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ORIGINAL PAPER

The arbuscular mycorrhizal fungus *Rhizophagus irregularis* affects arthropod colonization on sweet pepper in both the field and greenhouse

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Abstract In the present study sweet pepper plants, *Capsicum annuum*, were planted in greenhouse and open field conditions to test the effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* on phytophagous and predatory arthropod populations. Furthermore, we tested the hypothesis that AMF may increase the crop yield (number of fruits and their weight) and activity level of polyphenol oxidase (PPO) and peroxidase (POD), enzymes that seemingly decrease infestation by arthropod pests. The most abundant arthropod species found were the peach-potato aphid, *Myzus persicae*, western flower thrips, *Frankliniella occidentalis*, and the seven-spot ladybird, *Coccinella septempunctata*. Sweet pepper mutualism with AMF significantly reduced colonization by the peach-potato aphid under greenhouse conditions. Aphid density increased, however, on two of four pepper varieties tested under open field conditions. The density of ladybird predators did not appear directly influenced by AMF under greenhouse conditions, whereas a significantly higher predator density was found on three out of four pepper plant varieties with fungal mutualism tested under field conditions. Crop yield was significantly higher on plants with AMF mutualism under greenhouse conditions, but no clear effects were detected under field conditions. Both PPO and POD activity increased significantly and remained higher than controls until day 14 of the experiment under mutualism with AMF, although only in the greenhouse. The results suggest that under greenhouse conditions, pepper plant mutualism with AMF can increase pepper yield by reducing the numbers of the key pest, peach-potato aphid.

Keywords AMF · Cropping system · Enzyme activity · Mutualism · Peach-potato aphid · Plant resistance · Soil pH · Seven spotted ladybird · Western flower thrips

Key message

- The effects of the AMF *Rhizophagus irregularis* on arthropod populations were tested. Pepper plants with fungal mutualism had significantly fewer peach-potato aphids under greenhouse conditions.
- Both PPO and POD activity increased significantly under mutualism and remained higher for 14 days in the greenhouse.
- The results suggest that, under greenhouse conditions, pepper plant mutualism with AMF can increase pepper yield and reduce the numbers of the key pest, peach-

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potato aphid. This approach has potential economic benefits in reducing the usage and impacts of insecticides in pepper production.

Introduction

Several arthropod pests attack sweet pepper, *Capsicum annuum* L., under both greenhouse and open field conditions. The most serious pest in the greenhouse is the peach-potato aphid, *Myzus persicae* (Sulzer) (Insecta: Hemiptera: Aphididae) (Bass et al. 2014) (also known as the green peach aphid), but other pests including the two-spotted spider mite, *Tetranychus urticae* Kock (Arachnida: Acari: Trombidiformes: Tetranychidae), the western flower thrips, *Frankliniella occidentalis* (Pergande), and the onion thrips, *Thrips tabaci* (Linderman) (Insecta: Thysanoptera: Thripidae), may also occur and may prove problematic, whereas the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood) (Insecta: Hemiptera: Aleyrodidae) is rarely a problem on greenhouse sweet peppers (Howard et al. 1994). Besides aphids, thrips and spider mites, Lygus bugs (*Lygus* spp.; Insecta: Hemiptera: Miridae) can occur on sweet pepper under open field conditions (Howard et al. 1994; Jarvis 1992; Portree 1996). Of predators, the most important under both greenhouse and open field conditions are seven-spot ladybird beetles, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), predatory thrips (Family Aeolothripidae), predatory mites *Phytoseiulus persimilis* (Lombardini) (Mesostigmata: Phytoseiidae), minute pirate bugs *Orius* spp. (Hemiptera: Anthocoridae) and hymenopterous wasp parasitoids (Family Braconidae), all of which are important agents in biological pest control, under controlled greenhouse conditions (Malais and Ravensberg 2003; Gonzalez et al. 2016).

The peach-potato aphid is a polyphagous species and one of the world's major aphid pests on sweet pepper (Blackman and Eastop 2000; Emden and Harrington 2007), having a direct negative impact on this particular crop in both Europe and the USA (Silva et al. 2012). Aphid colonization of pepper plants occurs during the summer, causing chlorosis and sometimes necrosis of the plants. Other economic impacts result from pathogenic virus transmission (Verdugo et al. 2012). These include cucumber mosaic virus, tobacco etch virus, pepper mottle virus, potato virus Y and others that can result in flower and fruit abortion. Control of aphids with broad-spectrum insecticides has increased the incidence of resistance to insecticides in the peach-potato aphid over the past 50 years or so, at the same time reducing the numbers of natural enemies that control them (Amarawardana et al. 2007; Silva et al. 2012). Because peppers have no apparent genetic resistance to the damage caused by the peach-potato aphid, alternative methods of control are required to reduce colonization by these aphids during the growing period (Smith and Read 2010).

One possible method is the use of biological control agents to combat such damage and involving predators of the pest (Gonzalez et al. 2016). The most important predator of the peach-potato aphid, under both greenhouse and open field conditions, is the seven-spot ladybird beetle (Blackman and Eastop 2000; Smith and Read 2010; Emden and Harrington 2007). Ladybird beetles can respond to plants' emitted volatile organic compounds (VOCs) characteristic of general damage released following aphid damage (Hoballah and Turlings 2005; Hare and Sun 2011). However, there are situations when such arthropod natural enemies are not that effective. Under such circumstances, other kinds of biological control agent, i.e., based on microorganisms and/or on arbuscular mycorrhizal funguses (AMF), can provide a complementary strategy for protection (Gonzalez et al. 2016; Mutune et al. 2016).

