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Evidence of soil-located competition as the cause of the reduction of sunflower verticillium wilt by entomopathogenic fungi

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The increasing limitation of agrochemicals for disease control is a major challenge for European agriculture and a spur to developing environmentally friendly approaches such as biological control. Entomopathogenic fungi, which have been used in the control of insect pests for a long time, also have other uses, such as being antagonists of fungi, including plant pathogens. We determined the in vitro effect of three strains of *Metarhizium brunneum* and

two of *Beauveria bassiana* against *Verticillium dahliae* and *Cadophora helianthi*, causal agents of sunflower wilts. Both *M. brunneum* and *B. bassiana* were able to inhibit the mycelial growth of the sunflower pathogens and, according to the dual culture and microscopy results, two types of antagonism were observed as being dependent on the strain: competition and/or antibiosis. Greenhouse experiments showed that, after soil treatments with entomopathogens and plant inoculation by root immersion in conidial suspensions of *V. dahliae*, the entomopathogens were able to efficiently persist in the soil, and three of the four strains even significantly reduced the severity of symptoms in sunflowers. Interestingly, molecular analysis showed that all the strains were able to establish themselves as endophytes in sunflowers in the absence of *V. dahliae*. When the plants were inoculated with *V. dahliae*, we detected the pathogen, but not the entomopathogen, in the sunflowers by molecular methods. The results of this work suggest that the protection conferred by *M. brunneum* and *B. bassiana* against verticillium wilt might not be plant-located, but is probably the consequence of their competition with *V. dahliae* in the soil.

1.Introduction

Sunflower (*Helianthus annuus*) is the fourth largest oilseed crop worldwide. It is grown across all five continents but is particularly relevant in Europe, where over 20% of the world's production is located (FAOSTAT, 2020). Sunflower production is constrained worldwide by diseases. Although yield decreases largely depend on the pathogen, losses of up to 100% are frequently recorded when infections are severe (Gulya *et al*., 2019). Several sunflower pathogens have been described, but only about a dozen of them are important in economic terms (Gulya *et al*., 1997).

Verticillium dahliae, the causal agent of verticillium wilt and leaf mottle, is a soilborne ascomycete and a well-known vascular pathogen of several crop species that has been described in more than 200 different hosts (Pegg and Brady, 2002). Some of the most important hosts in economic terms are tomato, tobacco, potato, lettuce, cotton, olive tree, eggplant, artichoke, cauliflower, and sunflower. This pathogen is able to persist in the soil via microsclerotia for up to 14 years, affecting the yield of subsequent crops (Pegg and Brady, 2002). Yield losses due to *V. dahliae* are variable and largely depend on the affected crop, so that losses may range from 10% to 100% (Gulya *et al*., 1997; Klosterman *et al*., 2009), which makes this fungus one of the most important pathogens in many of its hosts, including sunflower (Martin-Sanz *et al*., 2018b).

The management of verticillium wilt is highly dependent on the host plant, and none of the available alternatives provide complete control of the pathogen (Pegg and Brady, 2002). Chemical control of verticillium wilt is based on fumigation or soil amendments with chemical (e.g., propamocarb) or organic (proteins, volatile fatty acids) compounds, yet this is only useful in certain crops (e.g., strawberry or pepper) and is limited by legal, economic, and/or environmental constraints (Rekanovic *et al*., 2007; Klosterman *et al*., 2009). Biological control, carried out by using fungi (a wide range of genera including *Fusarium* spp., *Phoma* spp., and many others) or bacteria (mainly pseudomonads), has been addressed by many authors (Varo *et al*., 2016), although none of the proposed methods have so far proven to be effective for disease control in field applications (Klosterman *et al*., 2009). Genetic resistance to verticillium wilt has been described and is widely used in crops like alfalfa, cotton, potato, tomato, strawberry, sunflower, oilseed rape, and lettuce (Klosterman *et al*., 2009).

In the case of sunflower, *V. dahliae* is one of the most important pathogens and is widespread in the USA, Argentina, Europe (Pegg and Brady, 2002; Gulya *et al*., 2019;

Molinero-Ruiz, 2019), and some regions of Canada (Erreguerena *et al*., 2019). The only

control measure available in sunflower is genetic resistance (Pegg and Brady, 2002), which

was first reported in this crop in Manitoba in 1957 (Putt, 1958) and was found to be race

specific and based on single genes (Fick and Zimmer, 1974). Since then, and due to further

breeding and genetics programmes, resistance to verticillium wilt has been widely explored

in sunflower, and several cultivars with resistance to the main pathogenic races have been

obtained or identified, including new resistance sources (Radi and Gulya, 2007). However,

the appearance of new races of *V*. *dahliae* that overcome the known resistance sources in the

USA, Argentina, and Spain greatly threatens this disease control method (Gulya, 2007;

Garcia-Ruiz *et al*., 2014).

Despite the undeniable importance of *V*. *dahliae*, there are recent reports of emerging

sunflower diseases that can threaten the crop in some cultivation areas (Martin-Sanz *et al*.,

2018a). *Cadophora helianthi* is of particular importance in Ukraine and Russia, where 26%

and 21%, respectively, of the world's sunflower oil is produced (FAOSTAT, 2020). Initially,

C. *helianthi* was mistakenly identified as *C*. *malorum* (Martin-Sanz *et al*., 2018a), but later it

was reported as a new species with special morphological and molecular traits (Crous *et al*.,

2019). Because no reports on *C*. *helianthi* existed until 2018, no control measures are yet

available. Moreover, its geographic distribution and economic importance remain unknown,

although it has been associated with increasing incidence of sunflower wilt in Russia (Martin-

Sanz *et al*., 2018a).

Traditionally, chemical control of plant diseases has relied heavily on synthetic

chemical compounds. However, these are not effective against certain diseases including

verticillium wilt (Klosterman *et al*., 2009). Furthermore, chemical treatments can induce the

resistance of the pathogen to active ingredients (Molinero-Ruiz *et al*., 2003; 2008). Biological

control is a topical option for effective disease management and, within it, entomopathogenic

fungi (EF) are a feasible alternative. The latter are pathogenic to insects, are naturally present in a wide number of environments, and are often associated with plants, via the rhizosphere or even as plant endophytes or epiphytes (Garrido-Jurado *et al.*, 2015). Certain EF have proved to be useful tools for controlling many insect pests within integrated pest management (IPM) strategies (Quesada-Moraga *et al.*, 2014). Thus, more than 170 fungal strains, most of which belong to *Beauveria bassiana* and *Metarhizium anisopliae*, are registered worldwide (de Faria and Wraight, 2007). Their efficacy lies both in the mortality caused by the mechanical damage produced during the propagation of fungal structures through the insect's organs and haemolymph, and in the secretion of insecticidal compounds (Quesada-Moraga *et al.*, 2014).

