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Biological control of Verticillium wilt of olive by *Paenibacillus alvei*, strain K165

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Abstract In the present study, the efficiency of the biocontrol agent *Paenibacillus alvei* (strain K165) to suppress Verticillium wilt of olive tree was evaluated in greenhouse and field experiments. *In planta* bioassays were conducted under greenhouse conditions and revealed that K165 significantly decreased symptoms on the susceptible cultivar 'Amfissis' by 44.5 and 51.6 % of the final disease severity index and relative area under disease progress curve (AUDPC), respectively. Thereafter, the suppressive effect of K165 against *Verticillium dahliae* was studied for two consecutive years (2007 and 2008) in a newly established olive orchard of the susceptible cv Amfissis and the resistant cv Kalamon, naturally infested with *V. dahliae*. The evaluation of K165 was carried out by recording symptoms, isolations and qPCR quantification of the pathogen in olive tissues. In both years, 'Amfissis' trees treated with K165 showed significantly lower final disease severity and relative AUDPC values compared to the non treated controls, whereas, in 2008 decreased symptom severity was associated with significantly lower *V. dahliae* DNA levels in plant tissues, indicating the suppressive effect of the biocontrol agent. However, no significant suppression was observed in 'Kalamon'. Pathogen isolations along with qPCR quantification revealed a seasonal fluctuation of *V. dahliae* biomass in olive tissues with higher amounts occurring in May, and lower amounts in February, August and November. This is the first report of biological control of Verticillium wilt of olive tree under field conditions, associated with reduced pathogen levels inside the xylem tissues.

Keywords Biological control · Olive · Suppression · *Verticillium dahliae* · Verticillium wilt

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Introduction

Verticillium wilt caused by the soilborne fungus *Verticillium dahliae* Kleb. is one of the most serious olive (*Olea europea* L.) diseases, worldwide, causing

severe losses and plant death (Jimenez-Diaz et al. 2012; Lopez-Escudero and Mercado-Blanco 2011). Microsclerotia (msc), the resting structures of *V. dahliae*, constitute the main potential infective inoculum of the pathogen in the field and persist in the soil for more than 20 years (Wilhelm 1955). The use of fungicides, besides having potentially toxic consequences and representing an environmental threat, have little effect on them (El-Zik 1985). The infectious hyphae that emerge from msc penetrate roots mainly in the areas of cell differentiation and in the root hair zone (Prieto et al. 2009). Therefore, the use of biocontrol agents (BCAs) capable of growing in the rhizosphere could constitute a potential disease management strategy for olive producers.

Numerous studies have shown the efficacy of various BCAs to suppress Verticillium wilt in different hosts (Berg et al. 2001, 2005; Mercado-Blanco et al. 2004; Tjamos et al. 2004). A limited number of those studies have been dedicated to biological control of *V. dahliae* in olive trees (Lopez-Escudero and Mercado-Blanco 2011). In one of those studies Mercado-Blanco et al. (2004) reported that the application of different strains of the *Pseudomonas fluorescens* complex reduced Verticillium wilt symptom development on the susceptible olive cv Picual under controlled conditions. Later, Prieto et al. (2009) determined the *in planta* interaction of the defoliating *V. dahliae* pathotype, with the endophytic biocontrol strain *P. fluorescens* PICF7. In a different study, application of *P. fluorescens* isolates reduced significantly disease incidence and severity on the susceptible olive cv Zard under greenhouse conditions (Sanei and Razani 2011). However, the effect of BCAs against Verticillium wilt on susceptible and/or resistant olive cultivars have not yet been investigated under field conditions. Furthermore, in all the aforementioned greenhouse experiments, olive plant material was artificially inoculated by using conidial suspension, instead of msc, that constitutes the main natural infective inoculum under field conditions.

An already known BCA is the *Paenibacillus alvei* strain K165 that has been isolated from the root system of tomato plants grown in Verticillium suppressive soil (Tjamos et al. 2004). Previous studies have shown the efficacy of strain K165 in reducing Verticillium wilt symptom development in eggplants and potato plants under glasshouse and field conditions (Markakis et al. 2008; Tjamos et al. 2004). The mode of action of K165 against *V. dahliae* has been mainly attributed to induced systemic resistance (ISR), in a salicylic acid-dependent pathway (Tjamos et al. 2005). Also, Antonopoulos et al. (2008) have reported that application of K165 resulted in the reduction of msc germination of *V. dahliae*, in the root tip and the zone of elongation of eggplants. However, the efficacy of K165 against *V. dahliae* has never been tested in olive trees, although the olive tree is one of the most susceptible hosts of the pathogen and also has a tremendous economical impact on the economy of the main olive producing countries, Spain, Italy and Greece (FAO 2012).