Some arbuscular mycorrhizal fungal species, such as *R. irregularis* N.C. Schenck and G.S. Sm. (Glomerales: Glomeraceae), are soil inhabitants that form widespread mutualistic symbioses with plant roots (Kloppholz et al. 2011). Previous studies have reported that the feeding and oviposition of the bean stem maggot (*Ophiomyia* spp.) (Insecta: Diptera: Agromyzidae) were significantly reduced in AMF-inoculated bean plants, which affected adult emergence of the pest fly compared with the control (Mutune et al. 2016). Many aspects of this AMF symbiosis are however still unclear, but previous research has shown that the plant-fungus interaction improves plant nutrition, thereby increasing biomass, and enhances the ability of the plant to suppress or resist some diseases (Odebode and Salami 2004; Hempel et al. 2009; Jiang et al. 2015). Odebode and Salami (2004) demonstrated that increases in the level of sugars and free amino acids in pepper seedlings inoculated with mycorrhizal fungi increased the defense of the plant against infection. These authors also showed that the low level of total phenol in mycorrhizal-inoculated plants apparently had a passive protective effect so that the plant did not build up so many chemical defense mechanisms against infection and perhaps indirectly against herbivorous insects like aphids. Recent studies have also revealed that *R. irregularis* secretes effector proteins that play a key role in mediating the accommodation process of the fungus within plant roots (Kloppholz et al. 2011). The same research showed that this particular fungus also secretes another protein, SP7, which interacts with the pathogenesis-related transcription factor ERF19 in the plant nucleus (Kloppholz et al. 2011). Overexpression of some ERF transcription factors was found to increase resistance to pathogen attack as well as to several abiotic stresses (Lee et al. 2004; Zhang et al. 2009). This increased resistance correlates with induced expression of various defense-related genes, including PR1, PR2, PR4, Osmotin and SAR8.2 (Lee et al. 2004; Zhang et al. 2009). Some effects on insect pests have also been reported. For example, inoculation of Timothy grass, *Phleum pratense* L. (Poales: Poaceae), with *R. irregularis* was found to decrease bird-cherry oat aphid *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae) populations by 47%, although at the same time, the rate of parasitism by wasp parasitoids (braconids) increased by 140% relative to control treatments (Hempel et al. 2009; Bonfante and Genre 2010).

Despite such promising attributes of AMF mutualism, the cascading effects of ladybirds on aphids under AMF mutualism of host plants have been rarely discussed (Fontana et al. 2009). Accordingly, AMF mutualism has been found to increase the emission of the green leaf volatile (*Z*)-3-hexenyl acetate from herbivore-damaged plants. Such AMF effects may have a positive role in predator attraction and further contribute to the efficiency of biological control. The idea that plant-induced polyphenol oxidase (PPO) and peroxidase (POD) enzymes may act as an anti-nutritive defense against phytophagous insects was first suggested by Felton et al. (1989) (reviewed by War et al. 2012). Evidence for an anti-herbivore role of PPO came from the discovery that the herbivore defense-inducing signal molecules systemin and methyl jasmonate (MeJA) induce both PPO activity and POD levels in tomato leaves (Constabel et al. 1995; Constabel and Barbehenn 2008). To this end, during the present study the main aims were to investigate colonization of sweet pepper by phytophagous arthropods, and especially by the peach-potato aphid and its predators, under different soil pH conditions (those generally used for pepper cultivation) in both greenhouse and open field conditions when plants are in mutualism with AMF. We aimed to test the following hypotheses:

1. Mutualism of sweet pepper plants with AMF *R. irregularis* can differentially influence colonization by phytophagous and predatory arthropods under certain cropping systems (greenhouse and open field).
2. Crop yield (number of fruits and their weight) can also be improved by AMF under certain cropping systems.
3. AMF may also increase activity levels of the polyphenol oxidase (PPO) and peroxidase (POD) enzymes, which may confer a degree of pest resistance.

Materials and methods

Greenhouse experiment

The experiment, performed during the 2011 growing season, consisted of 48 blocks of sweet pepper in a greenhouse (experimental area 0.3 ha) in a double-nested block design (Supplementary online material, Figure 1A). Two peppers, both Hungarian varieties, were used for the experiment. Cultivar Fehérözön (FO) is a short season variety that usually stops growing at the end of July, whereas the second, cv. Bibic (BC), grows until the end of August. These varieties were chosen because they are widely distributed and routinely cultivated under greenhouse conditions. From each variety, 24 randomly placed blocks, comprising 90 plants each (540 plants/variety and 1080 as total), were established (Supplementary online material, Figure 1A). Two young (two leaf) plants, previously grown under virus-free conditions by Blondy Romania SRL, were placed in 8-l pots containing sterile commercial soil (from Blondy Romania SRL) of three different pH levels, respectively (low, 4.5; medium, 5.5; high, 6.5). Thus, for both varieties, 8 blocks of 90 plants each were established under each of the three pH regimes. Four blocks from each variety/pH level were assigned to the mutualism treatment and inoculated with AMR spores purchased commercially (Italpollina SPA, Italy). Because spores were supplied encapsulated in polysaccharide capsules, 15 g inoculum (10% of the substrate weight, 50 spores per g^{-1}) of AMF were added per pot after dissolving in 100 ml water. The non-AMF control followed the same design, soil pH variation and number of plants (4 blocks/pH of 90 plants/block) (Supplementary online material, Figure 1A). The entire system was connected to an automatic drip irrigation source after 20 h, a period during which the mutualistic relationship between the plant and AMF was clearly established (controlled by carefully removing and assessing AMF-treated plant roots—three from each variety and pH—under a binocular microscope). From that moment on, irrigation was controlled conventionally for pepper production under greenhouse conditions, i.e., an average of 1 l water per pot per day at the beginning of the trials (10–30 May) and 2 l per pot per day during the summer (June–July). Standard nutrient applications (N 250, P 50, K 250, Ca 100, Mg 30, Fe 2, Mn 0.3, B 0.3, Cu 0.1, Zn 0.26 ppm) were applied automatically every 2nd week, while pH was controlled every 2nd day and if necessary adjusted (only a small decrease in pH at the end of the experiment was detected, whereupon 10 mg calcium carbonate was added to all plants and corrected to the initial values) to maintain constant treatment regimes. Temperature was maintained at 25 ± 6 °C, L16:D8 photoperiod, and 70% r.h. during the experiment. Fungal colonization of each plant was again confirmed for each variety and pH regime at the end of the experiments (Supplementary online material, Figure 2A, B). Opening the ventilation systems through roof ventilation and greenhouse doors from both sides on 10 May facilitated colonization of the pepper plants with aphids and predators. After 1 week of free colonization of arthropods (the assessment started on 17 May), 10 plants from the 90, selected at random from each block/treatment, were harvested (by carefully placing plants in plastic bags and then cutting the stems) for assessment and the exact numbers of peach-potato aphids, and if they occurred, other phytophagous species (mites and western flower thrips) and predator species (ladybird beetles, predatory thrips, mites and minute pirate bugs) and hymenopterous wasp (braconid) parasitized aphid mummies were counted in the laboratory.