However, EF can play other ecological roles, such as promoting plant growth (e.g., *Metarhizium brunneum* and *B. bassiana* promoting sorghum and sunflower growth; Raya-Diaz *et al.*, 2017a, 2017b); inducing systemic resistance (e.g., *B. bassiana* eliciting defensive responses in tomato; Shrivastava *et al.*, 2015); producing compounds used in the pharmaceutical industry (e.g., terpenoids, steroids, and phenols; Schulz *et al.*, 2002); enhancing plant nutrition (e.g., *M. brunneum* and *B. bassiana* improving Fe nutrition in sorghum and sunflower; Raya-Diaz *et al.*, 2017a, 2017b); and as antagonists of other fungi (Ownley *et al.*, 2010; Keyser *et al.*, 2016). Recent works have reported some EF as acting as antagonists of plant soilborne pathogens affecting, among others, olive and wheat, both in vitro (e.g., *M. brunneum* and *B. bassiana* inhibiting *Phytophthora inundata*; Lozano-Tovar *et al.*, 2013) and in vivo (e.g., *Clonostachys rosea* and *Metarhizium* spp. reducing wheat infection by *Fusarium culmorum*; Keyser *et al.*, 2016). The mechanisms underlying the antagonism between EF and other fungi are mainly associated with nutrient competition and antimicrobial metabolite production (Lozano-Tovar *et al.*, 2013, 2017), although the nature of those metabolites has not yet been explored. In sunflower, better vegetative growth and

nutrient absorption due to the inoculation with EF was reported (Raya-Diaz *et al*., 2017a), as well as a successful control of two sunflower insect pests: the maize leaf weevil *Tanymecus dilaticollis* (Coleoptera: Curculionidae; Takov *et al*., 2013), and the banded sunflower moth *Cochylis hospes* (Lepidoptera: Tortricidae; Barker, 1999). However, EF interactions with sunflower pathogens have not been studied so far.

The aim of this study was to determine whether five strains of two different EF species (three strains of *M*. *brunneum* and two of *B*. *bassiana*) could be suitable candidates as biological control agents against the sunflower pathogens *V*. *dahliae* and *C*. *helianthi*. Thus, we carried out both in vitro experiments, in which the EF were dually plated against both pathogens, and in planta ones, in which we monitored the effect of the EF on the severity of verticillium wilt symptoms in sunflowers. We also assessed the time lapse of EF in the substrate and performed a molecular detection of the fungi in the plants.

2. Materials and methods

2.1 Fungal isolates

All the isolates of the sunflower pathogens *V*. *dahliae* and *C*. *helianthi* and EF *M*. *brunneum* and *B*. *bassiana* included in this study are shown in Table 1. The five EF strains tested were selected from the culture collection in the Agricultural Entomology laboratory of the Department of Agronomy, University of Cordoba (Spain) and are deposited in the Spanish Collection of Culture Types at the University of Valencia (Spain) (Raya-Diaz *et al*., 2017a). These strains were selected on the basis of their efficacy against insect pests or plant pathogens (Lozano-Tovar *et al*., 2013) and their previous use in sunflower (Raya-Diaz *et al*., 2017a).

All *V. dahliae* isolates used were previously characterized in a multidisciplinary study (Martin-Sanz *et al.*, 2018b) and were selected on the basis of their different geographical origin and pathogenic race (Garcia-Carneros *et al.*, 2014; Martin-Sanz *et al.*, 2018b). *Cadophora helianthi* isolates were selected from the plant pathogen culture collection in the Field Crop Disease Laboratory at the Institute for Sustainable Agriculture from CSIC (IAS-CSIC) in Cordoba, Spain (Molinero-Ruiz, 2019).

2.2 Dual cultures

Dual cultures were carried out to challenge the sunflower pathogens *V. dahliae* and *C. helianthi* against five strains of the EF *B. bassiana* and *M. brunneum* (Table 1). Active cultures were obtained on fresh potato dextrose agar (PDA; BD) by streaking a loop followed by incubation at 25 °C in the dark. Pieces (2 mm^2) of actively growing seven-day-old colonies of either *V. dahliae* or *C. helianthi*, and EF, were dually plated on malt extract agar (MA; Scharlab S. L.) at a distance of 3 cm and incubated under the aforementioned conditions. The dual cultures were evaluated until the control treatment colonies reached the edge of the plate, 37 days after plating. The growth of every colony was recorded periodically by directly drawing its contour on the plate. After the last evaluation, photographs of every plate were taken. Pictures were analysed with the free software GIMP v. 2.8.22 (https://www.gimp.org/). The percentage of the mycelial growth inhibition (IMG) was calculated from the last measurement, corresponding to the definitive growth over the plate. The IMG was expressed as:

$$IMG = 100 \times \frac{\text{Colony diameter in the control } - \text{ Colony diameter in the treatment}}{\text{Colony diameter in the control}}.$$

Experiments were carried out, independently, for *V. dahliae* and *C. helianthi*, in completely randomized factorial designs with three replications (plates) for *V. dahliae* and four for *C. helianthi*, the EF strains and fungal isolates being the two factors.

2.3 Hyphal interaction

Interactions between hyphae of both the sunflower pathogens and the EF were observed under the microscope. For this purpose, a sterile microscope slide was covered with a thin layer of MA and isolates VdS0113 of *V. dahliae* or CadoSU01-17 of *C. helianthi* (Table 1), and the EF strains were placed dually at a distance of 3 cm perpendicular to the slide. The slides were incubated at 25 °C in the dark and monitored until the growth front of both fungi was perceived. They were then observed under a microscope.

2.4 Greenhouse experiment

An experiment was conducted to assess the effect of EF on the development of verticillium wilt symptoms in sunflower under greenhouse conditions. According to the results obtained in the dual culture assays, all the EF isolates in Table 1, except EAMb 01/158-Su (which did not significantly inhibit any of the pathogens), were included in the experiment. The isolate VdS0113 of *V. dahliae*, whose pathogenicity against sunflower was tested in our previous works (Gonzalez-Fernandez, 2015; Martin-Sanz *et al*., 2018b), and the sunflower breeding line RHA801, genetically susceptible to *V. dahliae* (Gonzalez-Fernandez, 2015), were used.

Sunflower seeds were surface-sterilized by a 10-min immersion in 10% sodium hypochlorite, rinsed twice with sterile deionized water and put into Petri dishes containing a thin layer of water-saturated perlite covered with sterile filter paper. Seeds were incubated in darkness at 25 °C for 48 hr until radicles 2–5 mm long developed. Then, the seedlings were transplanted into high density polyethylene trays (19.5 × 26.5 × 6.5 cm) containing sterile vermiculite (one treatment per tray). Each tray was watered with 100 ml tap water and then maintained in a greenhouse at 24 °C and a 14 hr photoperiod for 48 hr until treatments with EF were carried out on plantlets at the VE development stage (Schneiter and Miller, 1981), four days after sowing (DAS).

