 129, 61-73.

Paplomatas, E.J, Elena, K. (1998). Some general features of Verticillium wilts in trees. In: Hiemstra JA, Harris DC, 1998. A compendium of Verticillium wilts in trees: 31-32.

Pegg, G.F., Brady, B.L. (2002). Verticillium Wilts. Wallingford, UK: CABI publishing.

Piearce, G.D., Gibbs, J.N. (1981). Verticillium wilt of trees and shrubs. Arboricultural leaflet 9. Department of the Environment, Forestry Commission, HMSO, London.

Prieto, P., Navarro-Raya, C., Valverde-Corredor, A., Amyotte, S.G., Dobinson, K.F., Mercado-Blanco, J. (2009). Colonization process of olive tissues by *Verticillium dahliae* and its in planta interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7. Microbial Biotechnology 2(4), 499–511.

Rampersad S.N. (2010). A study of environmental factors that affect survival of pumpkin isolates of *Verticillium dahliae*. HortScience 45 (8), 1211-1217.

Riffle, J.W., Peterson, G.W. (1989). Diseases of trees in the Great Plains. Gun. Tech. Rep. RM-129. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Forest and Range Experiment Station; 1986. 149 p.

Rijkers, A.J.M., Hiemstra, J.A., Bollen, G.J. (1992). Formation of microsclerotia of *Verticillium dahliae* in petioles of infected ash trees. Netherlands Journal of Plant Pathology, 98, 261-264.

Rodríguez-Jurado, D., Blanco-López, M.A., Rappoport, H.F., Jiménez-Díaz, R.M. (1993). Present status of Verticillium wilt of olive in Andalucía (southern Spain). EPPO Bulletin 23, 513-516.

Schreiber, L.R., Green, R.J. (1963). Effect of root exudates on germination of conidia and microsclerotia of *Verticillium albo-atrum* inhibited by the soil fungistatic principle. Phytopathology 53, 260-264.

Schweingruber, F.H. (1990). Anatomy of European woods. An atlas for the identification of European trees, shrubs and dwarf shrubs. Paul Haupt Publishers, Berne and Stuttgart. 800 pp.

Schweingruber, F.H., Börner, A., Schulze, E.-D. (2013). Atlas of Stem Anatomy in Herbs, Shrubs and Trees. Springer Heidelberg Dordrecht London New York. Volume 2, VIII, 415 p. 1532 illus. ISBN 978-3-642-11637-7.

Shigo, A.L. (1984). Compartmentalization: A conceptual framework for understanding how tree grow and defend themselves. Annual Review of Phytopathology, 22, 189-214.

Sinclair, W.A., Smith, K.L., Larsen, A.O. (1981). Verticillium wilt of maples: Symptoms related to the movement of the pathogens in stems. Phytopathology 71, 340-345.

Sinclair, W.A., Lyon, H.H., Johnson, W.T. (1987). Diseases of trees and shrubs. Cornell University Press, Ithaca, New York. 574 p.

Smith, K.T. (2006). Compartmentalization today. Arboricultural Journal 29, 173-184.

Smith, I.M., Dunez, J., Lelliott, R.A., Phillips, D.H., Archer, S.A. (1988). European handbook of plant diseases. Blackewell Scientific Publications, Oxford etc., 583 S.

Talboys, P.W. (1968). Water deficits in vascular disease. In: Water Deficits and Plant Growth. Vol II: Plant Water Consumption and Response, (pp. 255–311) Academic Press, New York and London.

Taylor, J.B., Flentje, N.T. (1968). Infection, recovery from infection and resistance of apricot trees to *Verticillium albo-atrum*. New Zealand Journal of Botany 6, 417-426.

Tippett, J.T., Shigo, A.L. (1981). Barrier zone formation: a mechanism of tree defense against vascular pathogens. IAWA Bulletin n.s. 2, 163-168.

Townsend, A.M., Schreiber, L.R., Hall, T.J., Bentz, S.E. (1990). Variation in response of Norway maple cultivars to *Verticillium dahliae*. Plant Disease 74, 44-46.

Van Doorn, J., Pham, K.T.K., Hiemstra, J.A. (2009). Molecular detection of *Verticillium dahliae* in soil. 10th International Verticillium Symposium, abstract book p. 94.

Vigouroux, A., Castelain, C. (1969). La verticilliose de l'abricotier. I. Premières observations sur les symptoms et l'évolution de la maladie. Hypothèses sur quelques facteurs de variation.Annual Phytopathology 1, 427-448.

Weller, S.A., Elphinstone, J.G., Smith, N.C., Boonham, N., Stead, D.E. (2000). Detection of Ralstonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Applied Environmental Microbiology 66, 2853-2858.

Wilhelm, S., Taylor Bruce, J. (1965). Control of Verticillium wilt in olive trough natural recovery and resistance. Phytopathology 55, 310-316.

Worf, G.L., Spear, R., Heimann, S.M.F. (1994). Verticillium induced scorch and chlorosis in ash. Journal of Environmental Horticulture 12, 124-130.

Zimmermann, M.H. (1983). Xylem structure and the ascent of sap. Springer Verlag, Berlin etc. 143 pp.

Chapter 7

General discussion

Introduction

Verticillium wilt disease is one of the major constraints for olive (*Olea europaea* L.) plantations and tree nurseries, and causes substantial reduction in the production of olive orchards and high rates of tree mortality (Goud et al. 2011; Hiemstra and Harris 1998; Jiménez-Díaz et al. 2012). 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 (Hiemstra, 1998; López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al. 2012). Thus, as an important pre-planting measure, new plantations should not be stablished in or near fields with a known history of *Verticillium* infections (Jiménez-Díaz et al. 2012). Arguably, the best measure to control Verticillium wilt disease is by planting on soils 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 (Hiemstra et al. 2014; López-Escudero and Mercado-Blanco 2011; Jiménez-Díaz et al. 2012; Melero-Vara et al. 1995; Tjamos and Jiménez-Díaz 1998).

Use of healthy planting material

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 (Evans and Gleeson 1973; Mathre 1986; Malcolm et al. 2013). *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 (Karajeh and Masoud 2006). Moreover, nested-PCR analysis and plating assays have shown that seeds harvested from asymptomatic olive trees can transmit the pathogen to seedlings (Karajeh 2006). 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 (Malcolm et al. 2013; Petrini 1991). Currently, endophytic colonization of *V. dahliae* has been reported mainly from monocotoledonous plant species, such as barley, oat and wheat (Krikun and Bernier 1987; Mol 1995). 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. (Malcolm et al. 2013; Pegg and Brady 2002; Vallad et al. 2005). 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.

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 (Mercado-Blanco et al. 2003a; Prieto et al. 2009; Rodríguez-Jurado et al. 1993). In this thesis, *V. dahliae* could be detected in above-ground tissues of infected olive trees only one week after inoculation, while the first symptoms were only observed at about 5-7 weeks after inoculation depending on the level of susceptibility of the cultivar that was used (Chapter 5). 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.

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 (Markakis et al. 2009; Mercado-Blanco et al., 2003a; chapter 3). 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 (Rodríguez-Jurado, 1993). 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 (Levin et al. 2003a; Chapter 3), 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 (Mercado-Blanco et al. 2003b; Gramaje et al. 2013). However, also the sampling strategy may have a major influence. We demonstrate that the testing mixed samples instead of individual samples improves the robustness of detection methods (Chapter 3). Thus, exploitation of these PCR-based *in planta* detection methods, in combination with sampling strategies as suggsted here, facilitates robust testing of planting material for *V. dahliae* presence, aiming to provide pathogen-free planting material for establishing new plantations.

