Impact of Arbuscular Mycorrhizal Inoculation and Growth Substrate on Biomass and Content of Polyphenols in *Eclipta prostrata*

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Abstract. The aim of this study was to determine the influence of mycorrhizal inoculation and sand-peat composition as growth substrate on the biomass, and individual polyphenol concentration of *Eclipta prostrata*. Mycorrhizal inoculation alters some secondary metabolites of *E. prostrata*, showing significant differences in polyphenol contents between the treatments. Moreover, varying peat and sand rates, representing different nutrient supplies, had significant impacts on both mycorrhizal colonization and growth responses. Our results highlight that 60/40% (v/v) sand and peat ration is the best for a large-scale cultivation of *E. prostrata*, moreover supporting the highest total phenolic content. Through high-performance liquid chromatography (HPLC) analysis, nine individual phenolic components were analyzed, including wedelolactone and demethyl-wedelolactone at the highest concentration. Some of the identified compounds, such as 5-*o*-caffeoylquinic acid, quercetin-3-arabinoside, 4-*o*-caffeoylquinic acid, and protocatechuic acid have not been reported previously in *E. prostrata* cultivars. Using hierarchical cluster analysis, multiple groups are represented, suggesting the role of mycorrhizal inoculation, growth substrate, and their interaction on secondary metabolites of *E. prostrata*. Better understanding of the phenolic composition of *E. prostrata* and factors influencing it help to identify new industrial applications of this medicinal plant (together with arbuscular mycorrhizal fungi), and moreover, help to develop new strategies for the prevention and treatment of different diseases.

Eclipta prostrata belongs to the Asteraceae family, and it is distributed throughout the world in more than 83 countries (Holm et al., 1979). *E. prostrata* is valued for traditional Chinese herbal medicine to treat diseases such as diabetes type II, dizziness, haemoptysis, and liver diseases, among

others; to reduce lipid development; as antivenom against snakebite envenomation; and in human immunodeficiency virus-1 (HIV-1) management (Chung et al., 2017). The biological property of *E. prostrata* was first recorded by Holm et al. (1979) and described by Ho (2000) in Vietnam. Some previous studies have confirmed that *E. prostrata* contains different natural compounds such as flavonoids, alkaloids, triterpenoids, saponins, phenolics, essential oils, thiophenes, wedelolactone, and steroids (Han et al., 2013, 2015). The highest content of polyphenolic component reached 11.86%, followed by saponins, 1.7%; alkaloids, 0.34%; and flavonoids, 0.87%, in the work of Dhandapani and Sabna (2008). Triterpenoids

isolated from *E. prostrata* can play a crucial role in antiproliferative, antimicrobial potentials (Lee et al., 2008), and can suppress the growth of some gram-negative and positive bacteria (Cherdtrakulkiat et al., 2015). Wedelolactone have properties in against lung, breast, and cell prostate, antihepatotoxic, and antitrypsin activities as well (Benes et al., 2011). Moreover, Sarveswaran and co-workers (2012) suggested that wedelolactone induces a novel mechanism in the human body and may emerge as a novel therapeutic agent against prostate cancer.