The EF strains were applied similarly to the method described by Raya-Diaz *et al*. (2017b). They were plated in MA and incubated in the dark at 25 °C for 10–15 days. Fungal suspensions were prepared by scraping the mycelium with a sowing handle and suspending the fungus in 5 ml sterile deionized water. The suspensions were filtered through several layers of sterile cheesecloth to retain the mycelium, and homogenized by vortex. Concentrations were adjusted to 10^8 conidia/ml using a Neubauer chamber (Blau Brand). Each treatment (i.e., four plants; each plant a replication) was watered with 100 ml of the corresponding fungal suspension. Control plants were watered with 100 ml sterile deionized water. Plants were grown in the greenhouse under the aforementioned conditions for 1 month. During this time, they were given a nutrient solution (Hoagland and Arnon, 1950), 100 ml/plant, once a week and watered as needed.

Sunflowers were inoculated with *V. dahliae* 1 month after the treatment with the EF, 34 DAS, when plants were at V6 stage (Schneiter and Miller, 1981). Inoculation was carried out according to Martin-Sanz *et al*. (2018b) by manually uprooting the plants and immersing their root systems in 10^6 conidia/ml suspensions of *V. dahliae*, prepared as described above, for 30 min. Similarly, control plants were uprooted and immersed in sterile deionized water. After the inoculation, plants were individually transplanted into 0.7 L pots containing a mixture of sand:silt:peat (2:1:4) and incubated in the greenhouse under the aforementioned conditions for 1 month. The experiment was carried out in a completely randomized factorial design with four replications (plants), the two factors being *V. dahliae* (inoculated and noninoculated control) and EF (four strains and nontreated control). The severity of symptoms (Ss), expressed as the percentage of foliar tissue showing symptoms of wilting (Garcia-Ruiz *et al*., 2014), was assessed weekly for each plant until the end of the experiment, 4 weeks after inoculation with the pathogen and 64 DAS. By then, plants were at R5 (Schneiter and Miller, 1981). The sequential values were used to calculate the area under

the disease progress curve (AUDPC) by the trapezoidal integration method (Campbell and

Madden, 1990).

The population density of the EF isolates inside the pots was assessed weekly, from

the day on which plants were inoculated with *V. dahliae* (34 DAS) until the end of the

experiment (64 DAS) in accordance with the protocol described by Raya-Diaz *et al*. (2017a)

with slight modifications: briefly, a sample of 3 g substrate was collected at a depth of 0–3

cm randomly from the four replications (plants) of each EF treatment noninoculated with *V.

dahliae* and suspended in 30 ml sterile deionized water and then shaken with an orbital

shaker at 120 rpm for 90 min (Raya-Diaz *et al*., 2017a). The suspensions were diluted 10-fold

in sterile deionized water and 100 µl aliquots were plated on Sabouraud dextrose agar,

supplemented with 0.5 g/L chloramphenicol (SDAC; Scharlab, S. L.). Plates (four replicates

for each treatment) were incubated at 25 °C for 5–7 days. Colonies identified as *M. brunneum*

or *B. bassiana* were counted and cfu per g of substrate were calculated for each EF strain and

replication.

2.5 Microbiological and molecular detection of *V. dahliae* and entomopathogenic fungi in sunflower

The plant colonization of both *V. dahliae* and EF was assessed at the end of the greenhouse

experiment, when the fourth and last evaluation of Ss was done, 64 DAS. For *V. dahliae*, we

used the methodology described by Martin-Sanz *et al*. (2018b): briefly, 2-cm long fragments

of the stem base of each plant were cut and surface-sterilized by a 10 min immersion in 10%

sodium hypochlorite, then rinsed twice and dried under sterile air flow. The fragments were

divided into 2–5 mm pieces, which were plated on PDA and incubated at 25 °C for 5–7 days.

For the EF, we performed the isolation according to Gonzalez-Mas *et al*. (2019): leaves were

removed, sterilized by a 2 min immersion in 1% sodium hypochlorite, rinsed twice and dried

under sterile air flow. Then, fragments of 2 cm^2 were cut, plated on SDAC and incubated at 25 °C for 5–7 days.

For molecular diagnostic analyses, also at the end of the experiment 64 DAS, we surface-sterilized stem tissues as described above and lyophilized them. Total genomic DNA from lyophilized stem tissues of all the plants (noninoculated and nontreated controls, and plants only treated with the entomopathogen, only inoculated with *V. dahliae*, or both treated with the entomopathogen and inoculated with *V. dahliae*) was individually purified using the i-genomic Plant DNA Extraction NucleoSpin Plant II (Macherey-Nagel GmbH and Co. KG) according to the manufacturer's instructions. The quality and concentration of DNA samples were determined with a Qubit 3.0 fluorometer (Invitrogen). Finally, DNA samples were adjusted to a final concentration of 10 ng/µl and stored at −20 °C until required. The presence of fungi as endophytes in sunflower tissues was confirmed by amplification of the region consisting of the 5.8S ribosomal DNA and internal transcribed spacers 1 and 2 using the primer set ITS5/ITS4 (White *et al*., 1990). Optimized PCR assays were carried out in a final volume of 25 µl containing 0.4 µM of each primer, 800 µM dNTPs, 2.5 µl 10 × PCR buffer (800 mM Tris-HCl, pH 8.3–8.4 at 25 °C, 0.2% Tween 20 wt/vol), 0.75 U Horse-Power *Taq* DNA polymerase (Canvax Biotech), 2.5 mM MgCl$_2$, and 10 ng fungal DNA. The following profile was set for the amplifications: 3 min initial denaturation at 95 °C; 30 cycles of 30 s annealing at 56 °C, 2 min of extension at 72 °C and 30 s denaturation at 95 °C; and a final extension step of 10 min at 72 °C. Mycelial DNA of the fungi grown on PDA and sunflower DNA were used as positive controls, and water was used as a negative amplification control. All reactions were made in a T1 thermocycler (Whatman Biometra). Amplification products were separated by horizontal electrophoresis in 3.5% agarose gels containing 0.05 µl/ml GoodView nucleic acid stain (SBS Genetech Co., Ltd.) and visualized over a UV light

source. A 100–2,000 bp BrightMAX DNA ladder (Canvax Biotech) was included in the electrophoresis.

2.6 Data analysis

All the experiments in this work were repeated once and, after assessing the lack of any significant differences between the two replicates (McIntosh, 1983), data were pooled and analysed using Statistix 10 (Analytical Software).

Data expressed as percentages (IMG and Ss) were transformed using an arcsine transformation: $Y = arcsine\sqrt{\frac{Variable}{100}}$. Homoscedasticity (Brown and Forsythe test), normality (Shapiro–Wilk test) and randomization of residues (graphical test) were checked in order to perform an analysis of variance (ANOVA) of transformed IMG (dual culture experiments) and transformed Ss and AUDPC (greenhouse experiment). Dual culture experiments, as well as the greenhouse experiment, were statistically analysed according to completely randomized factorial designs. When significances were found for main factors and/or for their double interaction, means were compared using Fisher's least significant difference (LSD) test ($\alpha = .05$).