In view of all the above, the main objectives of this study were to assess the efficacy of strain K165 to protect olive plant material infested with *V. dahliae* msc inoculum under greenhouse conditions, as well as to evaluate the biocontrol potential against *V. dahliae* on resistant and susceptible olive cultivars under field conditions. It was also investigated whether symptom development is associated with fungal proliferation in vascular tissues, in different time periods in the field.

Materials and methods

Plant material

Plant material for greenhouse experiments, consisted of 8-month-old rooted cuttings of olive cv Amfissis, whereas for field experiments, 3-year-old rooted cuttings of cvs Amfissis and Kalamon were used. 'Amfissis' and 'Kalamon' have been characterized as susceptible and resistant to *V. dahliae,* respectively (Antoniou et al. 2008; Markakis et al. 2009, 2010).

Inoculum preparation

A highly-virulent non-defoliating *V. dahliae* isolate originated from a diseased 'Amfissis' olive tree was used in the greenhouse experiments (Antoniou et al. 2008). The isolate was cryopreserved by freezing a suspension of 10^7 conidia ml^{-1} in 25 % aqueous glycerol at −80 °C. Before being used, the fungus was transferred to potato dextrose agar (PDA; Merck) at 23 °C for 7 days. *Verticillium dahliae* msc were prepared in sucrose sodium nitrate (SSN) liquid medium. The liquid cultures were shaken in an orbital incubator at 23 °C for 3 weeks. Msc were centrifuged

at 10.000×*g* at 20 °C for 10 min to remove growth medium then air dried. Msc were resuspended in sterile distilled water and filtered through 70 μm mesh to select large (>70 μm) msc, which germinate easily and show high levels of pathogenicity (Hawke and Lazarovits 1994).

Biocontrol agent preparation

A spontaneous rifampicin-resistant derivative of strain of K165 (Tjamos et al. 2004) was used in the greenhouse experiments, while a wild-type K165 strain was applied in the field experiments. K165 was grown in nutrient broth plus glycerol (NG) liquid culture in Erlenmeyer flasks of 2 l capacity, containing 1 l of the medium, in an orbital incubator at 180 rpm at 30 °C for 48 h. A final concentration of 10^7 cfu ml^{-1} (measured by dilution plating) was obtained by water dilution.

V. dahliae: K165 bioassays under greenhouse conditions

Olive plants (8-month-old) cv 'Amfissis' were grown in plastic pots containing soil (Potground, Klasmann, Deilmann, Germany) and K165 was applied by root drenching (500 ml, 10^7 cfu ml^{-1}), 20 days before transplanting the plants to plastic pots containing 7 l soil infested with 20 *V. dahliae* msc g^{-1} soil. The olive plants were transplanted carefully without removing the soil of the rhizosphere and placed in a greenhouse, at 23 ± 5 °C with a 16-h light and dark cycle and neither herbicides nor pesticides were applied. Verticillium wilt symptoms were recorded every week for 80 days after plant transplanting to infested soil. The experiment was repeated three times with ten replicates (plants) per treatment and experiment.

Determination of the K165 rhizosphere population

The ability of K165 to colonize the rhizosphere of K165 treated olive plants was evaluated at the end of the greenhouse experiment (80 days post-inoculation, dpi), using the dilution plating technique (Harris and Sommers 1968). Ten plants per experiment were sampled and the experiment was performed three times (a total of 30 plants). To estimate rhizosphere populations, rhizosphere soil was collected and shaken for 45 min in 50 mM phosphate buffer (pH 7.02) containing Tween 20 (0.02 %). The suspension was plated onto PDA supplemented with rifampicin (100 μg ml^{-1}). After incubation at 30 °C for 48 h, the number of K165 colonies per g of rhizosphere soil was determined.

Experimental field

A naturally infested field, previously cultivated with highly susceptible *V. dahliae* vegetable crops was used to establish the experimental olive orchard. The *V. dahliae* susceptible crops (potato, tomato and egg-plant) grown in the field, exhibited severe Verticillium wilt symptoms in the past.

Verticillium dahliae msc levels were determined in the field by soil sampling from 30 arbitrary sites, following a 'Z' pattern extending to the whole area of the field. Approximately 100 g of soil were collected from each sampling point. Soil samples were dried in an oven at 30 °C and stored at room temperature. Subsequently, the soil was assessed for the number of msc using the modified Anderson sampler technique (Butterfield and DeVay 1977). In brief, 0.5 g of pulverized soil was distributed onto Petri plates containing *V. dahliae* selective medium (Ausher et al. 1975), using the modified Anderson sampler (Butterfield and DeVay 1977). Plates were incubated in the dark at 23 °C and *V. dahliae* msc were counted under the stereoscope after 4 weeks.