Arbuscular mycorrhizal fungi (AMF) are symbiotic fungi that associate with more than 85% of terrestrial plant species, including many important agricultural and horticultural taxa (Smith and Read, 2008). AMF provides benefits to host plants such as improved seedling survival, increase in growth and yield, resistance to abiotic or biotic stresses, and improving soil aggregates (Birhane et al., 2012; Duc et al., 2017; Gianinazzi et al., 2010). Recently, the application of AMF in the cultivation of some medicinal plants has been evaluated for their influence on the biologically active secondary metabolites. Fifty medicinal plant species from 19 families have been described as mycorrhizal dependent plants (Zeng et al., 2013) and Panwar and Tarafdar (2006) found six dominant AMF species such as *Funneliformis constrictum* (*Glomus constrictum*), *Rhizophagus fasciculatus* (*Glomus fasciculatum*), *Funneliformis geosporum* (*Glomus geosporum*), *Rhizophagus irregularis* (*Glomus intraradices*), *Funneliformis mosseae* (*Glomus mosseae*), and *Glomus rubiforme* in the roots of various medicinal plants. The enhanced dry weight of *Abelmoschus moschatus*, *Clitoria ternatea*, *Plumbago zeylanica*, *Psoralea corylifolia*, and *Withania somnifera* cultivars due to mycorrhizal inoculation cultivated in various soil types have also been investigated (Chandra et al., 2010). Besides increased growth, mycorrhizal fungi can promote the accumulation of some secondary metabolites, including beta-carotene and lycopene in tomato (Ulrichs et al., 2008), or carotenoid and anthocyanins in *Lactuca sativa* (Baslam et al., 2013). However, opposite results (no change in polyphenol concentration) have been published in *Ocimum basilicum* plants inoculated with *Rhizophagus irregularis* (Lee and Scagel, 2009). The mechanisms by which AMF alter the production of secondary metabolites in *E. prostrata* are still not clarified.

Sand and peat are the most commonly used substrates in agriculture showing different effects on both the growth and mycorrhizal efficiency of the target plants. The importance of peat as a growth substrate has been confirmed by Bunt (1974), who found the highest beneficial effect on growth of tomato plants in a 25:75% (v/v) sand and peat mixture. However, Chroboczek (1963) reported that a sand and peat mix substrate containing more than 50% of sand was also an amendment to cultivation of cucumber

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and tomato plants, missing information regarding *E. prostrata*.

Due to the increased demand for sustainable, large-scale cultivation of medicinal plants, the aims of this study were to investigate the effects of AMF inoculation on the biomass, total phenolic content, and polyphenol concentration of *E. prostrata* growing in different proportions of sand and peat.

Materials and Methods

Experiment design. Sand and peat were mixed at six different proportions representing various nutrient supplies (100/0; 80/20; 60/40; 40/60; 20/80; and 0/100%) (v/v), then sterilized by an autoclave. The moderately decomposed peat (H5) and sand characteristics are summarized in Table 1. Using 15 g of commercial mycorrhizal inoculant Symbivit® [a mixture of *Rhizopagus irregularis* (*G. intraradices*), *Funneliformis mosseae* (*G. mosseae*), *Claroideoglossum etunicatum* (*G. etunicatum*), *Claroideoglossum claroideum* (*G. claroideum*), *Rhizoglossum microaggregatum* (*G. microaggregatum*), and *Funneliformis geosporum* (*G. geosporum*)] containing infected roots, spores, and hyphae was settled into the planting hole representing mycorrhizal treated plants. The control plants received the same quantity of mycorrhizal inoculant, which was sterilized before use.

E. prostrata seeds were sterilized in 1% NaOCl, then washed with sterilized water several times and germinated on wet filter paper. Five pre-germinated seeds were sown to a 3 cm depth in each pot containing a different proportion of sand and peat substrate (750 cm³), then transferred to a climatic chamber EKOCHL 1500 (24/28 °C, 60% relative humidity, 16/8 h photoperiod, 600 µmol·m⁻²·s⁻¹ light intensity). After 7 d of growth, each pot thinned to one seedling. The plants were watered two times a week for 7 weeks. Each treatment had 10 biological replicates.

Assessment of plant growth rate and biomass. Plants were harvested after 7 weeks of growth; fresh and dry weight of shoots, roots, and leaf area were estimated. Leaf area was measured according to the method of Glozer (2008).

Determination of root colonization by mycorrhizal fungi. For the root staining, five plants were selected randomly from each treatment, then thirty root fragments (1 cm long) from each plant were collected (Trouvelot et al., 1986). The roots were cleared with 10% KOH for 10 min, acidified using 2% HCl and 0.05% Trypan blue in 1:1:1 water : glycerin : lactic acid overnight. The roots were then mounted on slides; root pieces were observed under a stereomicroscope at 100X magnification, and the root colonization was determined as described by Trouvelot et al. (1986), using MYCOCALC software.