Initially, all bags with plants were placed in a freezer at −20 °C for 24 h, whereafter the arthropods were counted. Every 2nd week, another ten plants from each block were assessed as previously described. This was done to ensure one assessment per aphid generation, because the generation time of the aphid is 10–14 days under these conditions. The procedure was repeated every 2 weeks until the end of July (six assessments in total), the period when FO pepper plants became too old and hence unsuitable for aphids. Because cv. Bibic (BC) grows until the end of August, a further assessment was made on this variety on 13 August, but because of the very low number of all arthropods found, this last assessment was not considered during analyses. Crop yield was estimated at the end of July for both cultivars and pH regimes by counting the number of fruits set and fresh fruit weight using a digital scale on ten randomly selected plants/block/treatment/variety/pH.

Field experiment

This was conducted during 2012–2013 using the four most popular pepper varieties (cvs. Long Chili, Sweet Bell, Sweet Flexum and Sweet Banana). Two varieties (cvs. Sweet Bell and Sweet Flexum) were closely related genetically to the varieties tested previously under greenhouse conditions. All varieties were relatively short season, usually maturing from June to late August. After germination in 100 ml germination pots (using standard seed starting/germinating mixes—ProMix Ultimate from Blondy Romania SRL) under virus-free greenhouse conditions, small two-leaf plants (360 plants from each variety) were placed outside for acclimatization (May 15 in both years). After 5 days in both years, these were taken from pots and transplanted to the field in double rows in a 0.7-ha field with soil of 5.5 pH in randomized blocks of 120 (2 rows of 60 plants with two varieties per row, i.e. 60 Long Chili followed by 60 Sweet Banana) (Supplementary online materials, Figure 1B). The next two rows contained the other two varieties spaced in the same way. All rows were irrigated with a drip system and mulched with black plastic. The field layout was conventional for cultivation of these varieties: 30 cm between adjacent plants, 40 cm between plants in a double row and 2 m between rows (Supplementary online materials, Figure 1B). Altogether 12 similarly designed double rows (each with 2 × 60 plants) were created containing a total of 1440 plants with four replicates for each variety. Four days later (24 May) replicates of 60 plants were randomly selected for each variety and assigned to treatments and received 50 g of the same AMF inoculum used in the greenhouse experiment. Following placing of the plants back in pots, and addition of the inoculum, the mutualism was checked. Mutualism was clearly visible (checked as during the greenhouse experiment); therefore, plants were transplanted back from pots to the field after 20 h. Two other groups of 60 plants each were assigned as pesticide treated, where commercially recommended insecticides and fungicides were periodically used to control aphid density and powdery mildew (Supplementary online material, Table 1). All pesticides were applied using a manual spray system very close to the plants. In this way, the spread of pesticides to adjacent and differently treated blocks was minimized. Another 2 × 60 group of plants of each variety was assigned as controls and did not receive any treatment. All plants were watered uniformly with 2 l water/day using a drip irrigation system until the end of the growing period. Nutrients were applied automatically every 2nd week as with the greenhouse experiment. Data assessment began 1 week after the experiment was set up, on 1 June in each year of the experiment. Ten randomly selected plants were assessed from each block every 2nd week using a similar method to the greenhouse experiment by cutting plants off at the ground level, freezing them and counting all arthropods found in the laboratory. Altogether, six assessments were performed bi-weekly to permit at least one generation of aphids to be produced between assessments in each year, until the end of August. At the end of the season, crop yield was assessed as in the greenhouse experiment. The entire procedure was repeated again in 2013.

PPO and POD enzyme extraction and activity assays

Leaf samples used for enzyme analyses were collected from both AMF-treated and control plants (from both greenhouse and 2012 field assessments) on day zero (i.e., 20 h after inoculation with AMF) and again on days 4, 8, 14, 28 and then every 2nd week. Samples were collected from the same plants used for assessment of aphids and predators in both experiments ($n = 10$ plants/block). Samples were held at −20 °C until enzyme extraction and activity assays. For extraction, 400 mg frozen leaves was homogenized in 1 ml of 50 mM phosphate buffer, pH 7.0, using a FastPrep Instrument high-speed benchtop homogenizer (MP Biomedicals). The homogenate was centrifuged at 1000 g for 30 min at 4 °C and the supernatant collected.

Protein concentration of the enzyme extract was determined by the Bradford method (Bradford 1976). The activity of polyphenol oxidase (PPO) was determined spectrophotometrically at a wavelength of 400 nm in a reaction mixture (1 ml) containing 50 mM phosphate buffer, pH 5.5, 10 mM 3-methyl-catechol and 150 µl enzyme extract. Enzyme activity was determined according to Cheema and Sommerhalter (2015). One unit of PPO activity was defined as the amount of enzyme producing 1 µmol of quinone per minute at 30 °C. Specific activity was expressed in U/mg protein. Peroxidase (POD) activity was determined by the method of Németh et al. (2009). The reaction mixture contained 955 µl of 50 mM phosphate buffer, pH 5.5, 10 µl of 0.01 g/l 3.3′-diaminobenzidine and 30 µl enzyme extract. The reaction was initiated by the addition of 5 µl 0.3% H_2O_2. The increase in absorbance at 480 nm was followed for 5 min, one unit of POD activity being defined as an absorbance change of 0.01 units/min.