V. dahliae: K165 bioassays under field conditions

Three-year-old rooted cuttings of 'Amfissis' and 'Kalamon' grown in plastic pots (12 l capacity) containing soil substrate (Potground, Klasmann, Deilmann, Germany) were planted in the experimental field, in October 2006. Sixty trees of each cultivar were planted and thirty of them were treated with K165 by root drenching approximately 3 l of 1×10^7 cfu ml^{-1} bacterial suspension per tree. The remaining thirty trees were root drenched with 3 l of non sterilized water and served as controls. The BCA was applied in January, March, May and September of two consecutive years (2007, 2008). Levin et al. (2003) proposed that *V. dahliae* infection cycle starts in autumn, with the reinvasion of the fungus probably caused by the germination of msc and the upward stream of conidia in the xylem during winter and spring, when disease expression is at a maximum, and

is completed when the fungal population decreases during summer, probably because of high temperatures (Tosi and Zazzerini 1998). Therefore, the months of January, March, May and September were considered as the most appropriate to apply K165, in order to suppress the disease. The experiment was performed with a randomized block design with three blocks and four experimental units (K165-treated 'Amfissis', control-'Amfissis', K165-treated 'Kalamon', control-'Kalamon') per block. Each experimental unit consisted of ten olive trees.

Disease assessment

Verticillium wilt symptoms in greenhouse experiments were recorded every week for 80 days after transplantation to the *V. dahliae* msc infested soil, while in field experiments symptoms were assessed in monthly intervals from February to November, for two consecutive years. In November 2007, diseased branches were removed by pruning and symptom recording was continued in February 2008, when new infections were observed. Disease incidence was estimated as the percentage of infected plants whereas mortality was estimated as the percentage of dead plants. The disease severity index of each branch was based on an arbitrary scale from 0 to 4 where 0 = healthy branch, 1 = dull green leaves, 2 = internally rolled leaves, 3 = necrotic leaves, and 4 = defoliated–dead branch. The percentage of disease index was calculated from the disease rating by the formula: disease severity index (%) = [Σ(rating no. × no. of branches in the rating)/total no. of branches × highest rating] × 100 % (Markakis et al. 2009). Disease ratings were plotted over time to generate disease progress curves. Subsequently the area under disease progress curve (AUDPC) was calculated by the trapezoidal integration method (Campbell and Madden 1990). Disease was expressed as a percentage of the maximum possible area with reference to the maximum value potential reached over the whole period of the experiment and is referred to as relative AUDPC.

Pathogen isolation and real-time quantitative PCR quantification

Two representative diseased or symptomless branches from each of eight randomly selected trees per treatment and experimental unit were collected in February, May, August and November of 2008. Ten wood chips from each branch, surface-disinfested with 95 % ethanol, were placed onto acidified potato dextrose agar (PDA, Merck). Several representative *V. dahliae* isolates recovered from symptomatic tissues were identified as the non-defoliating pathotype by multiplex PCR assay using the primer pairs D1/D2 and NDr/NDf (Mercado-Blanco et al. 2001, 2002), according to the procedure implemented in Markakis et al. (2009). The remaining branch tissues were used for DNA extraction. Branches were cut to form 7 to 12 mm long pieces, the bark was removed and subsequently the tissues were freeze-dried and ground to a fine powder by using an autoclaved mortar and pestle in the presence of liquid nitrogen. Total DNA was isolated according to Dellaporta et al. (1983), with the appropriate modifications (incubation time at 65 °C was increased to 1 h), and was quantified by spectrophotometry and agarose gel electrophoresis (1 % agarose in 1× TAE buffer, stained with ethidium bromide). Real-time quantitative PCR (qPCR) assays for quantification of *V. dahliae* DNA in olive tissues were conducted according to Markakis et al. (2009, 2010. In brief, polymerase chain reaction (PCR) products of the primer pair *Verticillium dahliae* internal transcribed spacer (ITS)1-F/ITS2-R cloned into the pGEM-Teasy vector. Standard curve ($y = -0.3471x + 17.36$, $R^2 = 0.99$) was generated from plotting the log of known DNA concentrations (10 ng to 1 fg of DNA), against the cycle threshold (Ct) values obtained from the quantitative PCR and served to calculate the relative amount of *V. dahliae* DNA in total genomic DNA samples extracted from infected olive tissues.