Chlorophyll a fluorescence determination. Chlorophyll a fluorescence, the maximum

efficiency of photosystem II (PSII) photochemistry (Fv/Fm), was determined after 30 min of dark-adaptation by using a Walz—PAM 2500 (Germany) fluorometer according to the method of Oxborough and Baker (1997) and Neményi et al. (1999). Four fully developed leaves from the shoot apex of a single plant in five biological replicates in each treatment were measured after 7 weeks of cultivation.

Proline determination. Proline concentration was quantified by reacting with ninhydrin acid, following the procedure of Bates et al. (1973). Briefly, each aerial part of *E. prostrata* fresh plant material (0.5 g) was homogenized in 10 mL of 3% aqueous sulfosalicylic acid and homogenate filtered through Whatman#1 filter paper. Then a 1:1:1 solution of proline, ninhydrin acid, and glacial acetic acid was added and incubated at 100 °C for 1 h. The reaction was arrested in an ice bath, the chromophore was extracted with 4 mL toluene, and its absorbance at 520 nm was determined in a BioMate spectrophotometer (Thermo Spectronic).

Determination of total phenolics components. Total phenolics (TP) concentration was measured by a Folin—Ciocalteu assay (Lister and Wilson, 2001). Briefly, 20 mL 60% ethanol was added to approximately 2 g of plant material (aerials part), mixed well, and then filtered. Folin—Denis reagent (0.5 mL) was added to a 1 mL filtered sample, and the content of the tube was mixed thoroughly. After 3 min, 1 mL of saturated Na₂CO₃ was added. The mixture was completed to 10 mL with distilled water and allowed to stand for 30 min at room temperature. Total phenolic contents absorbance was taken at 760 nm

and was expressed as mg gallic acid in 100 g fresh weight.

Determination of polyphenol by using high performance liquid chromatographic analysis. To extract polyphenols, each aerial part of *E. prostrata* fresh plant material (0.5 g, well-blended) were crushed in a crucible mortar in the presence of quartz sand. With the gradual addition of 20 mL of a mixture of 45% MeOH + 45% EtOH + 10% water, the crushed sample was transferred to an Erlenmeyer flask and subjected to an ultra-sonication force using an ultrasonic water bath device for 4 min. Then the sample was shaken by a mechanical (GLF3005) shaker for 15 min. The mixture was kept overnight at 4 °C, then filtered through a filter paper. It was further cleaned-up by passing through a 0.22 µm PTFE HPLC syringe filter before injection on to the HPLC column for the analysis of polyphenols. We used Nucleosil C18, 100, Protect-1 (Macherey-Nagel, Duren, Germany), 3 µm particle size, 150 × 4.6 column to separate phenolic compounds using a gradient elution of 1% formic acid in water (A) and acetonitrile (B), with a flow rate of 0.6 mL·min⁻¹. Gradient elution started with 2% B; it changed in 10 min to 13% B, then in 5 min to 25% B, and then in 15 min to 40% B, and finally, in 7 min it turned to 2% B.

The peaks were identified by comparing their retention times and spectral characteristics with available standards such as catechin, quercetin-3-glucoside, luteolin-glucoside, luteolin, wedelolactone, and demethyl-wedelolactone (Sigma-Aldrich Ltd., Hungary). For quantitation of phenolic compounds, the area of each peak was integrated at the maximum absorption wavelength, and the concentrations

Table 1. The main chemical characteristics of sand and peat substrate.

	pH	Phosphorus (mg/kg)	Potassium (mg/kg)	Carbonate (%)	Nitrogen (%)	Dry matter content (m/m%)
Peat	6.45 ± 0.06	1610 ± 14	3320 ± 24	42.93 ± 0.347	0.94 ± 0.018	15.7 ± 0.1
Sand	7.23 ± 0.03	62.16 ± 0.78	2485 ± 11	0.01 ± 0.0015	0.38 ± 0.081	79.4 ± 0.3

Fig. 1. Mycorrhizal colonization rate (%) of *E. prostrata* after 7 weeks growth at different rates of sand and peat as the growth substrate. Different letters indicate significant difference according to the Tukey post-hoc test ($P < 0.05$).