Data analyses

For the greenhouse data, intra-annual differences in the abundance of each arthropod species were first tested using multivariate analysis of variance (MANOVA) to account for arthropod population dynamics through the sampling period. Mean number of arthropods/ten plants/treatment/variety/pH regime were considered. No significant temporal difference was detected ($p < 0.01$). The most frequent pests were the peach-potato aphid followed by the western flower thrips, while the most frequent predator was the seven-spot ladybird, all three species occurring during the entire assessment period on both plant cultivar varieties, the data so collected being subject to further analyses. All other pests, predators and parasitized aphids were only present occasionally and in low numbers, so that statistical analyses were not possible, but even so, these insects are mentioned in the results. Second, arthropods (aphids, thrips and ladybirds) and crop yield data were tested for normality of errors (Kolmogorov-Smirnov test) and for equality of variance (Levene's test). Aphid data were normally distributed, so factorial ANOVA was used, followed by Tukey testing, and cultivar (FO and BC), treatment (control and AMF treated) blocks (to test block effects) and pH levels (4.5, 5.5 and 6.5) were used as fixed factors and aphid abundance as a random factor. Thrips and ladybird data did not meet the assumption of normality, so that the Kruskal-Wallis test was used, followed by the Mann-Whitney *U* test, and cultivar, treatments, blocks and pH levels were used as a fixed factors and thrips and ladybird abundance as random factors. Crop yield data were again found to be normally distributed, whereupon ANOVA followed by Tukey testing was also used and varieties, treatments, blocks and pH levels as fixed factors, and the average settled fruits and weight/ten plants as random factors were considered separately for each variety.

For the field data, inter-annual differences (tested separately for each year) in abundance of each arthropod were initially tested to account for population dynamics through the sampling period. Again, mean number of arthropods/ten plants/treatment/variety were considered. No significant temporal difference was detected ($p < 0.01$). Only peach-potato aphids and seven-spot ladybirds were consistently present together from 1 June until the end of August (the sixth assessment period) during both years of the experiment. Any other pests, predators and parasitized aphids were present only occasionally and in low numbers; as with the greenhouse experiment, statistics were hence not possible but the insects are again mentioned in the results. Aphid and predator data from the field experiment, along with crop yield data, were normally distributed (Kolmogorov-Smirnov and Levene's test), so factorial ANOVA followed by Tukey test was used with years (testing year effects), cultivars, treatments and blocks (testing block effects) as fixed factors, aphids and ladybird abundances, fruits set and their weights (averages per 10 plants/assessment) as random factors. All analyses were made using R version 3.0.1 (R Core Team 2013), and values below $p \leq 0.05$ were considered as statistically significant.

The PPO and POD enzyme activity values obtained were compared between the control and AMF-treated plants from day zero to 28; high decreases in both PPO and POD activity level (values near 0) were detected after day 14 until day 28 in both controls and treatments, except for POD at pH 6.5 in the greenhouse; therefore, we only used and graphically presented data between days 0–28 from both greenhouse and field samples (pepper leaves), including each cultivar, treatment and pH regime (for greenhouse). A complete randomized factorial ANOVA of PPO and POD specific activity values was performed to test for effects of treatment (control plants, plants inoculated with AMF and plants treated with pesticide for field experiment) and time (0, 4, 8, 14 and 28 days). The analyses involving the Tukey test with $p < 0.01$ and LSMEAN (minimal quadratic means), according to the statistical package SAS, were included.

Results

Arthropod abundance in the greenhouse

Mycorrhizal mutualism between pepper plants and AMF was observable when treatment and control plants were compared post-experiment (Supplementary online material, Figure 2A, B). Altogether a total of 24,248 peach-potato aphids, 464 western flower thrips and 687 seven-spot ladybirds were counted during the greenhouse experiment.

Statistical analysis failed to reveal any varietal block effect in aphid abundances ($F = 0.04$, $p = 0.9$). A significantly lower number of aphids colonized both FO and BC pepper varieties when plants were inoculated with AMF. This trend was stable at all soil pH levels tested for both pepper varieties (Fig. 1). Similar numbers of western flower thrips and predators were observed on control and AMF inoculated plants (Tables 1, 2), and again no block effects in thrips ($F = 0.9$, $p = 0.4$) and ladybird ($F = 0.2$, $p = 0.8$) abundance were detected. Only a low number of other phytophagous species (i.e., 42 two-spotted spider mites and 32 onion thrips) and predators [15 harlequin ladybird beetles (*Harmonia axyridis* Pallas), 14 predatory thrips (Family Aeolothripidae) and 11 minute pirate bug (*Orius* spp.)] was recorded during the whole experiment. A low number of mummified aphids ($n = 14$) was also observed.

Crop yield of AMF inoculated plants in the greenhouse

No varietal block effect in fruit numbers ($F = 1.6$, $p = 0.2$) and fruit weights ($F = 3$, $p = 0.08$) were detected. There were significantly more fruits on both FO and BC plants when they were inoculated with AMF. Similarly, the fresh weight of the fruits was significantly higher at all pH regimes when plants were AMF treated (Table 3).

PPO and POD activity in the greenhouse

The specific activity of PPO in pepper leaves (both varieties) grown under greenhouse conditions (Fig. 2a–c) showed significantly higher values ($F = 4.2$, $p < 0.01$) in inoculated plants at all pH regimes tested until day 14. From this day onwards, a decrease was detected until the end of the growing season; therefore, the PPO variations until day 28 were presented graphically (Fig. 2a–c). POD-specific activity in pepper leaves decreased from day 8 at pH 4.5, but remained significantly different ($F = 5$, $p < 0.01$) between AMF and control treatments (Fig. 3a). At pH 5.5, POD variation showed very similar trends as found for PPO activity, and again significant differences ($F = 4.8$, $p < 0.01$) between AMF inoculated and control plants were detected (Fig. 3b). A relatively constant level

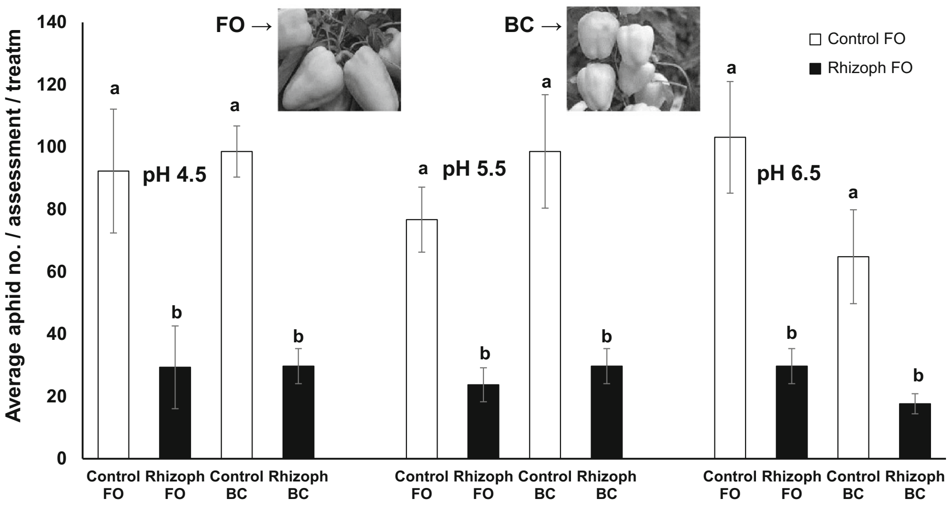

Fig. 1 The average number of peach-potato aphids per assessment per treatment per pepper cultivar at pH 4.5, 5.5 and 6.5 under greenhouse conditions. FO = cv. Fehérözön, BC cv. Bibic. *Bars* = ±1 SE. *Different letters* indicate statistically significant differences at $p < 0.01$ level (factorial ANOVA was used, followed by Tukey test)

