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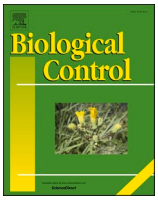

Combined effects of root-associated entomopathogenic and mycorrhizal fungi on the foliar pathogen *Botrytis cinerea* in tomato

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HIGHLIGHTS

- Entomopathogenic fungi (EPF) reduced foliar disease symptoms in tomato plants.
- Arbuscular mycorrhizal fungi (AMF) only affected plant growth, not disease.
- Fungal combinations showed functional complementarity for plant protection and growth.
- Effects in combinations were independent of root colonization pattern.
- Root inoculation by EPF decreased in presence of AMF, while AMF was unaffected by EPF.

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ABSTRACT

Many fungi live intimately associated with plants and may benefit or harm the host plant. Improved knowledge of such interactions is needed for increasing plant health and crop productivity by implementation of fungal inoculants. Co-inoculations of different beneficial fungi offer the possibility to understand complex plant–microbe interactions that may be functionally complementary for improved plant production and protection. Here, we studied the individual and combined effects of the arbuscular mycorrhizal fungus (AMF) *Funneliformis mosseae* with three isolates of entomopathogenic fungi (EPF), representing *Metarhizium brunneum*, *M. robertsii* and *Beauveria bassiana*, on protection against the foliar phytopathogen *Botrytis cinerea* and on plant growth. Seedlings of tomato (*Solanum lycopersicum* L. var. Moneymaker) were inoculated in the substrate with AMF or EPF alone and in dual combinations under greenhouse conditions. Inoculation with the different EPF isolates reduced lesion sizes of *B. cinerea* on inoculated tomato leaves, but only in the experimental repetition that showed highest level of disease severity. The AMF *F. mosseae* had no additional effect on *B. cinerea* lesion size in combinations with EPF. In the experimental repetition with least disease severity, the AMF treatment led to limited increase of *B. cinerea* lesion sizes. In general, *F. mosseae* caused an increase in plant biomass, and the co-inoculations of AMF and EPF did in some combinations increase plant growth. Below-ground interactions between AMF and EPF were observed, as the presence of AMF in the roots was associated with a decrease of EPF root colonization densities. However, AMF colonization rates were unaffected by EPF presence. The study indicated a functional complementarity between EPF and AMF by suppressing phytopathogens and increasing plant growth, respectively. However, it further revealed the challenge of obtaining consistent results of plant–microbe-phytopathogen interactions, which must be overcome for future implementation of beneficial fungi as inoculants in plant production.

Abbreviations: EPF, Entomopathogenic fungi; AMF, Arbuscular mycorrhizal fungi; Bb, *Beauveria bassiana*; Mb, *Metarhizium brunneum*; Mr, *Metarhizium robertsii*; Fm, *Funneliformis mosseae*.

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1. Introduction

The application of beneficial soil microorganisms in agriculture has received an increasing interest due to their potential to protect plants against pests and diseases as well as affecting plant growth (Jain et al., 2013; Kumar et al., 2016; Santos et al., 2019; Mondal et al., 2020). In this sense, the use of microbial combinations could lead to improved benefits from the microbes involved (He et al., 2019; Mondal et al., 2020; Sammauria et al., 2020, Minchev et al., 2021). Even though some works report on the multifaceted benefits from combinations of microbes (He et al., 2019; Vishwakarma et al., 2020; Aguilar-Paredes et al., 2020), there is still a lack of consistency when microorganisms are applied in combination under different conditions (Gadhave et al., 2016; Lee-Diaz et al., 2021). The differences in the effects of microbes on crops are explained by the biotic interactions with the host plants and their associated microorganisms (Gadhave et al., 2016), and the variable abiotic conditions that influence the plant–microbe interactions (Vimal et al., 2017).

Inoculation of a single microorganism has been the standard method to correlate the effect of the inoculant with the improvement of desirable traits in the plant. Nowadays, there is an increasing interest to use consortia of microbial inoculants (Canfora et al., 2021) and dual or multispecies inoculations constitute approaches to evaluate how the microbes interact with each other and with the plant for optimizing the effectiveness of combinations (Straub et al., 2008; Gadhave et al., 2016; Minchev et al., 2021). For instance, if the co-inoculation has a negative effect on the plant compared to the respective single inoculations, it should be determined if the constraints are due to competition between the inoculated strains, to antagonism of the inoculant with the host plant or to environmental conditions (Polis et al., 1989; Gadhave et al., 2016). Alternatively, if the co-inoculated microbes have more positive effects on the plant than when inoculated alone, the microbes may have complementary roles to each other (Hooper et al., 2005). Finally, if the effects of dual inoculation are neutral, i.e. similar to single inoculations, it could be explained by functional redundancy of the microbes in that particular context (Casula et al., 2006).

This knowledge area is still poorly explored, and while some studies observed similar effect against arthropod pests indicative of functional redundancy of the co-inoculants as compared with single inoculations (Canassa et al., 2019; Martinuz et al., 2012), in other works resulted in stronger effects in specific traits or measured variables when compared to use of individual microbes (Shrivastava et al., 2015; Oliveira et al., 2017; Takács et al., 2018). For example, dual fungal inoculations of tomato plants reduced larval weight of the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) more than each single inoculations of the same fungi (Shrivastava et al., 2015). Other studies found complementary effects on plants despite microbial antagonism between the inoculants (Zitlalpopoca-Hernandez et al., 2017), indicating the complexity of these interactions.

