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Effect of the endophytic plant growth promoting *Enterobacter ludwigii* EB4B on tomato growth

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Summary This study aims to develop a biocontrol agent against *Fusarium oxysporum* f.sp. *radicis-lycopersic*i (FORL) in tomato. For this, a set of 23 bacterial endophytic isolates has been screened for their ability to inhibit *in vitro* the growth of FORL using the dual plate assay. Three isolates with the most sound antagonistic activity to FORL have been qualitatively screened for siderophore production, phosphates solubilization and indolic acetic acid (IAA) synthesis as growth promotion traits. Antagonistic values of the three candidates against FORL were respectively: 51.51 % (EB4B), 51.18 % (EB22K) and 41.40 % (EB2A). Based on 16S rRNA gene sequence analysis, the isolates EB4B and EB22K were closely related to *Enterobacter ludwigii* EN-119, while the strain EB2A has been assigned to *Leclercia adecarboxylata* NBRC 102595. The promotion of tomato growth has been assessed *in vitro* using the strains EB2A, EB4B and EB22K in presence of the phytopathogen FORL. The treatments with the selected isolates increased significantly the root length and dry weight. Best results were observed in isolate EB4B in terms of growth promotion in the absence of FORL, improving 326.60 % of the root length and 142.70 % of plant dry weight if compared with untreated controls. In the presence of FORL, the strain EB4B improved both root length (180.81 %) and plant dry weight (202.15 %). These results encourage further characterization of the observed beneficial effect of *Enterobacter* sp. EB4B for a possible use as biofertilizer and biocontrol agent against FORL.

Additional keywords: Biocontrol, biofertilizer, *Enterobacter ludwigii*, PGPR

Introduction

The rhizospheric zone is rich in nutrients compared with the neighboring bulk soil due to the accumulation of a variety of organic compounds released by the roots through exudation, secretion and rhizodeposition. These organic compounds can be used as carbon and energy sources by microorganisms and microbial activity is particularly intense in the rhizosphere (Chauhan *et al*., 2015).

An alternative to increasing agricultural productivity in a sustainable way is the manipulation of micro-organisms that benefit the soil and plant health (Kloepper *et al*., 1989). For decades, rhizobacteria beneficial to plants are often referred to as plant growth promoting rhizobacteria (PGPR), which are characterized by at least two of the three following criteria: competitively root colonization, stimulation of growth and reduction of disease incidence (Reddy, 2013). Microbial-inoculants are being widely used to improve plant growth under controlled as well as natural field conditions (Nadeem *et al*., 2013).

Inoculants of PGPR bacteria improve root development through the production of certain phytohormones (Bloemberg and Lugtenberg, 2001), such as auxins including indole acetic acid (AIA), cytokinins and gibberellins (Vessey, 2003). Furthermore, many bacterial strains are able to improve the health of plants by limiting the saprophytic growth of phytopathogenic microorganisms, and some of them are used as biological control agents in agriculture (Bloemberg and Lugtenberg, 2001; Whipps, 2001). The great diversity of the mechanisms of action

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of these microorganisms is mainly related to their great ability to produce a wide range of secondary metabolites and to induce ISR in the host, making it less susceptible to subsequent infection by a pathogen (Van Loon, 2007; Weller *et al*., 2002).

PGPR include several bacterial endophytes with alleged positive effects on plant health and growth, which have been pursued mainly for agricultural applications to increase yields since three decades ago (Piccoli and Bottini, 2013). Endophytic bacterial strains selected on the basis of plant growth-promoting bacteria (PGPB) characteristics are useful for formulation of inoculants to improve growth and yield (Forchetti *et al*., 2010). Plant growth-promoting bacterial endophytes have been successfully used to induce fungal resistance in plants (Ji *et al*., 2014), and they are widely used in the developing areas of forest regeneration and phytoremediation of contaminated soils (Ryan *et al*., 2008).

PGPR modify the rhizospheric environment by producing antagonistic molecules with antibiotic or antifungal properties, or by synthesizing cell walls degrading enzymes and volatile organic compounds (VOC), which act against pathogens, disrupting bacterial cell–cell communication (*quorum sensing*) (Grobelak *et al*., 2015). In addition, many of these rhizobacterial strains can also improve plant tolerance against salinity, drought, flooding and heavy metal toxicity. Therefore, they enable plants to survive under unfavorable environmental conditions (Ma *et al*., 2011). Numerous works have reported beneficial effects of these rhizobacteria for improving plant growth under normal as well as stressful environment (Szepesi *et al*., 2009; Heidari and Golpayegani, 2012).

PGPR can additionally help plants by increasing their uptake of nutrient elements such as Phosphate (P). The low availability of this macronutrient to plants results from the fact that the P exists in insoluble forms (Kisiel and Kepczynska, 2016). Microbial phosphate solubilization is likely to be involved in a better plant growth, yield and nutrient uptake. Thus such trait is considered as a prospective tool for development of biofertilizers (Dey *et al*., 2004).

Siderophores are low molecular weight peptides or iron chelators (Santos-Villalobos *et al*., 2012). As soon as the complex siderophore-iron enters the cytosol the cells through specific siderophore receptors present in the cell membrane, the ferric iron gets reduced to a ferrous form which becomes free from the siderophore chelator complex. The released ferrous iron form is further used for metabolic processes (Venkat *et al*., 2017).

IAA is a secondary metabolite produced during the later stages of growth, after the stationary growth phase (Gupta *et al*., 2012), a phytohormone controlling many important physiological processes in plants such as cell division, tissue differentiation and root initiation (Khan *et al*., 2014).

Tomato, *Solanum lycopersicum* L. (Solanaceae) has been considered as one of the most important horticultural crop worldwide (Vos *et al*., 2014). However, tomato production has shown limitations arising from the use of cultivars susceptible to diseases and pests causing substantial production losses (Dias *et al*., 2017). Use of biological control agents, such as plant growth promoting rhizobacteria, can be a suitable approach in control of tomato diseases (Schmidt *et al*., 2004).

The main purpose of this study was to evaluate a) the antagonistic and potential biocontrol activities of the endophytic bacteria isolated from tomato roots against *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL), and b) their growth promoting ability in tomato at *in vivo* conditions.

Materials and methods

Isolation of endophytic bacterial strains

Bacterial strains were isolated from tomato roots of different hybrid varieties from Algerian soil (Algiers locality). The roots were washed and dried aseptically. Root samples were surface-disinfected to remove epiphytes, using 95% ethanol for 30 seconds, followed by a 10% sodium hypochlorite treatment for 2 min and then 75% ethanol for

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2 min. The root pieces were then rinsed three times with sterile distilled water to remove traces of disinfectant (Evans *et al*., 2003; Rubini *et al*., 2005). In a sterile mortar, the root pieces were crushed with sterile distilled water, to obtain a ground product including extracted bacterial cells. The enrichment was carried out by placing 1 g of the extract in 9 ml of nutrient broth. The culture was conducted at 30°C for 24 h, aiming to increase the initial biomass in order to recover a maximum bacterial charge (Bashan *et al*., 1993). 0.1 ml from each enrichment tube served to the isolation on nutrient agar at 30°C for 24 h. Once pure single colonies obtained, the conservation was achieved in glycerol at - 20°C.

Antagonistic activity of endophythic bacterial isolates against FORL

The test of the antifungal activity of the endophytic bacterial isolates against FORL was carried out on PDA medium using the dual culture technique described by Lee *et al*. (2010). A 6 mm fragment of FORL aged of 7 days was removed and deposited in the center of a new Petri dish containing the PDA medium and the bacterial suspensions were adjusted to 0.5 Mac Farland (prepared in nutrient broth). Then 5 µl of each tested bacterial suspension was placed at 1 cm from the edge of the same Petri dish. The experimental units were incubated at 25°C for 5 to 7 days. Control units included only the fungus without the tested bacteria. Each test was replicated three times. The inhibition rate was calculated as follows:

$$\text{Inhibition rate (\%)} = \frac{X-Y}{X} 100$$

Where:

X: Diameter of mycelium control (mm).
Y: Diameter of the mycelium in the presence of the bacterium (measured on the axis "fungus-bacterial colony" (mm).

Identification of endophytic bacterial isolates using 16S rRNA gene analysis

Total bacterial DNA was extracted according to the method of Liu *et al*. (2000). 16S rRNA gene fragments were amplified by PCR using an Invitrogen kit. Primers were as follows: 10-30 forward: 5′-GAG TTT GAT CCT GGC TCA-3′ and 1500 reverse: 5′-AGA AAG GAG GTG CAG ATC CC-3′. 5 µl of 10 x PCR buffer (Mg^{2+}), 8 µl of deoxynucleotide triphos-phate (200 mM of each dNTP), 100 pM of each primer, 2 µl of template DNA solution and 0.8 µl of Taq enzyme (5 U/µl). DNA fragments were recovered and purified. Sequence determination was performed by Beckman Coulter Genomics (United Kingdom). After 16S rRNA gene sequencing, identification of phylogenetic neighbors was done using BLASTN program against databases containing type strains located at the EzBioCloud database (https://www.ezbiocloud.net/). Neighbor-joining method under MEGA6.0 package was used for the construction of phylogenetic trees. The 16S rRNA gene sequences were submitted to the NCBI GenBank database.

Screening endophytic bacterial isolates for growth promotion traits

Phosphate solubilization

Each bacterial isolate was inoculated in Pikovskaya agar containing: 10 g glucose, 5 g tribasic calcium phosphate ($Ca_5HO_{13}P_3$), 0.5 g $(NH_4)2SO_4$, 0.2 g KCl, 0.1 g $MgSO_47H_2O$, trace of $MnSO_4$ and $FeSO_4$, 0.5 g yeast extract, and 15 g agar, in 1000 ml distilled water (Pikovskaya, 1948.). After 7 days of incubation, the presence of clear halos around bacterial colony was used to indicate positive Phosphate (P) solubilizing strains (Husen, 2003).

Indole-3-acetic acid production (IAA)

Bacterial strains were inoculated into nutrient broth containing: 5 g peptone, 1.5 g yeast extract, 1.5 g beef extract, and 5 g NaCl, in 1000 ml distilled water supplemented with L-tryptophan (500 mg/L) and incubated at 30°C for 5 days. The supernatant of the stationary phase culture was obtained by centrifugation at 10000 rpm for 15 min (Bric *et al*., 1991). An aliquot of 2 ml supernatant was transferred to a fresh tube to which 5 ml of Salkowski's reagent (1 ml of 0.5 M Fe-

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Cl_3 in 50 ml of 35% $HClO_4$) were added. After 25 min at room temperature, the absorbance of pink color developed was read at 530 nm. Varied amounts of pure indole-3-acitic acid were used as standard (Costacurta *et al*., 2006).

Siderophore production

All bacterial isolates were qualitatively screened for siderophore production by inoculation onto a Chrome Azurol Sulphonate (CAS) agar plate and incubation for 24 h at 28°C (Schwyn and Neilands, 1987). The change of the medium color from bluish to yellowish-orange after incubation indicates the presence of siderophores (Dias *et al*., 2017).

Impact of endophytic bacterial isolates on tomato plant growth

Seed treatments

Seeds of tomato (Isi Sementi S.p.A., Fidenza «Parma» Italy) were surface sterilized by soaking in 70% ethanol for 1 min followed by immersion in sodium hypochlorite (1%) solution for 10 min and rinsing at least five times with sterile distilled water. The seeds were germinated on sterilized filter paper sheets in the Petri dish (Piromyou *et al*., 2011). Each seed batch was inoculated with one of the selected isolates, using a suspension adjusted to 10^8 CFU/ml to evaluate their ability to modulate the plant response favorably (Piromyou *et al*., 2011).The bacterial treatment of the seeds was carried out under aseptic conditions by putting each batch of 22 seeds sterilized in the bacterial suspension for two different incubation periods (30 min and 60 min). The seeds of the "negative control" batches were inoculated with sterilized 0.85% NaCl solution and sown directly without bacterial treatment (Fallahzadeh-Mamaghani *et al*., 2009; Lee *et al*., 2010). The fungal suspension was made from fresh cultures of FORL, with a spore concentration of 10^6 conidia/ml (El Aoufir, 2001). The seeds were infested with FORL after the bacterial treatment, by immersing the batches in boxes filled with fungal suspension for a duration of 1 hour, then they have been recovered for sowing (Johansson *et al*., 2003).

Pot experiments

Pot experiments were conducted from March 2017 to April 2017. Tomato seeds which had undergone both bacterial and infestation treatment were sown in pots filled with a mixture of potting soil and sand (v/v) (approximately 100 g per pot) (Lee *et al*., 2010), Twenty two seeds per strain were used in four replicates. Pot plants were sprayed with distilled water using a spray gun and incubated at room temperature (20 to 25°C) and photoperiod 9:15 hours L:D. After 31 days of culture, the evaluation of the impact of fungal and bacterial treatment on the plant growth was made by measuring the length of the stem and main root, and the dry weight of each treated batch.

Statistical analysis

ANOVA with Duncan's multiple range test was used to detect significant differences of the experimental data using XLSTAT software, version 2014.3.01 (Adinosoft).

Results and discussion

Selection of antagonistic bacteria

The Dual Plate Assay revealed the presence of isolates capable of inhibition or restriction of the growth of FORL. The inhibition rates obtained were: EB4B (51.51%), EB22K (51.18%) and EB2A (41.40%) (Fig. 1), indicating that the strains possess significant antagonistic effect towards the pathogen. A relatively broader inhibition zone involves the synthesis of relatively potent antibiotics (Kadir, 2008). Lee *et al*. (2010) were able to isolate bacterial strains which were considered to have good antifungal potential with inhibition rates ranging from 0 to 45%. Mikani *et al*. (2007) results in the *Pseudomonas fluorescens* antagonism tests showed an inhibition of mycelial growth with an average of 59.8% for the best performing strain. Bacteria can produce many antifungal metabolites (Weller *et al*., 2007) such as phenazines, pyoluteorin, pyrrolnitrin, DAPG (2,4-diacetyl

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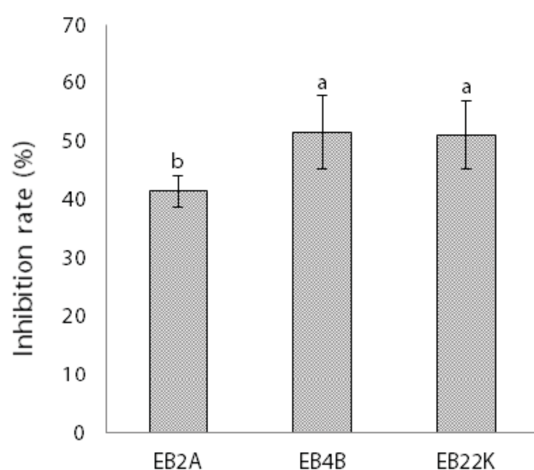

Figure 1. Dual Plate Assay against *Fusarium oxysporum* f.sp. *Radicis lycopersici*. Inhibition rate (%) caused by *Leclercia adecarboxylata* (EB2A), *Enterobacter ludwigii* (EB4B) and *Enterobacter ludwigii* (EB22K) isolates. Data are presented as means ±SE. ANOVA with Duncan's multiple range test was used to detect significant differences. Bars with different letters within the parameter indicate significant differences at $P \leq 0.05$.

phloroglucinol) and siderophores (pyoverdines or pseudobactins) which are the most frequently detected antifungals (Haas and Défago, 2005; Lemanceau *et al*., 2009).

Identification of selected PGPRs

The 16S rRNA gene sequences of the three bacterial strains reported in this paper are deposited in NCBI with the GenBank, with the following accession numbers: EB2A (MF693122), EB4B (MF693530) and EB22K (MF706259). Based on 16S rRNA gene sequence analysis, the isolates EB4B and EB22K were closely related to *Enterobacter ludwigii* EN-119 with an homology of 99.28% and 98.69%, respectively (Fig. 2).According to 16S rRNA sequence, the strain EB2A showed close proximity with *Leclercia adecarboxylata* NBRC 102595 (99.71%) (Fig. 2).

