

## Biological control of Verticillium wilt of olive within an integrated disease management framework

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**Abstract:** Verticillium wilt of olive (VWO) is one of the most serious biotic threats for olive cultivation, the disease being a severe problem in some traditional olive-cultivating regions within the Mediterranean Basin. Control of VWO is difficult and none of the available control measures is effective enough when applied individually. Therefore, an integrated disease management strategy is the only plausible framework for an effective control of VWO. The use of biological control agents (BCAs) can be considered either as a before-planting (preventive) or as a post-planting (palliative) action in established olive orchards. It is an approach that could be used in combination with other control tools. So far, only a few bacteria and fungi species have been examined as potential BCAs against VWO. Our studies have proved that some indigenous *Pseudomonas* spp. strains of olive roots, some of them with an endophytic lifestyle, are effective against VWO caused by the highly-virulent, defoliating (D) pathotype. An interesting field to be explored is the development of bioformulations which may combine BCAs with different biocontrol mechanisms operating synergistically; for instance, induction of systemic resistance triggered by an endophyte and antibiosis by a root-surface colonizer. However, how biocontrol actions can be effectively implemented within this integrated framework still needs of in-depth studies aimed to unravel the genetic and molecular basis underlying the olive/BCAs/*V. dahliae* tripartite interaction, as well as to understand how biotic factors may influence this system under field conditions.

**Key words:** biocontrol, defoliating pathotype, endophyte, *Olea europaea*, *Pseudomonas* spp. *Verticillium dahliae*

### Introduction

One of the major constraints for olive (*Olea europaea* L.) cultivation is Verticillium wilt, a disease caused by the soil-borne fungus *Verticillium dahliae* Kleb. Certainly, Verticillium wilt of olive (VWO) is nowadays considered one of the most serious biotic threats, the disease being reported in nearly all regions where this commodity is cultivated. Although an adequate assessment of the economic losses caused by VWO is difficult and systematic appraisals are generally lacking, latest surveys (Rodríguez *et al.*, 2009; Dervis *et al.*, 2010; López-Escudero *et al.*, 2010; Jiménez-Díaz *et al.*, 2011) conducted at distant geographical areas within the Mediterranean Basin are portraying a scenario where the disease is steadily expanding (reviewed by López-Escudero & Mercado-Blanco, 2011).

A number of diverse factors are contributing to the current importance, distribution and expansion of VWO which can be influential either on the pathogen, the host and/or the environment. Among these factors, the pathogen's propagules dispersal efficacy, the use of *V. dahliae*-infested soils and/or infected planting and propagation material, inadequate soil and crop management practices, growing of susceptible cultivars, fertilization and irrigation misuse, etc., can be playing decisive roles. It is worth mentioning that the current expansion of the disease in some areas has been associated with changes from traditional to new olive

growing habits (i.e., drip-irrigation, intensive culturing, etc.) (Dervis *et al.*, 2010; López-Escudero *et al.*, 2010). Moreover, a concerning observation from some important olive-growing regions (i.e., Spain and Turkey) is the rapid dispersal and increasing prevalence of *V. dahliae* isolates belonging to the highly-virulent, defoliating (D) pathotype. This suggests that several triggering causes are synergistically contributing to the current distribution and importance of VWO. Factors favoring the persistence, increase and dispersal of *V. dahliae* infective propagules (i.e., previous cultivation of *V. dahliae*-susceptible crops, long-term persistence of *V. dahliae* microsclerotia [MS], broad-range of hosts including weeds, use of infected planting material, or spreading of soil particles and/or plant debris harboring *V. dahliae* propagules by short- and long-distance means such as wind, cultivating machinery or irrigation water) have been recently reviewed (López-Escudero & Mercado-Blanco, 2011). The same accounts for agronomical (irrigation, fertilization, tree density and soil management) and environmental (temperature, edaphic features and biotic interactions) factors which have been proposed to help VWO onset, progress and severity, although their relative contribution have not been so far investigated in detail. Understanding these factors and their interactions is a key issue for the appropriate implementation of VWO control measures.