In the present work, two groups of root associated-fungi, arbuscular mycorrhizal fungi (AMF) and entomopathogenic fungi (EPF), were studied for their effects on tomato plant resistance against a phytopathogen and on plant growth under greenhouse conditions. AMF are obligate mutualistic symbionts colonizing plant roots (Thygesen et al., 2004; Fritz et al., 2006; Song et al., 2015; Mustafa et al., 2017; Bidellaoui et al., 2019; Ravnskov et al., 2020). They improve plant nutrient acquisition and abiotic stress tolerance, but AMF can also protect the plant against attack by necrotrophic pathogens and arthropod herbivores through the induction of systemic resistance (Pozo and Azcón-Aguilar, 2007). EPF are insect and mite pathogens, which infect through the cuticle to consume the internal tissues of the host, eventually leading to host death (Hajek and Meyling, 2018). However, several taxa of EPF in the phylum Ascomycota, order Hypocreales, can also associate with plants as rhizosphere colonizers and endophytes, potentially protecting the plant against pests and pathogens (Jaber and Ownley, 2018; Gange et al., 2019). Protection against arthropod herbivores is likely mediated by activation of plant defense mechanisms in the EPF-colonized plants (Raad et al., 2019; Ahmad et al., 2020; Rivas-Franco et al., 2020; Jensen et al., 2020; Cachapa et al., 2021; Rasool et al., 2021a,b), while protection against phytopathogens is less studied (Jaber and Ownley, 2018).

The combined effects of EPF and AMF as dual plant inoculants have been evaluated only in few studies (Gualandi et al., 2014; Shrivastava et al., 2015; Zitlalpopoca-Hernandez et al., 2017). However, positive effects of their combination on plant defense against insect herbivores have been observed by increased tolerance to attack (Zitlalpopoca-Hernandez et al., 2017). Specifically, the combination of native AMF populations and inoculation of the EPF *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) resulted in a higher tolerance in maize to damage by the root-feeding insect *Phyllophaga vetula* (Horn) (Coleptera: Scarabaeidae) (Zitlalpopoca-Hernandez et al., 2017). Similarly, dual inoculation of two AMF species (*Rhizophagus intrarradices* and *Gigaspora margarita*) and *B. bassiana* promoted plant growth more than single inoculation with the AMF in purple coneflower (Gualandi et al., 2014). So far, AMF have only been evaluated in combinations with isolates of *B. bassiana*, which is expected to colonize root systems more transiently than isolates of *Metarhizium* spp. (Hypocreales: Clavicipitaceae), which are found predominantly in the rhizosphere of many plants (Behie et al., 2015; Rasool et al., 2021a). These two groups of EPF may therefore produce different responses when combined with AMF.

Here, we tested the effects of the AMF *Funneliformis mosseae* (T.H. Nicolson & Gerd) (Glomeromycota: Glomerales) and three isolates of EPF each belonging to *B. bassiana, Metarhizium robertsii* J.F. Bisch., S.A. Rehner & Humber and *Metarhizium brunneum* Petch on plant protection against the foliar phytopathogen *Botrytis cinerea* Pers. (Ascomycota: Helotiales) when inoculating roots of tomato plants individually and in pairwise combinations. In addition, plant growth was also assessed by measuring final plant biomass. Since different mechanisms to increase plant biomass have been reported for the two fungal groups, AMF by transferring phosphorus (George et al., 1995; Ezawa and Saito, 2018) and EPF by transfer of nitrogen (Behie et al., 2012; Behie and Bidochka, 2014), we could expect complementary effects by dual inoculation of AMF and EPF on plant growth. Similarly, it could be predicted that the two fungi are linked with protection against foliar phytopathogens through the modulation of plant defenses (Pozo and Azcón-Aguilar, 2007; Jaber and Ownley, 2018; Raad et al., 2019; Sanmartin et al., 2020b), which could be induced in different ways. We specifically asked whether combined fungal inoculation would have more positive effects on plant performance (resistance against *B. cinerea* and plant growth) as compared to single inoculations, and we explored the compatibility of the two fungal groups during root colonization.

2. Materials and methods

2.1. Experimental design

An experiment with a randomized factorial design was set up under greenhouse conditions. The first factor included treatment with or without a strain of the AMF *F. mosseae.* The second factor included four levels of treatment with EPF: *Metarhizium brunneum* KVL 16–36, *M. robertsii* KVL 12–35, *B. bassiana* KVL 13–39, or no EPF (control). Each treatment included eight independent plants as biological replicates, giving 64 experimental units. The whole experiment was repeated twice (Experiment A and B). The two experimental repetitions had the same duration but were set up and harvested one week apart.

2.2. Greenhouse experiment

Seeds of tomato (*Solanum lycopersicum* L. var. "Moneymaker") were surface sterilized by immersion in ethanol (70%, 1 min), sodium hypochlorite (1%, 10 min), washing three times in sterilized water, and air drying for 30 min inside laminar air flow. The seeds were kept in the

fridge until use (maximum 3 months of storage). Seeds were sown in a disinfected plastic tray (25x35x10 cm) with sterilized vermiculite. After two weeks, seedlings were transplanted to 300 mL pots filled with sterilized substrate composed of vermiculite (fine grade 2) and sand (1:1 v/v). Seedlings were inoculated as described below:

The AMF *F. mosseae* (strain BEG12; formerly *Glomus mosseae*) from the International Bank of Glomeromycota was provided by the Mycorrhiza Lab at Estación Experimental del Zaidín (CSIC), Granada, Spain. The inocula are continuously maintained in an open-pot culture of *Trifolium repens* L. mixed with *Sorghum vulgare* Pers. (Steud.) Millsp. & Chase plants in a greenhouse. The inocula consist of substrate (vermiculite/sepiolite, 1:1), spores, mycelia, and infected root fragments from those cultures. Pots for mycorrhizal treatments were inoculated by adding 10% (v/v) of this *F. mosseae* inoculum. Un-inoculated control plants received a 3 mL aliquot of a filtrate (<20 μm) of the AMF inocula, in order to provide any microbial population free of AMF propagules and thereby eliminate the role of potential AMF-associated microbiota on treatments with AMF.