Non plant pathogenic endophytic bacteria can promote plant growth, improve nitrogen nutrition, and in some cases, are human pathogens such as enteric bacteria (Tyler and Triplett, 2008). PGPRs in different field crops are considered as human opportunistic pathogens (Dutta and Thakur, 2017). The third group of microorganisms that can be found in the rhizosphere are true and opportunistic human pathogenic bacteria, which can be carried on or in plant tissue and may enhance plant growth and health, in particular the *Enterobacteriaceae* that can invade the root tissue (Mendes *et al*., 2013). All strains isolated in this study should be further analysed using multi locus analysis to further species clarification. Thus, we can have a safer view of whether they are actually potential human pathogens or not.

Growth promotion traits of selected PGPRs

Bacterial isolates EB2A, EB4B and EB22K showed a distinct zone with the appearance of orange color indicating the production of siderophore which is beneficial to plants, via potential increase of iron availability. All isolated strains were able to solubilize the tricalcium P, resulting in large clear/halo zones.

Indolic acetic acid production was not remarkably different in the three bacterial strains (EB2A = 140 ± 0.10 µg/mL, EB4B = 152 ± 0.44 µg/mL and EB22K = 155 ± 0.78 µg/mL). Various plant growth promoting *Enterobacter* spp. such as *Enterobacter ludwigii* have been applied for plant development (Shoebitz *et al*., 2009; Madhaiyan *et al*., 2010; Kapoor *et al*., 2017). Shoebitz *et al*. (2009) and Gopalakrishnan *et al*. (2012) have also reported the exhibition of nitrogenase activity, phosphate solubilisation, IAA production and antifungal activity by a *E. ludwigii* strain. Previous reports had described some *Leclercia adecarboxylata* as efficient PGPR (Naveed *et al*., 2014; Melo *et al*., 2016; Kisiel and Kepczynska, 2016). Naveed *et al*. (2014) reported that inoculation with *Leclercia adecarboxylata* showed statistically significant greater biomass than the controls under environmental chamber conditions.

Siderophores production has been shown (CAS positive reaction), highlighting the potential of the strains belonging to *Enterobacter* genus to produce such secondary metabolites. Plants are known to use various bacterial siderophores as an iron source (Martinez-Viveros *et al*., 2010).

All three strains were capable of solubilizing the insoluble tricalcium phos-

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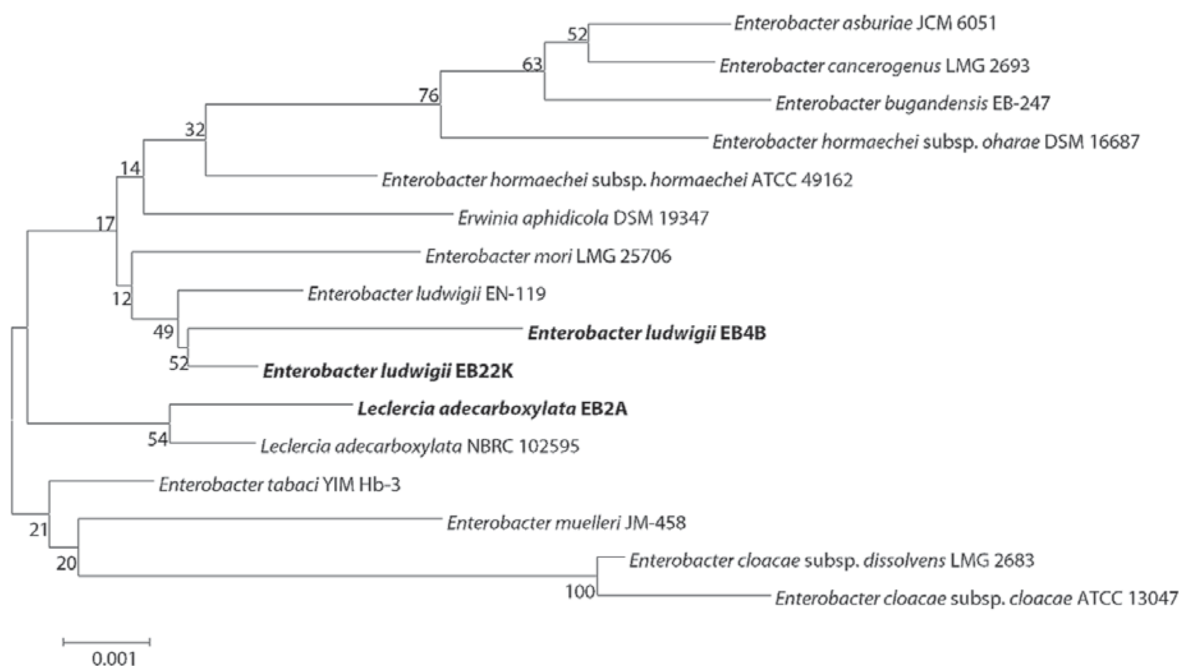

Figure 2. Phylogenetic analysis of *Leclercia adecarboxylata* (EB2A), *Enterobacter ludwigii* (EB4B) and *Enterobacter ludwigii* (EB22K) based on 16s rRNA gene sequencing. Neighbor joining tree was created using MEGA 6 (1000 bootstrap replicates) with a scale of 0.001 substitutions per nucleotide position.

phate ($Ca_5HO_{13}P_3$). Phosphate (P)-solubilizing bacter ia can exert a positive effect on tomato growth, increasing the phosphorus status (Van Veen *et al*., 1997) and significantly enhance photochemical activity accompanied by an increase of chlorophyll content in plants (Liu *et al*., 2017).

Production of siderophores (Loaces *et al*., 2011), solubilization of mineral phosphates (Ahemad *et al*., 2008), and synthesis of indolic acetic acid (IAA) (Khan *et al*., 2014) have beneficial effects on plant growth and are considered as frequent characteristics of PGPR (Gupta *et al*., 2012).

Effect of PGPRs on root size and plant biomass

The analysis of root length and dry weight after 31 days of culture showed that a period of 30 min of bacterization led to significantly better results than those with 60 min and revealed that inoculation of the PGPRs resulted in a significant increase in the biomass compared to the uninoculated controls. Inoculation with the strain EB4B significantly increased the root length compared to non-inoculated tomato seeds. The root length measurements showed a major increase of 326.60% for EB4B and 144.33% for EB22K (Fig. 3a) compared to control batches. The non-treated plants resulted in lower dry weight (Fig. 4a), while the treated exhibited an increase of 142.70% for EB4B and 79.94% for EB22K. After 31 days of cultivation, the plants of the “Control +” batch (Seeds in presence of the pathogen) experienced a low development compared to the “Control -” batch, which demonstrates the virulence of FORL and its incidence on tomato plants growth.

Treatment of tomato seeds with the endophytic strains in the presence of FORL resulted in significantly higher length of roots and plant dry weight compared to control, implying an increase of 180.81% for EB4B batch (Fig. 3b) whilst the average dry weight was enhanced by 202.15% for the same treatment (Fig. 4b). Thus, a significantly strong protective potential against FORL is indicated.

Overall, the strains exerted a particularly positive effect on the root system de-

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velopment by increasing *in vivo* root elongation and plant biomass (dry weight). The effect depended on the exposure time of the seeds to the inocula. A longer exposure (after 60 min of contact) produced a negative effect on root elongation and plant development, while a low bacterial concentration (after 30 min of contact) increased the root elongation and plant biomass. Kisiel and Kepczynska (2016) also reported that a higher density of the inoculum produced a negative effect on root elongation.

The increase in crop yield due to a bacterial culture results from two main beneficial effects: the stimulation of plant growth and the protection of plants against soilborne diseases. PGPR treatments are known to increase the percentage of germination, seedling vigor, emergence, root and stem development, total plant biomass, seed weight, early flowering, and yields of fruits and seeds (Ramamoorthy *et al*., 2001). The colonization of the roots by endophytic bacteria introduced on the seeds is distributed along the roots, including partial or complete colonization of the rhizosphere: inside the root, on the root surface and in the immediate soil of the rhizosphere (Landa *et al*., 2001). Kokalis-Burelle (2002) and Vessey (2003) reported that PGPR could increase yield of tomato by increasing the availability of nutrients in rhizosphere soil and promoting other beneficial plant-growth promoting bacteria. PGPRs can remarkably increase nutrient availability in inoculated plants in plots compared to non-inoculated ones (Adesemoye *et al*., 2008) and lead to a better tomato growth. Karlidag *et al*. (2013) demonstrated that inoculation of selected PGPR increased considerably the growth, chloro-

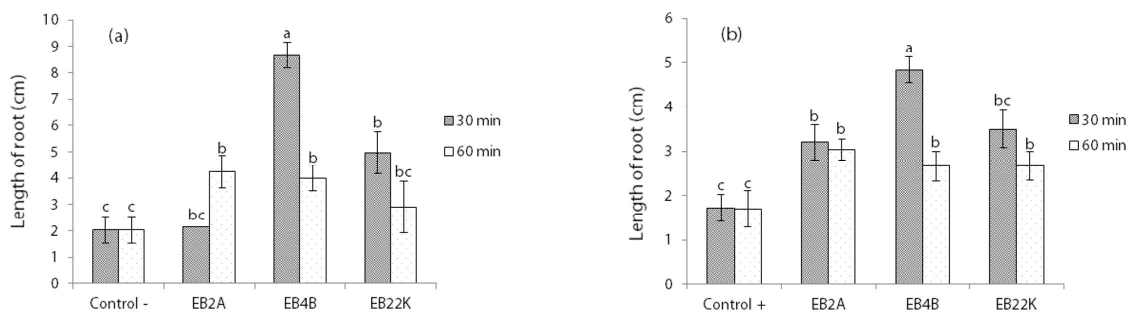

Figure 3. Growth promotion of tomato root induced by *Leclercia adecarboxylata* (EB2A), *Enterobacter ludwigii* (EB4B) and *Enterobacter ludwigii* (EB22K) isolates (30 min and 60 min of contact) a) in the absence of FORL and b) in the presence of FORL evaluated after 31 days. Data are presented as means ±SE. ANOVA with Duncan's multiple range test was used to detect significant differences. Bars with different letters within the parameter indicate significant differences at P ≤ 0.05.

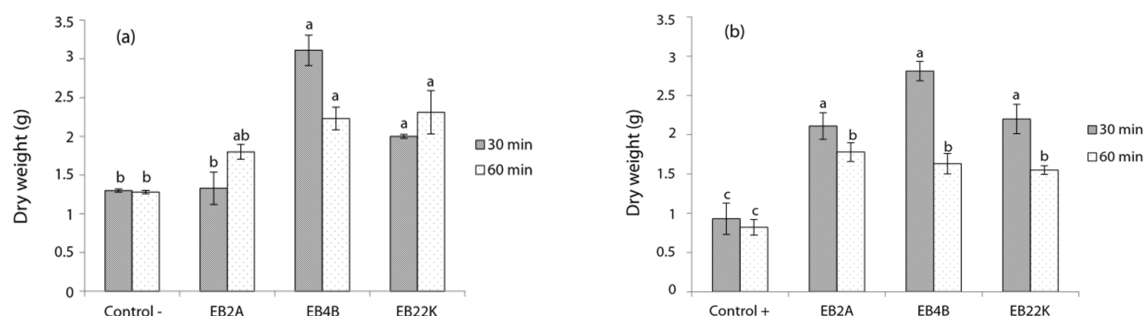

Figure 4. Dry weight promotion of tomato plants induced by *Leclercia adecarboxylata* (EB2A), *Enterobacter ludwigii* (EB4B) and *Enterobacter ludwigii* (EB22K) isolates (30 min and 60 min of contact) a) in the absence of FORL and b) in the presence of FORL evaluated after 31 days. Data are presented as means ±SE. ANOVA with Duncan's multiple range test was used to detect significant differences. Bars with different letters within the parameter indicate significant differences at P ≤ 0.05.

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phyll content and nutrient.

The treatment of tomato seeds with PGPR strains (*Leclercia adecarboxylata* and *Enterobacter ludwigii*) have led to a reduction in susceptibility to FORL, with a substantial promotion of tomato growth (length of root and dry weight). The protective effect of the strains could be explained by their ability to produce antifungal substances, as highlighted by previous antifungal potential characterization studies (bacteria producing salicylic acid, rhamnolipids, chitinase, cellulases) in addition to good competitiveness for carbon and energy sources (Haas and Défago, 2005).

In vivo tests indicate the protective effect of the isolated PGPR against FORL in the tested tomato cultivar, but also growth promotion with singular rates up to 200%. For such reason and for other reasons, root colonization is often considered as a limiting factor for biological control in the rhizosphere (Dekkers *et al*., 1997). However, selection of root colonizers has been an empirical process involving the random control of isolates. Herein, the relationship between growth promotion and protection of tomato will allow more targeted selection of strains for more effective use in biological control (Landa *et al*., 2001). The use of *Leclercia adecarboxylata* and *Enterobacter ludwigii* has already been reported for growth promotion and protection of tomato (Gopalakrishnan *et al*., 2012; Kisiel and Kepczynska, 2016), and the traits and genes that contribute to root colonization capacity have been extensively studied (Landa *et al*., 2001). Nevetheless, the relationship between protection and the growth promotion or yield enhancement by PGPR has been further established, as it appears that the reduction in the severity of the disease is accompanied by an increase in the yield after adequate bacterial treatments (Lemanceau and Alabouvette, 1991).

In conclusion, biological control of root and crown rot disease caused by the FORL soil pathogen in tomato, *via* the introduction of the tested endophytic bacterial strains of *Enterobacter* species, is proposed as a potential alternative to chemical substances. Based on antagonistic tests, the bacterial isolates with remarkable beneficial traits such as the combination of production of IAA, siderophores and phosphate solubilization could be a useful tool to enhance the sustainable production of tomato. *In vivo* trials demonstrated an efficient interaction of the three isolates with the tomato plant, promoting growth and induction of plant defense against FORL. Isolate EB4B appears to be a promising alternative for future bioformulation and field application.

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Επίδραση εφαρμογής του ενδοφυτικού βακτηρίου *Enterobacter ludwigii* EB4B στην προώθηση της ανάπτυξης φυτών τομάτας

M.E.A. Bendaha και H.A. Belaouni

Περίληψη Η μελέτη αυτή στοχεύει στην ανάπτυξη ενός βιολογικού παράγοντα έναντι του *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) στην τομάτα, ο οποίος παράλληλα προωθεί την ανάπτυξη των φυτών. Για το σκοπό αυτό, εξετάστηκε ένα σύνολο 23 ενδοφυτικών βακτηριακών στελεχών για την ικανότητά τους να αναστέλλουν την ανάπτυξη του FORL χρησιμοποιώντας τη δοκιμασία διπλής καλλιέργειας σε τρυβλίο. Τρία απομονωθέντα στελέχη με την πιο ανταγωνιστική δράση προς το FORL εξετάστηκαν επίσης ως προς την παραγωγή σιδηροφόρων, τη διαλυτοποίηση φωσφορικών αλάτων και τη σύνθεση ινδολοξικού οξέος (IAA) ως χαρακτηριστικά της προώθησης της ανάπτυξης. Η ικανότητα αναστολής της ανάπτυξης του FORL ήταν αντίστοιχα 51,51% (EB4B), 51,18% (EB22K) και 41,40% (EB2A). Με βάση την ανάλυση αλληλουχίας του γονιδίου 16S rRNA, τα στελέχη EB4B και EB22K κατατάσσονται φυλογενετικά πλησίον του στελέχους *Enterobacter ludwigii* EN-119, ενώ το στέλεχος EB2A έχει αποδοθεί στο *Leclercia adecarboxylata* NBRC 102595. Η επίδραση των στελεχών EB2A, EB4B και EB22K στην προώθηση ανάπτυξης των φυτών της τομάτας εξετάστηκε *in vitro* παρουσία του φυτοπαθογόνου FORL. Οι επεμβάσεις με τα επιλεγμένα στελέχη αύξησαν σημαντικά το μήκος της ρίζας και το ξηρό βάρος των

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φυτών. Τα πιο ενθαρρυντικά αποτελέσματα έδωσε το στέλεχος EB4B απουσία του FORL αυξάνοντας το μήκος της ρίζας κατά 326,60% και το ξηρό βάρος κατά 142,70% σε σύγκριση με τους μάρτυρες. Παρουσία του FORL, το στέλεχος EB4B βελτίωσε τόσο το μήκος της ρίζας (180,81%) όσο και το ξηρό βάρος (202,15%) των φυτών της τομάτας. Τα αποτελέσματα ενισχύουν την περαιτέρω μελέτη της παρατηρούμενης ευεργετικής επίδρασης του *Enterobacter* sp. EB4B για πιθανή χρήση του ως βιοδιεργερτικό παράγοντα και παράλληλα παράγοντα βιολογικής καταπολέμησης του FORL.

