Arbuscular mycorrhizal fungi are an alternative to the application of chemical fertilizer in the production of the medicinal and aromatic plant *Coriandrum sativum* L.

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ABSTRACT

The widespread use of agrochemicals is detrimental to the environment and may exert harmful effects on human health. The consumer demand for organic food plants has been increasing. There is thus a rising need for alternatives to agrochemicals that can foster sustainable plant production. The aim of this study was to evaluate the potential use of an arbuscular mycorrhizal (AM) fungus as an alternative to application of chemical fertilizer for improving growth performance of the medicinal and aromatic plant *Coriandrum sativum*. Plants were inoculated with the AM fungus *Rhizophagus irregularis* BEG163 and/or supplemented with a commercial chemical fertilizer (Plant Marvel, Nutriculture Bent Special) in agricultural soil. Plant growth, nutrition, and development of AM fungus were assessed. Plants inoculated with *R. irregularis* and those supplemented with chemical fertilizer displayed significantly improved growth performances when compared with controls. There were no significant differences in total fresh weight between plants inoculated with *R. irregularis* or those supplemented with chemical fertilizer. Leaf chlorophyll a + b (82%), shoot nitrogen (44%), phosphorus (254%), and potassium (27%) concentrations increased in plants inoculated with *R. irregularis* compared to controls. Application of chemical fertilizer inhibited root mycorrhizal colonization and the length of the extraradical mycelium of *R. irregularis*. Inoculation with *R. irregularis* was equally or more efficient than application of chemical fertilizer in promoting growth and nutrition of *C. sativum*. AM fungi may thus contribute to improve biologically based production of food plants and reduce the dependence on agrochemicals in agriculture.

The application of chemical fertilizers and pesticides is a major source of environmental pollution (Turra et al., 2010). Agrochemicals might lead to reduced ecosystem functioning, as well as soil and water degradation (Power, 2010). Agrochemicals might also exert deleterious effects on human health, particularly through exposure of workers in the agroecosystem (Meyer et al., 2010; Alavanja and Bonner, 2012; Freire et al., 2015) or the intake of contaminated food crops (Carvalho, 2006). Currently, the market share for ecologically produced crops is constantly increasing due to customer demands for safer and healthier food (Lim et al., 2014). Another emerging driving force for enhancement of more biologically based cultivation of agricultural crops is the continuous attempts within European Union regulations to reduce inputs of agrochemicals (European Commission, 2007; Skevas et al., 2013). There is thus a rising need to find alternatives instead of application of chemical fertilizers in plant production.

Arbuscular mycorrhizal (AM) fungi are a group of soil microorganisms that form a symbiotic association with plants (Oliveira et al., 2001). AM fungi colonize plant roots and produce hyphae that penetrate into the soil and create a network of extraradical mycelium (ERM). The ERM links colonized roots with the soil matrix and translocates nutrients from soil to roots (Oliveira et al., 2010). Therefore, AM fungi have the capacity to promote plant growth by

improving the uptake of nutrients, such as nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), sulfur (S), copper (Cu), and zinc (Zn), among others (Oliveira et al., 2005a, 2006; Willis et al., 2013). Studies showed that plants inoculated with AM fungi display not only improved growth, but also superior food quality properties, such as increased content of antioxidants, vitamins, and minerals (Albrechtová et al., 2012; Baslam et al., 2013). This AM fungi-mediated added value for food plants contributes to food quality and provides benefits to human health. In addition to their role in plant nutrition, AM fungi possess several other beneficial effects on plants, including improvement of drought tolerance (Augé et al., 2015) and protection against soil-borne plant pathogens (Sikes et al., 2009). Therefore, the potential use of AM fungi may improve biologically based production of food plants.

Coriandrum sativum L., Apiaceae (coriander), is a medicinal and aromatic plant species native to the Mediterranean region. *Coriandrum sativum* is cultivated globally due to its use in culinary practices and cosmetics (Maroufi et al., 2010). The leaves and the essential oil of the seeds of this economically important herb possess several pharmacological activities, such as antioxidant, antihyperglycemic, anxiolytic, diuretic, anti-inflammatory, antibacterial, antifungal, and anthelminthic (Mahendra and Bisht, 2011; Sahib et al., 2012). *Coriandrum sativum* is known to form symbiotic associations with AM fungi (Schroeder and Janos, 2004). However, few data are available on the outcomes of symbiosis for both plant and fungi in plant production systems.