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 (Taylor and Flentje 1968; Emechebe et al. 1974; Latorre and Allende 1983; Hiemstra 1995; Cirulli et al. 1998; Goud and Hiemstra 1998). Also in our work we 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 (Chapter 6). Interestingly, analysis of the distribution of the pathogen in the year after inoculation showed that new xylem sheahts in recovered ash trees were not infected by *V. dahliae*, whearas new xylem sheaths of both maple and symptomatic ash trees were infected (Chapter 6). 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 (López-Escudero and Blanco-López 2005; Wilhelm and Taylor 1965). 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

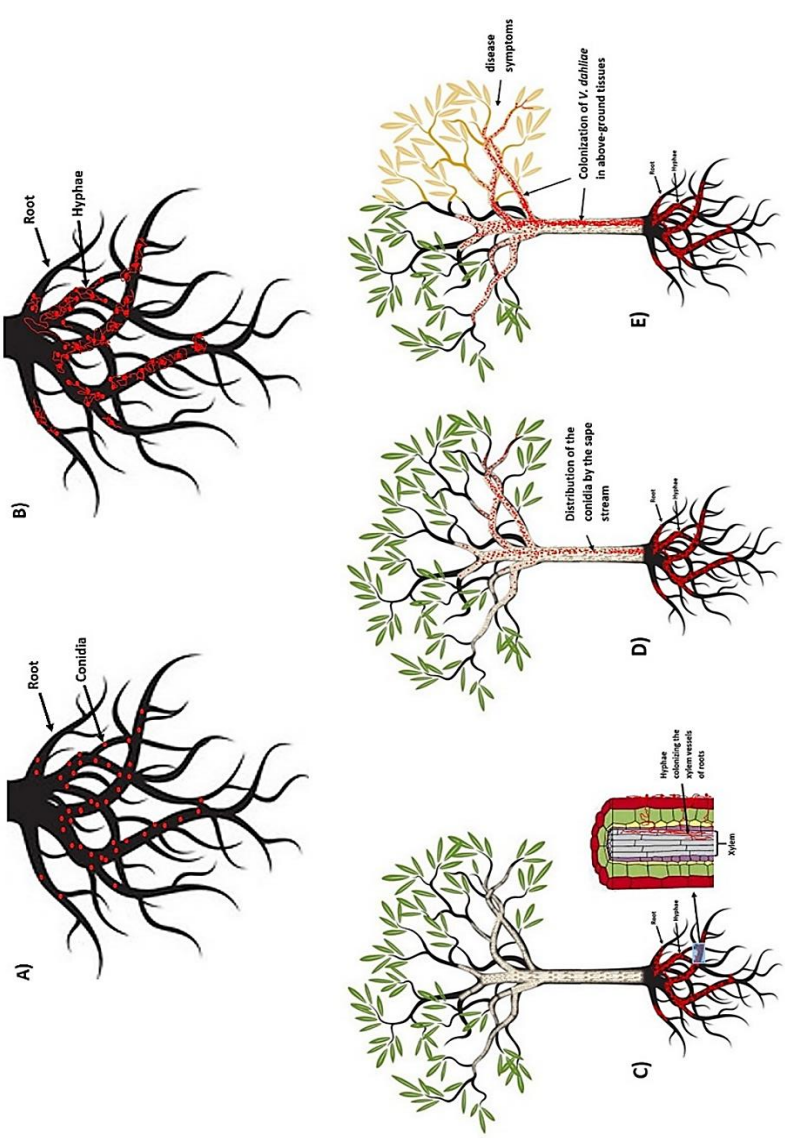


Figure 1. Progression of *V. dahliae* and disease symptoms in olive upon artificial inoculation. A) Attachment of conidiospores (red spots) to the root surface. B) Dense hyphal colonization of the root system. C) Hyphae colonizing the xylem vessels of roots. D) Distribution of conidia throughout the 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.

recovery (Jiménez-Díaz et al. 2012; López-Escudero and Mercado-Blanco 2011; Wilhelm and Taylor 1965). In this context, soil treatments such as soil solarization (Al-Ahmad 1993; Abu-Qamar and Al-Raddad 2001; Tjamos et al. 1991), soil fumigation (Conn et al. 2005; Gullino et al. 2002; Wilhelm et al. 1961), and organic or biological amendments (Blok et al. 2000; Goicoechea 2009; Hiemstra et al. 2013; Lima et al. 2008; Wilhelm and Taylor 1965) 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.

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. In chapter 6, we 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 (Hiemstra 1998; Nicole and Gianinazzi-Pearson 1996; Shigo 1984; Tippet and Shigo 1981). 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 (Shigo 1984). 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 (Garrett et al. 1979; Shain and Miller 1988; Smith 2006).

Recovery is also enhanced by producing new vascular tissue, which allows novel vegetative growth of affected stems and branches (Tjamos et al. 1991; López-Escudero and Blanco-López 2001). In trees in temperate climate zones every year a new zone of xylem elements (growth ring) is formed as long as 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) transdifferentiation which is defined as the conversion of one cell type into another with a different function (Okada 1991; Tosh and Slack 2002; Sugimoto et al. 2011), and 2) vascular hyperplasia which is generally defined as an induced increase in cell number as a result of infection (Talboys 1958; Jammes et al. 2005; Depuydt et al. 2009; Malinowski et al. 2012). 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 (Reusche et al. 2012). 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) (Demura et al. 2002; Kubo et al. 2005; Yamaguchi et al. 2010). 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 (Kubo et al. 2005; Reusche et al. 2012). 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 (Hu et al. 2010; Ohtani et al. 2011). Thus, studying the distribution of these genes or their homologs in other trees, and also 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.

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 (Blanco-López et al. 1998; Lopez-Escudero et al. 2004). Several experiments have been carried out to identify Verticillium wilt resistance in various tree species, such as maple (Townsend et al. 1990; Schreiber and Mayer 1992; Hiemstra 2000), pistachio (Ogawa and english 1991; Epstein et al. 2004), and olive (Bubici and Cirulli 2012; Mercado-Blanco & López-Escudero 2012; Antoniou et al. 2008; Trapero et al. 2013). Cultivars that have been introduced as resistant show reduction in disease progression when they are inoculated with *V. dahliae*, but can still be colonized by the pathogen as the pathogen could be isolated from inoculated trees. In this thesis, we studied distribution of *V. dahliae* in artificially inoculated 'Picual' and 'Frantoio' olive cultivars which are considered as highly susceptible and resistant to *V. dahliae*, respectively (López-Escudero et al. 2004). It was observed that 'Frantoio' plants not only showed signs of disease progression, but also were colonized by the pathogen, although the severity of disease symptoms and the amount of the pathogen detected were significantly lower than in 'Picual' (Chapter 5). 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 (Bubici and Cirulli 2011). 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 lycopersicum*) (Alexander 1972; Bae et al. 2008; Gulya 2007; Lynch et al. 1997; Schaible et al. 1951; Vallad et al. 2006; Klosterman et al. 2009). Nevertheless, for many other crops and tree species, genetic resistant is not readily available (Hiemstra and Harris 1998; Fradin and Thomma 2006). 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 (de Jonge et al. 2012; Fradin and Thomma 2009; Fradin et al. 2011; Kawchuk et al. 2001). Intriguingly, phylogenetic analysis showed that homologues of *Ve1* are widely distributed in plants (Song et al. 2016). So far, several *Ve1* homologous genes that confer race-specific resistance against *V. dahliae* have been reported such as *SlVe1* from *Solanum lycopersicoides* (Chai et al., 2003), StVe1 from *S. tuberosum* (Simko et al. 2004), *StVe* and *StoVe1* from *S. torvum* (Fei et al. 2004; Liu et al. 2012), *mVe1* from *Mentha longifolia* (Vining and Davis 2009), and *Vr1* from *Lactuca sativa* (Hayes et al. 2011). 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* (Zhang et al. 2012; Yang et al. 2014). 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* (Tang et al. 2016). However, genes conferring resistance to *V. dahliae* D and ND isolates have not been reported from tree hosts thus far.