Statistics

Analysis of variance (ANOVA) was used to determine the effects of treatment (control or K165) on disease incidence, final disease severity, mortality and relative AUDPC in greenhouse experiments. ANOVA was also applied to determine the effects of cultivar (Amfissis or Kalamon), treatment (K165 or control), sampling time point (February, May, August or November 2008) and their interactions on disease incidence, final disease severity, mortality, relative AUDPC, percentage of positive *V. dahliae* isolations and *V. dahliae* DNA level, in field experiments

Table 1 Analysis of variance for disease incidence (DI), final disease severity (FDS), mortality (M) and relative area under disease progress curve (RAUDPC), for Amfissis and Kalamon cultivars, treated with K165 or not (control) under field conditions, in 2007 and 2008

Source	df[b]	*F* values[a]							
		2007				2008			
		DI	FDS	M	RAUDPC	DI	FDS	M	RAUDPC
Cultivar	1	9.481*	15.373***	4.000	6.250*	7.042*	21.041***	10.667*	10.599**
Treatment	1	2.370	4.222*	4.000	8.570**	5.042	13.599***	24.000**	17.907***
Cultivar × treatment	1	0.000	4.222*	4.000	3.693	2.042	0.856	2.667	2.009
df[c]		8	116	8	116	8	116	8	116

[a] *, **, and *** significance at $P \leq 0.05$, 0.01, and 0.001 levels, respectively, according to the *F* test

[b] Degrees of freedom between groups

[c] Degrees of freedom within groups

Table 2 Analysis of variance for percentage of positive *Verticillium dahliae* isolations (*Vd* I) and DNA level (*Vd* DNA), for Amfissis and Kalamon cultivars, treated with K165 or not (control), in four sampling time points, under field conditions, in 2008

Source	df[a]	*F* values[b]	
		Vd I	*Vd* DNA
Cultivar	1	120.922***	46.490***
Treatment	1	5.858*	9.221**
Time	3	11.666***	14.869***
Cultivar × treatment	1	2.826	7.372**
Cultivar × time	3	3.016*	9.060***
Treatment × time	3	0.501	1.542
Cultivar × treatment × time	3	0.275	1.269
df[c]	–	368	368

[a] Degrees of freedom between groups

[b] *, **, and *** significance at $P \leq 0.05$, 0.01, and 0.001 levels, respectively, according to the *F* test

[c] Degrees of freedom within groups

(Tables 1, 2). Prior to ANOVA, homogeneity of variance across treatments was evaluated, and an arcsin transformation was applied to normalize variance. When a significant *F*-test was obtained for treatments ($P \leq 0.05$), the data were subjected to means separation by Tukey's honestly significant difference test. In greenhouse experiments, data on disease incidence, mortality, disease severity for each day of observation separately and relative AUDPC, were analyzed by carrying out a two-sample *t* test ($P \leq 0.05$).

Results

Suppression of Verticillium wilt symptoms in greenhouse experiments

Verticillium wilt symptoms, mainly chlorosis, started on day 30 in both K165 treated and control 'Amfissis' plants, whereas significant difference in symptom severity was observed no sooner than 44 dpi (Fig. 1). Subsequently, disease severity index in K165 treated plants was significantly lower compared to controls, for each day of observation. The relative AUDPC analysis along with the final disease severity and mortality demonstrated that symptom development in K165 treated trees was significantly lower than in controls, showing the suppressive effect of the biocontrol agent against *V. dahliae*, under greenhouse conditions (Table 3). The monitoring of the K165 population at the end of the experiment (100 days after K165 application), revealed that the BCA is an efficient olive root coloniser since its population number was $8.8 \pm 1.7 \times 10^3$ cfu g^{-1} rhizosphere.

Microsclerotia density in soil and suppression of Verticillium wilt symptoms in field experiments

Soil analysis of inoculum densities revealed high inoculum levels in the experimental field, since *V. dahliae* msc density was 10.62 ± 0.88 msc g^{-1} soil. The onset of symptom development was observed 4 months (February 2007) after transplanting (October 2006), mainly in 'Amfissis' trees (Fig. 2). The

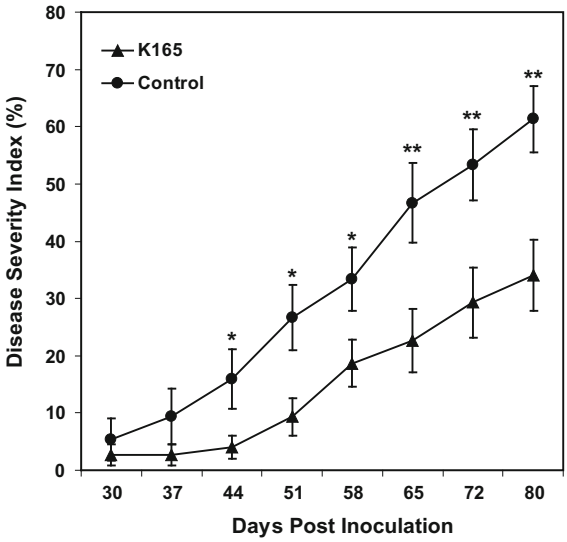

Fig. 1 Verticillium wilt disease severity index on olive cultivar 'Amfissis' treated with the biocontrol agent K165 (*filled triangle line*) or non treated controls (*filled circle line*) at 30, 37, 44, 51, 58, 65, 72 and 80 days after transplanting (dpi) to the *Verticillium dahliae* microsclerotia-infested soil. *Asterisk* and *double asterisk* significance at $P \leq 0.05$ and 0.01 levels, respectively, at each observation time-point, according to *t*-test. *Vertical bars* indicate SE