The aims of the present investigation were to (i) determine the effect of AM fungal inoculation on growth and nutrition of *C. sativum* and (ii) assess whether AM fungi may serve as a feasible alternative rather than application of chemical fertilizer (Plant Marvel, Nuticulture Bent Special, PMNBS) for sustainable production of food plants.

Materials and methods

Experimental design and setup

The two mericarps of *C. sativum* seeds were manually separated and surface sterilized with 0.5% (v/v) sodium hypochlorite for 10 min, placed between moist paper towels, and germinated at 20°C in the dark. After germination, seedlings of similar size were transplanted singly into 1-dm^3 pots containing agricultural soil. The soil was collected from the uppermost 10-cm layer of an organic farm in northern Portugal, sieved through a 4-mm mesh, and autoclaved twice (121°C for 25 min) on two consecutive days. The soil was a sandy loam with pH (1:2.5 w/v water) 6.3, electrical conductivity 0.14 dS/m, 3.3% organic matter, 0.24% total N, 51.5 mg/kg extractable (Egner–Riehm) P, 8.7 g/kg K, 2 g/kg Ca, 137 mg/kg magnesium (Mg), and 452 mg/kg sodium (Na). Microbial populations from the original nonsterile soil were reintroduced to each pot by adding 10 ml of filtrate as described in Oliveira et al. (2005a). A nitrocellulose membrane filter (24 mm diameter and 0.4 µm pore size; Pragopore, Pragochema Ltd., Czech Republic) was inserted vertically in each pot for future measurements of ERM length (Baláz and Vosátka, 2001). The experiment comprised 4 treatments: (i) control, (ii) plants supplemented with a commercial chemical fertilizer (PMNBS) (iii) plants inoculated with an AM fungus, and (iv) plants supplemented with PMNBS and inoculated with an AM fungus. All treatments were replicated 10 times. The AM fungal isolate used in this study was *Rhizophagus irregularis* BEG163. It was grown for 8 mo in a multispore pot culture containing a 1:1 (v/v) mixture of zeolite and sand with *Trifolium pratense* L. as host plant. At transplanting, each pot from the mycorrhizal treatments received 10 g inoculum consisting of colonized root fragments, hyphae, and spores in the mixture of zeolite and sand, placed 2 cm below the root system. Every pot from the nonmycorrhizal treatments received 10 g inoculum autoclaved twice (121°C for 25 min) on consecutive days. In order to eliminate differences in microbial populations introduced with the AM fungal inoculum, 5 ml of a suspension of AM fungal inoculum was added to each pot from the nonmycorrhizal treatments (Koide and Li, 1989). The suspension was prepared as described in Oliveira et al. (2010). Plants supplemented with chemical fertilizer received 25 ml of a commercial chemical fertilizer (200 mg/L N, 57 mg/L P_2O_5, 130 mg/L K_2O, 0.5 mg/L S, 0.4 mg/L Mg, 0.4 mg/L Cu, 0.4 mg/L Zn, 0.4 mg/L Mn, 1.4 mg/L B, 0.7 mg/L Fe, 0.007 mg/L

Mo) per pot 3 times per week. Nonfertilized plants received 25 ml deionized water. Plants were grown in a greenhouse under an average photoperiod of 12 h. Temperature and relative humidity ranges were 15–40 °C and 60–80%, respectively. Pots of different treatments were periodically rotated to different bench positions to minimize differences due to their location in the greenhouse.