A holistic approach for improvement of resistance to verticillium wilt disease

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 (e.g. Bubici and Cirulli 2011; López-Escudero and Mercado-Blanco 2011; Jiménez-Díaz et al. 2012; Mercado-Blanco and López-Escudero 2012). 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 (López-Escudero and Mercado-Blanco 2011; Jiménez-Díaz et al. 2012). 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 (Stuthman et al. 2007). 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 (Ramanatha Rao and Hodgkin 2001; Govindaraj et al. 2015). In the absence of sufficient diversity, mutagenesis followed by screening for enhanced resistance is a means to identify novel resistance traits (Schaible et al. 1951; Veronese et al. 2003). Actually, 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 (Shu et al. 2012). Several technologies have been developed for random mutation, e.g., radiation (gamma and X-ray) (Barakat et al. 2010; Nikam et al. 2014), chemical mutagens such as ethyl methanesulfonate and sodium azide and methylnitrosourea (Sikora et al. 2011), T-DNA- or transposon-based activation tagging

(Fladung and Polak 2012; Busov et al. 2011; Fladung et al. 2004; Harrison et al. 2007). 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 (van Harten 1998). 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 number of subcultures can be performed in a short time to increase the mutagenized population for selection (Penna et al. 2012). 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 olive genotypes that are developed in a breeding program, they should be challenged with the pathogen. López-Escudero et al. (2004) 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 particular 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 (Koike et al. 1994; Bhat and Subbarao 1999; Douhan and Johnson 2001) it is important to include isolates representing differential virulence in programs for evaluating host resistance to *V. dahliae* (Barbara et al. 1998).

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 olive and other tree species (Cerezo et al. 2011; Torreblanca et al. 2010). 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 kinase 2), and SOBIR1 (LRR-RLK

Suppressor Of BIR1-1) proteins (Fradin et al. 2009; Liebrand et al. 2013). Also, it has been reported that GhNDR1 and GhMKK2 are required for resistance mediated by the *GbVe1* and *Gbvdr5* genes in cotton (Gao et al. 2011). In tree hosts, however, many aspects of defence responses remain unknown and require investigation. With recent genomic and transcriptomic advances we are now better equipped to begin unravelling 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.

References

Abu-Qamar, M., and Al-Raddad, A. (2001). Integrated control of Verticillium wilt of olive with cryptonol in combination with a solar chamber and fertilizer. Phytoparasitica 29, 223-230.

Al-Ahmad, M. A. (1993). The solar chamber: An innovative technique for controlling Verticillium wilt of olive. Bulletin OEPP/EPPO Bulletin 23, 531-535.

Alexander, L.J., (1972). Susceptibility of certain Verticillium resistant tomato varieties to an Ohio isolate of the fungus. Phytopathology 52, 998-1000.

Antoniou, P. P., Markakis, E. A., Tjamos, S. E., Paplomatas, E. J., and Tjamos, E. C. (2008). Novel methodologies in screening and selecting olive varieties and root-stocks for resistance to *Verticillium dahliae*. European Journal of Plant Pathology 110, 79-85.

Bae, J., Halterman, D.A., Jansky, S.H. (2008). Development of a molecular marker associated with Verticillium wilt resistance in diploid interspecific potato hybrids. Molecular Breeding 22, 61-69.

Barakat, M.N., S.A.F. Rania, M. Badr, and M.G. Eitorky (2010). In vitro mutagenesis and identification of new variants via RAPD markers for improving Chrysanthemum morifolium. African Journal of Agricultural Research 5(8), 748-757.

Barbara, D.J., Paplomatas, E.J., Jiménez-Díaz, R.M., (1998). Variability in *V. dahliae*. In: Hiemstra, J.A., Harris, D.C. (eds.). A compendium of Verticillium wilts in tree species, pp. 43- 45. Ponsen and Looijen, Wageningen, The Netherlands.

Bhat, R.G., and Subbarao, K.V. (1999). Host range specificity in *Verticillium dahliae*. Phytopathology 89, 1218-1285.

Blanco-López, M.A., Hiemstra, J., Harris, D., López-Escudero, F.J., Antoniou, P. (1998). Selection and screening for host resistance. In: Hiemstra J, Harris D (eds) Compendium of Verticillium wilt in tree species. Ponsen & Looijen, Wageningen, pp 51-54.

Blok, W.J., Lamers, J.G., Termorshuizen, A.J., Bollen, G.J. (2000). Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. Phytopathology 90, 253-259.

Bubici, G., Cirulli, M. (2011). Control of Verticillium wilt of olive by resistant rootstocks. Plant Soil. DOI 10.1007/s11104-011-1102-9.

Busov, V., Yordanov, Y., Gou, J., Meilan, R., Ma, C., Regan, S., Strauss, S. (2011). Activation tagging is an effective gene tagging system in Populus. Tree Gen Genom 7, 91-101.

Cerezo, S., Mercado, J.A., Pliego-Alfaro, F. (2011). An efficient regeneration system via somatic embryogenesis in olive. Plant Cell Tiss Organ Culture 106, 337.

Chai, Y., Zhao, L., Liao, Z., Sun, X., Zuo, K., Zhang, L., Wang, S., Tang, K. (2003). Molecular cloning of a potential *Verticillium dahliae* resistance gene *SlVe1* with multi-site polyadenylation from *Solanum lycopersicoides*. DNA Sequence 14, 375-384.

Cirulli, M., Amenduni, M., Paplomatas, E.J. (1998). Verticillium wilt of major tree hosts. Stone fruits. In: Hiemstra J.A., Harris DC (eds) A compendium of Verticillium wilts in tree species. Ponsen and Looijen, Wageningen, pp 17-20.

Conn, K. L., Tenuta, M., and Lazarovits, G. (2005). Liquid swine manure can kill *Verticillium dahliae* microsclerotia in soil by volatile fatty acids, nitrous acid, and ammonia toxicity. Phytopathology, 95, 28-35.

Demura, T., Tashiro, G., Horiguchi, G., Kishimoto, N., Kubo, M., Matsuoka, N., Minami, A., Nagata-Hiwatashi, M., Nakamura, K., Okamura, Y., Sassa, N., Suzuki, S., Yazaki, J., Kikuchi, S., Fukuda, H. (2002). Visualization by comprehensive microarray analysis of gene expression programs during transdifferentiation of mesophyll cells into xylem cells. Proceedings of the National Academy of Sciences of the United States of America 99(24), 15794–15799.

Depuydt, S., Trenkamp, S., Fernie, A.R., Elftieh, S., Renou, J.-P., Vuylsteke, M., Holsters, M., Vereecke, D. (2009). An integrated genomics approach to define niche establishment by *Rhodococcus fascians*. Plant Physiology 149, 1366-1386.

Douhan, L.I., and Johnson, D.A. (2001). Vegetative compatibility and pathogenicity of *Verticillium dahliae* from spearmint and peppermint. Plant Disease 85, 297-302.

Emechebe, A.M., Leakely, C.L.A., Banage, W.B. (1974). Verticillium wilt of cacao in Uganda: wilt induction by mechanical vessel blockage and mode of recovery of diseased plants. The East African Agricultural and Forestry Journal 39, 337-343.

Evans, G., Gleeson, A. (1973). Observations on the origin and nature of *Verticillium dahliae* colonising plant roots. Australian Journal of Biological Sciences 26, 151-162.

Epstein, L., Beede, R., Kaur, S., and Ferguson, L. (2004). Rootstock effects on pistachio trees grown in *Verticillium dahliae*-infested soil. Phytopathology 94, 388-395.

Fei J., Chai Y., Wang J., Lin J., Sun X., Sun C., Zuo K., Tang K. (2004). cDNA cloning and characterization of the Vehomologue gene StVefrom Solanum torvum Swartz. DNA Sequence 15, 88-95.