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Effect of the olive fruit size on the parasitism rates of *Bactocera oleae* (Diptera: Tephritidae) by the figitid wasp *Aganaspis daci* (Hymenoptera: Figitidae), and first field releases of adult parasitoids in olive grove

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Summary The olive fruit fly *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) is the major pest of olives worldwide. The figitid wasp, *Aganaspis daci* (Hymenoptera: Figitidae), is a larval-prepupal endoparasitoid of fruit fly species, and it was found to successfully parasitize medfly larvae in field-infested figs in Greece. To assess the potential of *A. daci* as a biological control agent against *B. oleae*, we studied the effect of olive fruit size on parasitism rates of *A. daci* on 2nd and 3rd instar larvae of *B. oleae*, by using fruit of different size (cultivar 'Chalkidikis') and wild olive fruit. In addition, we conducted releases of *A. daci* females in a pilot olive grove in Volos, Magnesia. From July to October, we released 200 *A. daci* females/0.1 ha/week, followed by olive fruit sampling to estimate olive fruit infestation levels and the parasitism rates of *A. daci*. Laboratory trials revealed that fruit size and larvae instar were predictors of parasitism success of *A. daci*, with parasitism rates higher for small-size fruit of the cultivar "Chalkidikis" and the 3rd instar larvae of *B. oleae*. In field trials, no *A. daci* adults emerged from the olive fly infested fruit.

Additional keywords: biological control, larval instar, parasitism, olive fruit fly

Introduction

The olive fruit fly *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) is a monophagous species, of sub-Saharan African origin, that attacks fruits of African and Asian wild olive, *O. europaea* ssp. *cuspidate,* and the commercial olives *O. europaea* ssp. *europaea* (including wild olive fruit or naturalized 'oleasters') (Hoelmer *et al.*, 2011, Tzanakakis, 2006). Its presence has been well documented in the Mediterranean Basin, South and Central Africa, Middle East and India, and it has recently invaded California and northwestern Mexico (Nardi *et al.*, 2010). The olive fruit fly usually lays one or more eggs just below the surface of the fruit, and larvae form galleries while feeding on the pulp of the fruit. Besides secondary infestation from both bacteria and fungi, olive fruit fly infestation causes premature fruit drop and reduces fruit and olive oil quantity and quality (Manousis and Moore, 1987). Current management strategies for olive fruit fly populations rely primarily on insecticide application either through cover sprays or bait sprays that target adult fly. Resistance of *B. oleae* to insecticides has been documented both for olive fly populations from Greece (Kampouraki *et al.*, 2018) and California (Kakani *et al.*, 2010). Thus, *B. oleae* poses a serious threat to the olive industry worldwide and its control is challenging (Daane and Johnson, 2010).

Over nearly 100 years, considerable efforts have been made to manage the olive fruit fly in southern Europe by introducing primarily North African populations of *Psyttalia* (*Opius*) *concolor* (Szepligeti) (Hymenoptera: Braconidae), a koinobiont endoparasitoid of the second and third larval instar of the Mediterranean fruit fly (medfly), *Ceratitis capitata,* and the olive fly (Daane and Johnson, 2010). In Greece, initial trials in the island of Chalki gave promising results during the first year of release (18-37% parasitism rates) but parasitism rates declined

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(1-2%) the following years (Stavraki-Paulopoulou, 1966). In the island of Corfu, releases of *P. concolor* (from Tunisia) against heavily infested olive trees of the Lanolia variety during the spring revealed high parasitism rates (30-50%) even from the 1st week after the release (Kapatos *et al.*, 1977). The harvest period of olive fruit in Corfu (collected from the ground in the spring) differs from that of other regions in Greece (harvested in autumn). Apparently, unharvested fruit support an additional, spring generation of the olive fruit fly that gives adults late in May and/or June (Kapatos *et al.*, 1977). However, *P. concolor* rarely contributes to control of the olive fruit fly populations in spring without considering large inundative wasp releases (Liaropoulos *et al.*, 1977; Kapatos *et al.*, 1977).

In Spain, *P. concolor* was initially introduced in late 1970s, but failed to get established in several olive oil production areas (Jiménez *et al.*, 1998). More recently, high parasitism levels (>20%) of *B. oleae* pupae by *P. concolor* were recorded during August – September in organically-managed orchards in Majorca (Balearic Islands) (Miranda *et al.*, 2008). Apart from the Balearic Islands, *P. concolor* is also assumed to be established in southern Italy (Raspi and Loni, 1994). Moreover, field releases of the generalist, egg parasitoid of fruit flies, *Fopius arisanus* (Hymenoptera: Braconidae), revealed parasitism rates >20% during autumn when humidity levels are high in Italy (Moretti *et al.*, 2007).

In California (USA), numerous braconid parasitoids were screened from 2002 onwards as potential biological control agents of *B. oleae* and two larval endoparasitoids, *Psyttalia humilis* (from Namibia) and *P. lounsburyi* (from Kenya) have been released in olive groves of coastal and inland countries (Yokoyama *et al.*, 2011; Daane *et al.*, 2015). Recovery results revealed that the parasitoids have been established in coastal regions and, more importantly, *P. lounsburyi* can successfully overwinter and survive even at low *B. oleae* densities (Daane *et al.*, 2015).

The parasitoid *Aganaspis daci* (Hymenoptera: Figitidae, Eucoilinae) is another solitary, primary endoparasitoid that attacks the larval-prepupal stage of numerous fruit fly species in South-East Asia and Australia (Clausen *et al.*, 1965). It was first detected on parasitized larvae of *B. dorsalis* (Hendel) in Malaysia and Taiwan (Weld, 1951). In the Mediterranean basin, *A. daci* was first recovered from medfly puparia of field-infested figs in the Greek island of Chios in 1999, with the parasitism rate reaching 45% (Papadopoulos and Katsoyannos, 2003). Up to now, *A. daci* has been retrieved from medfly and *Rhagoletis cerasi* (Diptera: Tephritidae) pupae collected from field-infested citrus and cherry fruits in Thessaly and Attiki area, Greece (N.T. Papadopoulos and D. Papachristos, personal observations). In 2009, *A. daci* was recorded in Valencia, Spain, attacking medfly in fig and citrus fruits (Sabater-Muñoz *et al.*, 2012). Recently, *A. daci* was reported to be the predominant parasitoid of *C. capitata* pupae from field-infested loquat, grapefruit, peach and guava orchards in the coastal area of Tartous in Syria, with parasitism levels of 1.68%, 30.76%, 18.28% and 16.15%, respectively (Ali *et al.*, 2015; 2016).

In addition, *A. daci* has been introduced to many countries as a biological control agent of specific fruit fly species. Initially, it was introduced to Hawaii in 1948 for the biological control of *B. dorsalis,* where it was also successfully mass reared on medfly larvae/pupae (Clausen *et al.*, 1965). Releases for the control of the Caribbean fruit fly, *Anastrepha suspensa*, in Florida (USA) (introduced via Hawaii) resulted in establishment after three years (Baranowski *et al.*, 1993). In contrast, the parasitoid failed to become established in Mexico and Costa Rica (introduced via Hawaii) after being released against medfly and the Mexican fruit fly, *Anastrepha ludens* (Wharton *et al.*, 1998). In 2008, *A.daci* was introduced to Egypt (via Hawaii) for the biocontrol of *B. zonata*, with the initial parasitism rate ranging from 1.6 to 8% in a guava orchard and, interestingly, five years later, the parasitoid was detected in the wild in fruit fly infested citrus and guava fruits approximately 320km away from the initial release point (El-Heneidy *et al.*, 2019).

Even though *A. daci* is a native parasitoid

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of *Bactrocera* spp. and previous releases for the control of *B. dorsalis* and *B. zonata* have been attempted with some success (Clausen *et al.*, 1965; El-Heneidy *et al.*, 2019), biological control efforts, using this parasitoid, against the olive fruit fly have yet to be assessed. Preliminary results revealed that *A. daci* can successfully parasitize *B. oleae* larvae under laboratory conditions (N.T. Papadopoulos and Ch. S. Ioannou, unpublished data), but its response to infested olive fruit both in laboratory and field conditions remained unknown. It is well documented that the olive fruit size is a significant predictor of the parasitic success of the African parasitoids *P. ponerophaga* (Sime et al., 2007), *P. concolor* (Wang *et al.*, 2009a) and *P. lounsburyi* (Wang *et al.*, 2009b) on *B. oleae* host. To this end, we assessed in the laboratory performance of *A. daci* on infested olive fruit of different size. In addition, we included fruit of wild olive trees that can also support *B. oleae* populations in Europe (Bigler and Delucchi, 1981). Last, releases of *A. daci* were carried out in an olive grove in Greece for assessing its potential as a biological control agent against *B. oleae* host in field conditions.

Material and Methods

Insects

Laboratory colonies of *B. oleae* and *A. daci* were maintained in an insect room under controlled conditions (25 ± 1°C, 60 ± 10% R.H, and a photoperiod of L14: D10) in the laboratory of Entomology and Agricultural Zoology at the University of Thessaly, Volos, Greece.

Aganaspis daci specimens were obtained from a laboratory colony housed at the Valencian Institute of Agrarian Research [Instituto Valenciano de Investigaciones Agrarias (IVIA), Valencia, Spain]. This colony was established in 2010 with specimens obtained from medfly-infested figs in Bétera, Spain. Since then, a laboratory colony was maintained on the host *C. capitata* (de Pedro *et al.*, 2016). After transferring to Volos, *A. daci* was reared on mature (L_3) medfly larvae, which were reared in the laboratory on an artificial diet as it is described by Boller (1985).

Olive flies obtained from infested olive fruit of the “Pelion” cultivar collected from organic olive groves in Volos in June 2016. Flies were reared for 2 generations on olive fruit that had been collected at an appropriate ripening stage (full size, green color) and stored at low temperatures (6-7°C) until being used. All adults were maintained in wooden framed, nylon-mesh-screened holding cages (30 x 30 x 30 cm) and had free access to food (mixture of yeast hydrolysate and sugar in a ratio of 1:4) and water. Olive fruit were offered to females for oviposition, and mature larvae dropped from the fruit into a container placed below. Pupae were collected and kept under the same conditions.

Effects of olive fruit size on parasitism success

To assess the effect of fruit size on the parasitism rate of *A. daci* (the number of *A. daci* adults recovered per olive fly pupae), we used i) olives of the cultivar “Chalkidikis”, and ii) wild olive fruit (wild growing olive fruit of *O. europaea* ssp. *europaea*). The olive fruit size (length and width) and olive fruit thickness were measured for both “Chalkidikis” olives (small-sized and big-sized) and for wild olive fruit using a sample of 50 fruit per category. Pulp thickness (i.e., distance from the epidermis to pit) was averaged from three needle-probe measurements per fruit. Measurements were always taken from the mid-point along the longitudinal axis of a fruit. Fruit from “Chalikidikis” olives were mechanically categorized as small (length < 3.1cm) and big (length ≥ 3.1cm). All olive fruit were offered to mature, mated females of *B. oleae* for oviposition, allowing 1 - 2 oviposition stings per fruit. Larvae were left to develop inside fruit until reach 2nd or 3rd instar. Monitoring of larvae development was based on samples of dissected fruit. Olive fruit with 2nd and 3rd instar larvae were offered to *A. daci* females for parasitism. Five *A. daci* females without previous oviposition experience (5-10 days old) were trans-

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ferred to plexiglass cages (15x15x15cm) with ten olive fruits infested with 2nd or 3rd instar *B. oleae* larvae. *Aganaspis daci* females were allowed to oviposit to olive fruits for 24h having free access to food (sugar and honey) and water. Then, fruit were individually transferred to ventilated petri dishes for collecting parasitized olive fruit fly pupae. Petri dishes with pupae were kept in the insect room and checked daily until adult olive fly and parasitoid emergence ceased. For each fruit size and larvae instar (2nd or 3rd instar) treatment, we run ten replicates (10 infested fruits with 5 *A. daci* female/replicate). Five replicates of ten infested fruits remained in a parasitoid unit without parasitoid for 24h as control treatment to assess the natural olive fly larval/pupae mortality.

Induced mortality was evaluated by comparing % mortality in the treatment with % mortality in its control, using the Schneider-Orelli formula (Püntener, 1981) as follows:

Induced mortality (%) = [(treatment mortality - control mortality)/ (100 - control mortality)] x 100%.

Induced mortality refers to mortality of host pupae attributed to parasitoids, from which adults do not emerge.

Hence, population reduction is defined as the sum of induced mortality and percentage parasitism.

Field releases of *A. daci*

One rainfed, olive grove located in Nea Ionia Volos (Magnesia, Greece) (39°37'N; 22°93'E, 16m) was selected for *A. daci* field releases. The size of the olive grove was estimated at 0.5ha, and it was mainly planted with the cultivar "Pelion". The experimental field was surrounded by olive groves and wild growing olive trees and has been certified (according to EC 834/2007) as an organic farm for at least the last five year. To monitor the olive fly population, 10 McPhail traps loaded with ammonium sulphate 2% as attractant were deployed on 10th July, 2018. Adult captures recording and replacement of the attractant solution was conducted every week until the end of the experiment. Apart from male and female captures per trap per week, the percentage of fertile females caught was determined following dissection under a binocular microscope (Fletcher *et al.*, 1978). *Aganaspis daci* releases took place one week after the first capture of mature *B. oleae* females (bearing mature oocytes in ovaries) in the traps. Ten 10 parasitoid females (5-10 days old) per fertile olive fruit fly female captured were released, with a maximum release of 200 female parasitoids per 0.1 ha/week or 1000 female *A. daci*/week due to rearing constraints. One week after the first release, a total of 300 olive fruit were randomly collected, and kept under laboratory conditions until the emergence of *B. oleae* pupae. Then, collected pupae were placed in Petri dishes until the emergence of olive fruit flies and/or *A. daci* adults. The experiment was completed on 23rd October, 2018 because of the premature drop of almost all olive fruit caused by the heavy *B. oleae* infestation. Climatic data (temperature and humidity) during the pilot trials (from 10th July, 2018 to 23rd October, 2018) were obtained from the closest meteorological station located at Nea Anchialos Airport (39°21'N; 22°08'E, 25m a.s.l.) 17.8 km south from the pilot field site (Fig. 1).

Statistical analysis

The normality of data was assessed with the Kolmogorov–Smirnov test. Kruskal-Wallis test was used to compare olive fruit size metrics (length, width, pulp thickness). Kruskall–Wallis and Mann–Whitney tests were used to determine whether olive fruit size and larval instar (2nd and 3rd) had an effect on a) parasitism rate of *A. daci*, b) induced mortality and c) population reduction of olive fly, respectively. All statistical analyses were performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA).

Results

Effect of olive fruit size on parasitism success

The length of small and big categories of "Chalkidikis" fruit averaged 2.7 ± 0.3 cm and

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3.6 ± 0.3 cm, respectively; fruit width averaged 1.8 ± 0.1 cm and 2.5 ± 0.1 cm, respectively. Mean length and width of wild olive fruit were estimated to be 2.0 ± 0.2 cm and 1.6 ± 0.1 cm, respectively. Hence wild olive fruit were smaller than the small size "Chalkidikis" fruit. The average pulp thickness of "Chalkidikis" olive fruit ranged from 0.57 ± 0.11 cm (small fruit) to 0.85 ± 0.01 cm (big fruit), while pulp thickness of wild olive fruit was estimated at 0.62 ± 0.11 cm. Kruskal-Wallis tests revealed that olive fruit differ in length (χ^2=133.197, df=2 P<0.001), width (χ^2=132.104, df=2, P<0.001) and fruit thickness (χ^2=99.189, df=2, P<0.001).