The three isolates of EPF are stored in a culture collection at −80 °C at Department of Plant and Environmental Sciences, University of Copenhagen, Denmark. The EPF isolates were grown on Saboraud Dextrose Agar (SDA) for three weeks at 24 °C in darkness. Conidial suspensions were obtained by scratching the culture surface of the Petri dishes to release conidia into a sterilized solution of Triton X (0.05 %). After centrifuging twice (3000 rpm, 3 min) to discard mycelium and debris in the supernatant and resuspending in 0.05% Triton X, conidial concentrations were determined by serial dilutions and counting conidia in a hemocytometer (Fuchs-Rosenthal). For all suspensions, the conidial viability was estimated by adding 100 μL of each conidial suspension on two SDA plates, followed by incubation for 22 h at 24 °C in darkness, and observation under light microscopy (400x). Conidia were considered as germinated if germ tubes were more than twice the length of the conidia. Suspensions with > 90% germination rate were used for experiments. The suspensions were prepared to contain $1x10^8$ conidia/mL in 0.05% Triton-X. Inoculations were done on two-week-old tomato seedlings immediately after transplanting by adding 1 mL of either EPF suspension or control (0.05% Triton-X) directly to the roots by drenching.

2.3. Plant growth, harvest and analysis

Plants were randomly distributed and grown in a greenhouse at 24/18 °C with 16/8 hrs. day/night cycle and 70% humidity. Plants were watered daily (tap water) during six weeks after transplanting, and fertilized twice per week with 50 mL of a modified Long Ashton nutrient solution (Hewitt, 1966) containing 25% of the standard phosphorus (P) concentration.

Plants were harvested 6 weeks after inoculation. The roots were gently washed with tap water and the shoots separated from the roots by cutting. Fresh weight of roots and shoots was registered. Two subsamples of each root system were collected for quantifying colonization of EPF and for determination of AMF colonization, respectively.

2.4. Botrytis cinerea infection bioassay

Prior to the bioassay, *Botrytis cinerea* CECT2100 (Spanish collection of type cultures, Universidad de Valencia, 46,100 Burjassot, Spain) was grown for 3 weeks at room temperature on half strength Potato Dextrose Agar (PDA) plates. Conidia were collected from the PDA plates by scratching the surface with a spatula with half strength Potato Dextrose Broth (PDB) and pregerminated in darkness for 2 h in Gambor's B5 medium (Duchefa Biochemie) supplemented with 10 mM KH_2PO_4 and 10 mM sucrose. Inoculations of *B. cinerea* were applied to detached tomato leaves of 8-weeks old plants (6 weeks after the AMF/EPF inoculations), cut during harvesting of the plants (see section 2.3). The third or fourth true leaf of each plant was detached with a sharp blade, placed in a Petri dish (140 mm × 20 mm) on wet paper (5 mL sterilized water) and challenged with *B. cinerea* by applying 5 μL of conidia suspension (10^6 conidia/mL) onto each of the five leaflets per leaf. Petri dishes were randomly distributed and incubated at 24 °C, 70% relative humidity, for 5 days. Diameters of the lesions were determined for each leaflet with a digital Vernier caliper.

2.5. Quantification of root colonization by EPF

Root pieces of one subsample from each plant were cut into 1 cm sections and manually mixed. Fifteen randomly selected root sections from each subsample were then added to a glass tube containing 5 mL of sterile 0.05% Triton-X and homogenized with a pestle mounted on a drill for 10 s as described by Steinwender et al. (2015). From this suspension, 100 μL were spread in duplicate on selective agar media SDA (containing agar 6 g/L, glucose 10 g/L, peptone 5 g/L, Streptomycin 600 mg/L, Tetracycline 50 mg/L, Cycloheximide 50 mg/L and dodine 20 mg/L, pH 6.3–6.5) and incubated at 24 °C in darkness for 14 days. Fungal colonies with morphological resemblance to the inoculated EPF species were quantified as colony forming units (CFU) after 7 days and corroborated after 14 days.

2.6. Quantification of root colonization by AMF

Root pieces from the other subsample from each plant were cut into 2 cm segments and stained using the method described by Vierheilig et al. (1998) to verify the mycorrhizal colonization. The percentage of AMF colonization was determined through the line-intercept method developed by Giovannetti and Mosse (1980) in a Nikon Eclipse 50i microscope under bright-field conditions.

2.7. Statistical analysis

Lesion size of *B. cinerea* was determined by the average of the diameter of the five necrotic lesions per leaf (based on five leaflets per plant). Plant biomasses were analyzed as the fresh weight of roots and shoots separately, and the root:shoot (R/S) ratio were also analyzed. The root colonization by the EPF were log + 1 transformed before the analysis as some values were zero. The percentage of roots colonized by AMF were arcsine transformed prior to analysis.

Variance homogeneity was verified with the Levine test and the normality with the Shapiro-Wilk test. Based on previous ANOVA three-ways analyses (factors: experimental repetition, AMF, EPF), the interactive effect of experiment × EPF was found as significant for the response variables *B. cinerea* lesion size and plant biomass (root and shoot). Consequently, data for these variables were analyzed separately for the two experimental repetitions (Experiment A and B), while data from EPF and AMF root colonization were analyzed for both experimental repetitions combined as no interactive effects with experiment and other factors were found.

Two-way ANOVA were performed with the software IBM SPSS statistics 25 for analyses of the response variables *B. cinerea* lesion size, plant biomass (roots, shoots, R/S ratio), and EPF and AMF colonization. Post hoc analyses were made with LSD multiple range test when main factors or their interactions were found significant.