3. Results

3.1 Dual cultures

When cultured together with the sunflower pathogens, all the EF strains showed an antagonistic activity, as they significantly inhibited the mycelial growth of both *V. dahliae* ($p < .001$) and *C. helianthi* ($p < .0001$). Furthermore, in dual cultures involving *V. dahliae*, the mycelial growth of the pathogen was significantly dependent on the EF ($p < .0001$) and the

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pathogen isolate ($p < .001$), although the interaction EF × pathogen isolate was not significant ($p = .1761$). The percentage of IMG of *V. dahliae* ranged from 8.3% (*V. dahliae* VdS0216 co-cultured with EABb 04/01-Tip strain of *B. bassiana*) to 63.5% (*V. dahliae* VdS1016 co-cultured with EABb 01/33-Su strain of *B. bassiana*). Additionally, *M. brunneum* EAMb 01/158-Su presented the lowest IMG for four of the six *V. dahliae* isolates, whereas *B. bassiana* EABb 01/33-Su gave the highest inhibition against four of the six isolates (Table 2). Furthermore, EABb 01/33-Su strain caused an average IMG of 47.0% on the six *V. dahliae* isolates, EAMa 01/58-Su of 42.5%, EAMb 09/01-Su of 40.9%, EABb 04/01-Tip of 30.6%, and EAMb 01/158-Su of 24.3%. Lastly, the three *M. brunneum* strains caused an average IMG of 35.7% on the six *V. dahliae* isolates, whereas the two *B. bassiana* strains caused an average IMG of 38.6%.

In the case of *C. helianthi*, the EF significantly affected the pathogen's mycelial growth ($p < .0001$), which was not dependent on *C. helianthi* isolate ($p = .9808$); nor was the interaction between them significant ($p = .3386$). The IMG of *C. helianthi* ranged from 19.6% (EAMb 01/158-Su of *M. brunneum*) to 37.4% (EABb 01/33-Su of *B. bassiana*) (Table 3). The three *M. brunneum* strains caused an average IMG of 24.5% on the three *C. helianthi* isolates, whereas the two *B. bassiana* strains caused an average IMG of 34.8%.

Finally, inhibition halos were observed when *M. brunneum* strain EAMb 01/158-Su was co-cultured with any of the pathogens. In addition, the EF strains EAMb 09/01-Su and EABb 01/33-Su were able to overgrow the mycelium of the pathogens. The EF strain EAMa 01/58-Su was able to facultatively cause inhibition halos and overgrow the pathogens. Types of antagonism are shown in Figure 1: inhibition of *V. dahliae* and/or *C. helianthi* by *M. brunneum* (Figure 1a,c) and overgrowth of *B. bassiana* on *V. dahliae* and/or *C. helianthi* (Figure 1b,d).

3.2 Hyphal interaction

When slides of dual cultures were cut from the culture medium and observed under the microscope, with the exception of the lack of contact between EAMb 01/158-Su strain and either of the pathogens, contact between hyphae of *V. dahliae* or hyphae of *C. helianthi* and all the other EF strains was observed (Figure S1). No antagonistic interactions were observed.

3.3 Greenhouse experiment

Control plants not inoculated with *V. dahliae* did not show any symptoms of verticillium wilt and were excluded from data analysis. Initial symptoms of verticillium wilt were observed in the control plants inoculated with the pathogen 1 week after inoculation, and they reached 95% Ss at the end of the experiment (Figure 2a). Significant reductions in both Ss and AUDPC due to *V. dahliae* were associated with treatments with one strain of each EF species: *M. brunneum* EAMb 09/01-Su (53% and 1096, respectively) and *B. bassiana* EABb 01/33-Su (31% and 475, respectively) compared with the control plants (95% and 2226, respectively). The two remaining EF strains did not have a significant effect on verticillium wilt (either disease severity or AUDPC) (Figure 2a,b).

In the first assessment of the population density of the EF isolates, the cfu/g substrate varied between 8.25×10^5 (EABb 01/33-Su) and 1.2×10^5 (EABb 04/01-Tip) at the moment of inoculation with *V. dahliae* (Figure 3a). Four weeks later, these populations decreased down to 3.75×10^4 (EABb 01/33-Su) and 0 (EABb 04/01-Tip). All the strains showed a marked decrease in populations 1 week after inoculation with *V. dahliae*, with the exception of EABb 04/01-Tip, which showed very low levels throughout the five sampling weeks, and decreased drastically during the last week (Figure 3a). In contrast, the severity of wilt symptoms in plants treated with EF and later on inoculated with *V. dahliae* increased mainly

between the first and second week, and varied little until the end of the experiment (Figure 3b).

3.4 Microbiological and molecular detection of *V. dahliae* and entomopathogenic fungi in sunflower

At the end of the experiment, *V. dahliae* was successfully isolated from those plants that had been inoculated with the pathogen irrespective of the treatment with EF. The isolation percentages ranged from 32% to 64% in the treatments with EABb 01/33-Su and EAMb 09/01-Su, respectively (Table 4). Lastly, none of the EF was successfully isolated from sunflower leaves at the end of the experiment.

With regard to the molecular analyses, amplifications of samples from plants inoculated with *V. dahliae* yielded a 550 bp fragment, which is diagnostic of the pathogen (Figure 4a). However, we were unable to confirm the presence of any of the EF strains in sunflowers inoculated with *V. dahliae*. The most interesting finding was that, in the absence of *V. dahliae*, bands of similar sizes to those of *B. bassiana* and *M. brunneum* were amplified, showing that the four EF strains succeed in endophytically colonizing the plants (Figure 4b). As expected, all the samples yielded the 750 bp band diagnostic of sunflower (Figure 4a,b).

4. Discussion

In this work, we assessed the performance of five strains of EF as biological control agents against the sunflower pathogens *V. dahliae* and *C. helianthi*. Our results show that species of *Metarhizium* and *Beauveria* can play an active role as antagonists of those pathogens. Both the EF strain and the *V. dahliae* isolate had a significant effect on the mycelial growth of the pathogen when co-cultured with the EF, whereas under the same experimental conditions, the

growth of *C. helianthi* was only dependent on the EF strain. This is not surprising, as *V. dahliae* affecting sunflowers in Europe exhibits a wide diversity that is highly dependent on its geographical origin (Martin-Sanz *et al*., 2018b). As for *C. helianthi*, it was recently reported as being a pathogen of sunflower (Crous *et al*., 2019). Although species diversity information is still needed, heterogeneity was not expected within just the three isolates included in our experiments. The most revealing finding was that, in spite of EF inhibiting the growth of both pathogens, no trend in their effect was observed as being associated with either genus (*B. bassiana* or *M. brunneum*), and one *B. bassiana* strain was the most effective one (EABb 01/33-Su) against *C. helianthi* and most of the *V. dahliae* isolates. Similarly, the antagonism of EF against the olive pathogens *V. dahliae*, *Phytophthora megasperma* and *P. inundata* was largely dependent on the particular strain more than on any other factor (Lozano-Tovar *et al*., 2013).