Plant parameters analyses

After a growth period of 62 d, plants were removed from the pots. The root system was separated from the shoot and washed to remove adhered soil. Root and shoot were weighed and the sum of their masses yielded the total plant fresh weight. The root/shoot fresh weight ratio was calculated by dividing the root by shoot mass. A fresh subsample (0.02 g) was cut from the second mature leaf from the plant apex and the concentrations of chlorophyll a, chlorophyll b, and chlorophyll a + b determined after extraction with *N,N*-dimethylformamide according to Wellburn (1994). Shoots were dried at 70°C for 48 h and 0.3 g of finely ground material was digested as described in Novozamsky et al. (1983). Phosphorus and N concentrations in shoots were determined by colorimetry (Spectronic Genesys 10 Bio, Thermo Electron Corporation, USA), while K concentration was determined by flame atomic emission spectroscopy (Walinga et al., 1989).

Fungal parameters analyses

A subsample (0.2 g) of fresh roots of *C. sativum* was cut into 1-cm pieces and stained with trypan blue using a modified Phillips and Hayman (1970) protocol (Oliveira et al., 2005b). Percentage root length colonized (RLC) by AM fungi was assessed using the grid-line intersect method (Giovannetti and Mosse, 1980) under a stereomicroscope (Olympus SZ61, Japan). Stained root pieces were mounted on glass slides and examined with a compound microscope (Leica DM 750, Germany) (×100–400) to assess abundance of arbuscules in the mycorrhizal root segments (Trouvelot et al., 1986). Arbuscule abundance was determined using the software Mycocalc (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html) and expressed as percent of the colonized root length occupied by arbuscules. ERM length was determined by the inserted membrane technique (Baláz and Vosátka, 2001) followed by the grid-line intersect method under a compound microscope (Leica DM 750, Germany), using an ocular grid at ×200 magnification (Brundrett et al., 1994). Background lengths of mycelium found in non-mycorrhizal treatments were subtracted from the values obtained in the corresponding mycorrhizal treatments and the ERM length wqas expressed in centimeters of hyphae per 1 cm^2 of the inserted membrane filter.

Statistical analysis

Normality and homogeneity of variances were confirmed and data analyzed using two-way analysis of variance (ANOVA) for each dependent variable (plant parameters) versus the independent variables (fungal inoculation and fertilization). When a significant F value was obtained ($p < .05$), treatment means were compared using Duncan's multiple-range test. Fungal parameters data were analyzed without including the respective noninoculated control treatments using Student's t-test at a significance level of $p < 0.05$. All statistical analyses were performed with the SPSS 20.0.0 software package (IBM SPSS Statistics, USA).

Results and discussion

Plant growth

Both the application of chemical fertilizer and inoculation with *R. irregularis* significantly improved growth of *C. sativum* compared with controls (Figure 1). There were no significant differences in total fresh weight between plants supplemented with chemical fertilizer and those inoculated with *R. irregularis* (Figure 1C). Fungal inoculation exerted the same effect on shoot fresh weight of *C. sativum* as application of chemical fertilizer (Figure 1A). The shoots including stem and leaves are among the most valuable parts of *C. sativum* for culinary, cosmetic, and medicinal uses (Maroufi et al., 2010; Sahib et al., 2012). Although AM fungi are known to increase plant biomass and