Table 1 Comparison between the relative abundance of western flower thrips (n = number of individuals, median, ±25th and 75th quartiles, U and p values) on greenhouse pepper plants of two varieties grown at three levels of soil pH (4.5, 5.5 and 6.5) and receiving AMF inoculation (*Rhizophagus*) or not (control)

pH	*n* Control	n AMF	Median	25th/75th quart.	*U*	*p*
Feherozon (FO)						
4.5	35	42	1.5	0.2/4.2	0.1	ns
5.5	30	41	1.5	0.2/3.2	0.4	ns
6.5	46	43	2.5	0.2/4	0.1	ns
Bibic (BC)						
4.5	40	42	0.9	0.2/1.6	0.1	ns
5.5	35	42	1.4	0.1/1.5	0.5	ns
6.5	32	36	1.4	0.3/1.3	0.3	ns

Data were compared using Kruskal-Wallis followed by Mann-Whitney *U* test

ns not significant

Table 2 Comparison between the relative abundance of seven-spot ladybirds (n = number of individuals, median, ±25th and 75th quartiles, U and p values) on greenhouse pepper plants of two varieties grown at three levels of soil pH (4.5, 5.5 and 6.5) and receiving AMF inoculation (*Rhizophagus*) or not (control)

pH	*n* Control	*n* AMF	Median	25th/75th quart.	*U*	*p*
Feherozon (FO)						
4.5	71	59	1.1	0.2/1.4	1.1	ns
5.5	49	73	1.1	0.1/1.8	1.4	ns
6.5	65	63	1	0.4/1	−1	ns
Bibic (BC)						
4.5	50	62	0.8	0.3/1.5	−1.1	ns
5.5	45	52	1.1	0.2/1.7	−0.8	ns
6.5	42	56	1.3	0.5/1.5	−0.4	ns

Data were compared using Kruskal-Wallis followed by Mann-Whitney *U* test

ns not significant

of POD activity was observed at pH 6.5, but even so, highly significant differences between AMF treatment and the control were detected ($F = 3.9$, $p < 0.01$) (Fig. 3c).

Arthropod abundance in field

A total of 46,216 peach-potato aphids and 2018 seven-spot ladybirds were counted during the field experiment. Because of the high variation in aphids and ladybird abundance between cultivars (i.e., Sweet Bell), these data are presented as $\log_{10}$ transformed in Fig. 4a, b. No year effects were detected in aphids ($F = 0.4$, $p = 0.6$) and ladybird abundances ($F = 1.3$, $p = 0.2$). Also no block effects in aphid ($F = 0.3$, $p = 0.5$) and ladybird abundances ($F = 1.7$, $p = 0.1$) were observed. Furthermore, no clear effects of mycorrhizal mutualism on aphid colonization were detected between varieties. Numbers of aphids decreased significantly on cv. Long Chili when inoculated with AMF, but increased on cvs. Sweet Bell and Sweet Banana, whereas no observable effect was observed on cv. Sweet Flexum (Fig. 4a). In contrast, higher numbers of seven-spot ladybirds colonized cvs. Sweet Bell, Sweet Flexum and Sweet Banana when these were treated with AMF, although numbers were not significantly different between AMF-treated and the control on cv. Long Chili (Fig. 4b). Again, a low number of other phytophagous arthropod species (i.e., 39 two-spotted spider mites and 44 onion thrips) and predator species was detected in the field (32 harlequin ladybirds, 29 predatory thrips and 27 minute pirate bugs) during the entire experiment. Lastly, a low number of mummified aphids ($n = 26$) was detected.

Crop yield of AMF inoculated plants in the field

No varietal year (fruit numbers: $F = 1.8$, $p = 0.1$, fruits weight: $F = 2.1$, $p = 0.1$) and block effect (fruit numbers: $F = 0.6$, $p = 0.7$, fruits weight: $F = 1.9$, $p = 0.2$) were detected in crop yield under field conditions. Similar numbers of fruits were set by all four pepper varieties in all treatments (Table 4). The average fruit weight was lower for cv. Sweet Bell in the AMF treatment compared with pesticide-treated and control plots. The average fresh weight was lower at cv. Sweet Flexum control compared with AMF and pesticide treatments. Higher fruit weight for cv. Sweet Banana under AMF treatment was detected

Table 3 The average number of fruit sets and their average fresh weight (g) (means, SE, F and p values) compared between treatments in the greenhouse experiment

pH	Number of fruits set				
	Mean cont.	Mean AMF	SE	*F*	*p*
Feherozon (FO)					
4.5	15.9	33.7	1.2	**17**	**
5.5	12	29.3	2.2	**15.1**	**
6.5	9.2	27.9	1.3	**14**	**
Bibic (BC)					
4.5	9.2	20.1	0.9	**13**	**
5.5	7.4	21.1	1.1	**9.4**	*
6.5	7.9	22.2	1.4	**11**	*
pH	Average fruit fresh weight (g) at harvest				
	Mean cont.	Mean AMF	SE	*F*	*p*
Feherozon (FO)					
4.5	215	403.5	28.8	**4.8**	*
5.5	233.5	445	23.7	**6.9**	*
6.5	302.5	477.5	19.9	**8.3**	*
Bibic (BC)					
4.5	390	523	29.1	**10**	*
5.5	406.5	543.5	37	**11.6**	*
6.5	400	512	20	**8.6**	*

Data represent treatment per pepper variety in ten randomly selected plants per plot per treatment (ANOVA and Tukey test)

Statistically significant differences are in bold

ns not significant

* $p < 0.01$; ** $p < 0.001$

compared with the pesticide treatment and the controls (Table 4).