Fladung, M., Deutsch, F., Hönicka, H., Kumar, S. (2004). DNA and transposon tagging in aspen. Plant Biology 6, 5-11.

Fladung, M., Polak, O. (2012). Ac/Ds-transposon activation tagging in poplar: a powerful tool for gene discovery. BMC Genomics. doi:10.1186/1471-2164-13-61.

Fradin, E.F., Thomma, B.P.H.J. (2006). Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Molecular Plant Pathology 7, 71-86.

Fradin, E.F., Zhang, Z., Ayala, J.C.J., Castroverde, C.D.M., Nazar, R.N., Robb, J., Liu, C.M., Thomma, B.P.H.J. (2009). Genetic dissection of Verticillium wilt resistance mediated by tomato Ve1. Plant Physiology, 150, 320-332.

Fradin, E., Adb-El-Haliem, A., Masini, L., Van den Berg, G., Joosten, M., et al. (2011). Interfamily transfer of tomato *Ve1* mediates Verticillium resistance in Arabidopsis. Plant Physiology 156, 2255-2265.

Gao, X., Wheeler, T., Li, Z., Kenerley, C.M., He, P., Shan, L. (2011). Silencing GhNDR1 and GhMKK2 compromises cotton resistance to Verticillium wilt. Plant Journal 66 293–305.

Garrett, P.W., Randall, W.K., Shigo, A.L., Shortle, W.C. (1979). Inheritance of compartmentalization of wounds in sweetgum (*Liquidambar styraciflua* L.) and eastern cottonwood (*Populus deltoides* Bartr.). Res. Pap. NE-443. Broomall, PA: U.S. Department of Agriculture, Forest Service, Northeastern Forest Experiment Station. 4p.

Goicoechea, N. (2009). To what extent are soil amendments useful to control Verticillium wilt? Pest Management Science, 65,831-839.

Goud, J.C., Hiemstra, J.A. (1998). Other tree species. In: Hiemstra JA, Harris DC (eds) A compendium of Verticillium wilts in tree species. Ponsen and Looijen, Wageningen, pp 37-39.

Goud, J.K.C., Termorshuizen, A.J., van Bruggen, A.H.C. (2011). Verticillium wilt in nursery trees: damage thresholds, spatial and temporal aspects. European Journal of Plant Pathology 131, 451-465.

Govindaraj, M., Vetriventhan, M., & Srinivasan, M. (2015). Importance of Genetic Diversity Assessment in Crop Plants and Its Recent Advances: An Overview of Its Analytical Perspectives. Genetics Research International 14. doi: 10.1155/2015/431487.

Gramaje, D., Pérez-Serrano, V., Montes-Borrego, M., Navas-Cortés, J.A., Jiménez-Díaz, R.M., Landa, B.B. (2013). A comparison of real-time PCR protocols for the quantitative monitoring of asymptomatic olive infections by *Verticillium dahliae* pathotypes. Phytopathology 103, 1058-1068.

Gullino, M. L., Minuto, A., Gilardi, G., Garibaldi, A., Ajwab, H., and Duafala, T. (2002). Efficacy of preplant soil fumigation with chloropicrin for tomato production in Italy. Crop Protection 21, 741-749.

Gulya, T., (2007). New strain of *Verticillium dahliae* in North America. Helia, 30, 115-120.

Harrison, E.J., Bush, M., Plett, J.M., McPhee, D.P., Vitez, R., O'Malley, B., Sharma, V., Bosnich, W., Seguin, A., MacKay, J., Regan, S. (2007). Diverse developmental mutants revealed in an activation tagged population of poplar. Canadian Journal of Botany 85, 1071-**1087.**

Hiemstra, J.A. (1995). Recovery of verticillium-infected ash trees. Phytoparasitica 23, 64-65.

Hiemstra, J.A. (1998). Some general features of Verticillium wilts in trees. In: Hiemstra, J.A., Harris D.C. (eds). A compendium of Verticillium wilts in tree species. Ponsen and Looijen, Wageningen, pp 5–11.

Hiemstra, J.A. (2000). Screening for Verticillium Resistance in Norway Maple. In: Tjamos, E.C., Rowe, R.C., Heale, J.B. and Fravel, D.R. (eds). Advances in Verticillium Research and Disease Management. APS Press St Paul, Minnesota 212-213.

Hiemstra, J.A. (2014). Der schnelle Nachweis von Verticillium. Jahrbuch der Baumpflege 2014, 108-120.

Hiemstra, J.A., Harris, D.C. (Eds.) (1998). A compendium of Verticillium wilts in tree species. Wageningen: Centre for Plant Breeding and Reproduction Research (CPRO-DLO)/West Malling: HRI-East Malling.

Hayes, R. J., McHale, L. K., Vallad, G. E., Truco, M. J., Michelmore, R. W., Klosterman, S. J., Maruthachalam, K.,Subbarao, K. V. (2011). The inheritance of resistance to Verticillium wilt caused by race 1 isolates of *Verticillium dahliae* in the lettuce cultivar La Brillante. Theoretical and Applied Genetics 123(4), 509-517.

Hu, R., Qi, G., Kong, Y., Kong, D., Gao, Q. and Zhou, G. (2010). Comprehensive analysis of NAC domain transcription factor gene family in *Populus trichocarpa*. BMC Plant Biology 10, 145.

Jiménez-Díaz, R.M, Cirulli, M., Bubici, G., Jiménez-Gasco, M., Antoniou, P.P., Tjamos, E.C. (2012). Verticillium wilt a major threat to olive production: Current status and future prospects for its management. Plant Disease 96(3), 304-329.

Jammes, F., Lecomte, P., de Almeida-Engler, J., Bitton, F., Martin-Magniette, M.L., Renou, J.P., Abad, P., and Favery, B. (2005). Genome-wide expression profiling of the host response to root-knot nematode infection in Arabidopsis. Plant Journal 44, 447-458.

Karajeh, M.R. (2006). Seed transmission of *Verticillium dahliae* in olive as detected by a highly sensitive nested PCR-based assay. Phytopathologia Mediterranea 45, 15-23.

Karajeh, M.R., Masoud, S.A. (2006). Molecular detection of *Verticillium dahliae* Kleb. in asymptomatic olive trees. Journal of Phytopathology 154, 496-499.

Kawchuk, L.M., Hachey, J., Lynch, D.R., Kulcsar, F., Van Rooijen, G., et al. (2001). Tomato Ve disease resistance genes encode cell surface-like receptors. Proceedings of the National Academy of Sciences USA 98, 6511-6515.

Koike, S.T., Subbarao, K.V., Davis, R.M., Gordon, T.R., Hubbard, J.C., (1994). Verticillium wilt of cauliflower in California. Plant Disease 78, 1116-1121.

Krikun, J., and Bernier, C. C. (1987). Infection of several crop species by two isolates of *Verticillium dahliae*. Canadean Journal of Plant Pathology 9, 241-245.

Kubo, M., Udagawa, M., Nishikubo, N., Horiguchi, G., Yamaguchi, M., Ito, J., Mimura, T., Fukuda, H. and Demura, T. (2005). Transcription switches for protoxylem and metaxylem vessel formation. Genes and Development 16, 1855-1860.

Latorre, B.A., Allende, P.T. (1983). Occurrence and incidence of Verticillium wilt on Chilean avocado groves. Plant Diseases 67, 445-447.

Levin, A. G., Lavee, S., and Tsror, L. (2003). Epidemiology of *Verticillium dahliae* on olive (cv. Picual) and its effects on yield under saline conditions. Plant Pathology 52, 212-218.

Liebrand, T.W.H., van den Burg, H.A., Joosten, M.H. A.J. (2013). Two for all: receptor-associated kinases SOBIR1 and BAK1. Trends Plant Science 19(2), 123-132.