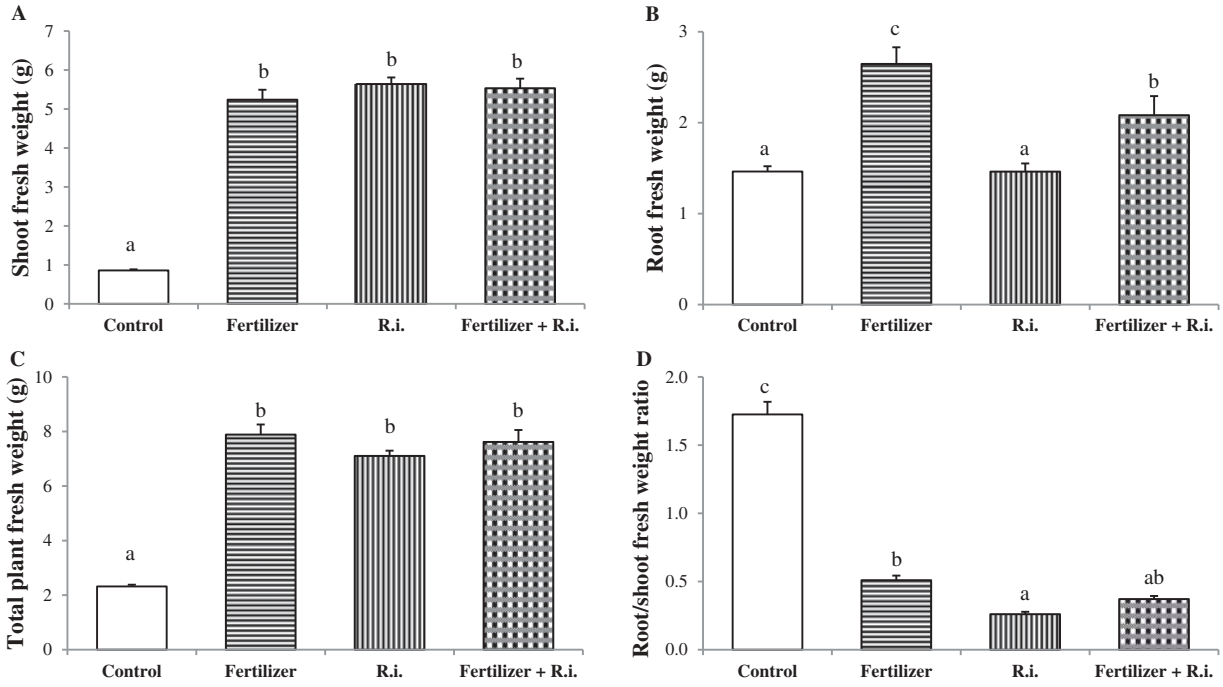

Figure 1. Shoot (A), root (B), and total plant (C) fresh weight and root/shoot fresh weight ratio (D) of *Coriandrum sativum* supplemented with chemical fertilizer and inoculated with *Rhizophagus irregularis*. Values are means ± 1 SE. Columns marked with the same letters are not significantly different according to Duncan's multiple range test at $p < .05$. R.i., *Rhizophagus irregularis*.

productivity in agroecosystems (Jeffries et al., 2003), their beneficial effects depend largely upon plant and fungal species involved in symbiosis (van der Heijden et al., 2015). Data demonstrated that *R. irregularis* improved the growth of *C. sativum* and therefore may be regarded as an alternative to application of PMNBS.

There was no marked improvement in shoot fresh weight and total fresh weight of plants simultaneously supplemented with chemical fertilizer and inoculated with *R. irregularis* compared with those only supplemented with PMNBS or only inoculated with *R. irregularis*. Evidence indicates that application of chemical fertilizer together with fungal inoculation does not enhance the growth of *C. sativum*. Therefore, inoculation with *R. irregularis* alone is preferred due not only to the observed effects on plant growth, but also to environmental benefits such that application of chemical fertilizer may be eliminated.

Plants supplemented with PMNBS displayed a significantly higher root fresh weight compared to any other treatment (Figure 1B). Inoculation with *R. irregularis* alone did not markedly influence root growth. However, plants simultaneously supplemented with chemical fertilizer and inoculated with *R. irregularis* showed a significantly higher root fresh weight compared with controls. Both fungal inoculation and application of chemical fertilizer markedly influenced root/shoot fresh weight ratio of *C. sativum* (Figure 1D). Plants from all mycorrhizal and fertilization treatments displayed a significantly lower root/shoot ratio than controls. The lowest root/shoot ratio was that of *C. sativum* inoculated with *R. irregularis*, showing that these plants had a significantly higher proportion of shoots. Smith et al. (2011) demonstrated that plants colonized by AM fungi possess lower root/shoot ratios. With the help of AM fungi, plants might take up soil nutrients more efficiently, and save energy and carbon for root production, concurrently increasing shoot growth in order to obtain higher photosynthetic rates (Maherali, 2014).

