Effect of the biological control strain *Serratia plymuthica* HRO-C48 on verticillium wilt of olive trees cv. Arbequina

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Abstract: Integration of biological control measures to protect olive planting material produced by nurseries could help managing Verticillium wilt in olive. Therefore, the application of *S. plymuthica* HRO-C48 to suppress *Verticillium dahliae* in seven-month old olive plants cv. Arbequina was investigated. The method of infestation with the pathogen, either by soil inoculation or by root dipping, determined the effect of the biocontrol agent. Using soil inoculation, HRO-C48 treatment reduced the disease severity. In addition, a statistically significant plant growth promoting effect was observed for HRO-C48 in non-pathogen stressed plants.

Key words: *Olea europea, Verticillium dahliae*

Introduction

Verticillium wilt, caused by *Verticillium dahliae*, is one of the most serious diseases affecting olive trees worldwide and is responsible for severe yield losses and plant death (Jiménez-Díaz et al., 1998). Until recently, effective control of *V. dahliae* was achieved, among other chemical control measures, by fumigating the soil with methyl bromide. This and other related substances show highly toxic effects to many non-target organisms as well as additional negative influence on the world climate. Therefore, in developed countries the application of methyl bromide was ultimately banned in 2005. The management of Verticillium wilt disease should be based on an integrated strategy, which involves primarily the choice of planting sites with low inoculum densities and increasing certainty that pathogen-free plants come from the nurseries. The beneficial rhizosphere microbiota can be enhanced for increased plant health by the introduction of beneficial microorganisms, and therefore offers an environmentally friendly alternative to control verticillium wilt. The beneficial Gram-negative bacterium *Serratia plymuthica* strain HRO-C48 was isolated from the rhizosphere of oilseed rape and selected as a biocontrol agent according to the following criteria: (a) high antifungal activity against fungal pathogens, e.g. *V. dahliae* and *Phytophthora cactorum in vitro*, (b) production of the plant growth hormone indole-3-acetic acid, (c) relative harmlessness to human health and the enviroment; and (d) low level of antibiotic resistence (Berg, 2000). The successful application of *S. plymuthica* HRO-C48 was shown for strawberries (Kurze et al., 2001). Here, the bacteria were able to colonize the rhizosphere to avoid an infection with *Verticillium dahliae*, and to enhance fruit yield. A product called RhizoStar® was developed for commercial strawberry production (Berg et al., 1999). Mercado-Blanco et al. (2004) have described the first-time employment of antagonistic

bacteria to protect olive nursery-plants against *V. dahliae*. In planting material of the highly susceptible olive cultivar 'Picual', the introduction of root-associated pseudomonads resulted in suppression of the defoliating (D) pathotype of *V. dahliae*. Engaging a similar experimental design, but using the susceptible cultivar 'Arbequina', the capacity of *S. plymuthica* HRO-C48 to protect olive planting stocks against *V. dahliae* was investigated.

Material and methods

Preparation of bacterial and fungal inocula

The inoculum of *Serratia plymuthica* HRO-C48 (DSMZ 12502) was grown on NA at 30°C for two days, scraped from the medium with sterile Drigalski spatula, and suspended in 10 mM $MgSO_4 \cdot 7H_2O$. Bacterial suspensions were centrifuged twice in 10 mM $MgSO_4 \cdot 7H_2O$ to remove residual metabolites ($10,500 \times g$, 20 min) and re-suspended in 10 mM $MgSO_4 \cdot 7H_2O$ solution. Bacterial concentration in the suspension were adjusted to $\log_{10}$ 8.7 CFU ml^{-1}.Monoconidial *V. dahliae* isolate V138I used in this study is a representative of the highly virulent, D patho-type originated from diseased cotton plants (Culture collection Instituto Agricultura Sostenible, CSIC, Córdoba, Spain). For bioassays, inoculum consisted of conidia from cultures in potato-dextrose broth (PDB) incubated in the dark for seven days at 24°C and 125 rpm. Conidia were harvested by filtering liquid culture through a layer of sterile cheesecloth and inoculum concentration was adjusted to $\log_{10}$ 7.3 conidia ml^{-1} by dilution with sterile water.