Lima, G., Piedimonte, D., de Curtis, F., Elgelane, A. A., Nigro, F., D'Onghia, A. M., Alfano, G., and Ranalli, G. (2008). Suppressive effect of cured compost from olive oil by products towards *Verticillium dahliae* and other fungal pathogens. Acta Horticulturae 791, 585-591.

Liu S.P., Zhu Y.P., Xie C., Jue D.W., Hong Y.B., Chen M., Hubdar A.K., Yang Q. (2012). Transgenic potato plants expressing *StoVe1* exhibit enhanced resistance to *Verticillium dahliae*. Plant Molecular Biology Reporter 30, 1032-1039.

López-Escudero, F.J., Blanco-López, M.A. (2001). Effect of a single or double soil solarization to control Verticillium wilt in established olive orchards in Spain. Plant Disease 85, 489-496.

López-Escudero, F. J., del Río, C., Caballero, J. M., and Blanco-López, M. A. (2004). Evaluation of olive cultivars for resistance to *Verticillium dahliae*. European Journal Plant Pathology 110, 79-85.

López-Escudero, F.J., Blanco-López, M.A. (2005). Recovery of young olive trees from *Verticillium dahliae*. European Journal Plant Pathology 113, 367-375.

López-Escudero, F.J., Mercado-Blanco, J. (2011). Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. Plant Soil 344, 1-50.

Lynch, D.R., Kawchuck, L.M., Hachey, J. (1997). Identification of a gene conferring high levels of resistance to Verticillium wilt in *Solanum chacoense*. Plant Disease 81, 1001-1014.

Malcolm, G.M., Kuldau, G.A., Gugino, B.K., Jiménez-Gasco, M.M. (2013). Hidden host plant associations of soilborne fungal pathogens: An ecological perspective. Phytopathology 103, 538-544.

Malinowski, R., Smith, J.A., Fleming, A.J., Scholes, J.D., and Rolfe, S.A. (2012). Gall formation in clubroot-infected Arabidopsis results from an increase in existing meristematic activities of the host but is not essential for the completion of the pathogen life cycle. Plant Journal 71, 226-238.

Markakis, E.A., Tjamos, S.E., Antoniou, P.P., Paplomatas, E.J., Tjamos, E.C. (2009). Symptom development, pathogen isolation and Realtime QPCR quantification as factors for evaluating the resistance of olive cultivars to Verticillium pathotypes. European Journal of Plant Pathology 124, 603-611.

Mathre, D.E. (1986). Occurrence of *Verticillium dahliae* on barley. Plant Disease, 70, 981-981.

Melero-Vara, J.M., Blanco-López, M.A., Bejarano-Alcázar, J., Jiménez-Díaz, R.M. (1995). Control of Verticillium wilt of cotton by means of soil solarization and tolerant cultivars in Southern Spain. Plant Pathology 44, 250-260.

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodríguez-Jurado, D., Jiménez-Díaz, R.M. (2003a). Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction. Physiological and Molecular Plant Pathology 63, 91-105.

Mercado-Blanco, J., Rodríguez-Jurado, D., Parrilla-Araujo, S., Jiménez-Díaz, R.M. (2003b). Simultaneous detection of the defoliating and nondefoliating *Verticillium dahliae* pathotypes in infected olive plants by duplex, nested polymerase chain reaction. Plant Disease 87, 1487-1494.

Mol, L. (1995). Formation of microsclerotia of *Verticillium dahliae* on various crops. Netherlands journal of agricultural science, 43, 205-215.

Nikam, A.A., Devarumath, R.M., Ahuja, A., Babu, H., Shitole, M.G., Suprasanna, P. (2015). Radiation-induced in vitro mutagenesis system for salt tolerance and other agronomic characters in sugarcane (*Saccharum officinarum* L.). The Crop Journal 3(1), 46-56.

Nicole, M., Gianinazzi-Pearson, V. (1996). Histology, Ultrastructure and molecular cytology of plant-microorganism interactions. Dordrecht: Kluwer. 261 pp.

Ogawa, J.M., English, H. (1991). Diseases of temperate zone tree fruit and nut crops. University of California and Division of Agriculture and Natural Resources Publiction 3345. 461 pp.

Ohtani, M., Nishikubo, N., Xu, B., Yamaguchi, M., Mitsuda, N., Goué, N., Shi, F., Ohme-Takagi, M., Demura, T. (2011). A NAC domain protein family contributing to the regulation of wood formation in poplar. The Plant Journal 67, 499-512.

Okada, T.S. (1991). Transdifferentiation: Flexibility in Cell Differentiation (Oxford, UK: Clarendon Press).

Pegg, G.F., Brady, B.L. (2002). Verticillium wilts. Wallingford, UK: CABI publishing.

Penna, S., Vitthal, S. B., Yadav, P.V. (2012). *In Vitro* Mutagenesis and selection in plant tissue cultures and their prospects for crop improvement. Bioremediation, biodiversity and bioavailability 6, 6-14.

Prieto, P., Navarro-Raya, C., Valverde-Corredor, A., Amyotte, S.G., Dobinson, K.F., Mercado-Blanco, J. (2009). Colonization process of olive tissues by *Verticillium dahliae* and its *in planta* interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7. Microbial Biotechnology 2(4), 499-511.

Petrini, O. (1991). Fungal endophytes of tree leaves. Pages 179-187 in: Microbial Ecology of Leaves. J. H. Andrews and S. S. Hirano, eds. Springer-Verlag, New York.

Ramanatha Rao, V., Hodgkin, T. (2002). Genetic diversity and conservation and utilization of plant genetic resources. Plant Cell, Tissue and Organ Culture 68(1), 1-19.

Reusche, M., Thole, K., Janz, D., Truskina, J., Rindfleisch, S., Drübert, C., Polle, A., Lipka, V., Teichmann, T. (2012). Verticillium infection triggers VASCULAR-RELATED NAC DOMAIN7–dependent de novo xylem formation and enhances drought tolerance in arabidopsis. The Plant Cell 24, 3823-3837.

Rodríguez-Jurado, D., Blanco-López, M.A., Rappoport, H.F., Jiménez-Díaz, R.M. (1993). Present status of Verticillium wilt of olive in Andalucía (southern Spain). European and Mediterranean Plant Protection Organization (EPPO) Bulletin 23, 513-516.

Schaible, L., Cannon, O.S., Waddoups, V. (1951). Inheritance of resistance to Verticillium wilt in a tomato cross. Phytopathology 41, 986-990.

Schreiber, L.R., Mayer, J.S. (1992). Seasonal variations in susceptibility and in internal inoculum densities in maple species inoculated with *Verticillium dahliae*. Plant Disease 76, 184-187.

Shain, L., Miller, J.B. (1988). Ethylene production by excised sapwood of clonal eastern cottonwood and the compartmentalization and closure of seasonal wounds. Phytopathology 78, 1261-1265.

Shigo, A.L. (1984). Compartmentalization: A conceptual framework for understanding how trees grow and defend themselves. Annual Review of Phytopathology, 22, 189-214.

Shu, Q.Y., Forster, B.P., Nakagawa, H. (2012). Plant mutation breeding and biotechnology. Plant breeding and genetics section joint FAO/IAEA division of nuclear techniques in food and agriculture international atomic energy agency, Vienna, Austria. 10.1079/9781780640853.0000.

Sikora, P., Chawade, A., Larsson, M., Olsson, J., & Olsson, O. (2011). Mutagenesis as a tool in plant genetics, functional genomics, and breeding. International Journal of Plant Genomics .doi: 10.1155/2011/314829.

Simko, I., Costanzo, S., Haynes, K.G., Christ, B.J., Jones, R.W. (2004). Linkage disequilibrium mapping of a *Verticillium dahliae* resistance quantitative trait locus in tetraploid potato (*Solanum tuberosum*) through a candidate gene approach. Theoretical and Applied Genetics 108, 217-224.