affected plants exhibited typical symptoms of the disease including chlorosis, wilting, defoliation and branch dieback. One month later, similar symptoms were observed on 'Kalamon' trees, but to a lower extent compared to 'Amfissis'. The disease severity index in 'Amfissis' and 'Kalamon' increased steadily until July, when a curbing in disease progress was noticed for 2 months (August–September 2007). A further increase of disease severity index was then recorded reaching 30.53 and 19.33 %, respectively in control and K165 treated 'Amfissis' cultivar trees, and 16.87 and 15.73 %, respectively in control and K165 treated 'Kalamon' cv trees, in November 2007 (Fig. 2). The final disease severity and relative AUDPC analysis demonstrated that K165 application reduced significantly symptoms in 'Amfissis' but not in 'Kalamon' trees (Table 3). Furthermore, symptom development in 'Kalamon' was significantly lower than in 'Amfissis', suggesting its resistance against *V. dahliae* under field conditions.

In November 2007, all symptomatic branches of affected trees were removed by pruning. In 2008, the first symptoms were observed in March and disease

Table 3 Values (±SE) of disease parameters of Amfissis and Kalamon cultivars, treated with K165 or not (control) in greenhouse and field experiments, in 2007 and 2008

Experiment	Treatment	Disease parameters[a]			
		DI (%)	FDS (%)	M (%)	RAUDPC (%)
Greenhouse	Amfissis-control	93.3 ± 3.3a	61.3 ± 5.8a	40.0 ± 5.8a	20.9 ± 3.7a
	Amfissis-K165	60.0 ± 15.3a	34.0 ± 6.2b	13.3 ± 6.7b	10.1 ± 2.5b
Field 2007	Amfissis-control	86.7 ± 3.3a	30.5 ± 4.8a	6.7 ± 3.3a	14.0 ± 1.8a
	Amfissis-K165	73.3 ± 3.3ab	19.3 ± 2.5b	0.0 ± 0.0a	7.4 ± 0.6b
	Kalamon-control	60.0 ± 11.6ab	16.9 ± 3.1b	0.0 ± 0.0a	8.0 ± 1.6b
	Kalamon-K165	46.7 ± 12.0b	15.7 ± 3.0b	0.0 ± 0.0a	6.6 ± 1.0b
Field 2008	Amfissis-control	93.3 ± 3.3a	59.0 ± 6.4a	33.3 ± 3.3a	30.1 ± 3.9a
	Amfissis-K165	63.3 ± 3.3ab	34.3 ± 5.8b	6.7 ± 3.3b	13.7 ± 2.2b
	Kalamon-control	60.0 ± 10.0ab	29.5 ± 5.7b	13.3 ± 6.7b	16.5 ± 3.3b
	Kalamon-K165	53.3 ± 12.0b	14.7 ± 2.6b	0.0 ± 0.0b	8.3 ± 1.6b

[a] Disease parameters were evaluated periodically on the basis of external symptoms during a period of 80 days after transplanting to the *Verticillium dahliae* microsclerotia-infested soil (20 msc g^{-1} soil), in greenhouse experiments. In field experiments, disease parameters were recorded periodically for 13 months after planting, until November 2007. Then, diseased branches were removed by pruning and symptom recording was continued for another 12 months, until November 2008. *DI* final disease incidence, *RAUDPC* relative area under the disease progress curve with reference to the maximum value potentially reached over each assessment period, *FS* final mean severity of symptoms, *M* mortality. Within experiments, values in columns followed by the same letter are not significantly different according to *t*-test and Tukey's HSD test at $P \leq 0.05$, in greenhouse and field experiments, respectively

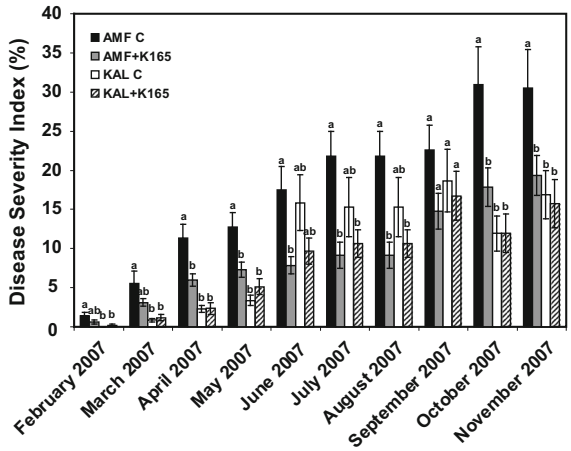

Fig. 2 Verticillium wilt disease severity index on olive cultivars 'Amfissis' and 'Kalamon' planted in naturally infested fields and treated either with the biocontrol agent K165 or water (control), in monthly intervals from February to November 2007. *Columns with different letters* refer to differences between treatments at each observation time point, according to the Tukey HSD test ($P \leq 0.05$). *Vertical bars* indicate SE