Table 2. How influence different treatments the biomass of shoot, roots, and leaf area of *Eclipta prostrata* (mean $\pm$ SD, n = 5) after 7 weeks of growth.

Treatment ^z		Shoot ^{yx}		Root ^{yx}		Leaf area (cm ² /plant) ^{zy}
Sand/Peat % (v/v)		Fresh (g/plant)	Dry (g/plant)	Fresh (g/plant)	Dry (g/plant)	
100/0	AMF+	1.99 $\pm$ 0.47 f	0.13 $\pm$ 0.027 ef	0.25 $\pm$ 0.09 d	0.06 $\pm$ 0.02 d	8.09 $\pm$ 0.32 e
	AMF-	0.39 $\pm$ 0.09 g	0.03 $\pm$ 0.022 f	0.18 $\pm$ 0.49 d	0.01 $\pm$ 0.005 d	2.06 $\pm$ 0.30 f
80/20	AMF+	19.38 $\pm$ 3.82 de	2.34 $\pm$ 0.73 bc	9.79 $\pm$ 1.21 ab	1.41 $\pm$ 0.16 b	18.04 $\pm$ 1.71abc
	AMF-	14.37 $\pm$ 3.84 e	1.91 $\pm$ 0.47 bcd	6.61 $\pm$ 0.59 c	0.72 $\pm$ 0.15 c	13.74 $\pm$ 1.00 d
60/40	AMF+	38.85 $\pm$ 3.92 a	3.93 $\pm$ 0.46 a	11.68 $\pm$ 0.43 a	1.75 $\pm$ 0.15 a	21.65 $\pm$ 3.05 a
	AMF-	27.43 $\pm$ 3.97 bc	2.74 $\pm$ 0.53 b	8.95 $\pm$ 1.78 b	0.92 $\pm$ 0.17 c	15.13 $\pm$ 2.28 cd
40/60	AMF+	14.83 $\pm$ 1.94 e	1.48 $\pm$ 0.27 cd	1.52 $\pm$ 0.25 d	0.14 $\pm$ 0.03 d	18.90 $\pm$ 0.82 abc
	AMF-	14.41 $\pm$ 3.87 e	1.27 $\pm$ 0.49 cd	1.32 $\pm$ 0.57 d	0.11 $\pm$ 0.025 d	16.59 $\pm$ 3.75 bcd
20/80	AMF+	33.41 $\pm$ 4.91 ab	3.89 $\pm$ 0.92 a	5.73 $\pm$ 0.54 c	0.70 $\pm$ 0.15 c	19.49 $\pm$ 1.35 ab
	AMF-	12.58 $\pm$ 2.97 e	1.15 $\pm$ 0.43 de	0.96 $\pm$ 0.17 d	0.08 $\pm$ 0.01 d	18.85 $\pm$ 2.79 abc
0/100	AMF+	23.43 $\pm$ 4.72 cd	2.2 $\pm$ 0.28 bcd	4.78 $\pm$ 0.67 c	0.62 $\pm$ 0.12 c	18.46 $\pm$ 2.39 abc
	AMF-	15.70 $\pm$ 1.55 e	1.28 $\pm$ 0.31 cd	0.79 $\pm$ 0.36 d	0.11 $\pm$ 0.04 d	16.91 $\pm$ 2.02 bcd
Growing media (GM)		***	***	***	***	***
Mycorrhizae (M)		***	***	***	***	***
M $\times$ GM		***	***	***	***	***

^zAMF+ represents the mycorrhizal; AMF- represents the nonmycorrhizal plants.

^yDifferent letters in each parameter indicate significant difference according to the Tukey test ($P < 0.05$) among sand and peat ration.