PPO and POD activity in the field

No observable differences in PPO activity were detected between cultivars (cv. Long Chili-Sweet Bell, $F = 1.2$, $p = 0.9$; Long Chili-Sweet Flexum $F = 1.6$, $p = 0.2$; Long Chili-Sweet Banana, $F = 0.9$, $p = 0.8$; Sweet Bell–Sweet Flexum, $F = 1.3$, $p = 0.6$; Sweet Bell–Sweet Banana, $F = 1.5$, $p = 0.5$; Sweet Flexum–Sweet Banana, $F = 1.7$, $p = 0.4$). Similarly, no differences between cultivars in terms of POD activity level were detected (Long Chili-Sweet Bell, $F = 1.1$, $p = 0.8$; Long Chili-Sweet Flexum $F = 1.4$, $p = 0.5$; Long Chili-Sweet Banana, $F = 0.2$, $p = 0.9$; Sweet Bell–Sweet Flexum, $F = 1.1$, $p = 0.9$; Sweet Bell–Sweet Banana, $F = 1.3$, $p = 0.6$; Sweet Flexum–Sweet Banana, $F = 1.2$, $p = 0.7$).

Discussion

Based on these results, it is clear that sweet pepper mutualism with AMF significantly reduces peach-potato aphid colonization under greenhouse conditions (Fig. 1). The

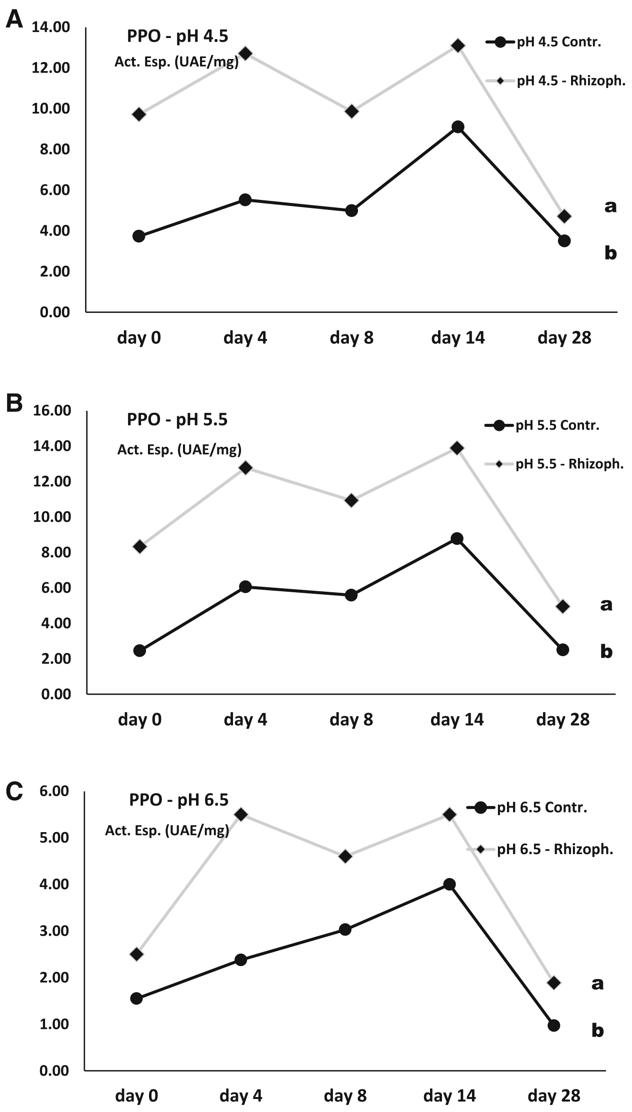

Fig. 2 Activities of polyphenoloxidases (PPO) in sweet pepper leaves, plants inoculated with AMF *R. irregularis* (*black line*) and non-mycorrhizal control (*gray line*) plants at pH 4.5 (**a**), 5.5 (**b**) and 6.5 (**c**) under greenhouse conditions. *Different letters* indicate statistically significant differences at the $p < 0.01$ level (factorial ANOVA was used, followed by Tukey test)

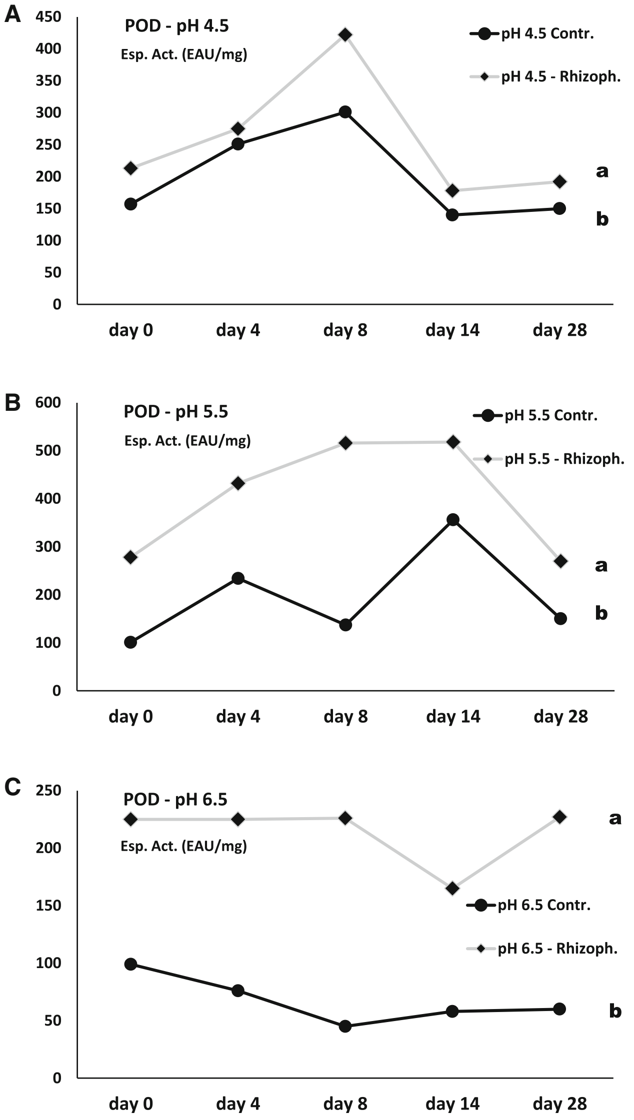

Fig. 3 Activities of peroxidase enzyme (POD) in sweet pepper leaves, plants inoculated with AMF *R. irregularis* (*black line*) and non-mycorrhizal control (*gray line*) plants at pH 4.5 (**a**), 5.5 (**b**) and 6.5 (**c**) under greenhouse conditions. *Different letters* mean statistically significant differences at $p < 0.01$ level (factorial ANOVA was used, followed by Tukey test)

fungus *R. irregularis* seemingly improved effective resistance under all three different soil pH regimes. The range of pH values tested encompasses those in which peppers are normally grown under greenhouse conditions. No effects on other phytophagous arthropod species (e.g., western flower thrips) were observed (Table 1).