Smith, K. T. (2006). Compartmentalization Today. Arboricultural Journal 29(3), 173-184.

Song, Y., Zhang, Z., Seidl, M. F., Majer, A., Jakse, J., Javornik, B., Thomma, B. P. H. J. (2016). Broad taxonomic characterization of Verticillium wilt resistance genes reveals ancient origin of the tomato Ve1 immune receptor. Molecular Plant Pathology, n/a-n/a. doi: 10.1111/mpp.12390.

Stuthman, D.D., Leonard, K.J., Miller-Garvin, J. (2007). Breeding crops for durable resistance to disease, p. 319-367, In D. L. Sparks, ed. Advances in Agronomy, Vol. 95. Academic Press, New york, USA.

Sugimoto, K., Gordon, S.P., and Meyerowitz, E.M. (2011). Regeneration in plants and animals: Dedifferentiation, transdifferentiation, or just differentiation? Trends Cell Biology 21, 212-218.

Tang, J., Lin, J., Yang, Y., Chen, T., Ling, X., Zhang, B., Chang, Y. (2016). Ectopic expression of a *Ve* homolog *VvVe* gene from *Vitis vinifera* enhances defense response to *Verticillium dahliae* infection in tobacco. Gene 576(1), 492-498.

Taylor, J.B., Flentje, N.T. (1968). Infection, recovery from infection and resistance of apricot trees to *Verticillium albo-atrum*. New Zealand Journal of Botany 61, 417-426.

Tippet, J.T., Shigo, A.L. (1981). Barrier zone formation: a mechanism of tree defence against vascular pathogens. IAWA Bulletin 2, 163-168.

Tjamos, E. C., Biris, D. A., and Paplomatas, E. J. (1991). Recovery of olive trees from Verticillium wilt after individual application of soil solarization in established olive orchards. Plant Diseases 75, 557-562.

Tjamos, E.C., Jiménez-Díaz, R.M. (1998). Management of disease. In: Hiemstra JA, Harris DC, 1998. A compendium of Verticillium wilts in trees 55-57.

Talboys, P.W. (1958). Association of tylosis and hyperplasia of the xylem with vascular association of the hop by *Verticillium* alboatrum. Transactions of the British Mycological Society 41, 249-260.

Torreblanca, R., Cerezo, S., Palomo-Ríos, E., Mercado, J. A., and PliegoAlfaro, F. (2010). Development of a high throughput system for genetic transformation of olive (*Olea europaea* L.) plants. Plant Cell Tissue Organ Culture 103, 61-69.

Tosh, D., and Slack, J.M.W. (2002). How cells change their phenotype. Nature Reviews Molecular Cell Biology 3, 187-194.

Townsend, A. M., Schreiber, L. R., Hall, T. J., and Bentz, S. E. (1990). Variation in response of Norway maple cultivars to *Verticillium dahliae*. Plant Disease 74, 44-46.

Trapero, C., Díez, C. M., Rallo, L., Barranco, D., López-Escudero, F. J. (2013). Effective inoculation methods to screen for resistance to Verticillium wilt in olive. Scientia Horticulturae 162, 252-259.

Vallad, G.E., Qin, Q-M., Grube, R., Hayes, R.J., Subbarao, K.V. (2006). Characterization of race-specific interactions among isolates of *Verticillium dahliae* pathogenic on lettuce. Phytopathology 96, 1380-1387.

Vallad, G. E., Bhat, R. G., Koike, S. T., Ryder, E. J., Subbarao, K. V. (2005). Weedborne reservoirs and seed transmission of *Verticillium dahliae* in lettuce. Plant Disease 89, 317-324.

Van Harten, A.M. (1998). Mutation breeding: Theory and practical applications. Cambridge University Press, Cambridge.

Vining K., Davis T. (2009). Isolation of a Vehomolog, *mVe1*, and its relationship to Verticillium wilt resistance in *Mentha longifolia* (L.) Huds. Molecular Genetics and Genomics 282, 173-184.

Veronese, P., Narasimhan, M.L., Stevenson, R.A., Zhu, J.K., Weller, S.C., Subbarao, K.V. and Bressan, R.A. (2003). Identification of a locus controlling Verticillium disease symptom response in *Arabidopsis thaliana*. Plant Journal 35, 574-587.

Wilhelm, S., Sorken, R. C., Sagen, J. E. (1961). Verticillium wilt of strawberry controlled by fumigation of soil with chloropicrin and chloropicrin-methyl bromide mixtures. Phytopathology 51, 744-748.

Wilhelm, S., Taylor, J.B. (1965). Control of Verticillium wilt of olive through natural recovery and resistance. Phytopathology 55, 310-316.

Yamaguchi, M., Goué, N., Igarashi, H., Ohtani, M., Nakano, Y., Mortimer, J.C., Nishikubo, N., Kubo, M., Katayama, Y., Kakegawa, K., Dupree, P., Demura, T. (2010). VASCULAR-RELATED NACDOMAIN6 and VASCULAR-RELATED NAC-DOMAIN7 effectively induce transdifferentiation into xylem vessel elements under control of an induction system. Plant Physiology 153, 906-914.

Yang, Y., Ling, X., Chen, T., Cai, L., Liu, T., Wang, J., Fan, X., Ren, Y., Yuan, H., Zhu, W., Zhang, B., Ma, D.P. (2014). A Cotton *Gbvdr5* Gene Encoding a Leucine-Rich-Repeat Receptor-Like Protein Confers Resistance to *Verticillium dahliae* in Transgenic Arabidopsis and Upland Cotton. Plant Molecular Biology Reporter 33(4), 987-1001.

Zhang, B.L., Yang, Y.W., Chen, T.Z., Yu, W.G., Liu, T.L., Li, H.J., Fan, X.H., Ren, Y.Z., Shen, D.Y., Liu, L., Dou, D.L., Chang, Y.H. (2012). Island cotton *Gbve1* gene encoding a receptor-like protein confers resistance to both defoliating and non-defoliating isolates of *Verticillium dahliae*. PLoS One, 7:e51091.

Summary

Vascular wilts caused by xylem-colonizing pathogens are among the most devastating plant diseases that affect a wide range of plant species worldwide. **Chapter 1** is the general introduction to the thesis outlining the process of infection by vascular wilt pathogens. Vascular wilt pathogens, which comprise bacteria, fungi and oomycetes, are generally soil-borne micro-organisms that infect their host plants through the roots. They traverse the cortex of the roots and enter the xylem vessels, after which they proliferate within these vessels, causing blockage of water and mineral flows that may result in partial or complete wilting or death of whole plants. Verticillium wilt disease caused by the soil-born fungus *Verticillium dahliae* Kleb. is among the major vascular wilt diseases in herbaceous crops as well as woody plants. Verticillium wilt is intensively studied in herbaceous hosts, whereas little is known about Verticillium wilts of woody hosts. This chapter is completed with an outline of the PhD research project.

Verticillium wilt is a serious problem in olive-growing regions and in tree nurseries worldwide. In **Chapter 2**, we review common and differentiating aspects of Verticillium wilt, caused by *V. dahliae*, in woody hosts, with further emphasis on olive, ash and maple. The establishment of new planting sites on infested soils, the use of infected plant material and the spread of highly virulent pathogen isolates are the main reasons of increasing problems with Verticillium wilt in tree cultivation. The use of practices to avoid spreading of the disease and to reduce soil inoculum levels, combined with resistant host plant cultivars if these are available, are discussed as the most effective measures to deal with Verticillium wilt disease. It is underlined that improvement of methods for early detection and accurate diagnosis of the pathogen in diseased trees, planting material and at cultivation sites prior to planting is essential in this regard.