### **Control of Verticillium wilt of olive by implementing an integrated disease management strategy**

Verticillium wilts, and VWO is not an exception, are diseases very difficult to control. Among the diverse factors contributing to this fact are: i) the broad range of hosts which can be colonized by the pathogen; ii) the genetic variability of *V. dahliae* populations residing in soils; iii) the capacity to produce MS able to persist in soils for prolonged periods of time; or iv) the lack of effective fungicide treatments due to the pathogen location within the xylem vessels. None of the available control measures is sufficiently effective when applied individually. Therefore, successful control of VWO requires the implementation of an integrated disease management strategy, based in the development and application of both preventive and palliative control measures (Tjamos, 1993; López-Escudero & Mercado-Blanco, 2011). Preventive (before-planting) measures are certainly the most plausible ones from an economic perspective, and they are basically aimed to pathogen exclusion (i.e., use of healthy plant material and free-pathogen soils) and/or pathogen eradication (i.e., biological, physical, cultural or chemical practices to reduce the number of propagules). After an olive orchard has been established, it is encouraged to implement physical, biological and cultural methods aimed to reduce the pathogen inoculum (i.e., soil solarization, organic amendments, etc.), avoid new infections (i.e., using resistant cultivars), and hamper the spread of the pathogen to neighboring orchards (i.e., reducing tillage). Indeed, diverse control actions recommended before planting (i.e., soil solarization) can also be used as palliative actions (for instance, when healthy plants are planned to substitute dead trees). Likewise, the use of microbial antagonists could be at the production stage (nurseries) or implemented as a post-planting measure in the field.

Concerning preventive control measures, the search for sources of genetic resistance, the evaluation of olive genotypes, the use of resistant olive rootstocks, and the development of breeding programs aimed to produce cultivars resistant to VWO likely constitute the most efficient approach within an integrated disease management strategy. Thus, several research groups are currently aiming to uncover effective sources of VWO resistance in different regions where olive cultivation is relevant. Similarly, the production of pathogen-free

certified olive plants is a key preventive control action to avoid pathogen dispersal to new areas. Of course, this action must be linked to the use of non-infested soils. Thus, the early and reliable *in planta* detection of the pathogen (particularly of the D pathotype) is of utmost importance for the management of VWO. Diverse qualitative and quantitative PCR-based protocols are already available to detect the presence of *V. dahliae* pathotypes *in planta* (Markakis *et al.*, 2009; Mercado-Blanco *et al.*, 2003a; 2003b) and in soil (Pérez-Artés *et al.*, 2005).

With regards to post-planting control measures, a number of physical, cultural, biological and chemical practices (i.e., soil solarization, biofumigation, elimination of weeds and/or infected plant debris, etc.) aimed to eliminate infective *V. dahliae* propagules within a plantation can be accomplished. Moreover, some of these measures can be complementary. For instance, soil solarization (an action that can be applied either before or after plating) can be used in combination with organic amendment (biofumigation) or with application of biological control agents (BCAs), whose populations can even be promoted in soil after this combined action (Tjamos & Paplomatas, 1987).

### **Biological control of VWO: Use of beneficial microorganisms**

As aforementioned, the use of BCAs can be implemented either before planting or as a palliative action in established orchards. Thus, introduction of antagonistic microorganisms during the propagation process in nurseries to protect pathogen-free certified plants from *V. dahliae* infections has been proposed (Tjamos, 1993; Mercado-Blanco *et al.*, 2004). Our studies have demonstrated that indigenous *Pseudomonas fluorescens* and *Pseudomonas putida* strains originated from olive roots are effective BCAs in nursery-produced olive plants. Some of these bacterial strains *in vitro* antagonize *V. dahliae* olive pathotypes, produce metabolites (i.e., siderophores, salicylic acid, or hydrogen cyanide) largely studied as involved in biocontrol activity by *Pseudomonas* spp. (Mercado-Blanco & Bakker, 2007), colonize and persist in the rhizosphere of olive plants, and control infections caused the D pathotype in different types of nursery-produced olives (Mercado-Blanco *et al.*, 2004). The mechanism(s) involved in VWO biocontrol by these strains have not yet been elucidated. However, some interesting findings have been recently gathered by using biotechnological and microscopy tools. Thus, autofluorescent protein-tagging of bacterial cells and confocal laser scanning microscopy enabled to demonstrate that *P. fluorescens* PICF7 and *P. putida* PICP2 have endophytic lifestyles, colonizing the intercellular spaces of the olive roots cortex (Prieto & Mercado-Blanco, 2008; Prieto *et al.*, 2011). Endophytism poses additional advantages to be exploited in biocontrol of VWO since endophytic BCAs are adapted to the ecological niche where beneficial effects can be displayed. Our studies suggest that effective VWO biocontrol by strain PICF7 needs of an effective colonization (superficial and endophytic) of intact olive roots, and that bacteria should colonize the roots before *V. dahliae* penetrates into the root interior (Prieto *et al.*, 2009). Recently, we have been able to demonstrate that root hairs seem to play a pivotal role in the internal colonization of the roots by strains PICF7 and PICP2 (Prieto *et al.*, 2011). Hameed *et al.* (2005) also informed of *in vitro* antagonism against *V. dahliae* of indigenous *Pseudomonas* strains from olive. However, no information on biocontrol activity has been further reported.