***Significant at $P < 0.001$.

Fig. 2. Fv/Fm quantum efficiency of photosystem II. (A), Proline concentration (B) in the leaves of *E. prostrata* after seven weeks growth, using different rates of sand and peat as the growth substrate. Black and grey columns refer to AMF treated (AMF+) and Control (AMF-) plants, respectively. Different letters indicate significant difference according to the Tukey post-hoc test ($P < 0.05$).

were calculated by relating the areas of peaks to those of the available external standards (Merken and Beecher, 2000). In the case of no standard materials available, the polyphenol compounds were tentatively identified based on their retention recognized on chromatogram

and spectral properties (Fang et al., 2015). The standard materials were singly injected as external standards and cochromatographed with the samples as well.

Statistical analysis. Statistical analysis was carried out using the SAS 9.1 (SAS

Institute, Cary, NC) package for Windows. Means were compared by Tukey post-hoc test at $P < 0.05$. Principal component analysis (PCA) as a statistical procedure was used to investigate patterns in polyphenolic data, and to highlight similarities and dissimilarities in phenolic contents of *E. prostrata* with and without arbuscular mycorrhizal fungi (AMF). Hierarchical cluster analysis (HCA) was performed to identify relative similarity among treatments, and the result was drawn as a dendrogram. The PCA and HCA were carried out by using an XLSTAT program.

Results

Mycorrhizal inoculation resulted in successful root colonization by mycorrhizal plants, while no infection was observed in noninoculant plants. The highest percentage of root colonization ($76.23\% \pm 15.6$) was found at 60/40% (v/v) sand/peat, followed by a higher sand proportion at the same rate (Fig. 1). There was no significant difference in root colonization at higher than 40% (v/v) peat rations. Mycorrhizal inoculation and growth substrate had significant influence on both the fresh and dry biomass of shoots and roots (Table 2). The total fresh and dry biomass of shoots in mycorrhizal treatments were 55% and 67% higher than in the control one, respectively. Moreover, mycorrhizal inoculation increased the totally fresh and dry root weight by 79% and 140%, respectively, compared with nonmycorrhizal plants (Table 2).

The highest leaf area of *E. prostrata* was measured in mycorrhizal plants at 60/40% (v/v) sand/peat substrate, and the lowest one was found with sand substrate in the non-mycorrhizal treatment. Both the mycorrhizal inoculant and growth substrate have a significant influence on the leaf area (Table 2).

The plants growing at different proportions of sand and peat varied in their Fv/Fm (Fig. 2A). The highest quantum efficiency of photosystem II was recorded in mycorrhizal treatment 60/40% (v/v) sand/peat mix;

Fig. 3. Total phenolic content (TP) in leaves of *E. prostrata* after 7 weeks growth at different rates of sand and peat as the growth substrate. Black and grey columns refer to AMF treated (AMF+) and Control (AMF-) plants, respectively. Different letters indicate significant difference according to the Tukey post-hoc test ($P < 0.05$).

however, no significant difference could be detected between different treatments. Both the mycorrhizal fungi and growth substrate have a significant influence on proline concentration of the leaves. Increasing the proportion of peat substrate resulted in a small-scale increase in proline concentration, reaching the highest level of both treatments in 100% peat (Fig. 2B).

Different proportions of peat had significant effects on the total phenolic component (TP) concentration of aerial parts, but mycorrhizal inoculation did not (Fig. 3). Peat concentration higher than 40% (v/v) decreased the phenolic content of plants. *E. prostrata* growing in a 60/40% (v/v) sand and peat substrate ration had the highest phenolic content with both treatments.