Information on the distribution of *V. dahliae* in infected trees helps to design an appropriate and efficient sampling method for reliable detection of the pathogen in diseased trees. In **Chapter 3**, the distribution of *V. dahliae* in young twigs and leaves of infected olive trees is studied by real-time quantification of *V. dahliae* DNA. Analysis of twig and leaf samples collected from different sides of the crown of infected olive trees showed a non-uniform distribution of the fungus within infected parts of diseased olive

trees. It was demonstrated that testing of combined samples comprising subsamples from at least 5 twigs from different sides of the tree, or 5-10 random leaves, can reliably detect the pathogen.

V. dahliae isolates that infect olive trees can be classified as defoliating (D) isolates that are highly virulent, or non-defoliating (ND) isolates that are generally less aggressive. Discrimination of these pathotypes is important in order to predict the severity of disease, and decide on appropriate disease management strategies. This is particularly important due to the alarming spread of highly virulent isolates of the D pathotype worldwide. In **Chapter 4**, a novel method is designed for accurate discrimination and sensitive detection of D and ND isolates of *V. dahliae.* Through comparative genomics of multiple D and ND isolates of *V. dahliae* a region was identified that is present in all sequenced ND isolates, while absent from all D isolates. Based on this presence-absence polymorphism, a set of primers was designed spanning this region that was able to generate differentially sized amplicons for isolates that belong to the different pathotypes. Additionally, a nested-PCR assay was designed to increase the sensitivity and improve detection of D and ND isolates *in planta.*

In **Chapter 5**, the relation of the dynamics in pathogen distribution in infected plants to the differences in extent and severity of disease caused by D and ND isolates in resistant and susceptible olive genotypes is studied. To this end, the distribution of a D (V117) and a ND (V4) isolate of *V. dahliae* in root-inoculated young plants of a susceptible (Picual) and a partially resistant cultivar (Frantoio) of olive and its relationship to the disease progression was investigated using real-time PCR. The amount of pathogen DNA detected in the two cultivars correlated with their susceptibility to Verticillium wilt, with lower quantities of V4 and V117 DNA detected in 'Frantoio' than in 'Picual'. Also quantities of pathogen DNA in V117-inoculated plants were higher than quantities of pathogen DNA in V4-inoculated plants. The distribution patterns of D and ND isolates in the lower, middle and top parts of tested olive cultivars showed that differences in symptom severity were related to amounts of the pathogen in lower and middle parts of the trees, since colonization of the pathogen in top parts of the stem of inoculated plants was minor and was not significantly different between treatments. Moreover, microscopic

analysis of infection and colonization processes of *V. dahliae* in olive plants inoculated with GFP-labelled isolates revealed that colonization of the above ground tissues of infected olive plants is by means of conidia transported upward with the xylem sap stream.

In **Chapter 6** we investigated the spatial and temporal distribution of *V. dahliae* in relation to disease progression and recovery in stem-inoculated maple and ash trees. These species differ strongly in vascular anatomy with maple having a diffuse porous xylem anatomy whereas ash has a ring porous xylem anatomy. Results showed that that differences in the xylem anatomy of ash and maple did not significantly affect the speed and extent of the upward spread of the pathogen in stem-inoculated trees. Nevertheless, the xylem of ash trees is much less supportive for growth and survival of *V. dahliae* than that of maple trees, as in the year after inoculation disease incidence and also quantities of *V. dahliae* detected in maple trees were significantly higher than in ash trees. Moreover, *V. dahliae* could not be reisolated at all from ash trees that had recovered from disease. However, it could be detected by PCR in some cases in the xylem formed in the year of inoculation, never in the xylem formed in the year after inoculation. Nevertheless, *V. dahliae* easily could be detected in the wood of diseased ash and maple trees in the year after inoculation. Notably, despite the presence of a layer of terminal parenchyma cells between growth rings, in ash trees showing disease symptoms in the year after inoculation *V. dahliae* was present in the xylem of the new growth ring. It was also observed that in stem-inoculated trees *V. dahliae* can move downward from the point of inoculation into the root collar, which may provide an avenue for infection of new growth rings in ash trees.

Finally, in **chapter 7** the major results described in this thesis are discussed and placed in a broader perspective.

Acknowledgments

Now that my PhD thesis has been finalized I would like to thank people that played an important role in my success. First of all, I would like to thank my family, particularly my parents, for their devotedness and support throughout my lifetime. I have no words to express my appreciation for your boundless support, I kiss your hands. Special thanks go to to my loyal wife, Zahra. I'll never forget your constant prayers and your love from the beginning of our common life till now. I admire your persistence, patience, kindness, strength and encouragement. I also thank the family of my wife for their encouragements and moral support. My son, Mohammad Reza was only a little boy when we arrived in the Netherlands. My dear son, you were really patient and easy going while we had a lot of movements and difficulties during the years when I was performing my PhD; I am proud of you.

I am thankful to my promoters Prof. dr. ir. B.P.H.J. Thomma and Prof. dr. ir. P.J.G.M de Wit. Bart, I thank you for giving me the opportunity to become a member of your group. I really appreciate the new window of science that you opened for me. Your guidance and supervision were always helpful to mature, to grow my mindset and keep on a right track throughout my thesis research; thanks Bart. My sincere gratitude also goes to Pierre for his valuable criticism and comments on my thesis. I would like to express my highest gratitude to my co-promoter Dr. Ir. J.A. Hiemstra, for his remarkable supervision. Dear Jelle, you were really patient and without your support and encouragement I would not have been able to complete my work. Dear Bart, Pierre, and Jelle, your efforts definitely helped me to develop my competence as an independent scientist, and I am truly happy to have been able to work with you.

I would like to thank my laboratory colleagues at the Institute of Applied Plant Research (PPO). Khanh Pham, Suzanne Breeuwsma and Joop van Doorn, I have benefited a lot from your knowledge and expertise and our scientific discussions were always very helpful. With you, I experienced a very calm and happy atmosphere in the laboratory.

I would also like to thank my laboratory colleagues of the Verticillium group, especially Parthasarathy Santhanam, Peter van Esse, Ronnie de Jonge, Anja Kombrink, Zhao Zhang, and Grardy van den Berg. I have learned a lot from your knowledge, thanks.

I also thank Yin Song for his assistance while performing greenhouse tests, as well as for his help during the final stages before my PhD defense. Dear Yin and Xiaoqian, I thank both of you for your help as my paranymphs. My dear friends, I wish all of you much success in your future scientific and personal lives.

I also thank Bert Essenstam and Henk Smid for taking care of my experiments in the greenhouse. You were excellent experts that properly managed my plants and treated them very well.

I would like to extend my gratitude to Gloria María García-Ruiz from the IFAPA Center Alameda del Obispo in Cordoba, and Mario Pérez-Rodríguez from the Department of Agronomy of Cordoba University, in Spain. Dear Gloria and Mario, I appreciate your contribution and sharing of your valuable expertise on Verticillium infections of olive during the period that you were in Lisse. Thank you very much.

Living in the Netherlands caused several ups and downs for my family and me, with several movements in Wageningen, and from Wageningen to Hillegom. Nevertheless, this period of my life yielded a lot of memorable experiences. Finding new Iranian and international friends, and getting familiar with new cultures by travelling through the Netherlands and to other European countries was a unique occasion in my lifetime that I will never forget. I would like the Netherlands with its friendly and hospitable people.