Plant nutrition

Inoculation with *R. irregularis* and application of chemical fertilizer significantly increased shoot N concentration of *C. sativum* compared with controls (Figure 2A). Plants inoculated with *R. irregularis* possessed the highest shoot N concentration, irrespective of the fertilization treatment. Plants

supplemented with chemical fertilizer, plants inoculated with *R. irregularis*, and plants simultaneously supplemented with PMNBS and inoculated with the AM fungus showed significantly higher shoot P concentrations than controls (Figure 2B). Shoot P concentration was significantly higher in plants inoculated with *R. irregularis* than in plants supplemented with chemical fertilizer. Plants from all mycorrhizal and fertilization treatments displayed a significantly higher shoot K concentration than controls (Figure 2C). No marked differences in shoot K concentration were obtained among all mycorrhizal and fertilization treatments. Shoot concentrations of N, P, and K increased in plants inoculated with *R. irregularis* by 44, 254, and 27%, respectively, compared with controls. The highest elevation was observed in shoot P concentration. Phosphorus is essential nutrient for plant growth; however, its low bioavailability in soils makes it difficult for plants to take up (Marschner, 2012). This limitation might be overcome by application of AM fungi. Improved P acquisition is one of the well-documented effects of

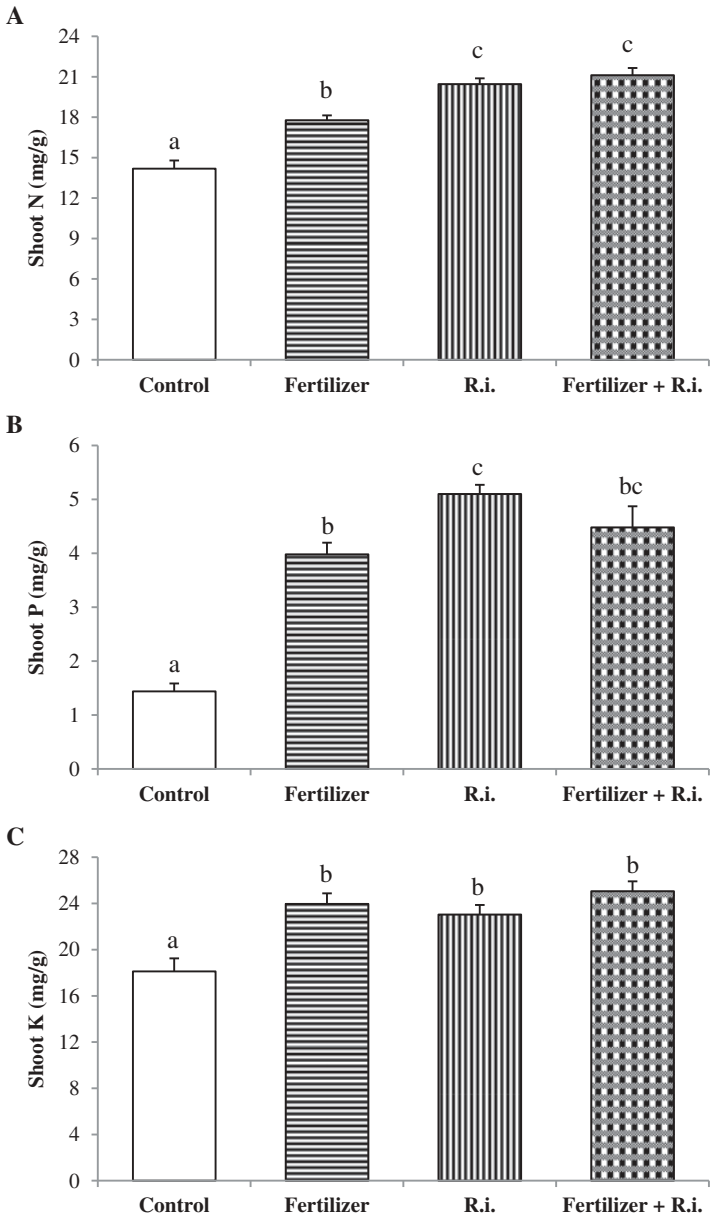

Figure 2. Shoot nitrogen (A), phosphorus (B), and potassium (C) concentration of *Coriandrum sativum* supplemented with chemical fertilizer and inoculated with *Rhizophagus irregularis*. Values are means ± 1 SE. Columns marked with the same letters are not significantly different according to Duncan's multiple range test at $p < .05$. R.i., *Rhizophagus irregularis*.