From the results of the dual culture experiments and the microscope observations, we identified two types of antagonism exerted by EF against plant pathogenic fungi. First, mycelial growth inhibition of the pathogens by two strains of *B. bassiana* (EAMb 09/01-Su and EABb 01/33-Su) and one of *M. brunneum* (EAMa 01/58-Su) was associated with overgrowth of the EF on both *V. dahliae* and *C. helianthi*. Not only do our EF strains have notoriously high growth rates (Quesada-Moraga *et al*., 2014; Raya-Diaz *et al*., 2017a; 2017b), but the ability of *M. brunneum* strain EAMa 01/58-Su to grow over the mycelia of olive root rot pathogens has already been reported (Lozano-Tovar *et al*., 2013). Similarly, Varo *et al*. (2016) carried out dual cultures to test the efficacy of several biological control agents against an isolate of *V. dahliae* that was pathogenic to olive. They identified two modes of antagonism: clear inhibition zones without *V. dahliae* mycelium, and the growth of the biocontrol agents over the pathogen. In our microscopy work, we observed contact and even intertwining of the hyphae of the confronted fungi in the absence of any mechanical

alteration or degradation, which is in agreement with competition as being the mode of action of *M. brunneum* and *B. bassiana*. Other authors have also proposed competition as at least one of the modes of action operating in disease suppression by EF (Ownley *et al.*, 2010; Lozano-Tovar *et al.*, 2013). Secondly, the presence of highly marked inhibition zones when EAMb 01/158-Su strain of *M. brunneum* was dually plated with the pathogens, and hyphae that were clearly distant from those of either *V. dahliae* or *C. helianthi* under the microscope, suggest that the detrimental effect of the strain is associated with the release of diffusible inhibitory substances. An antibiotic effect of *M. brunneum* against olive pathogens has already been reported by Lozano-Tovar *et al.* (2017). The chemical identification of compounds produced by EAMb 01/158-Su strain displaying antibiosis in our experiments is the subject of further work.

Regarding the development of verticillium wilt in sunflowers treated with *B. bassiana* or *M. brunneum* (strains EABb 01/33-Su and EAMb 09/01-Su, respectively) and thereafter inoculated with the pathogen, both entomopathogens were associated with significant disease reductions, with *B. bassiana* (EABb 01/33-Su) having the most pronounced effect. Although none of the EF strains were detected inside the plants in the presence of *V. dahliae*, an outstanding finding was that, in the absence of the pathogen, the four EF strains succeeded in establishing themselves in sunflowers. Despite these EF strains being well known as transient colonizers of different hosts when applied by foliar spraying (Resquín-Romero *et al.*, 2016; Garrido-Jurado *et al.*, 2017), in our work, with soil drenching applications, they were not only able to survive and settle in the substrate, but also to penetrate the plants and establish themselves as endophytes for weeks. Even though the entomopathogens were unable to compete against *V. dahliae* inside sunflowers, their ability to colonize the plants is an essential feature that could be useful against sunflower pathogens other than *V. dahliae* or even as plant growth promoters (Ownley *et al.*, 2010). Also, the protection conferred by

entomopathogenic fungi against verticillium wilt might be due, at least for the most part, to the direct competition between *V. dahliae* and *M. brunneum* (EAMb 09/01-Su) or *B. bassiana* (EABb 01/33-Su) in the soil. Both of these strains are highly adapted to the soil environment (Garrido-Jurado *et al*., 2017), which seems to favour their competition with *V. dahliae*. Varo *et al*. (2016) tested a wide variety of microorganisms against verticillium wilt in olive, reporting that both the antagonism in the soil/plant and systemic mechanisms could contribute to a relief of symptoms, thus controlling the disease. In this respect, Raya-Diaz *et al*. (2017a) reported that EF could promote sunflower growth and inflorescence production under controlled conditions due to an enhancement in Fe bioavailability. Whether this could be related to some extent to the triggering of systemic resistance in sunflower, consequently having a role in disease control, should be explored in future research.

In accordance with Raya-Diaz *et al*. (2017b), who determined that soil treatment was the best option for the application of EF because it resulted in good population recovery levels, we found that the four EF strains dramatically decreased during the first 4 weeks in the soil (from treatment to inoculation) and, thereafter, during the following 4 weeks. One of the strains (EABb 04/01-Tip) was even unable to remain in the soil as a stable population. Moreover, and as mentioned above, the persistence of EABb 01/33-Su and EAMb 09/01-Su populations in the soil could be the consequence of their adaptation to this environment and a first requirement for controlling verticillium wilt. Also, the transient colonization of melon plants by these same strains after foliar applications has been reported (Garrido-Jurado *et al*., 2017), but this does not seem to be the operating mechanism in our experiment in which treatments were conducted by soil drenching. Although present in the soil, none of the three strains was reisolated from plant samples, suggesting that the observed in vivo effect of EF against *V. dahliae* is not plant-located, but is most likely the consequence of the intense competition waged by *B. bassiana* (EABb 01/33-Su) and/or *M. brunneum* (EAMb 09/01-Su)

against the pathogen in the soil, as already mentioned. Moreover, as a result of the profuse growth of these EFs in comparison to that of *V. dahliae*, pathogen soil populations might be decreased, and root penetration and the development of eventual symptoms delayed in time, as suggested by the positive identification of *V. dahliae* in plants treated with the EF. In addition to the soil-mediated interplay between EABb 01/33-Su or EAMb 09/01-Su and *V. dahliae* suggested by the results of this work, further research should explore whether these strains are able to penetrate sunflower roots and inhibit *V. dahliae* in the host.

Few studies relating EF and insect pests of sunflower have been carried out so far. One by Takov *et al*. (2013) reported the presence of *B. bassiana* associated with a sunflower crop in Bulgaria. According to the authors, the indigenous EF caused high mortality rates in the sunflower pest *Tanymecus dilaticollis*. Similarly, Barker (1999) tested the efficacy of *Metarhizium anisopliae* (Metsch) and *B. bassiana* against the sunflower pest *Cochylis hospes* under laboratory conditions. In spite of the high efficacy in controlling sunflower insect pests reported in both works, the long-term survival of EF was also pointed out as being a priority for potential field applications.

In conclusion, both *M. brunneum* and *B. bassiana* are able to inhibit the mycelial growth of *V. dahliae* and *C. helianthi* and, according to the dual culture and microscopy results, two types of antagonism can operate: competition and/or antibiosis. In addition, although the entomopathogens were not found inside the plants in the presence of *V. dahliae*, they were when the pathogen was absent. In fact, they were able to establish themselves inside sunflowers, suggesting their potential utility in applications such as, for instance, those to control sunflower diseases other than verticillium wilt, or to promote sunflower growth. Finally, the ability of EF populations to persist in the substrate for several weeks, together with disease reduction observed in treatments with some strains, suggests that the protection

they confer against verticillium wilt might not be plant-located, but is more likely to be the consequence of their competition with *V. dahliae* in the soil.

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Conflict of interests

The authors declare that they have no competing interests.

Data availability statement

The data that support the findings of this study are available from the authors upon reasonable request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1 Hyphal contact between an entomopathogenic fungus (*Metarhizium brunneum* or *Beauveria bassiana*) and *Verticillium dahliae* or *Cadophora helianthi* in dual cultures. (a) *M. brunneum* versus *V. dahliae*; (b) *B. bassiana* versus *V. dahliae*; (c) *M. brunneum* versus *C. helianthi*; (d) *B. bassiana* versus *C. helianthi*.