3. Results

3.1. Effects against Botrytis cinerea infection

Disease severity was different in the two experimental repetitions, with experiment A displaying larger necrotic lesions than in experiment B (ranging from 14 to 24 mm in A and from 9 to 17 mm in B), so the data of the two experiments were analyzed separately (Fig. 1). The three EPF isolates significantly reduced *B. cinerea* lesions in Experiment A as compared to the non-inoculated control, regardless whether AMF were present or not ($P = 0.026$, $F = 3.32$, d.f. = 3/56; Fig. 1A, Table 1). In

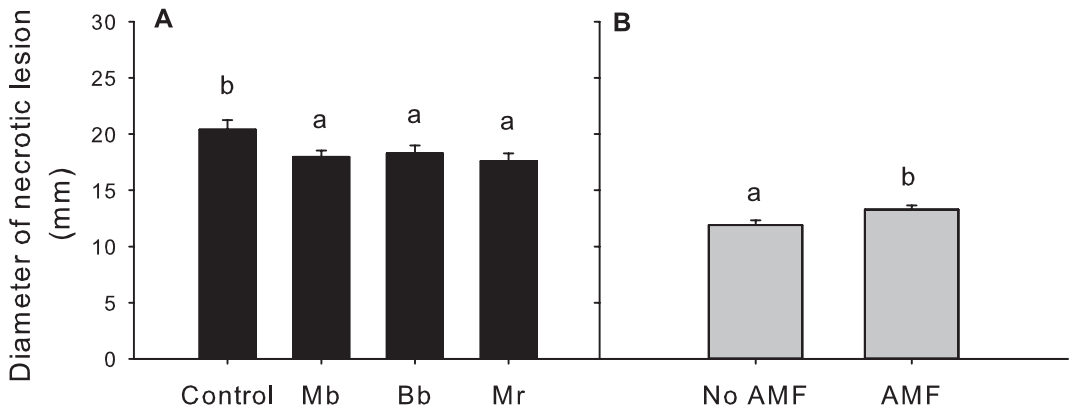

Fig. 1. Mean lesion sizes + SE (mm) by the phytopathogenic fungus *Botrytis cinerea* in detached leaves of eight-week tomato plants inoculated with the arbuscular mycorrhizal fungus *Funneliformis mosseae* (AMF) alone or combined with either of the entomopathogenic fungi (EPF) *Metarhizium brunneum* KVL 16–36 (Mb), *Beauveria bassiana* KVL 13–39 (Bb) or *Metarhizium robertsii* KVL 12–35 (Mr). Control plants were treated with Triton X (0.05%). Results represent two separate experimental repetitions (A) and (B). Different letters indicate significant differences between the treatments of EPF (A) or between treatments with or without AMF (B) (LSD, $P \leq 0.05$).

Table 1
Test values from two-way analyses of variance (ANOVA) of the response variable "Lesion size of *Botrytis cinerea*" for the two factors arbuscular mycorrhizal fungi (AMF), entomopathogenic fungi (EPF) and their interaction (AMF × EPF). ANOVA was conducted separately for each of the two experimental repetitions A and B (n = 8). Significant factors are highlighted in bold.

	Lesion size (mm)					
	Experiment A			Experiment B		
Factors	d.f.	F	*P*	d.f.	F	*P*
AMF	1/56	0.11	0.74	1/56	5.59	**0.022**
EPF	3/56	3.32	**0.026**	3/56	0.59	0.62
AMF × EPF	3/56	1.96	0.13	3/56	1.92	0.14

contrast, this effect was not observed in Experiment B, where inoculation with the AMF *F. mosseae* significantly increased lesions size of *B. cinerea* (P = 0.022, F = 5.588, d.f. = 1/56; Fig. 1B, Table 1). No significant interactions between AMF and EPF combinations were found in either of the two experiments (Table 1). Values of all treatment combinations are presented in Table 2.

3.2. Effects on tomato biomass

Inoculation with AMF increased root biomass, but the increase depended on the combination with EPF inoculations, reflected by significant interactions between AMF and EPF (Experiment A: P = 0.003, F = 5.123, d.f. = 3/56; Experiment B: P = 0.008, F = 4.319, d.f. = 3/56; Table 3). As such, root biomass was significantly increased in plants inoculated with *F. mosseae* in Experiment A compared to the plants not inoculated with AMF irrespective of combination with EPF. The exception was the root biomass of tomato plants inoculated with *M. robertsii* alone, which was also higher than the non-inoculated control plants (Fig. 2A). For experiment B, only the combination of *F. mosseae* and *M. brunneum* caused a significant increase in root biomass (Fig. 2B).

Table 2
Mean values (±SE) of *Botrytis cinerea* lesion size (mm) of experimental repetitions A and B for three isolates of entomopathogenic fungi (Mb = *M. brunneum* KVL 16–36; Bb = *B. bassiana* KVL 13–39; Mr = *M. robertsii* KVL 12–35), the arbuscular mycorrhizal fungus *Funneliformis mosseae* (Fm) and their combination treatments (n = 8).

Treatment	Experiment A	Experiment B
Control	21.41 ± 1.26	12.30 ± 0.79
Fm	19.46 ± 1.08	11.78 ± 0.62
Mb	19.22 ± 0.88	11.34 ± 0.71
Bb	17.26 ± 0.67	12.01 ± 1.19
Mr	16.86 ± 0.97	11.94 ± 0.93
Fm + Mb	17.31 ± 1.07	14.58 ± 0.42
Fm + Bb	18.70 ± 0.90	12.79 ± 0.63
Fm + Mr	18.34 ± 0.98	13.96 ± 1.04

As observed for the roots, inoculation with AMF was related to increased shoot biomass in both experiments (Experiment A: P < 0.0001, F = 18.42, d.f. = 1/56; Experiment B: P = 0.006, F = 8.326, d.f. = 1/56; Table 3). However, the effects depended on the EPF species co-inoculated with *F. mosseae* in Experiment A, as reflected by a significant interaction (P < 0.0001, F = 10.32, d.f. = 3/56; Table 3). Compared to the non-inoculated control, a significant reduction of shoot biomass was observed by *M. brunneum* and *B. bassiana* alone in Experiment A, which was compensated by the presence of *F. mosseae*, while the AMF treatment alone did not affect shoot biomass compared with the non-inoculated control (Fig. 2C). In contrast, shoot biomass of *M. robertsii* inoculated plants was unaffected by presence of AMF (Fig. 2C). In Experiment B, treatments with *F. mosseae* resulted in significantly increased shoot biomass (mean ± SE: 8.18 ± 0.15 g) compared with plants without AMF inoculation (mean ± SE: 7.55 ± 0.18 g) irrespective of EPF co-inoculation (Fig. 2D, Table 3).