Other bacteria and fungi have been examined as potential effective BCAs against VWO as well. In some cases, however, only antagonism *in vitro* has been reported, as for *Streptomyces plicatus* and *Frankia* sp. (Shahidi Bonjar & Aghighi, 2005). For others, biocontrol has been demonstrated. Thus, *Serratia plymuthica* HRO-C48 has been reported on

its ability to suppress the disease in *V. dahliae* artificially-inoculated olive plants (Müller *et al.*, 2007). This strain was able to colonize the rhizosphere of olive plants for a prolonged period of time. Interestingly, its biocontrol performance depended on the method used for pathogen inoculation. Indeed, disease suppression was observed when *V. dahliae* (D pathotype) was applied by soil inoculation rather than by root dipping (Müller *et al.*, 2007). A commercial bioformulation of the fungi *Trichoderma asperellum* and *T. gamsii* has also been tested against VWO produced by the D pathotype under conditions conducive for the disease (Jiménez-Díaz *et al.*, 2009). Biocontrol efficiency also depended on the method of pathogen inoculation: Effectiveness and consistency of biocontrol was higher in root-dipped inoculated plants than in plants transplanted to *V. dahliae*-infested soil. Under field conditions, suppression of VWO was also achieved to some extent by this *Trichoderma* spp. mixture. As for the previous cases, biocontrol mechanism(s) involved remain to be elucidated. Finally, the possibility to control VWO and other soil-borne pathogens by mycorrhizal fungi has also been explored (Karajeh & Al-Raddad, 1999; Porrás-Soriano *et al.*, 2006; Kapulnik *et al.*, 2010). However, results are variable and, in some cases, did not demonstrate tolerance improvement to *V. dahliae* (Kapulnik *et al.*, 2010).

## Conclusions and perspectives

VWO has become a major threat in some regions where olive is traditionally cultivated, and diverse epidemiological factors have contributed, likely in a synergistic way, to the present importance of the disease. Some factors are regrettable consequences of inadequate crop managing and plant propagation practices, others can be favored by changes in olive cultivation systems, and some are linked to environmental parameters. An integrated disease management strategy appears to be the only possible framework for an effective control of VWO that, in addition, should be based on sustainable agriculture criteria. It must be stressed that preventive measures are the most efficient and sustainable, and that development of additional control actions must be sustained on thoughtful understanding of the olive – *V. dahliae* pathosystem. Thus, to elucidate the molecular bases of the olive-*V. dahliae* and olive-*V. dahliae*-BCAs interactions, as well as to understand how diverse (a) biotic factors influence them should provide new avenues to develop robust control tools. Diverse studies encourage the use of BCAs, either at the olive propagation stage or during the first years after planting. Biocontrol measures should be viewed as complementary actions to other disease control actions: use of certified planting material, planting of tolerant/resistant olive cultivars, or the advantageous situation offered by the natural recovery phenomenon. An interesting perspective is the possibility to develop combinations of different microbial agents (bioformulations), especially those involving microorganisms that have different mechanisms of action and/or can be targeted to different root niches. For instance, bioformulations incorporating a beneficial endophyte together with other BCAs deploying biocontrol mechanisms on the root surface offer an interesting research field to be explored. However, development of commercial products combining different BCAs still needs of thorough studies to overcome potential competence among BCAs in the bioformulation, or between them and the natural beneficial microflora and/or the target plant when applied under field conditions.

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