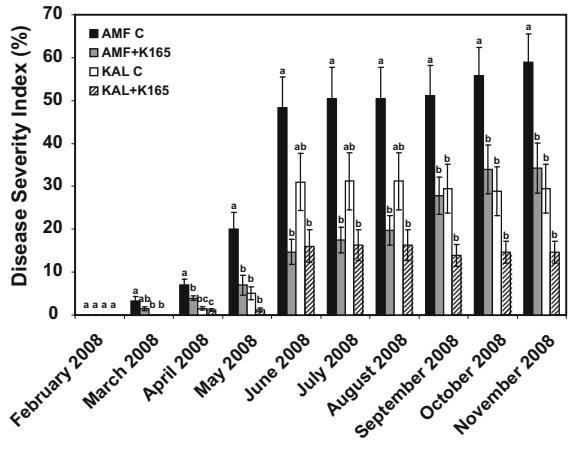

Fig. 3 Verticillium wilt disease severity index on olive cultivars 'Amfissis' and 'Kalamon' planted in naturally infested fields and treated either with the biocontrol agent K165 or water (control), in monthly intervals from February to November 2008. Columns with different letters refer to differences between treatments at each observation time point, according to the Tukey HSD test ($P \leq 0.05$). *Vertical bars* indicate SE

severity index was sharply increasing until June, reaching 48.27 and 14.71 %, respectively in control and K165 treated trees of 'Amfissis', and 31 and 16.07 %, respectively in control and K165 treated trees of 'Kalamon'. Afterwards, a slow progress of symptoms was observed, until November (Fig. 3). Like in 2007, final disease severity and relative AUDPC analysis for 2008 demonstrated that K165 application suppressed significantly symptom development in 'Amfissis'. Interestingly, the mortality of K165-treated 'Amfissis' trees was significantly lower compared to the non-treated control, during the second year in field experiment (Table 3). On the other hand, the application of K165 in the resistant 'Kalamon' did not result in significant reduction of symptoms compared to control trees.

Effect of K165 on *V. dahliae* isolation and DNA level in field experiments

The pathogen was isolated and quantified in olive tissues during all seasons of 2008. Representative *V. dahliae* isolates recovered from symptomatic tissues were identified as the non-defoliating pathotype by multiplex PCR assay (data not shown). Overall, the percentages of positive *V. dahliae* isolations observed in May (28.96 %), where significantly higher compared to February (9.79 %), August (11.56 %) and November (15.45 %). Furthermore, the percentage of positive *V. dahliae* isolations was significantly higher in branches of 'Amfissis' than 'Kalamon', in every month (Fig. 4). These results coincide with the qPCR results, which demonstrated a significantly higher amount of *V. dahliae* DNA in olive tissues in May compared to the other three months. Also, a significantly higher *V. dahliae* DNA level was observed in 'Amfissis' than in 'Kalamon' tissues (Fig. 5).

Although, no significant differences in the percentage of positive isolations were observed between K165 treated and non-treated trees within each cultivar (Fig. 4), qPCR results revealed significantly lower *V. dahliae* DNA levels in K165-treated 'Amfissis' trees compared to the rest of the treatments, in May, August and November (Fig. 5). In accordance to the previous data of symptom development and percentage of positive *V. dahliae* isolations, no significant differences in fungal DNA amounts were observed between K165 treated and non-treated trees of 'Kalamon'.

Discussion

Verticillium wilt has been often reported as the main phytopathological problem of olive trees in the orchards of Mediterranean countries and in California

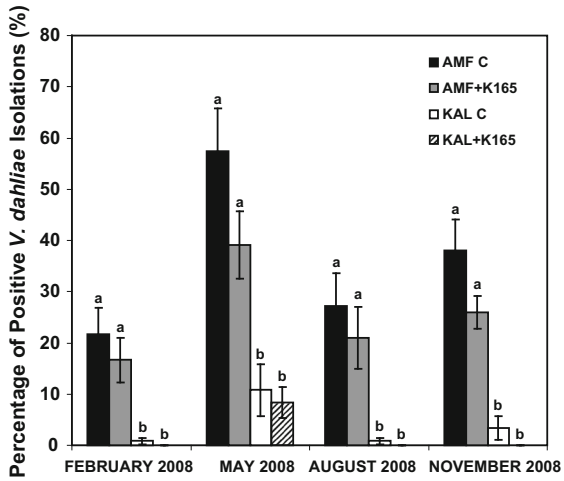

Fig. 4 Percentage of positive *Verticillium dahliae* isolations from branches of olive cultivars 'Amfissis' and 'Kalamon', planted in naturally infested fields, treated either with the biocontrol agent K165 or water (control), and sampled in February, May, August and November 2008. *Columns with different letters* refer to differences between treatments at each sampling time point separately, according to the Tukey HSD test ($P \leq 0.05$). *Vertical bars* indicate SE