All fungal inoculated plants had higher root:shoot ratios than the non-inoculated control plants in Experiment A, reflected by significant effects of the factors AMF, EPF and their interaction (Fig. 2E, Table 3). In Experiment B, there was also a significant AMF × EPF interaction (P = 0.012, F = 3.967, d.f. = 3/56; Table 3) on root:shoot ratios. Only the combination of *F. mosseae* and *M. brunneum* showed significantly higher root:shoot ratio than single inoculation with *M. brunneum*, while the opposite trend was seen for the treatments with *M. robertsii* (Fig. 2F). Values of biomasses for all treatments are presented in supplemental information (Table S1).

3.3. Root colonization

Data for fungal root colonization were merged for both experimental repetitions revealing that EPF colonization density was significantly affected by the EPF species, but also by inoculation with AMF (Table 4). Particularly, *M. brunneum* and *M. robertsii* were found in higher densities than *B. bassiana*, while both *Metarhizium* species decreased in density in a comparable manner when combined with *F. mosseae* (Fig. 3). Further, *B. bassiana* was only detected in tomato roots when inoculated alone and not detected when combined with *F. mosseae* (Fig. 3). The reduction of all three EPF species was statistically comparable in combination with *F. mosseae* (Fig. 3; Table 4) being reduced from an overall mean (±SE) of 1.96 (±0.17) logCFU mL^{-1} without AMF to 1.37 (±0.15) logCFU mL^{-1}

Table 3
Test values from two-way analyses of variance (ANOVA) of plant biomass, separately as root and shoot fresh weight (fw), and root:shoot ratio (R/S) for the two factors arbuscular mycorrhizal fungi (AMF), entomopathogenic fungi (EPF) and their interaction (AMF × EPF). ANOVA was conducted separately for each of the two experimental repetitions A and B (n = 8). Significant factors are highlighted in bold.

	Experiment A									Experiment B								
	Roots fw (g)			Shoots fw (g)			R/S			Roots fw (g)			Shoots fw (g)			R/S		
	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*
AMF	1/56	28.99	**<0.001**	1/56	18.42	**<0.001**	1/56	7.27	**0.009**	1/56	4.03	**0.05**	1/56	8.33	**0.006**	1/56	0.76	0.39
EPF	3/56	2.70	0.054	3/56	0.67	0.57	3/56	4.22	**0.009**	3/56	1.53	0.22	3/56	1.08	0.33	3/56	1.33	0.27
AMF × EPF	3/56	5.12	**0.003**	3/56	10.32	**<0.001**	3/56	7.04	**<0.001**	3/56	4.32	**0.008**	3/56	1.52	0.22	3/56	3.97	**0.012**

with AMF.

There was a trend towards decreased mycorrhizal root colonization in the presence of EPF (Fig. 4), but this effect was only marginally significant (P = 0.06; Table 4). The treatment with *F. mosseae* alone showed a mean colonization rate of 33.5%, while in co-inoculation with *B. bassiana* the mean mycorrhizal colonization rate was 23.5% (Fig. 4). Values of fungal root colonization for all treatments are presented in supplemental information (Table S2).

4. Discussion

Tomato inoculations with the arbuscular mycorrhizal fungus *F. mosseae* and three species of entomopathogenic fungi affected plant defense against the necrotrophic phytopathogen *B. cinerea* as well as plant growth, but the effects depended on the fungal combination and EPF isolate used. The results varied between the two experimental repetitions, supporting the notion of high context-dependency of plant–microbe interactions (Lee-Diaz et al., 2021). In general, we expected to observe an improvement of plant defense and increased biomass in fungal inoculated plants. However, the effect of the three different EPF isolates was predominantly on plant defense, while the effect of AMF was mainly related to plant growth.

Lesion sizes caused by *B. cinerea* were reduced by inoculation with EPF but not by *F. mosseae* in experiment A, whereas lesion sizes in experiment B, showing less overall disease severity, were increased by *F. mosseae* and not affected by EPF inoculations. In experiment A, where tomato leaves showed relatively high disease severity, *B. cinerea* lesion sizes were reduced by inoculation with EPF regardless of combination with *F. mosseae*, indicating no additional protective effect between the two fungal groups. In experiment B, with relatively low disease severity, EPF inoculations had no effect, while *F. mosseae* was associated with a slight increase of *B. cinerea* lesion sizes. Despite the variable results, this study illustrates the ability of root-associated EPF, including *Metarhizium* spp., to reduce *B. cinerea* disease progression in tomato leaves, supporting the increasing evidence of the capacity of EPF to protect plants against foliar pathogens (Jaber and Ownley, 2018; Raad et al., 2019) as well as arthropod herbivores (Cachapa et al., 2021; Canassa et al., 2019; Gange et al., 2019; Rasool et al., 2021a; Shrivastava et al., 2015; Ahmad et al., 2020).

Previous studies on plant disease effects by EPF were mainly based on *in vitro* experiments (reviewed by Jaber and Ownley, 2018). For instance, dual culture assays revealed growth inhibition of *B. cinerea* and other phytopathogens by different EPF as well as by their culture filtrates (Shin et al., 2016, 2017; Yun et al., 2017; Sammaritano et al., 2018), suggesting the production of antimicrobial compounds that inhibit pathogen growth (Lee et al., 2005; Sasan and Bidochka, 2013). Griffin (2007) also observed effects of *B. bassiana* against some fungal phytopathogens and suggested that competition could be a mechanism of disease control. However, all these interactions were registered *in vitro* and not in leaves of EPF colonized plants, thus they represent direct interactions on artificial substrates with limited resemblance with the effects against phytopathogens observed in the current study.