Parasitism rates of *A. daci* on 2nd and 3rd instar larvae of the olive fruit fly was lower than 11.4%, regardless of the size and cultivar. *Bactrocera oleae* population reduction was lower than 30% for 2nd and 3rd instar larvae in all olive sizes, except for L_3 on small "Chalkidikis" fruit (≈53%) (Table 1). Olive fly larvae developing in small olive fruit of the "Chalkidikis" cultivar seem to be more "vulnerable" to *A. daci* than those of the big fruit regardless of the larvae instar. No *A. daci* adults emerged from olive fly on wild olive fruit, irrespective of the larval instar. Olive fruit size was a significant predictor of parasitism rates (Kruskal-Wallis test, χ^2=14.885, df=2, P=0.001), as opposed to induced mortality (Kruskal-Wallis test, χ^2=0.687, df=2, P=0.709) and population reduction of olive fly (Kruskal-Wallis test, χ^2=1.749, df=2, P=0.417). Moreover, Mann-Whitney tests revealed that larval size (developmental stage) significantly affected parasitism rates (U=193.000, P=0.003) and population reduction (U=293.000, P=0.020), while it was marginally not significant predictor for the induced mortality caused by *A. daci* on olive fly populations (U=318.000, P=0.051).

Field releases of *A. daci*

Olive fruit fly adult captures were recorded from the first week of sampling. Captures peaked (300-500 adults per week) from 11 to 18 September, 2018 (Fig. 2). Fruit infestation was low (<15%) until the 11th of September and significantly increased after the 25th September, 2018 (>100% olive infestation) and remained high until the end of the survey. Percentage of mature females were higher than 85% during the first week of sampling and remained high (85-97%) until the end of the experiment. In total, 13,220 *A. daci* females were released from 24th July, 2018 to 23rd October, 2018. Fruit sampling revealed that 1,845 *B. oleae* adults emerged from 2,454 olive fly pupae; however, no *A. daci* adults emerged from the infested olives (Table 2). It is noted that no olive fruit

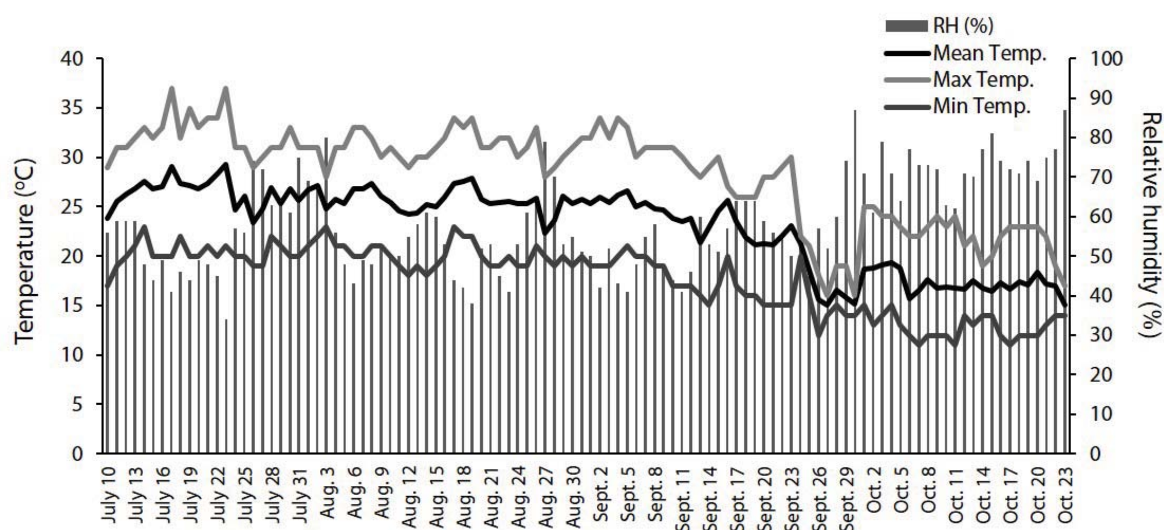

Figure 1. Climatic data from the pilot olive grove during the period of field releases. Data were obtained from the closest meteo station located at the airoport of Anchialos, 17.8 km away from the pilot field.

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Table 1. Parasitism rates of *Aganaspis daci*, induced mortality and population reduction of olive fly on L_2, and L_3 larvae.

Larval instar	Olive fruit	*A. daci* parasitism (%) (mean ± SE)	Induced mortality (%)* (mean ± SE)	Population reduction (%)** (mean ± SE)
	Wild olive fruit	0	24.4 ± 5.8	24.4 ± 5.8
L_3	Chalkidiki (small)	11.4 ± 2.8	41.3 ± 10.7	52.7 ± 10.8
	Chalkidiki (big)	5.5 ± 1.9	22.9 ± 7.9	28.4 ± 7.2
	Wild olive fruit	0	19.4 ± 5.4	19.4 ± 5.4
L_2	Chalkidiki (small)	3 ± 1.6	15.4 ± 3.9	18.4 ± 3.7
	Chalkidiki (big)	0	18.3 ± 11.9	18.3 ± 11.9

*Induced mortality (%) = [(treatment mortality - control mortality)/ (100 - control mortality)] x 100%.
**Population reduction is the sum of induced mortality and percentage parasitism.

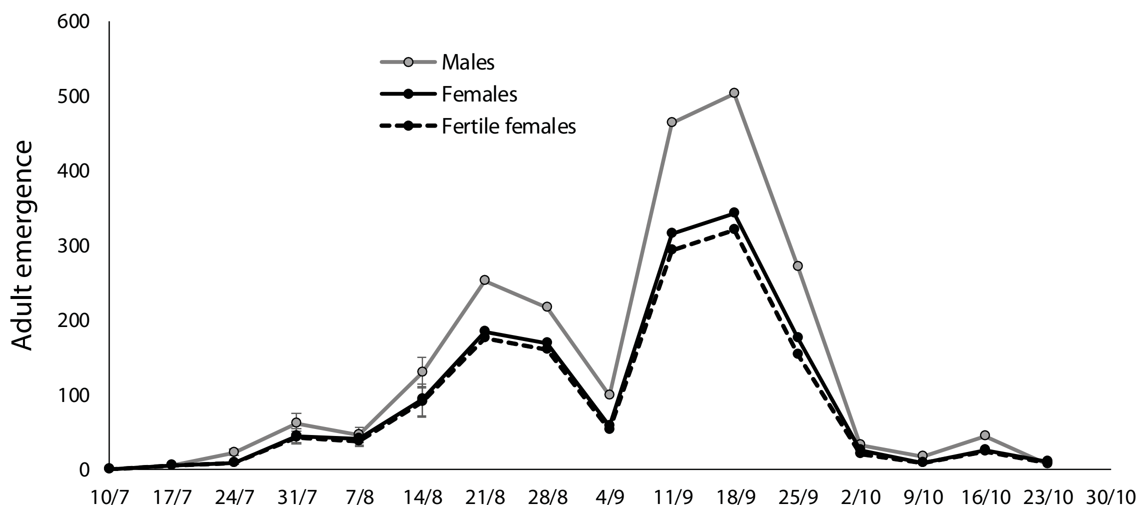

Figure 2. Mean captures (numbers ± SE) of olive fruit flies on McPhail traps per week during monitoring period. Males, females and fertile females are shown. Traps installed on 10th July, 2018.

were available for sampling following the last *A. daci* release conducted on 23rd October, 2018 due to the premature fruit drop caused by the heavy olive fly infestation.

Discussion

Our results suggest that parasitism success of *A. daci* on *B. oleae* larvae feeding on olive fruit was both affected by olive fruit size and larval instar under laboratory conditions. Specifically, the parasitism rates of the infested olive fruit of the "Chalkidikis" cultivar ranged from 11.4% to 3% for small and big fruit, respectively. Interestingly, *B. oleae* larvae were fully protected in wild olive fruit since no *A. daci* emergence was recorded. It is noted that wild olive fruit were smaller than those from the small "Chalkidikis" fruit; however, there was no difference in pulp thickness between wild olive and small "Chalkidikis" fruit. Moreover, both parasitism rates of *A. daci* and olive fly population reduction were higher for 3rd instar compared to 2nd instar larvae. In contrast,

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Table 2. Results of olive fruit infestation rates and parasitism rates of *Aganaspis daci* on olive fruit fly after releases during the period of 24th July – 23rd October, 2018.

A. daci release		Olive infestation levels		Number of emerged individuals (n)		% parasitism of *B. oleae*	% *B. oleae* infested olives*	% *B. oleae* adult emergence (con-side-ring formed pupae)
Release date	*A. daci* females	Fruit collection date	*B. oleae* pupae	*B. oleae*	*A. daci*			
24/7/2018	560	7/8/2018	25	17	0	0	8.3	68.0
31/7/2018	840							
7/8/2018	1000	14/8/2018	42	34	0	0	14	81.0
14/8/2018	1000	21/8/2018	34	21	0	0	11.3	61.8
21/8/2018	1000	28/8/2018	33	29	0	0	11	87.9
28/8/2018	1000	4/9/2018	36	26	0	0	12	72.2
4/9/2018	1000	11/9/2018	23	16	0	0	7.7	69.6
11/9/2018	1000	18/9/2018	41	30	0	0	13.7	73.2
18/9/2018	1000	25/9/2018	209	194	0	0	69.7	92.8
25/9/2018	1000	2/10/2018	431	325	0	0	143.7	75.4
2/10/2018	1000	9/10/2018	652	408	0	0	217.3	62.6
9/10/2018	1000	16/10/2018	448	352	0	0	149.3	78.6
16/10/2018	820	23/10/2018	480	393	0	0	160	81.9
23/10/2018	1000							
Total	13220		2454	1845	0			

*Sample of 300 olive fruit per sampling date

olive fruit size and larvae instar have no impact on induced mortality. Our field trials revealed that olive fruit fly adult captures in traps were high from the middle to the end September, resulting in high infestation rates of olive fruit from the end of September to the end of October, when our trials ceased due to premature olive fruit drop. Zero parasitoid recovery rates reveal that *A. daci* failed to get established and parasitize the olive fruit fly in the pilot orchard under the prevailing conditions.

Effect of olive fruit size on parasitism success

Our laboratory experiments support that *A. daci* adults are not able to emerge from infested wild olive fruit, being in line with a previous field study that revealed that wild olive is not an appropriate host of olive fly parasitoids in Crete (Neuenschwander *et al.*, 1983). However, olive fruit size and larval instar have been found to be significant predictors of the parasitism rates of *A. daci* on *B. oleae* larvae. For *Psyttalia* species, fruit size of the domesticated olives is known to have a negative impact on their parasitism success on *B. oleae* larvae (Sime *et al.*, 2007; Wang *et al.*, 2009a; 2009b). Given that *Psyttalia* species follow "a drill and sting" strategy for parasitizing their host (2nd and 3rd instar larvae), their ovipositor length is expected to be a significant predictor of parasitism success, in line with Latiere's hypothesis (Latiere, 1917). This is particularly true for the 2nd instar of *B. oleae* larvae that tend to tunnel deeper in large, fleshier fruit of European cultivars (Wang *et al.*, 2009a). On the other hand, *A. daci* females follow "an ingress and sting" strategy, which means that they enter the fruit in order to parasitize the host larvae favored by the smooth, compressed body shape. In this context, the relative ovipositor length of *A. daci* females seems to be

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less important for the parasitism success of *B. oleae* on different-sized fruit, compared to the opiines' parasitoid. Indeed, pulp thickness of all three study olive fruit (5.7 – 8.5mm) is significant higher than the ovipositor length of *A. daci* (NT Papadopoulos, unpublished data) but still *A. daci* is capable of parasitizing *B. oleae* larvae. As a result, variability in *A. daci* parasitism efficiency in olive fruit cannot be explained by the ovipositor length. In addition, the 3rd instar *B. oleae* larvae were found to be more vulnerable to parasitism by *A. daci.* This is partly attributed to the fact that the L_3 (positioned closer to surface because on their way to either pupate (summer generation] or exit the fruit and pupate in soil (autumn winter generation) are more exposed to *A. daci* attacks than the L_2 larvae (Tzanakakis, 2006; Wang *et al.*, 2009b).

Field releases of *A. daci*

Although *A. daci* can successfully parasitize infested olive fruit in laboratory conditions, our results support no activity of *A. daci* in field conditions, despite of the prevailing optimal conditions for the field released wasps in terms of host availability. In general, field studies have so far revealed very low fertility rates and high immature mortality of *A. daci*, driven mainly by low (<20°C) or high (>30-35°C) temperatures (de Pedro *et al.*, 2016; 2017), even though exceptions exist (Papadopoulos and Katsoyannos, 2003; Ali *et al.*, 2016). Nevertheless, parasitism rates under low temperatures are expected to be high only under low humidity levels, while warm and wet conditions favor parasitism rates of *A. daci* during summer, suggesting that both temperature and relative humidity affect parasitism rates of *A. daci* in the Mediterranean basin (de Pedro *et al.*, 2017). For instance, in the coastal area of Syria, field parasitism of *A. daci* was recorded from May to September with a peak during June and August, when temperatures ranged from 22°C to 26°C (Ali *et al.*, 2016). In our study, high temperatures and low relative humidity prevailing in the area during July and August may resulted in high mortality rates of the released parasitoids. In addition, low temperatures (<20°C) during the last month of the field trial are likely to extend the duration of the immature stages of the parasitoid and decrease survival rates of *A. daci*, despite of the high olive fly infestation that offered an increased host availability (Tormos *et al.*, 2013).

Given that olive cultivar has an effect on parasitism rates of *A. daci*, it is possible to assume that the parasitism failure in pilot trials is attributed to cultivar type, although there is no data regarding *A. daci* response to olive fly larvae feeding on the "Pelion" cultivar.. In this sense, further studies are needed for assessing the parasitism rates of *A. daci* on olive fly infested fruit of the "Pelion" cultivar under both laboratory and field conditions following more controlled experimental approaches. Moreover, taking into account the oviposition strategy and the searching behavior of *A. daci*, it is expected that the figitid wasp will predominantly parasitize larvae in fallen fruits due to the easy entry through cracks of wounded fallen fruit, as previous studies have documented for the fruit fly parasitoids *A. pelleranoi* and *Odontosema anastrephae* (Hymenoptera: Figitidae) (Aluja *et al.*, 2009). In our study, high *B. oleae* infestation (particularly during the last month of the field trial) resulted in large numbers of fallen olive fruit but parasitism of olive fly larvae hosted in these fruit was not evaluated. Moreover, it is important to note that samples of *B. oleae* pupae that gave no adults (*B. oleae* or *A. daci*) were not examined for immature stages of *A. daci* that did not manage to reach adult stage. Hence, the possibility that parasitism exists on fallen fruit or pupae in the soil cannot be excluded.

Associative learning is well documented, particularly for the opiine parasitoid species (Giunti *et al.*, 2015). Laboratory experiments revealed that early adult learning affects host preferences (*B. oleae vs C. capitata*) in the medfly mass-reared *P. concolor* (Canale and Benelli, 2012; Giunti *et al.*, 2016). Even though parasitoid behavioral studies in the field are still scarce (Randlkofer *et al.*, 2010; Kostenko *et al.*, 2015), host plant appears to

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be another important source of information for parasitoids' foraging capacity (Giunti *et al.*, 2015; Segura *et al.*, 2012; 2016). Recently, it was proved that host-fruit odor learning influences foraging capacity of the fruit fly parasitoid, *Diachasmimorpha krausii* (Hymenoptera: Braconidae), against the tephritid host fly *Bactrocera tryoni* in nectarines and tomatoes (Masry *et al.*, 2019). The role of olive trees and olive fruits on the performance of *A. daci* and other olive fruit fly parasitoids remain totally unexplored, despite of the long term efforts for the biological control of olive fruit fly (Daane and Johnson, 2010). In this sense, it is plausible to suggest that parasitism failure of *A. daci* against the heavily infested olive fruit may be partly attributed to olive trees/fruit recognition issues. Further studies are necessary for assessing the factors that affect foraging capacity of *A. daci* in olive groves.