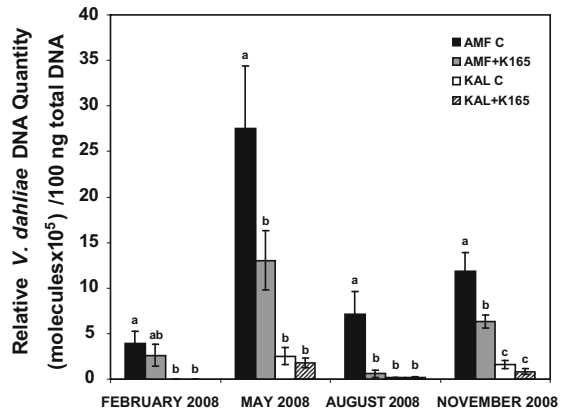

Fig. 5 Relative *Verticillium dahliae* DNA amount in tissues of olive cultivars 'Amfissis' and 'Kalamon' (molecules $\times$ 10^5 per 100 ng total DNA), planted in naturally infested fields, treated either with the biocontrol agent K165 or water (control), and sampled in February, May, August and November 2008. *Columns with different letters* refer to differences between treatments at each sampling time point separately, according to the Tukey HSD test ($P \leq 0.05$). *Vertical bars* indicate SE

(Lopez-Escudero et al. 2010; Snyder et al. 1950). Since Verticillium wilt cannot be currently controlled by chemicals, the use of resistant or tolerant olive cultivars is considered as the most efficient method for disease management (Lopez-Escudero et al. 2004).

Management of Verticillium wilts of woody hosts such as olive tree is difficult and should be based on an integrated disease control strategy (Lopez-Escudero and Mercado-Blanco 2011; Tjamos 1993; Tjamos and Jimenez-Diaz 1998). Exploiting the potential of microbial antagonists for the protection of olive planting material has been proposed as a desirable pre-planting measure for the integrated management of Verticillium wilt (Mercado-Blanco et al. 2004). Therefore, several *Pseudomonas* spp. strains have been described as beneficial for olive tree (Mercado-Blanco et al. 2004; Prieto et al. 2009; Sanei and Razani 2011). The mode of action of the most efficacious of those Gram negative *Pseudomonas* strains, PICF7, has been suggested to be the induction of host defence (Gómez-Lama Cabanás et al. 2014), like in the case of the Gram positive sporulating *P. alvei* strain K165 (Tjamos et al. 2005). However, the efficiency of these bacterial strains under field conditions or their suppressive effect on resistant or tolerant cultivars has never been tested before. In the present study, the efficacy of the biocontrol agent *P. alvei* strain K165 was evaluated to protect planting material of the highly susceptible olive 'Amfissis' and the resistant 'Kalamon' against *V. dahliae*, under greenhouse and field conditions. In the greenhouse experiments, an attempt was made to simulate the natural infection process of Verticillium in olive trees in the field by using msc as the infective inoculum, in contrast to previous studies where the root system of olive trees was dipped into a conidial suspension of the fungus (Mercado-Blanco et al. 2004; Prieto et al. 2009; Sanei and Razani 2011).

Data on the highly virulent defoliating pathotype of *V. dahliae* suggest than inoculum densities greater than 3 msc g^{-1} in naturally infested soils are considered very high for olive trees, since disease incidence can reach values greater than 50 % in susceptible cultivars (Lopez-Escudero and Blanco-Lopez 2007; Trapero et al. 2013). Furthermore, in artificial inoculation experiments with the non-defoliating pathotype of *V. dahliae* it was shown that the use of 10 and 20 msc g^{-1} soil, did not result in significant differences in symptom development in the susceptible 'Amfissis' and the resistant 'Kalamon' (Antoniou et al. 2008). In the present study, the average inoculum density in the experimental field was 10.62 and 20 msc g^{-1} soil in the greenhouse experiments. According to the aforementioned literature, these msc densities

constitute a significant infectious inoculum for olive trees.

The present data showed that K165 suppressed significantly disease symptoms on the susceptible 'Amfissis', under controlled and field conditions, and the observed decrease of disease severity index in the field was associated with significant reduction of fungal biomass in olive tissues. In a previous study, the application of K165 resulted in significant reduction of *V. dahliae* msc germination, in the root tips and the zone of elongation of eggplants compared to the control (Antonopoulos et al. 2008). Therefore, the observed decrease of fungal biomass in the olive tissues can be possibly attributed to the reduction of msc germination, which subsequently resulted in decreased fungal invasion in olive tissues and lower symptoms severity on 'Amfissis'. In contrast, neither symptom development nor *V. dahliae* DNA reduction was observed in the K165 treated 'Kalamon' trees, indicating a non significant suppressive effect of this biocontrol agent on the resistant cultivar.