Potential factors that may increase plant resistance need to be considered, such as the nutrient limitation of aphids on mycorrhizal plants and plant defense compounds. Aphids feed directly from the plant's phloem (in contrast with thrips, which feed directly from the plant cells) and thus may encounter some limitation of nutrients or digestion of some compounds on mycorrhizal plants, mechanisms partially described elsewhere (e.g., Hempel et al. 2009; Ruscitti et al. 2011; Kloppholz et al. 2011).

The significantly higher PPO and POD activity levels (Figs. 2, 3a–c) found under greenhouse conditions may further explain the lower aphid abundances on treated plants. In other studies, PPO and POD defenses were also found to be strongly induced by methyl jasmonate (MeJA) and oligogalacturonic acid, major plant defense signaling compounds (Constabel et al. 1995). Such responses have, however, been more widely investigated in tomato, potato and soybean plants (Constabel and Barbehenn 2008). For example, it has been shown that PPO induction in tomato occurs as a result of multiple signals acting in parallel with a suite of other anti-herbivore proteins, including several types of protease inhibitors (PIs) and the anti-nutritive enzymes arginase deaminase and threonine deaminase. PPO is thought to play a similar role in defense against

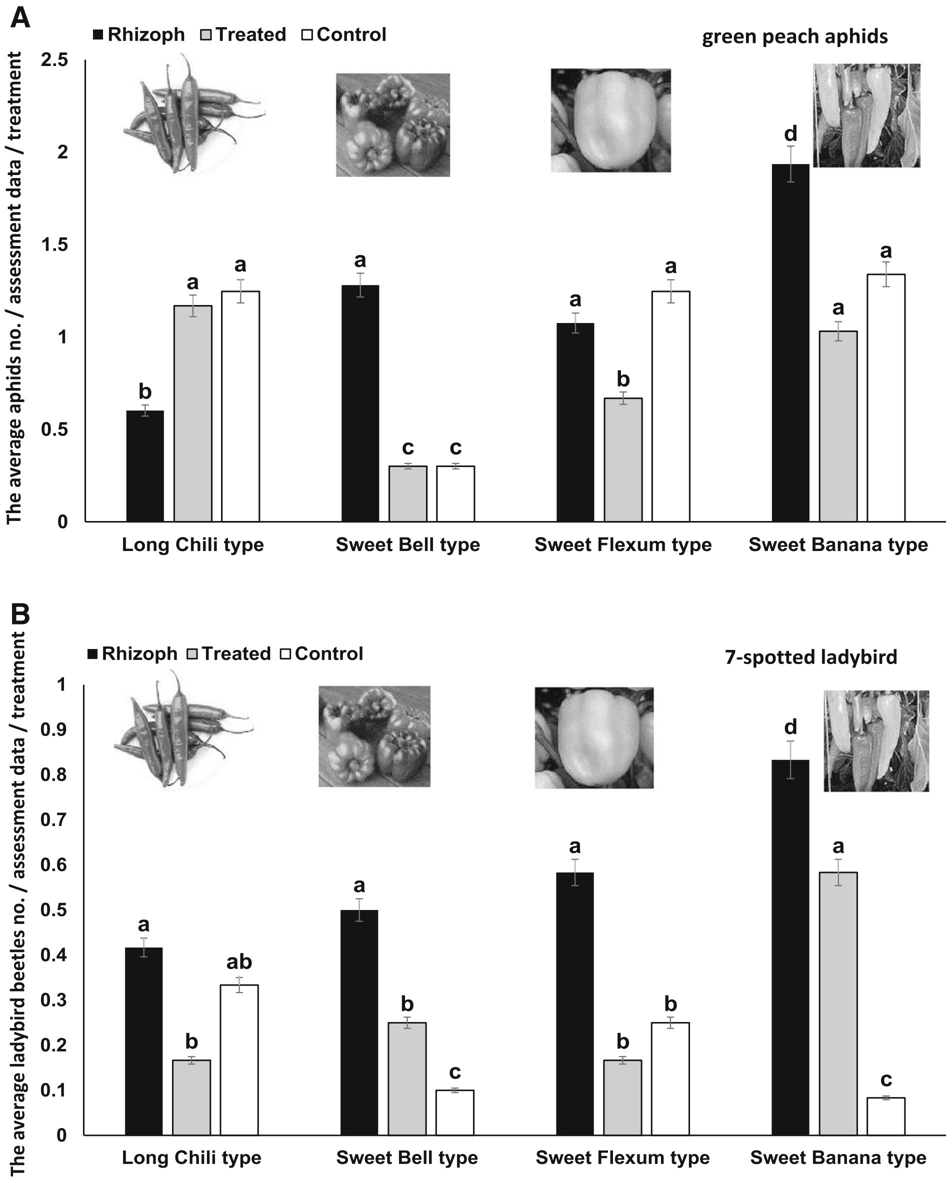

Fig. 4 The average number ($\log_{10}$ transformed) of peach-potato aphids (**a**) and seven-spot ladybird beetles (**b**) per assessment per treatment per ten pepper plants/cultivar under field conditions. *Error bars* = ±1 SE. *Different letters* indicate statistically significant differences at the $p < 0.01$ level (factorial ANOVA was used, followed by Tukey test)