Figure legends

Figure 1 Antagonism exerted by entomopathogenic fungi *Metarhizium brunneum* and *Beauveria bassiana* on sunflower pathogens *Verticillium dahliae* and *Cadophora helianthi* in

dual cultures. (a) Inhibition on *V. dahliae* VdS0216 by *M. brunneum* EAMa 01/58-Su; (b)

overgrowth of *B. bassiana* EABb 01/33-Su on *V. dahliae* VdS0916; (c) inhibition on *C.*

helianthi CadoSU01-17 by *M. brunneum* EAMb 01/158-Su; (d) overgrowth of *B. bassiana*

EABb 01/33-Su on *C. helianthi* CadoSR02-16.

Figure 2 Verticillium wilt severity, expressed as (a) percentage of the foliar tissue affected

(%) 4 weeks after pathogen inoculation (64 days after sowing); and (b) area under disease

progress curve (AUDPC) at the same time, in sunflower plants inoculated with *Verticillium*

dahliae and treated with different entomopathogenic fungi. Control plants were inoculated

with *V. dahliae* alone. Letters on the bars indicate homogeneous groups according to LSD

post hoc test ($p < .05$). Analyses of verticillium wilt severity (a) were performed on

transformed data using an arcsine transformation.

Figure 3 (a) Populations of four entomopathogenic fungi recovered from the substrate of

pots in which sunflower plants were grown. Inoculation of sunflower plants with *Verticillium*

dahliae was performed in week 0. Verticillium wilt symptoms were assessed between weeks

1 and 4. (b) Time course expression of verticillium wilt symptoms (severity of symptoms, %)

in EF-treated plants from weeks 1 to 4 after inoculation with *V. dahliae*. Vertical bars

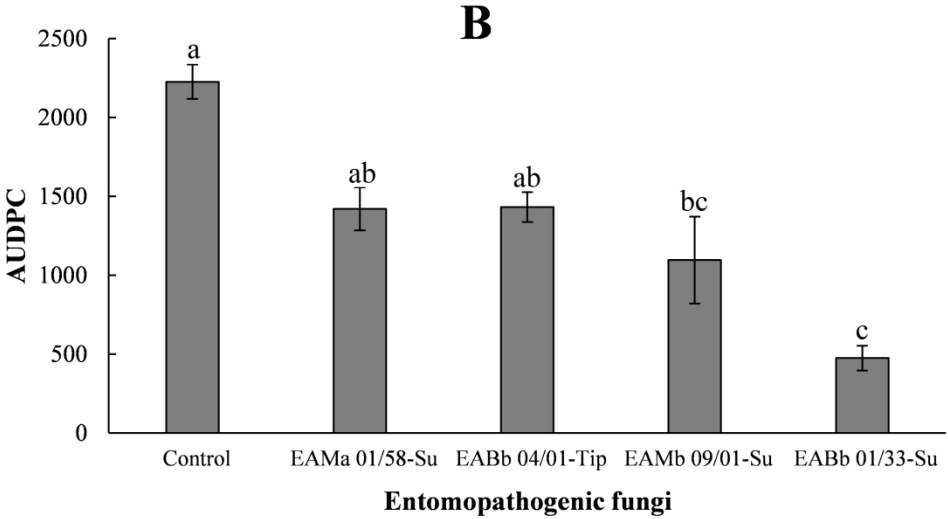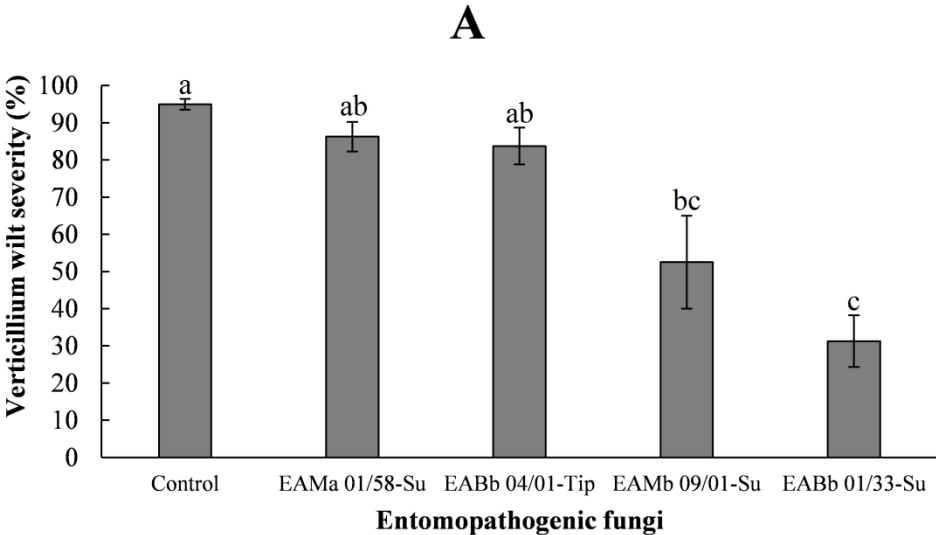represent the severity of symptoms in inoculated plants averaged across EF treatments.

Figure 4 Band patterns obtained after PCR amplification of the ribosomal DNA region (5.8S

rDNA and internal transcribed spacers 1 and 2) with the universal primer set ITS5 and ITS4

from individual sunflower plants. DNA samples were obtained from lyophilized stem tissue

of sunflower plants (breeding line RHA801, R5 stage, 64 days after sowing) treated with: (a)

four entomopathogenic fungi (EF) strains: *Beauveria bassiana* EABb 01/33-Su and EABb

04/01-Tip, and *Metarhizium brunneum* EAMa 01/58-Su and EAMb 09/01-Su (always in the

same order). Lanes 1–6, DNA from sunflower breeding line RHA801, *V. dahliae* isolate