In the HPLC analysis, the gradient elution applied was able to efficiently separate a wide range of phenolic compounds such as protocatechuic acid; 5-*o*-caffeoylquinic acid; demethyl-wedelolactone; 4,5-dicaffeoylquinic acid; 3,5-dicaffeoylquinic acid; quercetin-3-arabinoside; luteolin; 4-*o*-caffeoylquinic acid; and wedelolactone (Tables 3 and 4; Fig. 4), with demethyl-wedelolactone and wedelolactone being abundant in all of the different samples examined. The level of the individual polyphenols was affected, to a high extent, by the proportions of peat and sand in the growing media. In the inoculated plants, such a tendency held true only for protocatechuic acid, 5-*o*-caffeoylquinic acid, quercetin-3-arabinoside, and 3,5-dicaffeoylquinic acid. In both inoculated and noninoculated samples, peat proportions between 60% and 80% caused a drastic decrease in the content of all polyphenols detected in the extracts. Notably, a drastic decrease in the polyphenol content did not occur with 100% peat in both inoculated and control samples. With AMF inoculation, the concentration of luteolin was 45.74 $\mu\text{g/g}$ at a 0/100% (v/v) sand and peat mixture, which was significantly higher than that determined in the other treatments ($P < 0.05$).

The content of luteolin; 3,5-dicaffeoylquinic acid; wedelolactone; 4-*o*-caffeoylquinic acid; and protocatechuic acid was higher by 75%, 37%, 10%, 41%, and 67%, respectively, in mycorrhizal inoculated plants as compared with their levels in the control ones. Whereas the content of 5-*o*-caffeoylquinic acid; demethyl-wedelolactone; 4,5-dicaffeoylquinic acid; and quercetin-3-arabinoside was lower by 25%, 13%, 47%, and 31%, respectively. The highest level of protocatechuic acid (41.87 $\mu\text{g/g}$) was recorded in a 60/40% (v/v) sand and peat mixture by AMF+ (Tables 3 and 4). In addition, the highest levels of wedelolactone, the major polyphenol, were found in plants grown in peat proportion between 0% and 40% in both inoculated and control treatments.

The principal component analysis (PCA) was applied to assess the data on phenolic content in *E. prostrata* plants determined by HPLC. As demonstrated in Fig. 5A and B, principal component 1 (Factor 1) explains up to 55.58% of the total variance and is characterized mainly by protocatechuic acid; 5-*o*-caffeoylquinic acid; demethyl-wedelolactone; 4,5-dicaffeoylquinic acid; quercetin-3-arabinoside; wedelolactone; and 3,5-dicaffeoylquinic acid. Principal component 2 (Factor 2), explaining 12.52%, is contributed mainly by luteolin-glucoside. The PCA scatterplot showed 68.10% of the total variability in the phenolic data set. Through PCA and AHC, it is important to note that the C treatment cluster group has significant differences of polyphenolic contents compared with other treatments (Figs. 5A, B, and 6).

Discussion

There are several studies reporting the advantages, prospects, and feasibility of introducing AMF to plant production (Birhane et al., 2012; Gianinazzi et al., 2010; Ryan and Graham, 2002). Moreover, AM symbiosis has been found to increase the active bio-compounds of some medicinal plants (Zeng

et al., 2013); but data regarding *E. prostrata* has not been published until now. The increased demand for sustainable cultivation of *E. prostrata*, together with its high concentration of secondary metabolites used in human medicine and plant protection, draws attention to AMF.

Our results indicate that AMF inoculation beneficially affected the growth parameters and some secondary metabolites of *E. prostrata* (Tables 2 and 3). In general, AM symbiosis significantly increased the fresh and dry weights of shoots and roots compared with the control, nonmycorrhizal plants (Table 2). The highest dry weight of shoots was found in the presence of mycorrhizal fungi both at 60/40% (v/v) and 20/80% (v/v) sand/peat proportion. In nonmycorrhizal treatment, only 60/40% (v/v) sand/peat mixture showed maximum values for shoot and root growth. The beneficial effect of peat on growth of plants could be attributed to the increased aerobic conditions and better water-holding capacity than sand, in addition to the influence on the spread and colonization of AMF (Linderman and Davis, 2003). Moreover, higher phosphorus and other nutrients in peat than in sand could explain the increased growth compared with plants growing in sand. Our results indicated that the greatest extent of mycorrhizal colonization found at 60/40% (v/v) sand and peat ration is correlated with the effects of AMF on plant growth, thus agreeing with the findings of Stevens et al. (2011). Higher than 40% peat concentration had a negative effect on root colonization by AMF, similar with the works of Ma et al. (2007) and Marschner et al. (2006); but an opposite result was also reported by Vosatka (1995). On the other hand, some researchers showed that the effect of AMF symbiosis does not correlate with colonization level (Toussaint, 2007) and was most effective when root colonization scored from 20% to 30% (Feldmann et al., 2009).