insects (Bergey et al. 1996; Chen et al. 2005; Constabel and Barbehenn 2008). In our case, the high PPO activity at all pH values may explain the pepper defense against aphids if plants are acting in a mutualistic relation with AMF *R. irregularis*. The optimum pH of PPOs is broad, while the reactivity of quinones with amino acids in an acidic medium is greatly reduced (Felton et al. 1992). POD activity consequent upon mutualism with *R. irregularis* has also been observed under greenhouse conditions. Other studies have reported that plants (barley) infested with greenbug aphids, *Schizaphis graminum* (Rondani), increased the total soluble peroxidase activity in cv. Frontera, with a maximum level of hydrogen peroxide (H_2O_2,) activity observed after 20 min of infestation (Argandoña et al. 2001). This finding suggests that POD is directly involved in the stress responses of plants induced by insect (here cereal aphid) infestation. During its mutualism with plants, the fungus secretes effector proteins in a very short time (after 20 h), which remains active even after 14 days. Kloppholz et al. (2011) showed that *R. irregularis* secretes SP7, a protein that interacts with the pathogenesis-related transcription factor ERF19 in the plant nucleus (Kloppholz et al. 2011). This increased resistance correlates with induced expression of various defense-related genes, including PR1, PR2, PR4, Osmotin and SAR8.2 (Lee et al. 2004; Zhang et al. 2009), and may significantly decrease aphid colonization on these plants.

No clear effects of AMF mutualism on aphid colonization were detected under open field conditions however (Fig. 4a). Furthermore, no differences in PPO and POD activity among inoculated, pesticide-treated and control pepper plant growth under open field conditions were observed. Other studies have also shown that the effect of *R. irregularis* under open field conditions is low or even unobservable and that the successful establishment of mutualistic interaction with plant roots in field soils depends on the indigenous fungal species present in the soil (Hepper et al. 1988; Engelmoer et al. 2014). The low competitiveness of *R. irregularis* with other soil microorganisms may explain the low and insignificant PPO and POD activity between treatments and the low or zero effects of peach-potato aphid colonization on plants under open field conditions.

In contrast with other studies (Hempel et al. 2009), the density of ladybird beetles was not directly influenced by *R. irregularis* under greenhouse conditions (Table 2). This can be explained in a different way. A possible explanation is that ladybirds, which use both visual and olfactory cues to hunt their aphid prey, become confused visually in terms of prey selection under greenhouse conditions (Bond 2007). Accordingly, visual predators tend to focus on less cryptic color morphs and neglect the others. This can however be difficult under greenhouse conditions where little or no UV radiation is present and the color perception is different compared with that in open field conditions (Johansen et al. 2011). Under field conditions, the effects of AMF on ladybird abundance were observable in three of four varieties (Fig. 4b). Earlier studies reported that mycorrhizal infection increased the emission of the green leaf volatile (*Z*)-3-hexenyl acetate and sesquiterpenes, which may have important implications for the indirect defense (through predators) of the plant (Fontana et al. 2009). Under field conditions this effect, similarly to emissions from herbivore-damaged plants, probably elicits a positive attractive effect on ladybirds; therefore, their abundance increased in three of the four cultivar varieties under AMF treatments.

Considering crop yield, the number of fruits and their average weight were significantly higher in both varieties (FO and BC) inoculated with AMF in the greenhouse (Table 3). Even so, no clear effects of the mutualism on crop yield were detected in the field (Table 4). Similar researches by Selvakumar and Thamizhiniyan (2011) have shown that AMF-treated chili plants show maximal yield when compared to control under saline conditions. Because of low AMF manifestation due to its low competition under

Table 4 Comparison between the average number of fruits sets and their average fresh weight (g) (means, SE, *F* and *p* values) per treatment per pepper variety in ten randomly selected plants per plot per treatment in the field experiment (comparisons were made using ANOVA and Tukey test)

	Number of fruit sets				Av. weight (g) at harvest			
	Mean	SE	*F*	*p*	Mean	SE	*F*	*p*
Long Chili								
Rhizoph.	37.3	1.9	1.2	ns	183.6	12.2	2.1	ns
Treated	39.3	0.7	0.9	ns	179.4	11	1.7	ns
Control	44.4	0.3	1.1	ns	181.3	11.9	1.9	ns
Sweet Bell								
Rhizoph.	24.1	1.5	2.3	ns	**168.7**	9.2	3.9	+
Treated	20.2	0.9	2	ns	373.7	17.7	2.3	ns
Control	21.6	1.3	1.9	ns	364.1	15	2.1	ns
Sweet Flexum								
Rhizoph.	24.3	1.6	0.8	ns	830.8	27.3	1.6	ns
Treated	22.7	0.7	0.4	ns	970.8	29.2	2.5	ns
Control	22.6	1.1	0.3	ns	**307.8**	14.1	4.7	*
Sweet Banana								
Rhizoph.	25.9	2.7	1.2	ns	**537.9**	29.1	5.01	*
Treated	28.7	1.5	2.1	ns	277.9	11.1	1.9	ns
Control	26.9	0.5	1.6	ns	335.0	15.2	2.6	ns

Statistically significant differences are in bold

ns not significant

$^{+}$ $p < 0.05$; * $p < 0.01$

open field conditions (Hepper et al. 1988; Engelmoer et al. 2014), such effects probably do not occur.

Conclusions

In conclusion, the present study clearly reveals that the mutualistic interaction of pepper plants with the AMF *R. irregularis* is effective under greenhouse conditions in increasing induced plant host resistance to peach-potato aphids. The positive effects of the beneficial predator, the seven spot ladybird, were, however, only observable under open field conditions (our first hypothesis was partially confirmed), thereby additionally providing an economic and environmental benefit by reducing synthetic pesticide use. The higher PPO and POD activity and the higher crop yield were seen only in the greenhouse (our second and third hypotheses were again confirmed, partially at least). All these results reveal that the effects of AMF on sweet pepper plants are various and depend on several factors (environmental, biotic) that need to be further developed to achieve a practical method for AMF use in sweet pepper production.

Author contributions

AB, JB and KB performed the field experiments; AB, KASz and ED performed the PPO and POP analyses; AB, KTJR, JB and HDL performed the data analyses; AB and JB prepared figures and tables; HDL and AB wrote the main manuscript text; HDL edited and corrected the language of the manuscript. All authors reviewed the manuscript and agreed with the submission.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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