Bioassay experimental design

The growth chamber trial was arranged in a two-stage setup. For the first 90-day stage, seven-month old, micropropagated olive plants cv. Arbequina, provided by Cotevisa (Valencia, Spain), were carefully uprooted from the substrate, their roots thoroughly washed in tap water, and dipped into a *S. plymuthica* HRO-C48 cells suspension for 10 min. For the control treatment, plants were dipped in 10 mM $MgSO_4 \cdot 7H_2O$. Plants used for assessment of plant growth promotion (16 per treatment) were transplanted (one per pot) into clay pots with a diameter of 15 cm containing an autoclaved (twice on consecutive days) soil mixture (sand/loam, 2:1, v/v). For the biocontrol activity evaluation, 20 plants were grown in soil which was infested by thoroughly mixing 100 ml of a conidial suspension of *V. dahliae* V138I with 1.0 kg of soil mixture to obtain a final concentration of $\log_{10}$ 6.3 conidia g^{-1} soil. The experiment was conducted in a randomized block design. Plants were incubated in a controlled growth chamber at 23±1°C, 60 to 90% relative humidity, and received a 14 h photoperiod for three months. Plants were watered as needed, and fertilized weekly with a hydro-sol fertilizer 20-5-32+microelements (Haifa Chemicals, LTD, Haifa, Israel). During the 90 days, no symptoms of Verticillium wilt could be observed. Thereafter, plants, which were grown in infested soil, were again exposed to the pathogen in two different ways. For one hand, ten plants per treatment were uprooted and replanted into 20-cm-diameter clay pots filled with autoclaved sand/loam mixture (2:1, v/v) infested with $\log_{10}$ 7.0 conidia g^{-1}. On the other hand, soil-free root bales of another ten plants each treatment were trimmed using sterile scissors, dipped into conidial suspension ($\log_{10}$ 7.0 conidia ml^{-1}), and transplanted into 20-cm-diameter containing sterile sand/loam mixture. Finally, four plants each treatment from the plant growth-promoting assay were also replanted into 20-cm-diameter containing sterile sand/loam mixture after they were uprooted. Olives were arranged in a randomized block design and were grown for another 79 days under same conditions described before.

Assessment of disease development and plant growth

Disease reaction was assessed by the percentage and severity of symptoms on a 0-4 rating scale according to the percentage of affected leaves and twigs (0 = no symptoms, 1 = 1–33%,

2 = 34-66%, 3 = 67-100%, and 4 = dead plant) at weekly intervals after the first symptoms occurred. Data were subjected to analysis of variance. Percentage values were arcsine transformed $(Y/100)^{0.5}$ before analyses. Data on disease severity were used to calculate the following: (i) a disease severity index (*DII*) determined as $DII = (\Sigma \, Si * Ni)/(4 * Nt)$, where Si is the symptoms severity, Ni is the number of plants with Si symptoms, and Nt is the total number of plants; and (ii) the standardised area under disease progress curve of *DII* plotted over time (AUDPC) calculated as described previously (Mercado-Blanco et al., 2004). Treatment means were compared with those of the control using the Dunnett's test at $P = 0.05$. For assessing plant growth promotion, total plant length, calculated by summing length of stem and lateral branches, was scored. Initial measurements were done after transplanting treated olives. The plant growth promotion experiment was terminated after 90 days, and total plant length was recorded again to calculate the relative plant growth. Four plants were grown another 79 days after replanting into a new pot and measured once again.

Results and discussion

S. plymuthica HRO-C48 was shown to be able to colonize the rhizosphere as well the endorhiza of olive plants until 168 days post-inoculation (data not shown). After HRO-C48 treatment and planting in *V. dahliae*-infested soil, no symptoms caused by the D isolate V138I evolved in olive plants during 90 days. Thus, the experimental plants were once more exposed to the pathogen. Fig. 1 illustrates the course of the disease development from the moment of the second inoculation. For *S. plymuthica* HRO-C48 bacterized plants, a suppression of the Verticillium wilt was found when *V. dahliae* was applied by soil inoculation rather than by root dipping. Using soil infestation, all plants developed symptoms characteristic to those caused by the D pathotype. In control plants, Verticillium wilt developed by 32.9 days reaching a final *DII* of 0.64 and an AUDPC of 27.8. Bacterization with HRO-C48 reduced the *DII* by 23.4%, and AUDPC by 40.6% (Fig. 1A). Results obtained by immersing olive roots into conidial suspension were different to those of soil infestation. In non-bacterized control plants, first symptoms appeared by 25.7 days post-inoculation reaching a final *DII* of 0.71 and AUDPC of 31.2. The disease development in *S. plymuthica*-treated plants started by 24.9 days, and the final *DII* (0.81) as well the AUDPC (38.6) were slightly higher compared to the control plants (Fig. 1B). The ability of the bacteria to delay the development of symptoms caused by the D patho-type of *V. dahliae* depended on the inoculation procedure. Compared to the soil infestation method, it is apparent that the rhizosphere of plants dipped into the conidial suspension was disturbed by this procedure. It could be speculated that strain HRO-C48 cells, which operates from the outer surface, were washed off by the immersion and were thus unable to prevent the root from penetration by fungal hyphae. Incorporation of the fungal inoculum into the growth substrate represents the more realistic form of *Verticillium* infestation. In that case, the rhizosphere and its bacterial community remain intact. The putative 'protective shield' formed by *S. plymuthica* was able to impede the fungal attack.