VdS0113, and the four EF strains; lanes 7–14, DNA from two sunflowers only treated with the four EF strains. (b) EF followed by inoculation with *Verticillium dahliae* isolate VdS0113. Lanes 1–5, DNA from sunflower, and the four EF strains; lanes 6–13, DNA from two sunflowers treated with the four EF strains and inoculated with *V. dahliae* 1 month later. M, 100–2,000 bp BrightMAX DNA ladder (Canvax Biotech).
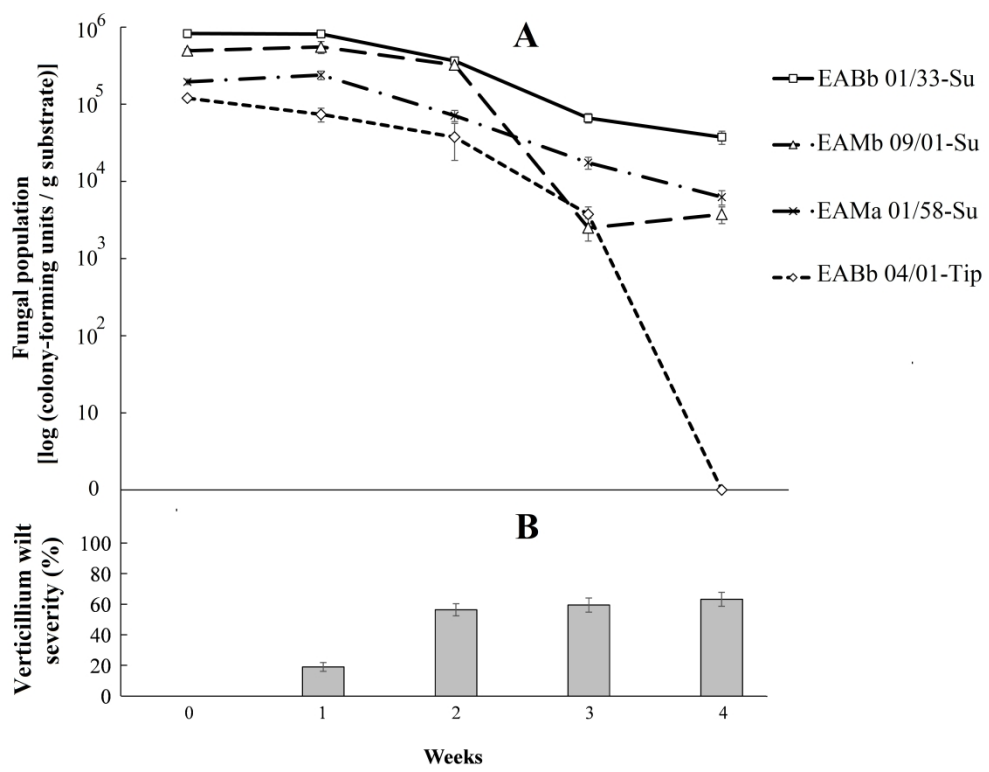
426x421mm (96 x 96 DPI)
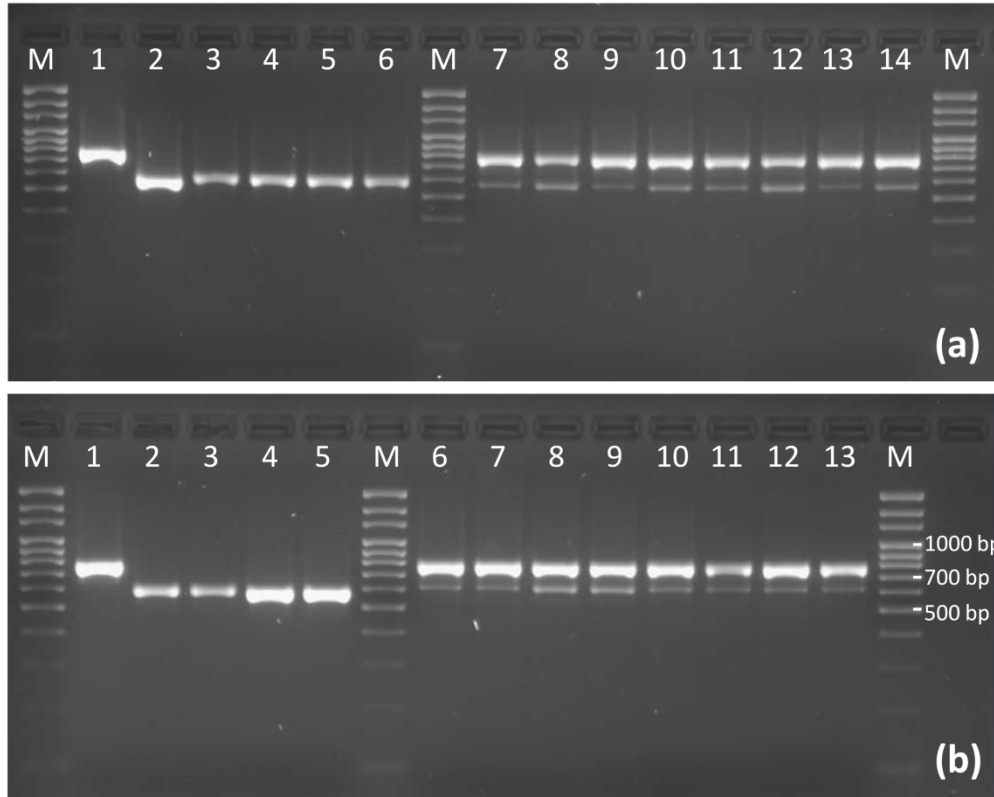
A

Verticillium wilt severity (%) vs Entomopathogenic fungi

B
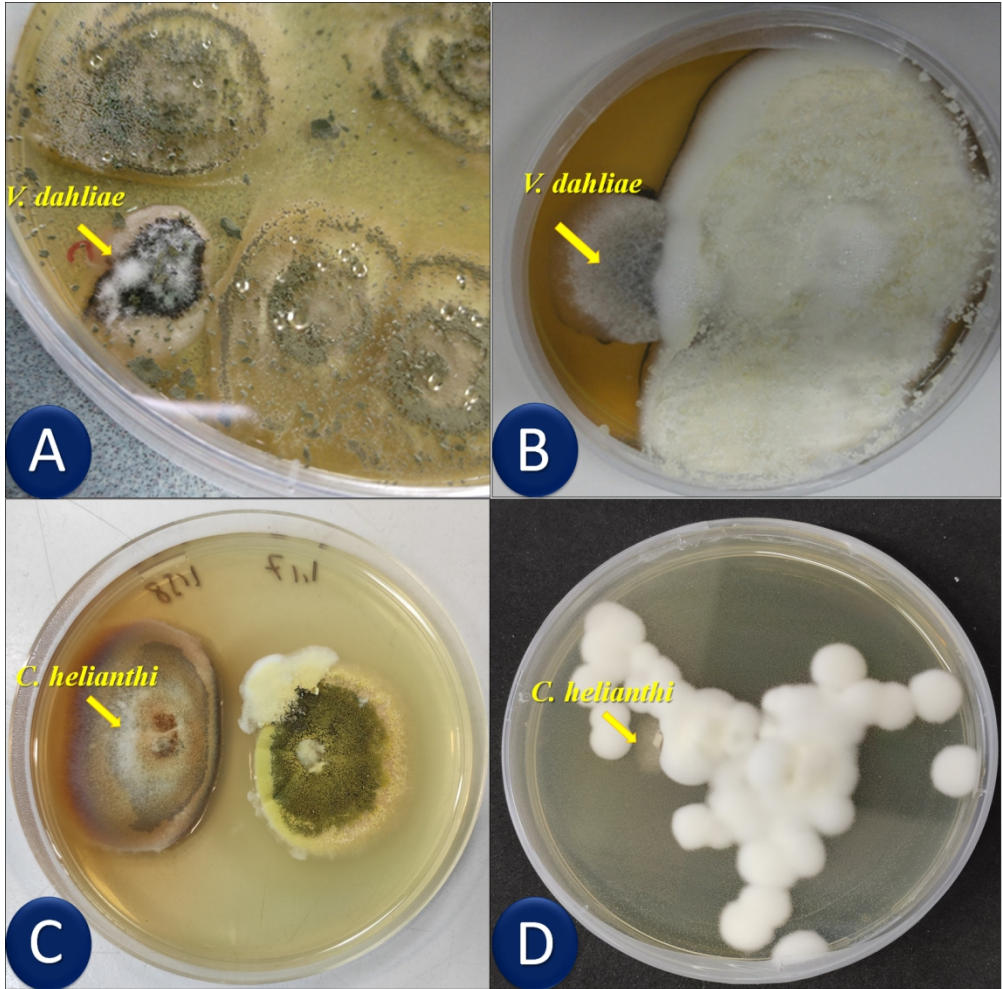
AUDPC vs Entomopathogenic fungi

886x1024mm (96 x 96 DPI)