Conclusions

To summarize, our study aimed at evaluating the potential of the figitid wasp, *A. daci*, as a biological control agent of the olive pest, *B. oleae*. To this end, we assessed the effects of olive fruit size and *B. oleae* larval instar on its parasitism success under laboratory conditions, and then we examined *A. daci* performance on *B. oleae* larvae in a pilot olive grove in Greece. Laboratory studies revealed that small-sized olives infested with 3rd instar larvae of olive fruit fly are most vulnerable to *A. daci* attacks. However, infested wild olive fruit found not to be an appropriate host for *A. daci*. Our first attempt of field control of olive fruit fly raised the well documented inherent difficulties of classical biological control of *B. oleae*, resulting in no parasitism. Overall, further studies are needed, both in laboratory and field conditions, for getting a better insight of *A. daci* potential in olive fly biological control programs.

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Επίδραση του μεγέθους του ελαιοκάρπου στον παρασιτισμό του *Bactocera oleae* (Diptera: Tephritidae) από το παρασιτοειδές *Aganaspis daci* (Hymenoptera: Figitidae) και πιλοτική εξαπόλυση του παρασιτοειδούς σε ελαιώνα

Κ.Α. Μωραΐτη, Γ.Α. Κυρίτσης και Ν.Τ. Παπαδόπουλος

Περίληψη Ο δάκος της ελιάς, *Bactocera oleae* (Diptera: Tephritidae), αποτελεί τον σημαντικότερο εχθρό της ελαιοκαλλιέργειας παγκοσμίως, του οποίου η βιολογική αντιμετώπιση παραμένει μάλλον ανεπιτυχής στον αγρό. Το *Aganaspis daci* (Hymenoptera: Figitidae) είναι ένα ενδοπαρασιτοειδές που προσβάλλει μεγάλης ηλικίας προνύμφες των ειδών της οικογένειας Tephritidae και βρέθηκε να παρασιτεί επιτυχώς προνύμφες της μύγας της Μεσογείου σε προσβεβλημένα στον αγρό σύκα

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στην Ελλάδα. Προκειμένου να εξεταστεί η δυνατότητα αξιοποίησης του *A. daci* ως παράγοντα βιολογικής καταπολέμησης του δάκου της ελιάς, μελετήθηκε η επίδραση του μεγέθους του ελαιοκάρπου στον παρασιτισμό προνυμφών 2ης και 3ης ηλικίας του δάκου της ελιάς, χρησιμοποιώντας διαφορετικού μεγέθους καρπούς της ποικιλίας «Χαλκιδικής» και καρπούς αγριελιάς. Επιπρόσθετα, πραγματοποιήθηκαν εξαπολύσεις του *A. daci* σε πιλοτικό ελαιώνα στην ευρύτερη περιοχή του Βόλου, Μαγνησίας. Κατά την περίοδο Ιουλίου – Οκτωβρίου 2018, πραγματοποιήθηκαν σε εβδομαδιαία βάση εξαπολύσεις 200 θηλυκών ατόμων/στρέμμα με παράλληλες συλλογές ελαιοκάρπων (300 ελαιόκαρποι/εβδομάδα) για έλεγχο του ποσοστού προσβολής των καρπών από τον δάκο της ελιάς και του ποσοστού παρασιτισμού από το *A. daci*. Από τα αποτελέσματα προκύπτει ότι τόσο το μέγεθος του ελαιοκάρπου όσο και το στάδιο ανάπτυξης της προνύμφης του δάκου της ελιάς είναι καθοριστικοί παράγοντες για τον επιτυχή παρασιτισμό από το *A. daci*. Υψηλά ποσοστά παρασιτισμού καταγράφηκαν στους μικρού μεγέθους καρπούς της ποικιλίας «Χαλκιδικής» και στις προνύμφες 3ης ηλικίας του δάκου της ελιάς σε εργαστηριακές συνθήκες. Αντίθετα, δεν προέκυψαν ενήλικα του *A. daci* από προσβεβλημένους ελαιόκαρπους κατά τη διάρκεια των πιλοτικών δοκιμών στον αγρό.

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SHORT COMMUNICATION

Lavandula angustifolia and *Oxalis pes-caprae*, hosts of *Meloidogyne hapla* and *Meloidogyne javanica* - A note for *Meloidogyne luci* in Greece

A.R. Gonçalves[1], I.L. Conceição[1], M. Kormpi[2] and E.A. Tzortzakakis[3]*

Summary Root-knot nematodes (RKN), *Meloidogyne* spp., have a wide host range and are common in the Mediterranean area. Cultivated lavender (*Lavandula angustifolia*) was found naturally infested by *M. hapla* in Kozani area, the first documented infestation of this crop by RKN in Greece. *Oxalis pes-caprae*, a common winter weed in Crete, was found to be a host of *M. javanica* under artificial inoculation. This weed acts as a potential winter host of the nematode in fields cultivated with vegetable crops. Two populations of *M. ethiopica* were found in kiwi and maize in Greece in the past. Recently, populations of *M. ethiopica* from Europe were re-classified as *M. luci*, based only on the population isolated from kiwi for Greece. In the current work, the RKN populations originating from kiwi and maize and maintained on tomato, were identified as *M. luci*. Nematode species identification was determined by electrophoretic analysis of protein extracts obtained from females.

Additional keywords: Esterase phenotypes, identification, root-knot nematodes, weed

Root-knot nematodes (RKN), *Meloidogyne* spp., are amongst the most economically important nematodes in agriculture, having a wide host range and being common in the Mediterranean area (Lamberti, 1981; Moens *et al*., 2009). Species identification using morphological characteristics is complex, difficult and time consuming. In Greece, RKN have been found in several areas and their identification was based on morphological and morphometric characters and/or differential host tests until the middle of 1990's (Tzortzakakis *et al*., 2011). In the last 20 years, the four major RKN species (*Meloidogyne arenaria* (Neal) Chitwood, 1949, *Meloidogyne hapla* Chitwood, 1949, *Meloidogyne incognita* (Kofoid & White) Chitwood, 1949 and *Meloidogyne javanica* (Treub) Chitwood, 1949), *Meloidogyne luci* (Carneiro, Correa, Almeida, Gomes, Mohammad Deimi, Castagnone – Sereno & Karssen, 2014) (initially identified as *Meloidogyne ethiopica* Whitehead, 1968) and *Meloidogyne hispanica* (Hirschmann, 1986) have been identified in Greece using molecular and/or biochemical markers (Conceição *et al*., 2012; Tzortzakakis *et al*., 2011, 2014, 2016, 2019; Gerič Stare *et al*., 2017).

Herein *Meloidogyne* spp. from populations isolated from cultivated plants in Greece, were identified by electrophoretic analysis of protein extracts obtained from RKN females, using the Mini-Protean III system (Bio-Rad) according to Esbenshade and Triantaphyllou (1985a,b) and Pais *et al.* (1986), with some modifications. The analysis revealed new host plant species for *M. hapla* and *M. javanica* and also confirmed the identity of *M. luci* in two populations maintained on tomato.

In autumn of 2018, some plants of cul-

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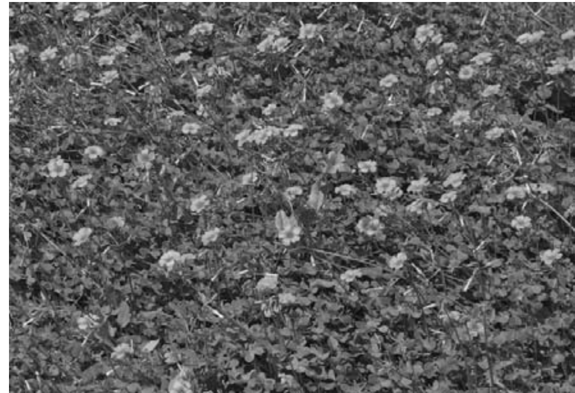
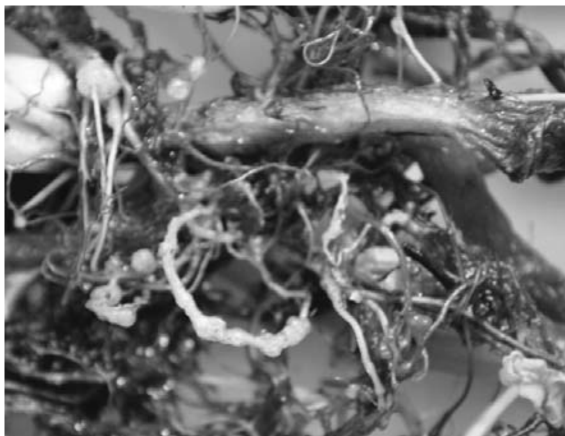
tivated lavender (*Lavandula angustifolia*) from the area of Kozani, North Greece, indicated symptoms of stunting. Roots were examined and galls typical for RKN infestation were detected (Fig. 1). Egg masses were collected and used to inoculate tomato plants (*Solanum lycopersicum*, cv. ACE) grown in pots filled with commercial compost soil and maintained in a growth room with 16h photoperiod and temperature ranging from





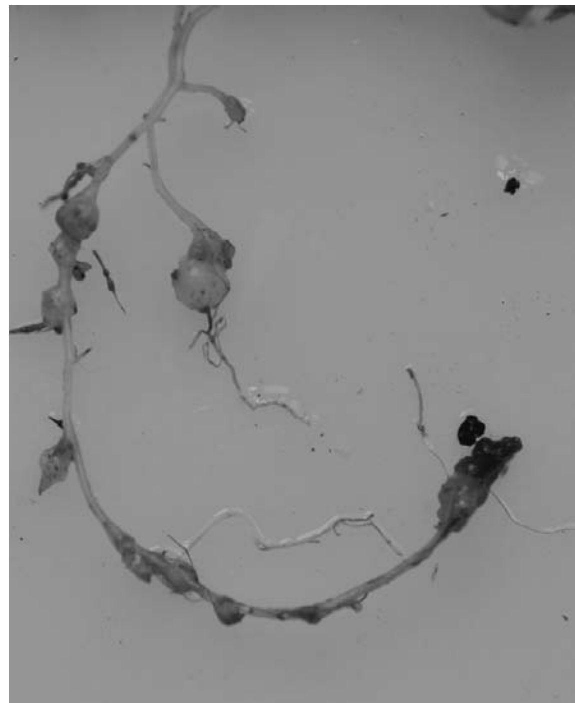


Figure 1. Top left: Root of cultivated lavender *(Lavandula angustifolia)* with galls caused by *Meloidogyne hapla.* Top right: *Oxalis pes-caprae,* a quite common winter weed in Crete, Greece. Bottom: Root of *Oxalis pes-caprae* with galls caused by *Meloidogyne javanica* after artificial inoculation.

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21 to 24°C. The RKN population originating from lavender was identified as *M. hapla*. This species has been previously reported in Greece based only on morphological identification characteristics (Hirschman *et al.*, 1966; Koliopanos, 1980; Pyrowolakis, 1980; Vovlas and Antoniou, 1987; Vlachopoulos, 1994). Recently it was found in Crete infecting the mountain tea variety "Malotira" (*Sideritis syriaca* L.) and that was the first molecular identification of this species in Greece (Tzortzakakis *et al.*, 2019). The current work represents the first report of natural infestation of cultivated lavender by *M. hapla* in Greece.

Oxalis pes-caprae (Fig. 1) is a common winter weed in Crete. Bulbs of *O. pes-caprae* were collected from soil samples originating from vineyards in autumn and stored. In October, they were planted in pots filled with commercial compost soil and inoculated with pieces of heavily galled roots of a tomato plant found infested with RKN in a home garden. The pots were maintained in a glasshouse without artificial light or heating from October to April, when the plants were let to dry. Air temperature fluctuated from 3°C at winter nights to as high as 35°C in spring. The roots were washed from the soil and revealed presence of galls with egg masses (Fig. 1). Several egg masses were collected and inoculated to tomato plants (cv. ACE) grown in pots in a growth room as described previously. After approximately two months, the plants were uprooted and RKN females isolated from the roots were used for species identification.

The population that infested and reproduced on *O. pes-caprae* in pots was identified as *M. javanica* which is a prevalent species of RKN in Crete (Tzortzakakis *et al.*, 2011). The related species *Oxalis corniculata* has been reported as a host of *Meloidogyne* species (Dabaj and Jenser, 1990; Belle *et al.*, 2016; Santos *et al.*, 2019). In Italy, *M. javanica* has been found parasitizing *O. pes-caprae* in citrus groves (Ciancio *et al.*, 1992). The current work indicates that this weed could act as a potential winter host of this nematode species, especially in fields cultivated with vegetable crops in the period from spring to autumn.

Meloidogyne luci was recently described from populations originating from Brazil, Chile, Iran (Carneiro *et al.*, 2014) and later from Guatemala and Portugal (Janssen *et al.*, 2016; Maleita *et al.*, 2018; Santos *et al.*, 2019). Gerič Stare *et al.* (2017) stated that all reports of *M. ethiopica* from Europe (Slovenia, Italy and Greece) refer to the species *M. luci*. The first report of *M. ethiopica* in Greece was based on the identification of two populations, one isolated from kiwi and the other from maize (Conceição *et al.*, 2012). Both of these populations have been cultured in potted tomatoes at the Nematology Laboratory of the Institute of Olive Tree, Subtropical Crops and Viticulture, Heraklion, Crete, since 2009. In 2017, Gerič Stare *et al.* identified the population isolated from kiwi as *M. luci*. In the present work, the RKN populations originating from kiwi and maize were both identified as *M. luci* confirming the presence of this species in two different areas of Greece. Recently, 26 different plant species were recognized as hosts of *M. luci* and because it has been misidentified as *M. ethiopica* for several years, a broader distribution is currently documented (EPPO, 2017).

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Lavandula angustifolia και *Oxalis pes-caprae*, ξενιστές των κομβονηματωδών *Meloidogyne hapla* και *Meloidogyne javanica* - Σημείωση για τον *Meloidogyne luci* στην Ελλάδα

A.R. Gonçalves, I.L. Conceição, Μ. Κορμπή και Ε.Α. Τζωρτζακάκης

Περίληψη Οι κομβονηματώδεις (*Meloidogyne* spp.) έχουν ένα μεγάλο εύρος ξενιστών και είναι ευρέως διαδεδομένοι στην Μεσόγειο. Στην περιοχή της Κοζάνης βρέθηκε καλλιεργούμενη λεβάντα (*Lavandula angustifolia*) με προσβολή από τον νηματώδη *Meloidogyne hapla* με την συγκεκριμένη καταγραφή να είναι η πρώτη στην Ελλάδα. Η οξαλίδα, *Oxalis pes-caprae*, αποτελεί ένα συνηθισμένο χειμερινό ζιζάνιο στην Κρήτη και βρέθηκε να είναι ξενιστής του είδους *M. javanica* σε συνθήκες τεχνητής μόλυνσης. Επομένως μπορεί να αποτελέσει ένα χειμερινό ξενιστή του νηματώδη σε αγρούς με καλλιέργεια κηπευτικών. Δύο πληθυσμοί του νηματώδη *M. ethiopica* είχαν βρεθεί σε καλλιέργεια ακτινιδίου και αραβόσιτου στην Ελλάδα παλαιότερα. Πρόσφατα, επιβεβαιώθηκε ότι όλες οι αναφορές του *M. ethiopica* στην Ευρώπη αφορούν το είδος *M. luci*, λαμβάνοντας υπόψη μόνο τον πληθυσμό στο ακτινίδιο για την Ελλάδα. Οι πληθυσμοί που είχαν απομονωθεί από την καλλιέργεια του ακτινιδίου και του αραβόσιτου στην Ελλάδα ταυτοποιήθηκαν ως *M. luci*. Η ταυτοποίηση των ειδών των νηματωδών έγινε με ηλεκτροφόρηση πρωτεϊνών προερχόμενες από θηλυκά άτομα.