AM fungi in plants (Oliveira et al., 2010; Smith et al., 2011). Increased tissue N and K concentrations are also common in mycorrhizal plants (Oliveira et al., 2005a; Willis et al., 2013). Data from the present study indicate that *R. irregularis* contributed to improve the nutritional status of *C. sativum*. This nutritional benefit may have also contributed to enhanced growth performance, which was observed in mycorrhizal *C. sativum* plants.

Leaf chlorophyll concentration was significantly higher in plants from all mycorrhizal and fertilization treatments compared with controls (Figure 3). Plants inoculated with *R. irregularis* demonstrated significantly higher leaf chlorophyll a and chlorophyll a + b levels than plants supplemented with PMNBS (Figures 3A and 3C), while no marked difference in leaf chlorophyll b concentrations was found between plants inoculated with *R. irregularis* and those supplemented with chemical fertilizer (Figure 3B). The concentration of chlorophyll is a physiological indicator of the photosynthetic potential of plants. AM fungi

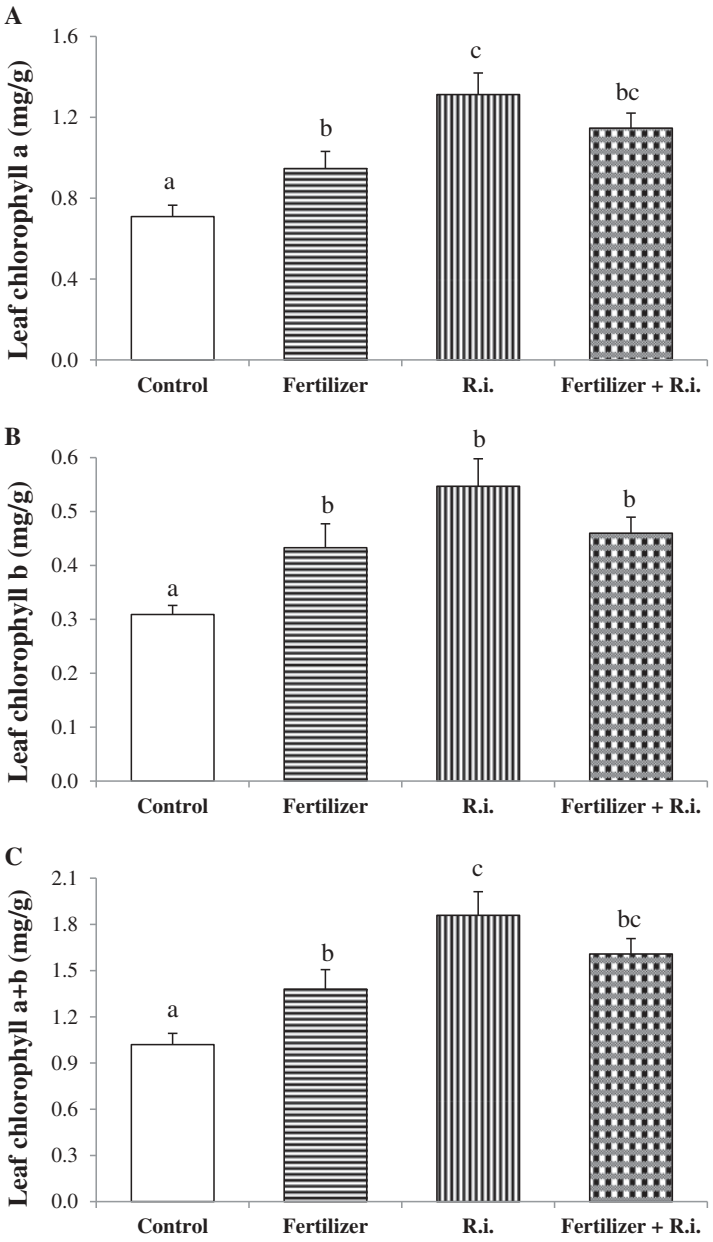

Figure 3. Leaf chlorophyll a (A), chlorophyll b (B), and chlorophyll a + b (C) concentration of *Coriandrum sativum* supplemented with chemical fertilizer and inoculated with *Rhizophagus irregularis*. Values are means ± 1 SE. Columns marked with the same letters are not significantly different according to Duncan's multiple range test at $p < .05$. R.i., *Rhizophagus irregularis*.

