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Involvement of Induced Systemic Resistance in control of Verticillium wilt by fluorescent *Pseudomonas* spp.

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Abstract: Verticillium wilt, caused by *Verticillium dahliae* Kleb., is a problem in many crops and the disease is difficult to control. Strains of *Pseudomonas fluorescens* and *P. putida* were previously isolated from root tissues of olive trees, cv. Picual. Some of them are endophytic and can control the highly-virulent, defoliating (D) pathotype of *V. dahliae* in olive. One mode of action of disease suppression by fluorescent *Pseudomonas* spp. is induced systemic resistance (ISR). *Pseudomonas* spp. strains were tested for ISR in a system using *Arabidopsis thaliana* and the pathogens *P. syringae* pv. *tomato* and *Botrytis cinerea*. To include *V. dahliae* in these studies we inoculated *A. thaliana* Col-0 with several isolates of this pathogen belonging to different vegetative compatibility groups (VCG). Isolate V937I (VCG1A, D pathotype) produced severe symptoms in Arabidopsis, and *P. fluorescens* PICF7 was able to control the disease caused by this virulent isolate. The use of non-ISR expressing accessions and mutants of *A. thaliana* will allow to evaluate involvement of ISR in control of Verticillium wilt.

Key words: *Pseudomonas* spp., Induced Systemic Resistance, *Verticillium dahliae,* Arabidopsis

Introduction

Verticillium wilt of olive (VWO), caused by the soil-borne plant pathogenic fungus *Verticillium dahliae* Kleb., is an increasing problem. Its control is problematic due to: the pathogen's ability to produce microsclerotia which are persistent in soils, the broad host range, the inefficiency of chemical treatments, and the genetic diversity of the pathogen (López-Escudero & Mercado-Blanco, 2011). Effective control of VWO requires the implementation of an integrated disease control strategy (Tjamos, 1993), with emphasis on preventive measures. Among the various disease control measures, biological control agents (BCAs) have received scant attention. BCAs may act against the pathogen through competition or antibiosis. However, they can also be effective by indirect mechanisms, for example by inducing host plant resistance responses. Induced resistance is a state characterized by an enhanced defensive capacity in plants after appropriate stimulation. Certain non-pathogenic bacteria present in the rhizosphere the so-called plant growth-promoting rhizobacteria (PGPR) (Kloepper et al., 1980; Schroth & Becker, 1990), can elicit Induced Systemic Resistance (ISR) (van Loon et al., 1998). Since many ISR-inducing rhizobacteria can inhibit the growth of the pathogen in a direct way, it has to be demonstrated that both microorganisms (i.e., the BCA and the pathogen) are spatially separated for the duration of experiments designed to determine the existence of an ISR response (Bakker et

al., 2003). The study aimed to assess the ability of several *Pseudomonas* spp. strains native to olive roots, to elicit ISR against different pathogens in the model plant *Arabidopsis thaliana*.

Material and methods

Arabidopsis thaliana/*BCAs bioassays*

Strains *P. fluorescens* PICF4 and PICF7 and *P. putida* PICP2 (Mercado-Blanco et al., 2004), and their respective spontaneous rifampicin-resistant (Rf^R) mutants were studied for their abilities to elicit ISR in *A. thaliana* against *P. syringae* pv. *tomato* (Pst) and *B. cinerea*. Different genotypes of *A. thaliana* were used: Col-0 (wild type); *Myb*-72 (R2R3-MYB-like transcription factor protein mutant unable to elicit ISR response) and *Sid*-2 (salicylic acid mutant which cannot express SAR) (Segarra et al., 2009). All bioassays were performed as described by Pieterse et al. (1996), except for the *V. dahliae* bioassays (see below). The *Pseudomonas* strains from olive roots and *P. fluorescens* WCS417r (van Peer et al., 1991) were introduced (except for *V. dahliae* bioassays) by soil inoculation with bacterial suspensions (10^6 cfu/g soil) prior to transplant of *Arabidopsis* seedlings (Djavaheri, 2007). Plants were grown at 21°C, 70 % relative humidity and a 8-h photoperiod of fluorescent light at 200µEm^{-2} s^{-1}.

I) Pst strain DC3000 was used (Whalen et al., 1991) according to the methodology described by Pieterse et al. (1998), and Ton et al. (2002). Briefly, *Arabidopsis* plants (20-25) were grown either in control soil or in *Pseudomonas* treated soil. After 6 to 7 weeks, plants were challenge inoculated by dipping the leaves in a Pst suspension (2.5 x 10^7 cfu/ml). Three to four days after inoculation the percentage of leaves with symptoms (necrotic or water-soaked spots surrounded by chlorosis) was scored. Data were subjected to analysis of variance ($\alpha = 0.05$).