Besides shoot and root weight, the leaf area is also important from the agronomical point of view and is influenced by various nutrient supplies (Taranet et al., 2017) or/and mycorrhizal inoculation (Liang et al., 2018). AMF had positive influence on leaf area of *E. prostrata*, a finding that is similar with the results of Birhane et al. (2012), who reported enhancing leaf area, biomass, and stomatal conductance by *Boswellia papyrifera* inoculation with AMF.

In addition to the beneficial effects of AMF on nutrient uptake, mycorrhizal symbioses often gives a balance to different stress conditions (Estrada et al., 2013), driving electron transport to an excited PSII reaction center. However, in our work, no differences were found in chlorophyll a fluorescence (Fv/Fm) between mycorrhizal and nonmycorrhizal plants; similar with the work of Parádi et al. (2003), significantly higher proline content of both treatments (AMF+, AMF-) was measured in 100% peat substrate. Proline plays an important role in plants: it protects the plants from various stresses and also helps plants to recover

Table 3. Change in the concentration of polyphenol compounds (mean $\pm$ SD, n = 3) under different treatments.

Sand/Peat % (v/v)	Inoculation ^z	Protocatechuic acid ($\mu\text{g/g}$) ^{yx}	5- <i>o</i> -caffeoylquinic acid ($\mu\text{g/g}$) ^{yx}	Demethyl wedelolactone ($\mu\text{g/g}$) ^{yx}	4- <i>o</i> -caffeoylquinic acid ($\mu\text{g/g}$) ^{yx}
100/0	AMF+	3.59 $\pm$ 1.61 c	65.09 $\pm$ 18.32 bc	989.00 $\pm$ 246.84 a	67.95 $\pm$ 35.92 b
	AMF-	4.43 $\pm$ 0.4a c	173.34 $\pm$ 53.47 a	826.09 $\pm$ 330.35 ab	69.85 $\pm$ 38.47 b
80/20	AMF+	19.20 $\pm$ 4.4 b	57.15 $\pm$ 26.21 c	776.55 $\pm$ 146.96 abc	70.73 $\pm$ 20.29 b
	AMF-	23.57 $\pm$ 5.9 b	136.02 $\pm$ 32.7 ab	1255.23 $\pm$ 124.6 a	253.49 $\pm$ 3.78 a
60/40	AMF+	41.87 $\pm$ 9.7 a	169.89 $\pm$ 36.16 a	1001.86 $\pm$ 187.47 a	55.53 $\pm$ 39.32 bc
	AMF-	5.46 $\pm$ 1.34 c	56.09 $\pm$ 29.22 ac	1066.79 $\pm$ 265.17 a	50.39 $\pm$ 16.58 bc
40/60	AMF+	4.11 $\pm$ 2.2 c	9.26 $\pm$ 1.29 c	214.55 $\pm$ 63.36 c	UDL
	AMF-	4.63 $\pm$ 2.69 c	13.44 $\pm$ 6.51 c	401.05 $\pm$ 53.13 bc	UDL
20/80	AMF+	1.88 $\pm$ 0.47 c	16.22 $\pm$ 2.2 c	231.42 $\pm$ 110.9 c	15.49 $\pm$ 11.45 bc
	AMF-	3.76 $\pm$ 1.22 c	12.34 $\pm$ 1.08 c	260.81 $\pm$ 68.38 bc	UDL
0/100	AMF+	3.19 $\pm$ 1.78 c	8.11 $\pm$ 0.24 c	711.73 $\pm$ 95.18 abc	UDL
	AMF-	2.33 $\pm$ 0.39 c	45.4 $\pm$ 8.81 c	722.81 $\pm$ 283.03 abc	25.47 $\pm$ 12.4 bc
Growing media (GM)		***	***	***	***
Mycorrhizae (M)		***	*	NS	***
M $\times$ GM		***	***	NS	***