enhance the photosynthetic potential of their host plants by increasing the concentration of photosynthetic pigments (Oliveira et al., 2005a) and/or developing photosynthetically active tissue (Adolfsson et al., 2015). Vafadar et al. (2014) found significant elevation in chlorophyll a, b, and a + b levels of the medicinal plant *Stevia rebaudiana* inoculated with *R. irregularis*. Inoculation with the same AM fungal species was also shown to increase leaf chlorophyll concentrations of other plant species (Oliveira et al., 2005a; Zhu et al., 2012). In the present study, inoculation with *R. irregularis* was equally or more efficient than application of PMNBS in enhancing the nutritional status and chlorophyll levels of *C. sativum*, indicating that AM fungi might serve as an effective alternative to chemical fertilizers.

Effect of chemical fertilizer on fungal development

There was no AM fungal colonization in the roots of noninoculated plants. Inoculation with *R. irregularis* resulted in 25 and 38% RLC in plants with or without application of PMNBS, respectively (Table 1). These values are within the range of 11–43% reported by Schroeder and Janos (2004) for *C. sativum*. The percent RLC, arbuscule abundance, and ERM length were significantly reduced with application of chemical fertilizer. The increased availability of nutrients, especially P, resulting from application of chemical fertilizers is known to inhibit AM development by reducing root colonization (Willis et al., 2013) and length of the ERM (Olsson et al., 2014). Data from the present study showed that application of chemical fertilizer was detrimental to development of *R. irregularis* associated with *C. sativum*.

Table 1. Mycorrhizal colonization and length of the extraradical mycelium of *Rhizophagus irregularis* with and without the application of chemical fertilizer.

Treatment	AMF colonization (%RLC)	Arbuscule abundance (%)	ERM length (cm/cm^2)
Rhizophagus irregularis	38 ± 4	45 ± 4	10 ± 2
Fertilizer + *Rhizophagus irregularis*	25 ± 3	20 ± 5	5 ± 1
Student's *t*-test significance	*	*	*

Note. Values are means ±1 SE. Asterisk indicates significant effect at the level of $p < .05$. AMF, arbuscular mycorrhizal fungal; RLC, root length colonized; ERM, extraradical mycelium.

Conclusions

Coriandrum sativum inoculated with *R. irregularis* displayed higher shoot and total weight than noninoculated controls. This effect was probably due to enhanced uptake of soil nutrients mediated by AM fungus. Inoculated plants possessed also greater leaf chlorophyll concentrations, indicating enhanced photosynthetic potential. Mycorrhizal *C. sativum* plants showed improved growth and nutrition without input of chemical fertilizer. There were no significant differences in shoot and total weight between *C. sativum* inoculated with *R. irregularis* and those supplemented with PMNBS. Therefore, inoculation with AM fungi may be regarded as an alternative ecotechnological approach to application of chemical fertilizer in production of *C. sativum*. Further studies are required to determine the effects of AM fungi and chemical fertilizer on the essential oil content of *C. sativum*. AM fungi display potential to reduce dependence upon agrochemicals in agriculture, thus contributing to prevent environmental degradation and improve food quality, and consequently resulting in benefits to human health.

Funding

R. S. Oliveira and Y. Ma acknowledge the support of Fundação para a Ciência e a Tecnologia (FCT) through the research grants SFRH/BPD/85008/2012 and SFRH/BPD/76028/2011 and Fundo Social Europeu (FSE). I. Rocha was supported by the FCT grant BI-EXPL/AGR-TEC/1204/2013. M. F. Carvalho acknowledges the Investigator FCT program supported by FCT, FSE, and Programa Operacional Potencial Humano (POPH). This work was financed by national funds through FCT under Project EXPL/AGR-TEC/1204/2013 and by Fundo Europeu de Desenvolvimento Regional (FEDER), Eixo I do Programa Operacional Fatores de Competitividade (POFC) of Quadro de Referência Estratégica Nacional (QREN) (COMPETE: FCOMP-01-0124-FEDER-041572).

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