With best regards

Mojtaba Keykhasaber

Curriculum Vitae

Mojtaba Keykasaber was born on April 8, 1979, in Zabol, Sistan and Baloochestan province, Iran. He did the primary school in Zabol, and the secondary and high school in Mashhad, Iran. After he obtained his high school diploma in 1997, he successfully passed the national exam for universities in 1998 and enrolled at Ferdowsi University of Mashhad to study "Plant Protection" in the College of Agriculture. He obtained his BSc in 2002. Subsequently, he passed the national exam and started to study plant biotechnology at Mazandaran University, Sari, in 2002. He performed his MSc thesis on "Production of ice nucleation deficient (Ice-) mutants of the epiphytic strains of *Erwinia herbicola*" and he graduated in 2005. After his MSc degree, Mojtaba started working in plant biotechnology and breeding department at Zabol University as a researcher and a lecturer. In 2010, he was awarded an overseas scholarship from Ministry of Science, Research, and Technology (MSRT) of Iran to perform his PhD. He was accepted by the Laboratory of Phytopathology of Wageningen University and started his PhD on June, 2011 in the group of Verticillium. From 2012 he kept his PhD at Wageningen plant research and he worked on aspects of spatial and temporal distribution of *Verticillium dahliae* in olive, maple and ash trees and improvement of detection methods which has become the main subject of his PhD thesis. After his PhD he will join the Department of Plant Biotechnology in Zabol University in Iran.

List of publications

M. Keykhasaber, H. Rahimian, N. Babaeian, M. Bolouri moghadam (2007). Production of ice nucleation deficient (Ice^-) mutants of the epiphytic strains of *Erwinia hrbicola*. *Iranian jurnal of biotechnology*. 5 (3), 153-157.

M. Keykhasaber, A. A. V. Sedehi, A. Zakeri (2009). Study of expression of low-temperature responsive genes for selected barley accessions. *Biotechnology*, 8(1):176-179.

Ronnie de Jonge, H. Peter van Esse, Karunakaran Maruthachalam, Parthasarathy Santhanam, Mojtaba Keykhasaber, Zhao Zhang, Toshiyuki Usami, Bart Lievens, Krishna V. Subbarao, and Bart P.H.J. Thomma (2012). Tomato Immune Receptor Ve1 Recognizes Effector of Multiple Fungal Wilt Pathogens Uncovered by Genome and RNA Sequencing. *Proceedings of the National Academy of Sciences of the United States of America* 109, 13. - ISSN 0027-8424 - p. 5110 - 5115.

Parthasarathy Santhanam, Jordi C. Boshoven, Omar Salas, Kyle Bowler, Md Tohidul Islam, Mojtaba Keykhasaber, Grardy C. M. Van Den Berg, Maor Bar-Peled, Bart P.H.J.Thomma (2016). Rhamnose synthase activity is required for pathogenicity of the vascular wilt fungus *Verticillium dahliae. Molecular Plant Pathology*. DOI: 10.1111/mpp.12401.

M. Keykhasaber, K.T.K. Pham, B.P.H.J. Thomma, J.A. Hiemstra (2016). Reliable detection of unevenly distributed *Verticillium dahliae* in diseased olive trees. *Plant Pathology*. Doi: 10.1111/ppa.12647.

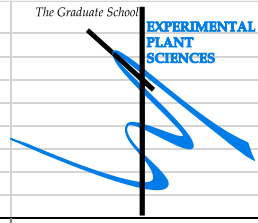
M. Keykhasaber, B.P.H.J. Thomma, J.A. Hiemstra (2016). Distribution and persistence of *Verticillium dahliae* in the xylem of Norway maple and European ash trees. *European Journal of Plant Pathology* (accepted).

M. Keykhasaber, B.P.H.J. Thomma, J.A. Hiemstra (2017). *Verticillium* wilt of woody plants with emphasis on olive and shade trees. *European Journal of Plant Pathology* (Review paper; submitted).

M. Keykhasaber, G.M. Garcia-Ruiz, M. Pérez-Rodríguez, B.P.H.J. Thomma, J.A. Hiemstra (2017). Distribution of defoliating and non-defoliating *Verticillium dahliae* isolates in root-inoculated olive trees that display differential levels of susceptibility. *Plant pathology* (submitted).

Education Statement of the Graduate School

Experimental Plant Sciences



Issued to:	**Mojtaba Keykha Saber**
Date:	**1 March 2017**
Group:	**Laboratory of Phytopathology, and Applied Plant Research**
University:	**Wageningen University & Research**

1) Start-up phase		*date*
►	**First presentation of your project**	
	Title: Identifying novel pathogenicity genes in Verticillium dahliae using Agrobacterium tumefaciens-mediated gene transfer (ATMT) method	Oct 16, 2011
►	**Writing or rewriting a project proposal**	
	Title: Unravelling aspects of spatial and temporal distribution of Verticillium dahliae in olive, maple and ash trees and improvement of detection methods	Dec 2011
►	**Writing a review or book chapter**	
	Title: Verticillium wilt caused by Verticillium dahliae in woody plants with emphasis on olive and shade trees; European Journal of Plant Pathology, submitted	2017
►	**MSc courses**	
►	**Laboratory use of isotopes**	
	Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure		*date*
►	**EPS PhD student days**	
	EPS PhD student day, Wageningen (NL)	May 20, 2011
	EPS PhD student day, Amsterdam (NL)	Nov 30, 2012
	EPS PhD student day, Leiden (NL)	Nov 29, 2013
►	**EPS theme symposia**	
	EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', Amsterdam (NL)	Feb 25, 2014
	EPS Theme 4 symposium 'Genome Biology', Wageningen (NL)	Dec 03, 2014
►	**Lunteren days and other National Platforms**	
	Platform Molecular Genetics, Lunteren (NL)	Oct 10-11, 2013
►	**Seminars (series), workshops and symposia**	
	Meeting at the Max-Planck institute for Terrestrial Microbiology, Marburg, Germany	Oct 09-11,2011
	EPS Symposium 'Omics Advances for Academia and Industry'	Dec 11, 2014
	Molecular and Developmental Genetics: Reprogramming Photosynthesis in Arabidopsis thaliana, Leiden	Jan 08, 2015
►	**Seminar plus**	
►	**International symposia and congresses**	
	11th international Verticillium symposium 2013, Gottingen, Germany	May 05-08, 2013
	7th International Symposium of "Plant Protection and Plant Health in Europe, Berlin, Germany	Mar 12-13, 2015
►	**Presentations**	
	Poster: 11th international Verticillium symposium 2013, Gottingen, Germany	May 05-08, 2013
	Poster: Spring School 'Host-Microbe Interactomics	Jun 02-04, 2014
	Poster: 7th International Symposium of 'Plant Protection and Plant Health in Europe', Berlin, Germany	Mar 12-13, 2015
►	**IAB interview**	
	Meeting with a member of the International Advisory Board of EPS	Jan 05, 2015
►	**Excursions**	
	Subtotal Scientific Exposure	*9.1 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Autumn School 'Host-Microbe Interactomics'	Nov 01-03, 2011
	Course: Bioinformatics, A User's Approach	Aug 27-31, 2012
	Spring School 'Host-Microbe Interactomics'	Jun 02-04, 2014
	Course: Microscopy and Spectroscopy in food and plant science	May 06-09, 2014
	Course: Genome assembly	Apr 28-29, 2015
►	**Journal club**	
	Participation in literature discussion group at Phytopathology	2011-2015
►	**Individual research training**	
	Working with microtome and microscopy imaging (confocal and epi microscopes), WUR - Cell Biology, dr. ing. NCA de Ruijter	Oct 07-08, 2013, Aug 07,18, 2014, Sep 01, 03,16-17, 2014
	Subtotal In-Depth Studies	*9.9 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Course: Project and time management	Oct 30-Dec 11, 2013
	Course: Techniques for Writing and Presenting a Scientific Paper (TWP)	Dec 03-06, 2013
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*2.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**35.2**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits	

** A credit represents a normative study load of 28 hours of study.*

This research was conducted at the Wageningen Plant Research and at the Laboratory of Phytopathology of Wageningen University. This research was financially supported by the Ministry of Science, Research, and Technology (MSRT) of Iran and by Wageningen University.

Cover and layout design: Mojtaba Keykhasaber

Printed by: Proefschriftmaken.nl || Digiforce Vianen, NL