Mercado-Blanco (2012) suggested the use of BCAs either as a before-planting (preventive) or as a post-planting (palliative) action in established orchards combined with other control tools. Even though K165 post-planting application did not result in significant reduction of relative AUDPC or *V. dahliae* biomass in the resistant cv Kalamon, a considerable decrease by nearly 50 % of the final disease severity index, relative AUDPC and fungal DNA levels was recorded in the K165 treated trees compared to the control trees, in November 2008. Thus, the use of resistant cultivars combined with K165 or other BCAs could potentially be an efficient approach in the effort to control Verticillium wilt of olive tree.

The results of symptom development and fungal isolations along with qPCR suggest a seasonal activity of Verticillium wilt. Disease symptoms progressed rapidly from the end of winter to the middle of summer and the highest *V. dahliae* amounts were detected in the end of spring. Subsequently, the observed curbing of disease severity index was associated with the lower levels of the pathogen in olive tissues, as it was revealed by pathogen isolations and qPCR, symptoms further progressed from the middle to the end of autumn. This failure to increase after May probably reflects reduced sporulation of the fungus and coincides with the cyclical periods of fungal elimination that characterize the lifestyle of *V. dahliae* in the vascular system of trees and plants (Heinz et al. 1998; Mercado-Blanco et al. 2003; Markakis et al. 2009, 2010). Other researches have likewise reported the curbing or decrease of disease severity (Wilhelm and Taylor 1965; Levin et al. 2003). This has been attributed to the natural phenomenon of recovery associated with mechanisms that allow trees to overcome injury and decay, and can be activated after infections caused by vascular pathogens such as *V. dahliae* (Lopez-Escudero and Blanco-Lopez 2005; Markakis et al. 2009, 2010). However, natural recovery in itself is unlikely to be exploited in a control strategy if soil inoculum remains present (Bubici and Cirruli 2014). Eventually, it might be used as a control measure after a drastic soil inoculum reduction or under moderate or low disease pressure. Similarly, Lopez-Escudero and Blanco-Lopez (2007) reported that periods of increased disease incidence in olive trees were mainly in spring and autumn, whereas Navas-Cortés et al. (2008) reported that highest infection rate occurred in the winter to spring period and decreased to minimum values in the summer to fall period.

In addition, seasonal changes in the colonization of the trees by the fungus were observed, since the highest percentage of positive isolations and DNA levels of the pathogen were detected in May, whereas significantly lower *V. dahliae* amounts were noticed in February, August and November. These findings are partially in accordance with a previous study (Levin et al. 2003), where the highest isolation rates in diseased trees were in winter and spring (45 and 34 %, respectively), and the lowest were in autumn and summer (19–20 %). The low pathogen amounts detected in the end of winter (February 2008) could possibly be explained by the fact that diseased branches were removed by pruning in November 2007, and the second infection cycle of vascular colonization had not yet taken place by actively growing *V. dahliae*, probably because of the low temperatures in winter (Tosi and Zazzerini 1998). Therefore, the present data support the hypothesis of Levin et al. (2003) that Verticillium wilt of olive tree follows an infection cycle that starts in autumn with the activation of the fungus by the germinating msc, conidia are produced in the xylem and spread from roots through the transpiration stream to the upper parts of the trees during winter and spring, reaching a maximum disease expression. The cycle is completed when the fungal population decreases during summer, probably because of high temperatures (Tosi and

Zazzerini 1998), exhibiting a curbing in disease symptoms and recovery (Lopez-Escudero and Blanco-Lopez 2005).

The present study reveals for the first time the efficiency of a BCA to control Verticillium wilt of olive tree in naturally infested soils. K165 successfully suppressed disease symptoms and fungal biomass in tissues of the susceptible 'Amfissis', while a remarkable but no statistically significant decrease was observed in the resistant 'Kalamon', under high inoculum densities. The success of biological control requires an understanding of the mode of action of the antagonist, its interactions with the plant and the pathogen, and also the dose of application (Alabouvette et al. 2009). It also depends on the ecological fitness of the BCAs, especially when they target soilborne plant pathogens. Further study is therefore needed to determine the mode of action of the antagonist, its ecological fitness and an efficient and economically feasible delivery system, adapted to olive trees. One step forward would be to investigate the efficiency of K165 to protect other susceptible, tolerant or resistant cultivars, in relation to various soil types and climatic conditions. The use of host resistance combined with BCAs under an integrated disease management strategy is the only plausible framework for the effective control of one of the most serious olive tree diseases (Mercado-Blanco 2012).

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