1439x1132mm (96 x 96 DPI)

745x599mm (96 x 96 DPI)

Plant Pathology

Table 1 Fungal isolates used in this work, with a list of the experiments in which they were used

Fungal species	Isolate	Host of isolation	Origin	Experiments
Verticillium dahliae	VdS0113	Sunflower	Cadiz, Spain	Greenhouse experiment
	VdS1014	Sunflower	Valu lui Traian, Romania	Dual cultures
	VdS0216	Sunflower	Montech, France	Dual cultures
	VdS0316	Sunflower	Macerata, Italy	Dual cultures
	VdS0616	Sunflower	Slava Rusa, Romania	Dual cultures
	VdS0916	Sunflower	Kastamonu, Ahmetbey, Turkey	Dual cultures
	VdS1016	Sunflower	Manvelivka, Dnepropet, Ukraine	Dual cultures
Cadophora helianthi	CadoSR02-16	Sunflower	Orenburg, Russia	Dual cultures
	CadoSR03-16	Sunflower	Orenburg, Russia	Dual cultures
	CadoSU01-17	Sunflower	Kiev, Ukraine	Dual cultures
Metarhizium brunneum	EAMa 01/58-Su	Wheat	Cordoba, Spain	Greenhouse experiment, dual cultures
	EAMb 01/158-Su	Olive	Seville, Spain	Dual cultures
	EAMb 09/01-Su	Wheat	Seville, Spain	Greenhouse experiment, dual cultures

Beauveria bassiana	EABb 01/33-Su	Olive	Cadiz, Spain	Greenhouse experiment, dual cultures
	EABb 04/01-Tip	*Iraella luteipes*	Seville, Spain	Greenhouse experiment, dual cultures

Table 2 Effect of different strains of the entomopathogenic fungi (EF) *Metarhizium brunneum* and *Beauveria bassiana* on the vegetative growth of *Verticillium dahliae*, expressed as inhibition of the mycelial growth (IMG)

Isolate	EF	EF strain	IMG (%)[a]
VdS1014	*M. brunneum*	EAMa 01/58-Su	53.8 ± 7.0 a
		EAMb 01/158-Su	27.0 ± 9.2 b
		EAMb 09/01-Su	53.9 ± 6.7 a
	B. bassiana	EABb 01/33-Su	45.6 ± 6.8 ab
		EABb 04/01-Tip	41.8 ± 9.4 ab
VdS0216	*M. brunneum*	EAMa 01/58-Su	37.8 ± 10.1 a
		EAMb 01/158-Su	18.7 ± 5.9 ab
		EAMb 09/01-Su	23.9 ± 5.2 ab
	B. bassiana	EABb 01/33-Su	39.5 ± 10.2 a
		EABb 04/01-Tip	8.3 ± 4.3 b
VdS0316	*M. brunneum*	EAMa 01/58-Su	38.7 ± 1.9 ab
		EAMb 01/158-Su	23.4 ± 3.3 b
		EAMb 09/01-Su	38.7 ± 7.2 ab
	B. bassiana	EABb 01/33-Su	41.1 ± 5.7 a
		EABb 04/01-Tip	29.9 ± 5.9 ab
VdS0616	*M. brunneum*	EAMa 01/58-Su	44.0 ± 6.4 a
		EAMb 01/158-Su	28.8 ± 6.7 ab
		EAMb 09/01-Su	43.9 ± 6.1 a
	B. bassiana	EABb 01/33-Su	42.9 ± 11.4 ab
		EABb 04/01-Tip	22.0 ± 5.8 b
VdS0916	*M. brunneum*	EAMa 01/58-Su	33.8 ± 6.6 b
		EAMb 01/158-Su	24.3 ± 5.4 b
		EAMb 09/01-Su	46.1 ± 12.3 ab
	B. bassiana	EABb 01/33-Su	57.2 ± 6.5 a
		EABb 04/01-Tip	55.6 ± 3.5 a
VdS1016	*M. brunneum*	EAMa 01/58-Su	47.5 ± 5.9 ab
		EAMb 01/158-Su	23.4 ± 6.9 c
		EAMb 09/01-Su	34.8 ± 5.6 bc
	B. bassiana	EABb 01/33-Su	63.5 ± 8.2 a

EABb 04/01-Tip 36.4 ± 9.7 bc

Note. Plates were incubated at 25 °C in the dark for 37 days, the time needed for the control of each *V. dahliae* isolate to reach the edge of the plate. For each *V. dahliae* isolate, means with a common letter are not significantly different according to Fisher's least significant difference test. Analyses were performed on transformed data using an acrsine transformation.

[a]Mean $\pm$ *SE* of three replications of IMG expressed as percentages.

Table 3 Effect of different strains of the entomopathogenic fungi (EF) *Metarhizium brunneum* and *Beauveria bassiana* on the vegetative growth of three isolates of *Cadophora helianthi*, expressed as inhibition of the mycelial growth (IMG)

EF	EF strain	IMG (%)[a]
M. brunneum	EAMa 01/58-Su	30.4 ± 3.2 ab
	EAMb 01/158-Su	19.6 ± 2.9 c
	EAMb 09/01-Su	23.5 ± 3.0 bc
B. bassiana	EABb 01/33-Su	37.4 ± 3.1 a
	EABb 04/01-Tip	32.2 ± 2.7 a

Plates were incubated at 25 °C in the dark for 37 days, the time needed for the control of each *C. helianthi* isolate to reach the edge of the plate. Means with a common letter are not significantly different according to Fisher's least significant difference test. Analyses were performed on transformed data using an acrsine transformation.

[a]Mean ± *SE* of four replications of IMG expressed as percentages.

Table 4 Isolation of *Verticillium dahliae* (%) from sunflower plants inoculated with *V. dahliae* only (control) or with *V. dahliae* and one strain of entomopathogenic fungi, four weeks after inoculation with the pathogen

Strain	Isolation of *V. dahliae* (%)
Control plants	60.7 ± 9.0
EABb 01/33-Su	32.1 ± 6.8
EABb 04/01-Tip	42.9 ± 14.3
EAMb 09/01-Su	64.3 ± 9.2
EAMa 01/58-Su	46.4 ± 14.7

Note. Data show mean ± *SE*.