In the present study, EPF were applied to the root system, so no direct interaction between the EPF and the foliar pathogen are expected during the bioassays. Some works have focused on the inhibition of root pathogens when isolates of EPF were inoculated by seed coating (Griffin, 2007; Ownley et al., 2008; Keyser et al., 2016; Rivas-Franco et al., 2020). In these scenarios, suppression of pathogens due to direct interactions are likely. However, in the present study the effects of root-inoculated EPF were observed against an above-ground pathogen. Thus, direct antagonistic mechanisms are unlikely, and indirect plant mediated effects are probably underlying the reduced disease severity observed, suggesting the induction of systemic defense mechanisms by EPF. Although endophytic establishment of the EPF in above-ground tissues were not assessed, we expect no or limited endophytic colonization in the tomato leaves, based on previous results of the same system (Rasool et al., 2021b). Particularly, plant associations of *Metarhizium* spp. are mainly limited to the root system (Meyling et al., 2011; Behie et al., 2015), while *B. bassiana* potentially could have colonized the above-ground tissues (Fang and St Leger, 2010; Pava-Ripoll et al., 2011; Rasool et al., 2021b). However, given the comparable effects observed with both *Metarhizium* spp. and *B. bassiana*, it is likely that the mechanisms for *B. cinerea* suppression are plant mediated and not associated to direct antifungal effects of the EPF.

There are limited reports focused on the mechanisms behind protection against above-ground phytopathogens induced by EPF inoculations. Raad et al. (2019) used two isolates of *B. bassiana* to counteract the infection of *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*. The authors revealed that *B. bassiana* induced transcriptional reprogramming of defense pathways and differential production of specialized metabolites in the plant (Raad et al., 2019), supporting the ability of EPF to induce plant defenses.

The induction of systemic resistance as a potential mechanism triggered by the root-inoculation of EPF is further reinforced by recent studies showing changes in the expression of genes related to the biosynthesis of the defense related phytohormones jasmonic acid (JA), salicylic acid and ethylene (Ahmad et al., 2020; Raad et al., 2019; Rivas-Franco et al., 2020), and by reported accumulation of defense related specialized plant metabolites (Cachapa et al., 2021; Rasool et al., 2021a, b). It is possible that the EPF induced these mechanisms of plant protection in an isolate specific manner (Rasool et al., 2021b).

It is reported that AMF inoculated tomato plants can protect against foliar pathogens through the activation of JA dependent plant defenses, metabolic changes and primed callose accumulation (Sanmartin et al., 2020a, 2020b). However, we did not find protection against *B. cinerea* by the AMF inoculation alone, rather a slight increase in disease severity in one experimental repetition, and no additional protective effects were observed by co-inoculation with EPF. This is in contrast to previous studies reporting of AMF inoculated tomato plants showing protection against *B. cinerea* infections (Sanchez-Bel et al., 2016; Sanmartin et al., 2020b) and against other fungal phytopathogens in different plant hosts (Thygesen et al., 2004; Fritz et al., 2006; Song et al., 2015; Mustafa et al., 2017; Bidellaoui et al., 2019; Ravnskov et al., 2020).