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Effect of rhizobacteria strains on the induction of resistance in barley genotypes against *Cochliobolus sativus*

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Summary Enhancement of the resistance level in plants by rhizobacteria has been proven in several pathosystems. This study investigated the ability of four rhizobacteria strains (*Pseudomonas putida* BTP1 and *Bacillus subtilis* Bs2500, Bs2504 and Bs2508) to promote the growth in three barley genotypes and protect them against *Cochliobolus sativus*. Our results demonstrated that all tested rhizobacteria strains had a protective effect on barley genotypes Arabi Abiad, Banteng and WI2291. However, *P. putida* BTP1 and *B. subtilis* Bs2508 strains were the most effective as they reduced disease incidence by 53 and 38% (mean effect), respectively. On the other hand, there were significant differences among the rhizobacteria-treated genotypes on plant growth parameters, such as wet weight, dry weight, plant height and number of leaves. *Pseudomonas putida* BTP1 strain was the most effective as it significantly increased plant growth by 15-32%. In addition, the susceptible genotypes Arabi Abiad and WI2291 were the most responsive to rhizobacteria. This means that these genotypes have a high potential for increase of their resistance against the pathogen and enhancement of plant growth after the application of rhizobacteria. Consequently, barley seed treatment with the tested rhizobacteria could be considered as an effective biocontrol method against *C. sativus*.

Additional keywords: Biocontrol, *Hordeum vulgare,* ISR, PGPR, spot blotch

Introduction

Plants have various mechanisms of resistance against pathogens. It has been proven that plant growth-promoting rhizobacteria (PGPR) capable of improving the growth and yield of crops by fixing atmospheric nitrogen, solubilizing insoluble phosphates and secreting hormones, such as IAA (Majeed *et al*., 2015; Ahmed *et al*., 2017), are also able to enhance plant resistance against pathogens by inducing the systemic resistance (ISR) (Pieterse *et al*., 2002). This phenomenon can be systemic as PGPR are in soil on the plant roots while their positive effects appear on the above-ground plant parts. ISR is long-lasting and not conducive for developing resistance in the targeted pathogen. In addition, the activation of ISR-mediated defensive mechanism is overexpressed upon the subsequent pathogen challenge. Therefore, ISR phenomenon can be the basis of integrated pest management strategies in both field and greenhouse crops (Van Loon *et al*., 1998; Ramamoorthy *et al*., 2001; Zehnder *et al*., 2001; Saravanakumar *et al*., 2007).

This phenomenon has been proved in several plants against a broad range of bacterial, fungal and viral diseases, as well as against insects and nematodes (Van Loon *et al*., 1998; Durrant and Dong, 2004; Bakker *et al*., 2007; Choudhary and Johri, 2009; De Vleesschauwer and Höfte, 2009; Reglinski, 2009). Most PGPR-elicited ISR in plants belong to the genera *Pseudomonas* and *Bacillus* (Kloepper *et al*., 2004; Choudhary and Johri, 2009). In the same context, a non-pathogenic *Pseudomonas putida* BTP1 strain has shown to enhance resistance in cucumber against *Pythium aphanidermatum*, and in bean and tomato against *Botrytis cinerea* (Ongena *et al*., 1999; Ongena *et al*., 2004; Adam *et al*., 2008). In addition, the same effect has been shown on grapevine and potato plants against phylloxera (*Daktulosphaira vitifoliae*) and potato tuber moth (*Phthorimaea operculella* Zeller), respectively (Adam *et al*., 2012; Adam *et al*., 2013; Adam *et al*., 2016). On the other hand, *Bacillus subtilis*

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Bs2500, Bs2504 and Bs2508 strains have also been demonstrated to induce systemic resistance in tomato and barley against *B. cinerea* and *Pyrenophora graminea*, respectively (Ongena *et al.*, 2008; Adam *et al.*, 2017).

The protective effect of PGPR was often proved in dicots, such as cucumber, tobacco and *Arabidopsis*. However, the efficacy of PGPR in monocots against necrotrophic pathogens has been demonstrated only in a few cases (Van Loon, 2007; Van Wees *et al.*, 2008; Vlot *et al.*, 2008; Pinedra *et al.*, 2010). *Cochliobolus sativus* (Ito & Kurib.) Drechsl. ex Dast. [anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.], the fungus causing spot blotch, is a common foliar pathogen of barley (*Hordeum vulgare*) and responsible for large economic losses in grain yield of cereals in North America (Mathre, 1990), Australia (Meldrum *et al.*, 2000) and Syria (van Leur *et al.*, 1997). Effective control of *C. sativus* can be achieved by the introduction of resistant cultivars as an important component of integrated disease management (Ghazvini and Tekauz, 2008).

Therefore, this work aimed mainly at studying the effect of four rhizobacteria strains (*P. putida* BTP1 and *B. subtilis* Bs2500, Bs2504, and Bs2508) against *C. sativus* on barley genotypes (Arabi Abiad, Banteng and WI2291) and on their ability to promote plant growth.

Materials and methods

Microbial strains and inoculum preparation

Rhizobacterial strains used in this study, *P. putida* BTP1 and *B. subtilis* Bs2500, Bs2504 and Bs2508, were provided by Prof. Philippe Thonart (Wallon Center for Industrial Biology, University of Liège, Belgium). *Pseudomonas putida* strain and *B. subtilis* strains were maintained for a short period at 4°C on King's B agar (King *et al.*, 1954) and 868 agar Petri dishes (20 g/l glucose, 10 g/l peptone, 10g/l yeast and 20 g/l agar) medium (Jacques *et al.*, 1999), respectively. For long-term maintenance, strains were stored at -80°C in cryotubes according to the manufacturer recommendations (Microban K; Prolab Diagnostic, Richmond Hill, Canada). For utilization, *P. putida* strain was grown on Casamino acids (CAA) broth medium (5 g/l CAA, 0.9 g/l K_2HPO_4 and 0.25 g/l $MgSO_4$) (Ongena *et al.*, 2002) for 24 h at 30±1°C, whereas *B. subtilis* strains were grown on 868 broth medium (20 g/l glucose, 10 g/l peptone and 10g/l yeast) for 48 h at 30±1°C. The cultures were then centrifuged at 10,000 rpm for 10 min. Supernatants were removed and bacterial cells were collected and resuspended in 10 mM $MgSO_4$ to a final concentration of 10^8 colony-forming units (CFU) per ml before use.

The fungal pathogen *Cochlibolus sativus* isolate Pt4 was provided by Dr. M.I.E. Arabi (Atomic Energy Commission of Syria). It was isolated from naturally infected barley leaves as described by Arabi and Jawhar (2003). The fungus was grown in 9 cm Petri dishes containing potato dextrose agar (PDA, Difco, Detroit, MI, USA) for 10 days at 22±1ºC in the dark. The conidial suspension was prepared by harvesting conidia with 10 ml of sterile distilled water. After removing mycelial debris by filtration through several layers of cheesecloth, the conidial suspension was centrifuged for 5 min at 5,000 g and conidia were resuspended in 0.01% X-triton-100 to a final concentration of $2x10^4$ conidia/ml.

Barley genotypes

The three barley (*Hordeum vulgare* L.), genotypes, Arabi Abiad, Banteng and WI2291, used in the present study, were provided by Dr. M.I.E. Arabi (Atomic Energy Commission of Syria). They were chosen for their differential reaction to artificial inoculation with *C. sativus* (Arabi, 2005). WI2291 (susceptible) originated from the Waite Institute (Glen Osmond, Australia), Banteng (resistant) is a German genotype and Arabi Abiad (moderate resistant) is a local genotype (heterogeneous landrace).

Assays for induction of resistance

Barley seeds of the three genotypes were surface sterilized by dipping in sodium hypochlorite (5%) for 5 min and washed

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with sterilized distilled water three times for 3 minutes each time. Prior to sowing, sterilized seeds were soaked for 10 min in suspensions of 10^8 CFU/ml 0.01M $MgSO_4$ of the rhizobacteria strains. Untreated (control) seeds were soaked for 10 min in distilled water. Then, seeds were sown in 10 cm-plastic pots (four seeds of each genotype per pot) containing a sterilized potting substrate (Brill Substrate GmbH, KG, Germany). To ensure good colonization with the rhizobacteria, the soil substrate was mixed after its sterilization with the rhizobacteria suspensions (approximately 1l of 10^8 CFU/ml rhizobacterial suspension of each strain per 3 kg of substrate to obtain a final concentration of $3.3x10^7$ CFU/g soil substrate) or with an equal volume of sterilized distilled water per 3 kg of substrate for the untreated (control) seeds. Pots were arranged in a randomized complete block design with six pots per treatment and genotype as replicates (total of 24 plants). Plants were placed in a growth chamber at temperatures 22±1ºC (day) and 17±1ºC (night) with a 12-h photoperiod and 80-90 % relative humidity. All

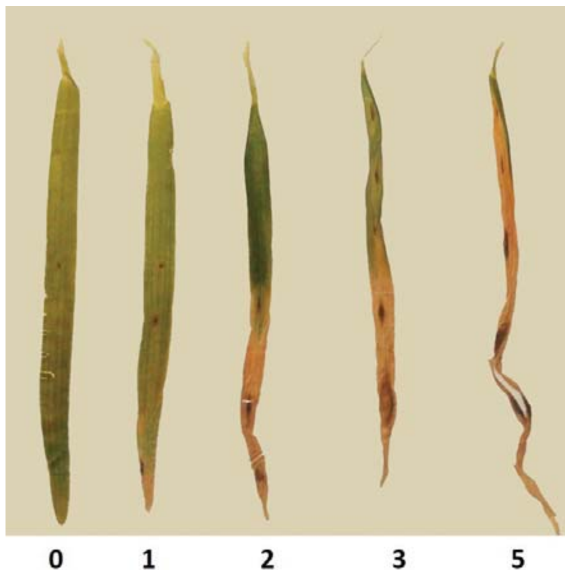

Figure 1. Development of symptoms over a period of 14 days on the second leaf of barley plants (Arabi Abiad genotype) inoculated with five drops (5μl each) of a conidial suspension (2 x 10^4 conidia/ml) of *Cochliobolus sativus*. Disease incidence was expressed as percentage of inoculation sites per leaf developing lesions (one lesion = 20%, 2 lesions = 40%, 3 lesions = 60%, 4 lesions = 80% and 5 lesions = 100%).

plants emerged from both treated and untreated seeds were inoculated with *C. sativus* at growth stage GS13 [three emerged leaves, based on the growth scale developed by Zadoks *et al.* (1974)] by depositing five drops (5μl each) of a conidial suspension ($2x10^4$ conidia/ml) on the second leaf of each plant. Experimental plants were initially incubated for 48h at 20°C, in darkness and under high humidity (> 90 %). Subsequently, they were placed in a growth chamber at 22 ± 1ºC (day) and 17±1ºC (night) with a 12-h photoperiod and 80-90% relative humidity. Fourteen days after inoculation, disease incidence was assessed and expressed as percentage of inoculation sites per leaf developing lesions (one lesion = 20%, 2 lesions = 40%, 3 lesions = 60%, 4 lesions = 80% and 5 lesions = 100%) (Fig. 1).

Estimation of rhizobacterial populations on barley roots

One gram of roots was collected from the rhizobacteria-treated barley genotypes 28 days after sowing and prior to their inoculation with *C. sativus*. Then, roots were washed with sterilized distilled water and crushed in sterilized pestle mortar with 2 ml sterilized distilled water to release the bacteria from tissues. One ml of the root solution was added to test tube containing 9 ml of 0.85% NaCl, and successive serial dilutions were prepared. Aliquots of 100 μl from dilution tubes were spread onto Luria-Bertani (LB) agar medium Petri dishes and incubated at 28ºC for 1-2 days. Bacterial populations were estimated as CFU/g of roots.

Testing leaves for the presence of rhizobacteria

Small leaf samples were excised from rhizobacteria-treated plants. The samples were surface sterilized with 5% sodium hypochlorite for 3 min and washed three times (for 3 min each time) with sterilized distilled water. Samples were left to dry on sterile filter paper. Then they were transferred under aseptic conditions onto Petri dishes containing LB agar medium. The dishes were incubated for 72 h at 28±1°C in the dark.

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Assessment of growth of rhizobacteria-treated barley plants

Six weeks after sowing, the experimental barley plants were harvested and plant growth parameters, such as plant height (measured from the soil level to the top of the main plant stem), number of leaves, wet weight (measured on the above soil level plant parts) and dry weight (above soil level plant parts were dried at 65ºC for 48h and weighted), were recorded.

Statistical analysis

Statistical analyses were performed using STAT-ITCF programme at 0.05 significance level (P=0.05) (Anonymous, 1988). Data were subjected to analysis of variance (ANOVA) for the determination of differences in the means between treatments. Differences between means were tested for significance using the Student-Newman-Keuls Test.

Results

Resistance induced by rhizobacterial strains in barley plants

The results showed that all tested rhizobacterial strains (*P. putida* BTP1 and *B. subtilis* Bs2500, Bs2504, and Bs2508) used in this study exhibited a protective effect on barley genotypes Arabi Abiad, Banteng and WI2291 against *C. sativus*. There was a significant difference among these strains concerning their ability to increase the resistance level in barley. However, *P. putida* BTP1 and *B. subtilis* Bs2508 strains were the most effective, as they significantly reduced the mean disease incidence on the three genotypes by 53 and 38%, respectively, compared to the control plants (Table 1). In addition, a significant difference was observed between barley genotypes used in this study. Banteng was the least susceptible genotype, while WI2291 and Arabi Abiad were very susceptible (Table 1). Moreover, of all the three genotypes tested, Arabi Abiad and Banteng were the most and the least responsive genotypes to rhizobacterial strains, respectively (Table 1). More specifically, the disease incidence in Arabi Abiad plants treated with BTP1 Bs2500, Bs2504, and Bs2508 strains decreased by 58, 41, 27 and 50%, respectively, as compared to control plants, whereas in Banteng plants, the disease incidence decreased by 41, 10, 13 and 15%, respectively, as compared to control plants (Table 1).

The density of the rhizobacterial population on the roots of the experimental barley plants ranged between 2.2 and 11.1x10^6 CFU/g of roots (Table 2), which shows that the rhizobacterial strains were readily established and maintained on barley roots. Furthermore, the present study showed that the rhizobacteria did not migrate from the roots to the leaf tissues, as no rhizobacteria were isolated from the plant leaves. Therefore, the inducing agent and the phytopathogen remained localized on different

Table 1. Effect of rhizobacterial strains of *Pseudomonas putida* (BTP1) and *Bacillus subtilis* (Bs2500, Bs2504 and Bs2508) on the disease incidence (%) on barley genotypes Arabi Abiad, Banteng and WI2291 inoculated with *Cochliobolus sativus*.

Treatment	Disease incidence (%)			Mean
	Arabi Abiad	Banteng	WI2291	
Control*	70.4** ± 3.8 a***	31.2 ± 3.8 a	74.4 ± 5.6 a	58.7 ± 3.4 a
BTP1	29.6 ± 3 d	18.4 ± 3.2 b	35.2 ± 3.7 d	27.7 ± 2.1 d
Bs2500	41.6 ± 4.3 bc	28 ± 3.5 ab	60 ± 4.2 bc	43.2 ± 2.7 b
Bs2504	51.2 ± 3.7 b	27.2 ± 3.8 ab	57.6 ± 4.4 bc	45.3 ± 2.7 b
Bs2508	35.2 ± 3.3 cd	26.4 ± 3.6 ab	48 ± 3.8 c	36.5 ± 2.3 c

* Plants inoculated with *C. sativus* and not treated with rhizobacteria
** Mean of 6 replicates
*** Means followed by the same letters do not differ significantly at P<0.01 according to Newman-Keul's test

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Table 2. Population density (CFU/g of roots) of rhizobacterial strains *Pseudomonas putida* (BTP1) and *Bacillus subtilis* (Bs2500, Bs2504 and Bs2508) on the roots of barley genotypes Arabi Abiad, Banteng and WI2291, as estimated 28 days after sowing and prior to their inoculation with *Cochliobolus sativus*.