II) *B. cinerea* bioassays were performed as described by Djavaheri (2007). Six to seven week old *Arabidopsis* plants (grown either in control soil or in *Pseudomonas* treated soil) were inoculated by applying 5-µl droplets of a conidial suspension of *B. cinerea* (7.5 x 10^5 conidia/ml half strength PDB) to six to eight well-developed leaves. The inoculated plants were maintained at 100% relative humidity, and 3 to 4 days after challenge inoculation disease symptoms were scored according to the scale: 0, no symptoms; 1, small, non-spreading lesion, 2, small, non-spreading lesion with chlorosis, 3, spreading lesion with chlorosis, 4, spreading lesion and leaf completely chlorotic or dead. Data were subjected to χ^2 test ($\alpha = 0.05$).

III) Pathogenicity of two *V. dahliae* olive isolates (V789I, belonging to the "Vegetative Compatibility Group" (VCG) 4B, non-defoliating [ND] pathotype, and V937I, VCG1A, defoliating [D] pathotype [Collado-Romero et al., 2006]) on *Arabidopsis* was studied. Three-week-old Col-0 plants were inoculated by dipping their roots in a conidial suspension (10^6 conidia/ml) or in 10mM $MgSO_4$ as a control. Disease incidence was scored as the percentage of diseased leaves of the total number of leaves per plant at regular time intervals after inoculation. Isolate V937I was virulent on *Arabidopsis* and therefore selected to conduct biocontrol experiments. *Arabidopsis* plants grown for three weeks in control soil or in soil amended PICF7 (5 x 10^7 cfu/g soil) were inoculated by dipping their roots in a conidial suspension (4.2 x 10^6 conidia/ml 10mM $MgSO_4$). Disease symptoms were scored at 14 and 24 days after infection according to the following scale: 0, no symptoms; 1, wilting in 25% of leaves; 2, wilting in 50% of leaves; 3, wilting in 75% of the plant and 4, dead plant. Bioassays included at least 20 repetitions (plants). Data were subjected to χ^2 test ($\alpha = 0.05$).

Colonization of Arabidopsis rhizosphere by* Pseudomonas *spp.
Three root systems of six to seven-week-old plants (from accession Col-0, and the mutants *Myb*-72 and *Sid*-2) per treatment were harvested and shaken for 1 minute in 5ml of 10mM $MgSO_4$ containing 0.5g of glass beads. Serial dilutions of root macerates were then plated onto KB plates (50µl) supplemented with chloramphenicol (13mg/l) ampicillin (50mg/l) and delvocid (100mg/l) and grown 48 h at 28°C. Rifampicin (50mg/l) was also added for counts of Rf^R derivatives.

Results and discussion

Pseudomonas syringae *pv.* tomato *and* Botrytis cinerea *bioassays*
Mean disease incidence and severity (measured as percentage of diseased leaves per plant) were similar regardless the treatment. None of the BCA examined in this work showed biocontrol activity against Pst or *B. cinerea* under the experimental conditions used.

Verticillium dahliae *bioassays*
Isolate V789I (VCG4B, ND defoliating in olive) did not induce any visible Verticillium wilt symptom in *A. thaliana* Col- 0 plants. However, isolate V937I (VCG1A, D pathotype in olive) caused clear disease symptoms (chlorosis and wilting) in Col-0 plants. No symptoms were observed in non-inoculated control plants at any time.

Isolate V937I was used to examine biocontrol activity of strain PICF7. Twenty four days after inoculation with *V. dahliae*, 70% of the non-bacterized plants showed very severe symptoms (3-4 in the symptoms scale). On the contrary, only 50% of PICF7-treated plants showed severe symptoms at the same time point.

Colonization of* Arabidopsis *rhizosphere by* Pseudomonas *spp. strains
All *Arabidopsis* genotypes used in this work had native rhizosphere bacterial populations that displayed different colony morphologies, preventing accurate counts of strains PICP2 and PICF4 and their rifampicin spontaneous mutants. Strain PICF7 and its Rf^R derivative mutant could be discriminated based on morphology and fluorescence on KB plates under UV and black light, and their counts showed that they colonized and persisted in the rhizospheres of *A. thaliana* Col-0 and its mutants *myb72* and *sid2*.

Further studies will focus on biocontrol activities of PICF7 and some specific mutants (defective in either siderophore production, motility, or antagonism against *V. dahliae*) of this strain. Strain PICP2 will also be included because it is effective as a biocontrol agent of VWO in nursery-produced olive plants (cvs. Picual and Arbequina) (Mercado-Blanco et al., 2004) and intriguingly it was recently demonstrated to colonize root hairs of propagated olive (Prieto et al., 2011). The involvement of ISR will be studied using non ISR expressing mutants in the Col-0 background and accessions RLD and WS-0 that are also do not express rhizobacteria-mediated induced resistance. Alternatively bacteria will be applied to the leaves and the pathogen to the roots, or split root systems will be developed, to ensure spatial separation between the inducers and the challenge pathogen.

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