Only few studies explored the co-inoculation of AMF and EPF for

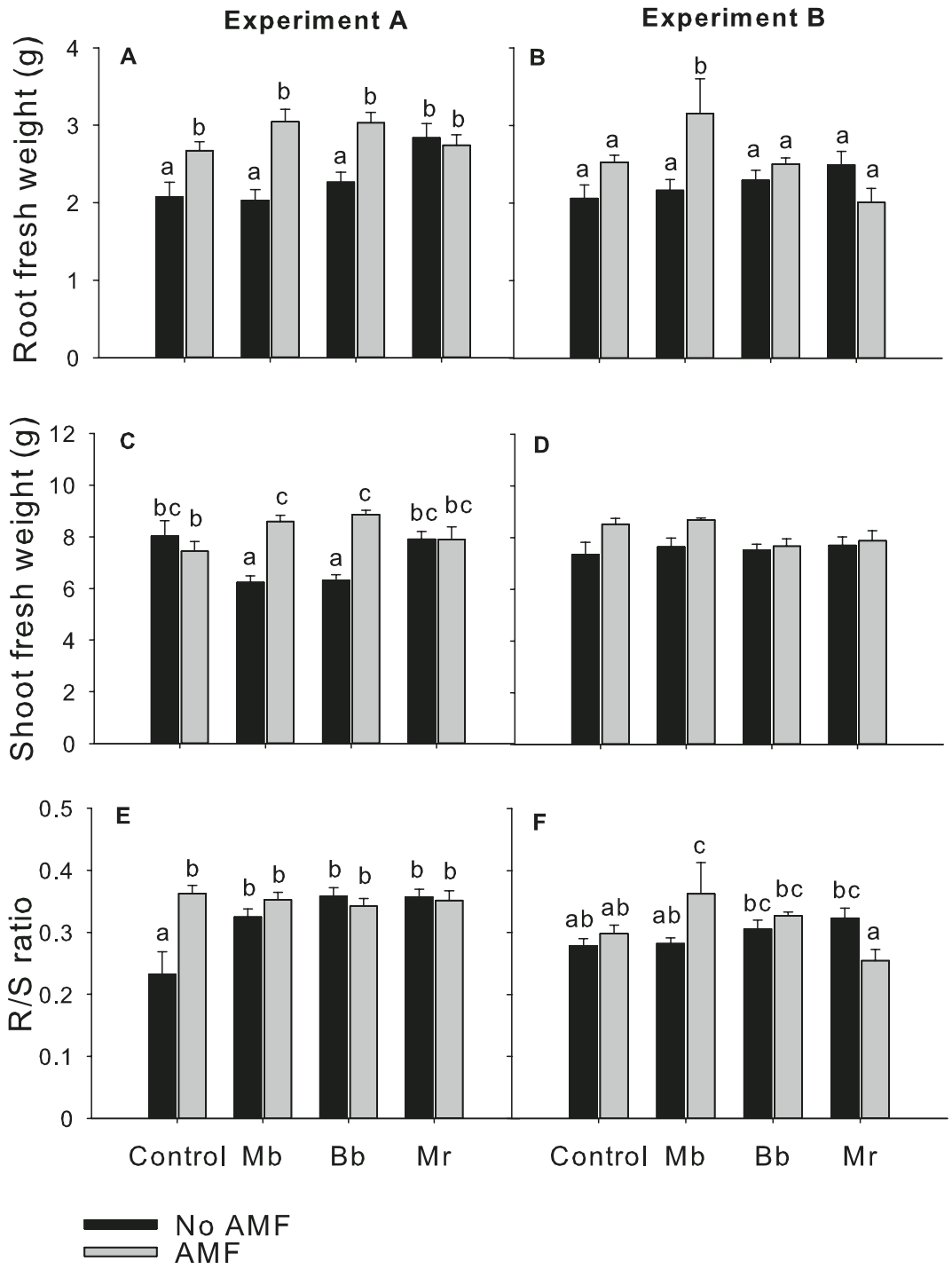

Fig. 2. Mean plant biomass (+SE) of eight week tomato plants divided by experimental repetitions A (left panel) and B (right panel). Root and shoot fresh weight (g, A-D) and the root:shoot (R/S) ratio (E-F) of plants grown with inoculations of the arbuscular mycorrhizal fungus *Funneliformis mosseae* (AMF) alone or in combination with entomopathogenic fungi (EPF) *Metarhizium brunneum* KVL 16–36 (Mb), *Beauveria bassiana* KVL 13–39 (Bb) or *Metarhizium robertsii* KVL 12–35 (Mr). Different letters above bars within each sub-figure indicate significant differences between combinations of AMF and EPF treatments (A, B, C, E, F) (LSD, $P \leq 0.05$). In (D), plants receiving treatments with AMF had significantly higher shoot weight than plants without AMF (No AMF = 7.55 ± 0.18 g; AMF = 8.18 ± 0.15 g; $P = 0.006$).

plant protection. Specifically, Shrivastava et al. (2015) coated tomato seeds with *B. bassiana* in combination with the AMF *Rhizophagus intraradices* and observed increased levels of terpenoids in the inoculated plants, which were related with reduced larval growth of the chewing insect herbivore *S. exigua*. Remarkably, both AMF and *B. bassiana* induced the production of terpenoids, and while the combination did not increase most of the studied compounds further, the combined treatment reduced larval weights more than the individual inoculations (Shrivastava et al. 2015).

Both AMF and EPF have individually been reported as plant growth promoters for maize, soybean, beans and wheat (George et al., 1995; Behie et al., 2012; Behie and Bidochka, 2014; Zitlalpopoca-Hernandez et al., 2017; Dara and Dara, 2017; Tall and Meyling, 2018) and tomato (Diop et al, 2003; Bona et al., 2017; Barra-Bucarei et al., 2020). Here, we investigated the effect of the single and dual inoculation on shoot and root biomass. We expected complementary effects by dual

Table 4
Test values from (left) two-way analysis of variance (ANOVA) of the response variable 'Root colonization of entomopathogenic fungi (EPF)' for the two factors arbuscular mycorrhizal fungi (AMF), EPF and their interaction (AMF × EPF). Test values (right) for one-way ANOVA of the response variable 'Root colonization of AMF' for the factor EPF (n = 16) Data from the two experimental repetitions A and B were combined for both analyses. Significant factors are highlighted in bold.

	Fungal root colonization					
	EPF (log + 1 CFU/mL)			AMF (arcsin percent)		
Factors	d.f.	F	*P*	d.f.	F	*P*
AMF	1/90	48.51	**<0.001**	–	–	–
EPF	2/90	297.7	**<0.001**	3/60	2.61	0.06
AMF × EPF	2/90	1.89	0.157	–	–	–

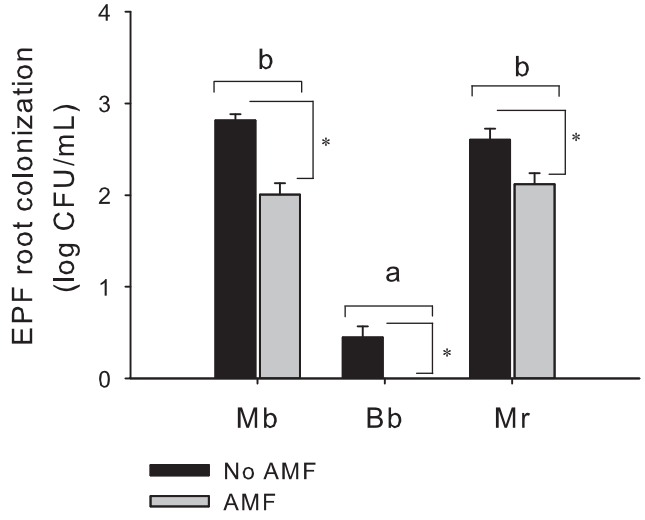

Fig. 3. Densities (log-transformed colony forming units, CFU) of the entomopathogenic fungi (EPF) *Metarhizium brunneum* KVL 16–36 (Mb), *Beauveria bassiana* KVL 13–39 (Bb) and *Metarhizium robertsii* KVL 12–35 (Mr) (mean log CFU mL^{-1} suspension + SE) in root suspensions plated on selective agar media. Different letters above the paired bars indicate significant differences between the EPF treatments. Asterisks indicate that treatments without the arbuscular mycorrhizal fungus *Funneliformis mosseae* (AMF) (1.96 ± 0.17 log CFU mL^{-1}) were significantly higher than treatments with AMF (1.37 ± 0.15 log CFU mL^{-1}), irrespective of EPF treatment (LSD, P ≤ 0.05).