Genotype	Population density of rhizobacteria strains (CFU/g roots)			
	BTP1	Bs2500	Bs2504	Bs2508
Arabi Abiad	9.4	11.1	4.2	8.6
Banteng	4.7	10.9	2.2	3.9
WI2291	7.2	4.7	3	8.8

plant organs showing that the disease suppression was due to induction of resistance in the host plant.

Effect of rhizobacteria on plant growth parameters

Effect on wet weight

The results showed that all rhizobacterial strains (BTP1, Bs2500, Bs2504, Bs2508) had a positive effect on the wet weight of the three barley genotypes (Arabi Abiad, Banteng and WI2291). Bacterial strains differed in their effect on the wet weight, but BTP1 and Bs2508 strains were the most effective, as they significantly increased the mean wet weight by 32, 28.8 and 36.2% and by 22.7, 11.1 and 27.3% in Arabi Abiad, Banteng and WI2291 genotypes, respectively, compared to the control plants (Table 3). On the other hand, Arabi Abiad genotype was more responsive to rhizobacterial treatments than the two other genotypes (Banteng and WI2291), as the general effect of all rhizobacterial strains on wet weight increased by 23.7% in Arabi Abiad genotype (Table 3).

Effect on dry weight

The results showed that all rhizobacterial strains (BTP1, Bs2500, Bs2504, Bs2508) had a positive effect on the dry weight of the three barley genotypes (Arabi Abiad, Banteng and WI2291). However, BTP1 and Bs2508 strains were the most effective, as they significantly increased the mean dry weight by 27.9, 17.6 and 24% and by 23.3, 8.8 and 18% in Arabi Abiad, Banteng and WI2291 genotypes, respectively, compared to the control plants (Table 3).

Effect on plant height

There were significant differences in the ability of the rhizobacterial strains tested (BTP1, Bs2500, Bs2504, Bs2508) to increase the mean plant height of Arabi Abiad, Banteng, and WI2291 genotypes. However, BTP1 and Bs2508 strains were the most effective as they significantly increased the mean plant height by 15.2, 8 and 17.6% and by 11.7, 7.3 and 13.3% in Arabi Abiad, Banteng and WI2291 genotypes, respectively, compared to the control plants (Table 3).

Effect on number of leaves

The results showed that all rhizobacterial strains (BTP1, Bs2500, Bs2504, Bs2508) increased significantly the mean number of leaves of the three barley genotypes (Arabi Abiad, Banteng and WI2291). However, BTP1 and Bs2508 strains were the most effective, as they increased significantly the mean number of leaves by 26, 18.8 and 27.2% and by 13.7, 6.3 and 17.3% in Arabi Abiad, Banteng and WI2291 genotypes, respectively, compared to the control plants (Table 3).

Discussion

Several studies reported that some rhizobacterial strains could be used as biocontrol agents against pests (Zehnder *et al.*, 1997; Zehnder *et al.*, 2001; Haas and Défago, 2005; Reglinski, 2009). In the present work, the protective effect of rhizobacteria

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Table 3. Effect of rhizobacterial strains of *Pseudomonas putida* (BTP1) and *Bacillus subtilis* (Bs2500, Bs2504 and Bs2508) on plant growth parameters of barley genotypes Arabi Abiad, Banteng and WI2291 inoculated with *Cochliobolus sativus*.

Genotype	Treatment	Wet weight (g)	Dry weight (g)	Plant height (cm)	No of leaves
Arabi Abiad	Control*	2.69** ± 0.1 c***	0.43 ± 0.01 c	37.6 ± 0.5 c	7.3 ± 0.2 c
	BTP1	3.55 ± 0.1 a	0.55 ± 0.02 a	43.3 ± 0.7 a	9.2 ± 0.3 a
	Bs2500	3.24 ± 0.1 b	0.52 ± 0.01 ab	40.3 ± 0.7 b	7.8 ± 0.3 bc
	Bs2504	3.22 ± 0.1 b	0.48 ± 0.02 bc	38.4 ± 0.8 bc	7.6 ± 0.3 bc
	Bs2508	3.3 ± 0.1 b	0.53 ± 0.01 a	42 ± 0.5 a	8.3 ± 0.1 b
	General effect****	23.7	20.93	9	12.67
Banteng	Control*	2.22 ± 0.1 d	0.34 ± 0.01 b	31.4 ± 0.4 b	9.6 ± 0.2 b
	BTP1	2.86 ± 0.1 a	0.4 ± 0.02 a	33.9 ± 0.4 a	11.4 ± 0.3 a
	Bs2500	2.3 ± 0.1 c	0.36 ± 0.02 a	32.2 ± 0.5 b	10 ± 0.2 b
	Bs2504	2.33 ± 0.1 c	0.35 ± 0.02 b	32.5 ± 0.6 b	9.7 ± 0.2 b
	Bs2508	2.57 ± 0.1 b	0.37 ± 0.01 a	33.7 ± 0.5 a	10.2 ± 0.3 b
	General effect****	13.29	8.82	5.33	7.55
WI2291	Control*	2.82 ± 0.1 d	0.5 ± 0.01 b	39.8 ± 0.5 c	8.1 ± 0.2 c
	BTP1	3.84 ± 0.1 a	0.62 ± 0.02 a	46.8 ± 0.6 a	10.3 ± 0.3 a
	Bs2500	3 ± 0.1 cd	0.53 ± 0.03 b	45 ± 0.7 ab	8.8 ± 0.2 bc
	Bs2504	3.1 ± 0.1 c	0.51 ± 0.03 b	41.4 ± 0.8 c	8.4 ± 0.3 c
	Bs2508	3.59 ± 0.1 b	0.59 ± 0.02 a	45.1 ± 0.6 ab	9.5 ± 0.2 b
	General effect****	19.92	12.5	12	14.19

* Plants infected with *C. sativus* but not treated with rhizobacteria
** Means of 6 replicates
*** Means followed by the same letters do not differ significantly at $P<0.01$ according to Newman-Keul's test.
**** Increase of mean growth of plants treated with different rhizobacterial strains compared to the control plants.

strains *P. putida* BTP1 and *B. subtilis* Bs2500, Bs2504 and Bs2508 on three barley genotypes against *C. sativus* was demonstrated. Results showed that treatment of barley seeds with any of the rhizobacterial strains tested led to a significant reduction in disease incidence in Arabi Abiad and WI2291 genotypes. However, in the case of Banteng genotype, a significant reduction in disease incidence was observed only on plants treated with BTP1 strain. These results are in agreement with precedent studies carried out with the same rhizobacterial strains on tomato and bean against *Botrytis cinerea,* and on barley against *Pyrenophora graminea* (Ongena *et al*., 2004; Adam *et al*., 2008; Ongena *et al*., 2008; Adam *et al*., 2017). *In vitro* studies showed that *P. putida* BTP1 strain could not inhibit *C. sativus* mycelial growth, which implies that there was no direct antagonism between these two organisms (rhizobacterium and fungal pathogen) (unpublished data). This is further supported by precedent work demonstrating that *P. putida* BTP1 did not excrete any fungitoxic compounds (Ongena *et al*., 1999). Thus, the resistance induced by BTP1 strain is unlikely to be related to the production of any antibiotic molecule with plant defense-stimulating activity. In the present study, all rhizobacterial strains used colonized very well the barley roots (between 2.2 and 11.1 x 10^6 CFU/g of roots). These results are in agreement with our precedent studies on tomato, which showed that *P. putida* BTP1 cell density was 3.0 (± 2.1) x 10^6 CFU/g on the roots at the time of inoculation of plants with *B. cinerea* (Adam *et al*., 2008). Raaijmakers *et al*. (1995) showed that the threshold population density of *P. putida* strain WCS358 and *P. fluorescens* strain WCS374 for a significant suppression of Fusarium wilt of radish was

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approximately 10^5 CFU/g of roots (Raaijmakers *et al.*, 1995). Previous studies on tomato and bean (Ongena *et al.*, 2002; Adam *et al.*, 2008; Ongena *et al.*, 2008) support our results that rhizobacterial strains are not able to migrate from the roots to the leaf tissues through the plant. Thus, both the resistance-inducing agent and the plant pathogen seem to remain localized on different plant organs, indicating that disease suppression could be due to induction of a systemic resistance phenomenon in the plant. Rhizobacterial strains *P. putida* BTP1 and *B. subtilis* Bs2500, Bs2504 and Bs2508 differed in their protective effect on barley genotypes Arabi Abiad, Banteng, and WI2291 against *C. sativus*. However, *P. putida* BTP1 and *B. subtilis* Bs2508 were the most effective strains. In addition, landrace genotype Arabi Abiad was the most responsive to rhizobacterial treatments with respect to the increase in resistance compared to Banteng and WI2291 genotypes. Therefore, there is a potential to increase the level of resistance of genotype Arabi Abiad to infection by *C. sativus* by using rhizobacteria. Banteng genotype was less susceptible to *C. sativus* compared to the other two genotypes. Generally, rhizobacteria strains could induce some resistance mechanism in Banteng genotype. Our results are in agreement with precedent studies, which showed that host genotypes differ in their expression of induced resistance, and that the highly susceptible genotypes were more responsive to induced resistance than the resistant genotypes (Dann *et al.*, 1998; Resende *et al.*, 2002; Tucci *et al.*, 2011; Walters *et al.*, 2011a; Córdova-Campos *et al.*, 2012; Adam *et al.*, 2017).

On the other hand, our results showed that rhizobacterial strains stimulated some of the plant growth parameters (i.e. wet weight, dry weight, plant height and number of leaves) under pathogen pressure in all barley genotypes tested. These results are in agreement with those of Orhan *et al.* (2006) studies on raspberry, which showed that colonization of plant roots and rhizosphere with rhizobacterial strains increased significantly the plant growth in terms of yield, cane length, number of clusters per cane and number of berries per cane. Several studies reported that applying *P. fluorescens* Pf5 on sugar beet, barley, corn, blueberry and tomato led to findings similar to those of our studies (De Silva *et al.*, 2000; Cakmakci *et al.*, 2001; Ataoglu *et al.*, 2004; Turan *et al.*, 2004). Furthermore, several PGPR may affect plant growth through the production and release of gibberellins as phytohormones; the growth of red pepper plants and alder plants was enhanced by treatment with some PGPR strains producing gibberellins (Gutiérrez-Mañero *et al.*, 2001; Joo *et al.*, 2005). Çavuşoğlu and Kabar (2008) demonstrated that some plant growth regulators (PGRs), such as gibberellic acid (GA_3), kinetin (KIN), benzyladenine (BA) and ethylene (E), overcome the negative effect of salt stress on percentage of seed germination, radicle elongation and fresh weight. Previous studies showed that *P. putida* BTP1 strain secretes N-alkylated benzylamine derivative (NABD), an elicitor who plays an important role in the elicitation of the ISR phenomenon in bean and tomato plants against *B. cinerea* (Ongena *et al.*, 2008). Thus, we could suggest that the benzylamine derivative produced by BTP1 and which is similar to benzyladenine, might play an important role in stimulation of the plant growth.

Our studies showed that the effect of *P. putida* BTP1 and *B. subtilis* Bs2508 strains on stimulation of the plant growth and resistance to *C. sativus* in barley plants was greater than that of the other strains (Bs2500 and Bs2504). This is in agreement with recent studies which reported that PGPR could be used to replace chemical fertilizers/pesticides and to stimulate the growth of tomato plants directly or indirectly via availability of many essential plant nutrients, phytohormones, or through suppression of plant diseases (Ahmed *et al.*, 2017). Several studies showed that the application of rhizobacterial strains in rice, potato and cotton crops reduced the incidence of charcoal root rot (*Macrophomina phaseolina*), late blight (*Phytophthora infestans*) and bacterial leaf blight (*Xanthomonas citri* pv. *malvacearum*), respectively. Furthermore, they increased the yield

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compared to untreated plants (Yasmin *et al.*, 2016; Rizvi *et al.*, 2017; Adrees *et al.*, 2019).

Consequently, the present study investigated for the first time the effect of rhizobacteria on induction of resistance and promotion of plant growth on three barley genotypes. Induced resistance by seed treatment with PGPR is considered one of the most important biocontrol methods against diseases, especially for crops that are grown over large areas. Finally, more research is needed to determine the effects of PGPR strains on barley plants under field conditions and on the defense mechanisms responsible for resistance to *C. sativus*.

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Επίδραση στελεχών ριζοβακτηρίων στην επαγωγή ανθεκτικότητας γονοτύπων κριθαριού εναντίον του μύκητα *Cochliobolus sativus*

A. Adam

Περίληψη Η ενίσχυση του επιπέδου αντοχής των φυτών από τα ριζοβακτήρια έχει αποδειχθεί σε πολλά παθοσυστήματα. Στην παρούσα μελέτη διερευνήθηκε η ικανότητα τεσσάρων στελεχών ριζοβακτηρίων (*Pseudomonas putida* BTP1 και *Bacillus subtilis* Bs2500, Bs2504 και Bs2508) να προάγουν την ανάπτυξη σε τρεις γονοτύπους κριθαριού και να τα προστατεύουν έναντι του φυτοπαθογόνου μύκητα *Cochliobolus sativus*. Τα αποτελέσματά μας έδειξαν ότι όλα τα στελέχη ριζοβακτηρίων που δοκιμάστηκαν είχαν προστατευτική επίδραση στους γονοτύπους κριθαριού Arabi Abiad, Banteng και WI2291. Ωστόσο, τα στελέχη *P. putida* BTP1 και *B. subtilis* Bs2508 ήταν τα πιο αποτελεσματικά καθόσον μείωσαν τη συχνότητα της ασθένειας κατά 53 και 38% (μέση επίδραση), αντίστοιχα. Από την άλλη πλευρά, υπήρξαν σημαντικές διαφορές μεταξύ των γονοτύπων κιθαριού που δέχτηκαν την επέμβαση με ριζοβακτήρια ως προς διάφορες παραμέτρους ανάπτυξης των φυτών, όπως το νωπό βάρος, το ξηρό βάρος, το ύψος των φυτών και ο αριθμός των φύλλων. Το στέλεχος *P. putida* BTP1 ήταν το πιο αποτελεσματικό καθόσον αύξησε σημαντικά την ανάπτυξη των φυτών κατά 15-32%. Επιπλέον, οι ευπαθείς στο παθογόνο γονότυποι κριθαριού Arabi Abiad και WI2291 εμφάνισαν την καλύτερη ανταπόκριση στα ριζοβακτήρια. Αυτό σημαίνει ότι οι συγκεκριμένοι γονότυποι έχουν υψηλό δυναμικό για αύξηση της αντοχής τους στο παθογόνο και ενίσχυση της ανάπτυξής τους μετά από εφαρμογή ριζοβακτηρίων. Ως εκ τούτου, η επέμβαση σε σπόρους κριθαριού με τα παραπάνω ριζοβακτήρια μπορεί να θεωρηθεί ως μια αποτελεσματική μέθοδος βιολογικής αντιμετώπισης του φυτοπαθογόνου μύκητα *C. sativus*.

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The scale insect *Coccus pseudomagnoliarum* (Kuwana) (Hemiptera: Coccomorpha: Coccidae) on citrus in Greece

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Summary Phenology and parasitism of the scale insect, *Coccus pseudomagnoliarum* (Kuwana) (Hemiptera: Coccomorpha: Coccidae), infesting *Citrus sinensis* (Rutaceae), were studied in Papagou area, in northeastern Athens, from June 2015 to June 2017. *Coccus pseudomagnoliarum* is a univoltine, viviparous, parthenogenetic species. It overwintered as settled 1st instar nymph on the shoots of the trees. The 2nd instar nymphs appeared between the beginning of April and the end of May, and the mature females were recorded from the beginning of May until the middle of June. The crawlers appeared between the middle of May and the middle of June and the 1st instar nymphs settled on the shoots at the end of May, where they remained during the whole summer period, winter, until the beginning of April next year. Parasitism of the scale was recorded between the beginning of May and the middle of May and reached a maximum rate of 35%. The recorded parasitoid species were *Coccophagus shillongensis* Hayat and Singh (Hymenoptera: Aphelinidae), *Coccophagus* spp. and *Metaphycus dispar* (Mercet) (Hymenoptera: Encyrtidae).