The ability to promote plant growth in the absence of the pathogen was determined by scoring stem and branch length after 90 and 169 days (Fig. 2). No statistically significant differences between the bacterial-treated and non-bacterized (control) plants were observed after 90 days. The relative length growth of the control plants amounted to 313±79% compared to 339±71% of plants inoculated with HRO-C48. After 169 days, however, the bacterization of olive roots with strain HRO-C48 resulted in a statistically significant increase of stem and branch growth of 837±78% on average, compared to the control plants (657±54%). Woody plants such as olives are known to grow relatively slowly. Accordingly, growth effects originating from bacterial activity ought to manifest in a long-term scale.

Hence, experiments to evaluate plant growth promotion should be of duration longer than five months. In conclusion, under conditions similar to those in the olive-nursery industry, the effect of *S. plymuthica* HRO-C48 on disease development and plant growth was demonstrated. Importantly, even three months after root bacterization, HRO-C48 was shown to provide some degree of disease protection. It can be suggested, that once HRO-C48 is introduced, the biocontrol agent may provide a long-term protection against soil-borne fungi, even when the plant is replanted into new, potentially infested soil.
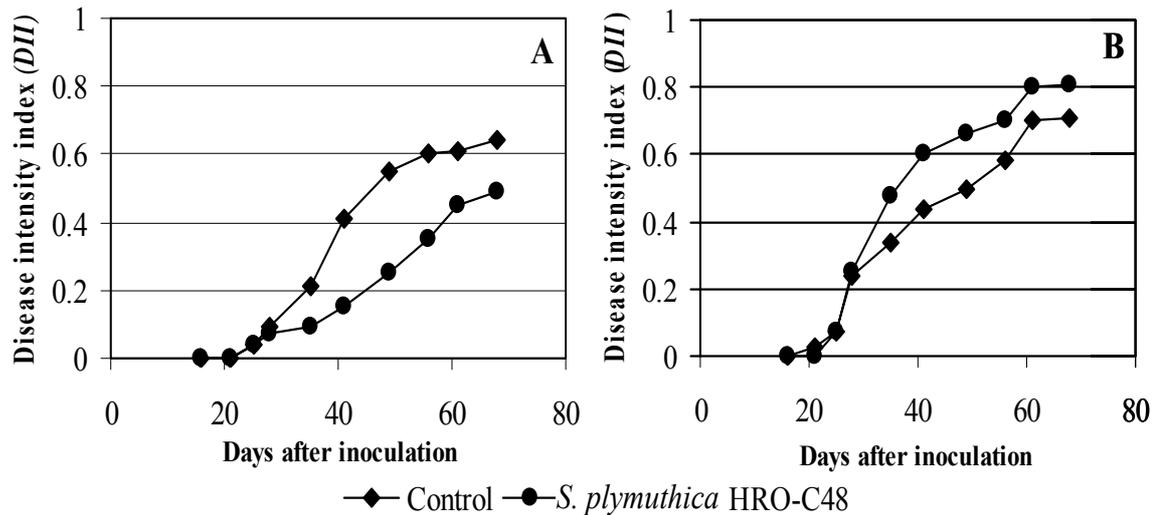
Figure 2. Effect of *S. plymuthica* HRO-C48 on the development of Verticillium wilt of olive cv. Arbequina. *Verticillium dahliae* was inoculated by either soil infestation (A) or root dipping. The disease intensity index (*DII*) ranging from 0-1 was calculated with data on incidence and severity of symptoms recorded at 7-days intervals.
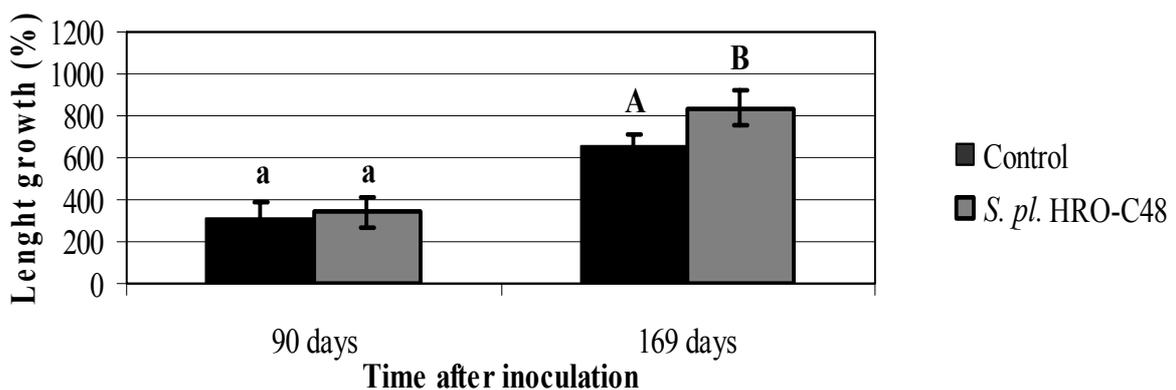
Figure 3. Plant growth promotion effect of *S. plymuthica* HRO-C48 on olive cv. Arbequina assessed by monitoring total plant length. Bars with same letters were not significantly different according to Fisher's protected LSD test ($P \leq 0.05$).

Acknowledgements

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