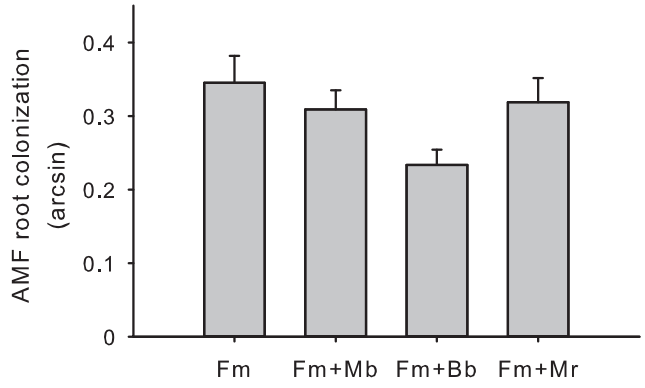

Fig. 4. Mean root colonization percent (+SE) of the arbuscular mycorrhizal fungus (AMF) *Funneliformis mosseae* (Fm) after inoculation alone or in combinations with entomopathogenic fungi: Mb = *Metarhizium brunneum* KVL 16–36, Bb = *Beauveria bassiana* KVL 13–39, Mr = *Metarhizium robertsii* KVL 12–35. No significant differences between treatments were found (P = 0.06).

inoculation on plant growth, as the two fungal groups have different mechanisms to improve plant nutrition acquisition, AMF by improving phosphorus uptake (George et al., 1995; Ezawa and Saito, 2018) and EPF by the transfer of nitrogen (Behie *et at.*,2012; Behie and Bidochka, 2014). The potential complementary roles of AMF and EPF in resource allocation could be observed for root biomass, most consistently for the *M. brunneum* isolate KVL 16–36. An isolate of *M. brunneum* with similar origin has previously been shown to increase tomato plant growth through seed inoculation (Rasool et al., 2021b), but in the present study this effect was only seen when combined with AMF. In contrast, effects of *M. robertsii* KVL 12–35 remained unchanged whether applied as single or dual inoculations. All fungal inoculations caused an increase in the root:shoot ratio in experiment A. This means that the relative allocation to root growth was higher than to shoots in the presence of EPF and/or AMF. Both types of fungi are well-established in roots (Smith and Read 2008; Fang and St Leger, 2010; Pava-Ripoll et al., 2011). However, when EPF and AMF were combined, the EPF densities of all three isolates decreased. Despite this reduction of EPF colonization, root growth stimulation by the inoculants may be helpful to increase the nutrient acquisition and to cope with abiotic stresses for the host plant (Porras-Soriano et al., 2009).

Functional compatibility between AMF and EPF is therefore not directly related to fungal quantification of root system colonization, and although AMF root colonization rates were unaffected by the presence of EPF, a near-significant trend towards a reduction was observed, mostly by *B. bassiana*. Similarly, Zitlalpopoca-Hernandez et al. (2017) reported that presence of native AMF populations negatively affected root colonization of an inoculated isolate of *B. bassiana*. Potentially, the presence of AMF could affect the colonization ability of EPF due to specificity of the plant-fungi interactions through competition or by altering plant defenses or composition of root exudates. As the AMF are obligate plant symbionts (Smith and Read 2008), higher specificity with the host plant could make these fungi more competent to establish in the roots compared to hypocrealean EPF, which are facultative plant associates (Vega, 2008; Vega et al., 2009). So far, it is unknown if products synthesized by the plants colonized by AMF, e.g. root exudates or specific volatiles, could explain the reduced colonization ability of EPF during co-inoculation. Some works have reported the modification of rhizosphere microbial communities and a decline of fungal and bacteria microbial inoculants when AMF were root-inoculated, while the other inoculants did not modify AMF colonization levels (Vázquez et al., 2000; Roesti et al., 2006; Rodríguez-Caballero et al., 2017). These interactions could be explained by the modification of root exudates (Vázquez et al., 2000), volatiles (Lioussanne et al. 2010), or flow of inorganic compounds (Zhang et al., 2018) of the inoculated plants.

The inoculated isolates of *Metarhizium* spp. and *B. bassiana* colonized tomato roots differently, and we did not observe a relationship between EPF root colonization levels and protection against *B. cinerea*. Similarly, Rasool et al. (2021b) found that the levels of plant tissue colonization by *B. bassiana, M. brunneum* and *M. robertsii* were not associated with the ability of inoculated tomato plants to increase defenses against herbivore populations. This further supports that protection by EPF inoculations are related to systemic changes in plant defenses rather than direct interactions between endophytic EPF and phytopathogens.

It was not possible to explain the differences between the divergent results of our two experimental repetitions. Both experiments had similar fungal colonization and comparable plant sizes, while the main difference was observed for the *B. cinerea* disease severity, which was most pronounced in experiment A. Probably the protection may vary with the severity of the disease, the effect being limited in experiment B. Both experiments had same duration and were set up under similar experimental conditions only one week apart. This suggests that plant–microbe interactions may be sensitive to changes of the microenvironment and the beneficial effects of fungal inoculations may become inconsistent in otherwise comparable scenarios. Such variable results can represent constraints to the future uptake and use of

beneficial microbes in plant production (Lee-Diaz et al., 2021). In addition, it should be noted that detached leaf bioassays may yield more variable results, as reported by Gange et al. (2019) after a *meta*-analysis of studies where plant-associated EPF were tested against arthropod herbivores, indicating that whole plant assays should be preferred.

In conclusion, our results showed that single inoculation of the three EPF isolates of *M. brunneum, B. bassiana* and *M. robertsii* improved plant protection against the phytopathogen *B. cinerea* in tomato, irrespective of root colonization ability. The AMF strain of *F. mosseae* had no ability to reduce *B. cinerea* lesion sizes, while the AMF inoculation was mainly associated with increased plant growth. Co-inoculation with AMF did not result in any additive effects of the observed plant protection by EPF. The results indicate a functional complementarity between the EPF by reducing plant disease and the AMF by increasing plant growth. The study emphasizes the need for selection of compatible microorganisms for future development of microbial-based plant protection, but also that beneficial effects may be inconsistent and context dependent. Thus, further research is needed on the complex regulation of plant defenses upon microbial inoculation in order to optimize the efficiency of beneficial microorganisms in crop management.

CRediT authorship contribution statement

Guadalupe Zitlalpopoca-Hernandez: Data curation, Formal analysis, Investigation, Conceptualization, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Maria J. Pozo:** Conceptualization, Methodology, Writing – review & editing. **Thure P. Hauser:** Funding acquisition, Writing – review & editing. **Nicolai V. Meyling:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2022.105034.

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