Additional keywords: *Coccus pseudomagnoliarum*, Coccidae, Greece, parasitism, phenology

Introduction

The citricola scale *Coccus pseudomagnoliarum*, has been recorded in Greece and in other regions of the world such as America (Arizona, California, Maryland), Australia, Azerbaijan, Croatia, Cyprus, France, Georgia, Iran, Israel, Italy, Japan, Korea, Russia, Sicily, Slovenia, South Spain, Syria, Turkey and Turkmenistan (García Morales *et al*., 2019).

Its host plants belong to the families: Apocynaceae (*Nerium oleander* L.), Berberidaceae (*Berberis* L.) Cannabaceae (*Celtis australis* L., *C. occidentalis* L., *C. sinensis* Pers.), Juglandaceae (*Juglans regia* L.), Lauraceae (*Laurus nobilis* L.), Lamiaceae (*Clerodendrum trichotomum* Thunberg), Lythraceae (*Punica granatum* L.), Myrtaceae [*Acca sellowiana* (Berg) Burret], Rhamnaceae (*Rhamnus pallasii* Fischer and Meyer), Rutaceae (*Citrus aurantium* L., *C. limon* (Linn.) Burm, *C. paradisi* Macfad, *C. reticulata* Blanco, *C. trifoliata* (L.), *Phellodendron amurense* Rupr.), Tamaricaceae (*Tamarix* L. sp.) and Ulmaceae (*Ulmus* L. sp., *Zelkova serrata* Thumb.) (García Morales *et al*., 2019).

Although *C. pseudomagnolarum* is not considered an important insect pest, in some countries serious damages are reported on citrus, recording high infestation densities of the scale. It has been referred as one of the most serious pest in the early 1990's on citrus in USA, Arizona and California [(Quayle, 1938; Kennett, 1988) in Trumble *et al*., 1995], but it was largely constrained in the 1940's due to applications of DDT and subsequent organochemical insecticides (Elmer *et al*. 1980). Its economic importance increased later again, because these insecticides lost their effectiveness due to resistance development (Trumble *et al*., 1995). Dreistadt (1996) studied the scale during 1991-1994 in USA, California and found that females and crawlers on Chinese hackberry *Celtis sinensis* (Cannabaceae) on untreated trees increased each year in comparison to previous years. The citricola scale was 5-25 times more abundant than the *Parthenolecanium* species combined: *Parthenolecani-*

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um corni (Bouché), *P. pruinosum* (Coquillett) and *P. cerasorum* (Cockerell) (Hemiptera: Coccidae).

The citricola scale completes one generation per year in several countries, as in USA (California) (Gill, 1988), Greece (Argyriou and Ioanides, 1975), Israel (Ben-Dov, 1980) and Australia (Smith *et al*., 1997). Gill (1988) reports that although males have been mentioned in bibliography, their actual existence is questionable and the scale reproduces parthenogenetically laying up to 2,000 eggs per female over a 1-2 month period. It is mentioned as an oviparous species overwintering at the stage of nymph (Gill, 1988; Argyriou and Ioannides, 1975).

The main natural enemies of *C. pseudomagnoliarum*, are many hymenopteran parasitoids species belonging to the families: Aphelinidae, Encyrtidae, Eulophidae and Pteromalidae (García Morales, *et al*., 2019; Mohamed *et al*., 2012).

In Greece, the citricola scale was recorded by De Lotto (1973) by the synonym *Coccus aegaeus* De Lotto, who found the scale on citrus in the island Rhodes in 1972. After its first record, Argyriou and Ioannides (1975) and Argyriou (1983) reported the presence of the scale in Greece, in Aegean islands Astypalaea and Chios on *Citrus* spp. Many years later, Japoshvili and Stathas (2017), recorded the citrocola scale in Athens on citrus, recording new parasitoid species for Europe infesting the scale. In the present study additional data are provided for the scale, concerning its biology, phenology and natural enemies in Greece. These data will contribute to enrich the existing information of citricola scale in Greece, since no further study on the scale was conducted in Greece during the last 40 years.

Materials and methods

The phenology and parasitism of *Coccus pseudomagnoliarum* were studied on infested *Citrus sinensis* (Rutaceae) in Papagou area, in northeastern Athens, from June 2015 to June 2017. Samples of fifteen infested shoots, 15 cm long, were taken every 15 days and were transferred in plastic bags to the laboratory, where examined under the stereoscope. The number of each developmental stage of the scale, the parasitized scales and the scales with encapsulated eggs of parasitoids were recorded. As parasitized were counted the scales containing parasitoids' larvae or nymphs and the scales with parasitoid exit holes. In each sampling, the number of each developmental stage of the scale, the parasitized scales and the scales containing encapsulated eggs of parasitoids were expressed as percentage (%) of the total number of scales.

The parasitized scales were kept in cylindrical plastic cages (diameter 3cm, hight 4cm, covered by organtin) under controlled conditions in incubators in the laboratory (temperature 25±1ºC, R.H. 65±2% and 16 hours light/day), until the emergence of the parasitoid adults.

The identification of the scale was made in the Laboratory of Entomology and Agricultural Zoology of the Technological Educational Institute of Peloponnese, using the key of Gill (1998). The species of the scale was confirmed by Professor Giuseppina Pellizzari (Dipartimento di Agronomia, Animali, Alimenti), University of Padua, Italy. The parasitoid species were identified by Professor George Japoshvili, (Institute of Entomology, Agricultural University of Georgia, Tbilisi).

Results

Coccus pseudomagnoliarum completed one generation per year. It is a parthenogenetic species, because no male larva, nymph or adult was recorded in the examined samples; it is viviparous because no egg or chorion of egg was found under the examined females during the present study. It overwintered as settled 1st instar nymph on the shoots of the trees (Fig. 1b). The 2nd instar nymphs appeared between the beginning of April and the end of May (Fig. 1c), and the mature females were recorded from the beginning of May until the middle of June (Fig.

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1d, e). The crawlers appeared between the middle of May and the middle of June (Fig. 1a) and the 1st instar nymphs settled on the shoots at the end of May, where they remained during the whole summer period, winter, until the beginning of April next year. (Fig. 1b).

Parasitism of the scale was recorded to occur between the beginning of April and the middle of June in both years of the study, mainly on 2nd instar nymphs and less on preovipositing females (Fig. 2). The highest parasitism rate reached 35% at the end of May 2017. The recorded parasitoid species were: *Coccophagus shillongensis* Hayat and Singh (Hymenoptera: Aphelinidae) (Fig. 3), and *Metaphycus dispar* (Mercet) (Hymenoptera: Encyrtidae). In addition, *Coccophagus* spp. were found. Scales with encapsulated eggs of parasitoids were found between the beginning of April and the end of May in both years and reached a maximum of 13% in the middle of May 2017 (Fig. 1f). Encapsulation was recorded mainly on the 2nd instar nymphs of the scale, which contained 1 to 6 encapsulated eggs (Fig. 4).

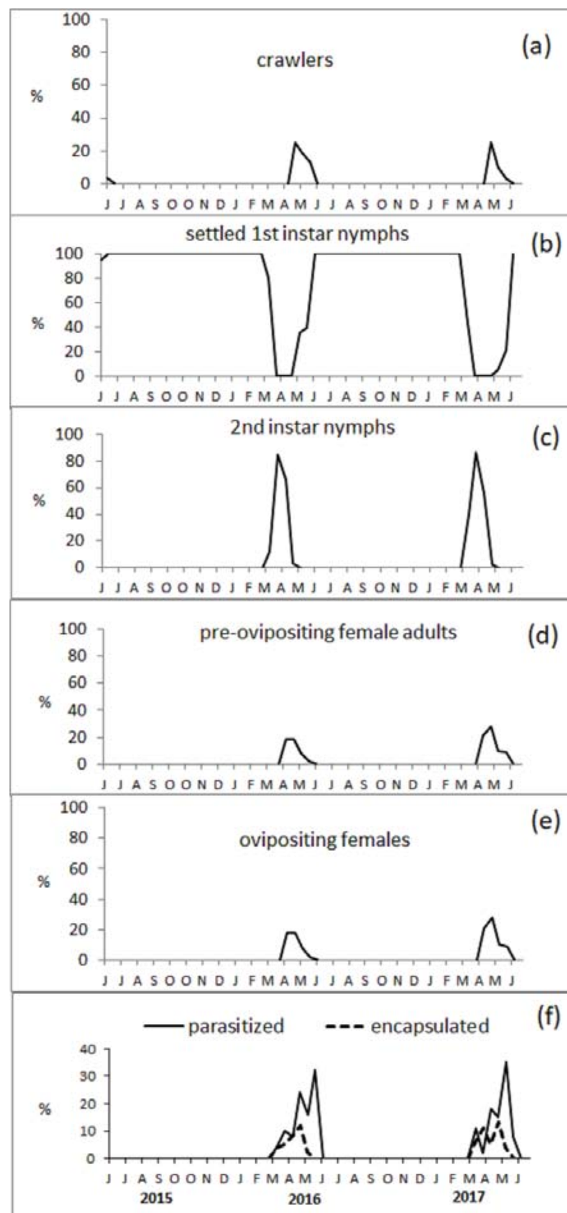

Figure 1. Percentage of developmental stages of *Coccus pseudomagnoliarum*, parasitism and encapsulation of the scale (associated parasitoid species *Coccophagus shillongensis*, *Coccophagus* spp., *Metaphycus dispar*) on *Citrus sinensis* in Papagou, Athens, from June 2015 to June 2017.

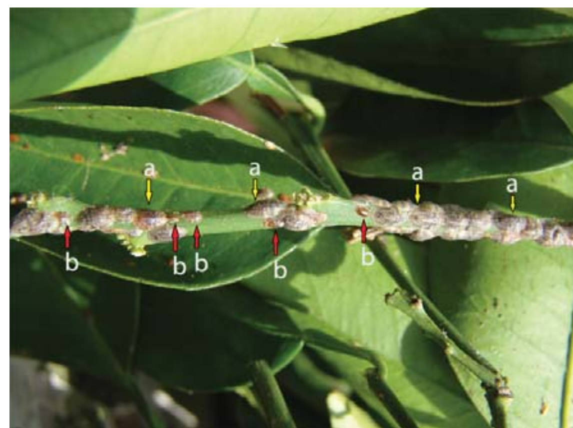

Figure 2. *Coccus pseudomagnoliarum* on *Citrus sinensis*: (a) preovipositing females; (b) parasitized scales.

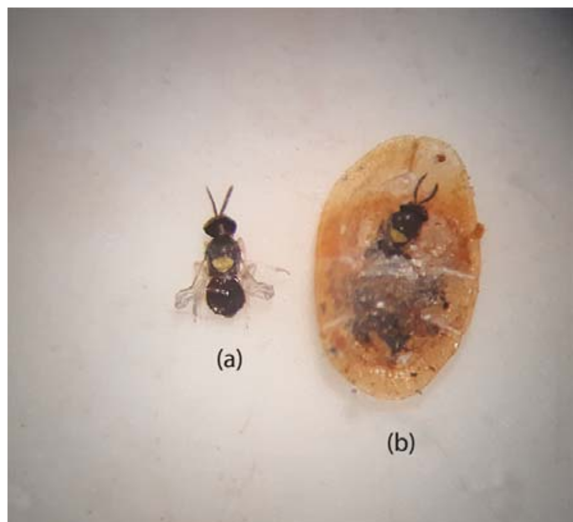

Figure 3. Parasitoid *Coccophagus shillongensis*: (a) adult; (b) in parasitized nymph of *Coccus pseudomagnoliarum*.

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Figure 4. Encapsulated eggs of parasitoids (associated parasitoid species *Coccophagus shillongensis, Coccophagus* spp., *Metaphycus dispar*) by second instar nymphs of *Coccus pseudomagnoliarum*.

Discussion

The voltinism found for *C. pseudomagnoliarum* (one generation per year) in this study coincides with the findings for the scale in other studies, on the Greek island of Rhodes (Argyriou and Ioannides, 1975), in Israel (Ben-Dov, 1980) and in California (Gill, 1988).

According to the present study, females reproduce parthenogenetically as also reported by Ben-Dov (1993) and García Morales *et al.* (2019). Gill (1988) refers that males have been mentioned in the literature but their actual existence is questionable at the time. In the present study the scale appeared to be viviparous, because no eggs or chorion of eggs was found under the examined scales during both years of the study. Argyriou and Ioannidis (1975) reported oviposition by females and empty eggs after hatching of crawlers. Similarly, Gill (1988) stated that the crawlers hatch immediately from the eggs or over a period of several days.

Coccus pseudomagnoliarum was found parasitized by *C. shillongensis, M. dispar* and *Coccophagus* unidentified species at the location near Athens. This record of *C. shillongensis* has actually been the first one in Europe (Japoshvili and Stathas, 2017). Argyriou and Ioannides (1975) have reported *Coccophagus licimnia* Walker (Hymenoptera: Aphelinidae) and *Metaphycus* sp. near *insidiosus* Mercet (Hymenoptera: Encyrtidae) with a maximum parasitism rate of 25% on the Greek island of Rhodes as well as the predators *Chilocorus bipustulatus* L. and *Exochomus quadripustulatus* L. (Coleoptera: Coccinellidae).

Encapsulation of parasitoid eggs by *C. pseudomagnoliarum* is related to the species *C. shillongensis, M. dispar* and *Coccophagus* spp. Tena and Garcia-Mari (2008) report high encapsulation levels of *Metaphycus helvolus* (Compere) (Hymenoptera: Encytridae) by the citricola scale on citrus in Spain, once the scale length reaches 2 mm at the end of spring, which reduce the efficiency of the parasitoid. A further study on the ecology of the recorded parasitoid species is considered important in order to clarify the contribution of each parasitoid species to the total parasitism of the scale, the relationship among the parasitoid's population and the influence of encapsulation on each species.

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Το κοκκοειδές έντομο *Coccus pseudomagnoliarum* (Kuwana) (Hemiptera, Coccomorpha, Coccidae) σε εσπεριδοειδή στην Ελλάδα

Γ.Ι. Σταθάς και Χ.Φ. Καριπίδης

Περίληψη Μελετήθηκε η φαινολογία και ο παρασιτισμός του κοκκοειδούς εντόμου *Coccus pseudomagnoliarum* (Kuwana) (Hemiptera: Coccomorpha: Coccidae), σε προσβολές εσπεριδοειδών *Citrus sinensis* (Rutaceae), κατά την περίοδο Ιουνίου 2015 - Ιουνίου 2017, στο βορειοανατολικό προάστιο Παπάγου της Αθήνας. Το *C. pseudomagnoliarum* συμπληρώνει μία γενεά το έτος, είναι είδος ζωοτόκο και παρθενογενετικό. Διαχείμασε ως σταθεροποιηθείσα νύμφη 1ης ηλικίας επί των προσβεβλημένων βλαστών. Οι νύμφες 2ης ηλικίας παρατηρήθηκαν από τις αρχές Απριλίου έως τα τέλη Μαΐου και τα ώριμα θήλεα από τις αρχές Μαΐου μέχρι τα μέσα Ιουνίου. Έρπουσες παρατηρήθηκαν από τα μέσα Μαΐου έως μέχρι τα μέσα Ιουνίου και οι σταθεροποιηθείσες νύμφες 1ης ηλικίας περί τα μέσα Μαΐου, όπου παρέμειναν στο στάδιο αυτό επί των βλαστών καθ’ όλη τη θερινή και χειμερινή περίοδο, μέχρι τις αρχές Απριλίου του επόμενου έτους. Παρασιτισμός του κοκκοειδούς παρατηρήθηκε από τις αρχές έως τα μέσα Μαΐου και ανήλθε σε ποσοστό 35%. Τα είδη των παρασιτοειδών που βρέθηκαν ήταν τα *Coccophagus shillongensis* Hayat and Singh (Hymenoptera: Aphelinidae), *Coccophagus* spp. και *Metaphycus dispar* (Mercet) (Hymenoptera: Encyrtidae).

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