^zAMF+ represents the mycorrhizal; AMF- represents the nonmycorrhizal plants.^yDifferent letters in each parameter indicate significant difference according to the Tukey test ($P < 0.05$) among sand and peat ration.^xNS = contrast is nonsignificant, *significant at $P < 0.05$, ***significant at $P < 0.001$. UDL = under detection limit.Table 4. Change in the concentration of polyphenol compounds (mean $\pm$ SD, n = 3) under different treatments.

Sand/Peat % (v/v)	Inoculation ^z	Quercetin-3- arabinoxide ($\mu\text{g/g}$) ^{yx}	Luteolin ($\mu\text{g/g}$) ^{yx}	3,5-dicaffeoylquinic acid ($\mu\text{g/g}$) ^{yx}	Wedelolactone ($\mu\text{g/g}$) ^{yx}	4,5-dicaffeoylquinic acid ($\mu\text{g/g}$) ^{yx}
100/0	AMF+	146.62 $\pm$ 30.52 bc	39.74 $\pm$ 4.58 ab	99.97 $\pm$ 17.89 a	3263.99 $\pm$ 950.64 a	27.04 $\pm$ 17.8 ab
	AMF-	74.52 $\pm$ 28.52 cd	21.45 $\pm$ 6.26 bc	87.69 $\pm$ 15.38 ab	2376.25 $\pm$ 604.10 abc	10.23 $\pm$ 4.55 bc
80/20	AMF+	161.66 $\pm$ 18.09 b	22.52 $\pm$ 11.82 abc	62.63 $\pm$ 10.48 bc	1362.73 $\pm$ 79.05 cde	17.57 $\pm$ 10 abc
	AMF-	253.90 $\pm$ 49.28 a	23.08 $\pm$ 4.84 abc	43.85 $\pm$ 8.79 cd	1953.80 $\pm$ 407.92 bcd	29.50 $\pm$ 8.18 ab
60/40	AMF+	169.17 $\pm$ 24.25 b	22.58 $\pm$ 7.54 abc	62.90 $\pm$ 10.61 bc	3063.97 $\pm$ 524.18 ab	38.03 $\pm$ 12.06 a
	AMF-	155.27 $\pm$ 27.54 b	12.35 $\pm$ 0.3 c	43.70 $\pm$ 6.17 cd	2588.27 $\pm$ 399.36 ab	15.13 $\pm$ 5.4 bc
40/60	AMF+	1.51 $\pm$ 0.38 d	1.39 $\pm$ 0.58 c	7.18 $\pm$ 3.91 ef	1010.37 $\pm$ 141.22 de	1.90 $\pm$ 0.08 c
	AMF-	UDL	12.73 $\pm$ 4.85 c	4.91 $\pm$ 2.79 ef	957.39 $\pm$ 129.32 de	2.28 $\pm$ 0.67 c
20/80	AMF+	UDL	14.10 $\pm$ 3.73 c	4.45 $\pm$ 2.11 f	757.71 $\pm$ 113.33 de	2.39 $\pm$ 0.71 c
	AMF-	133.21 $\pm$ 47.5 bc	5.03 $\pm$ 3.04 c	UDL	659.31 $\pm$ 86.17 e	2.39 $\pm$ 0.9 c
0/100	AMF+	21.89 $\pm$ 3.53 d	45.74 $\pm$ 18.94 a	37.37 $\pm$ 17.33 cde	1188.25 $\pm$ 90.34 cde	8.09 $\pm$ 1.75 bc
	AMF-	112.23 $\pm$ 23.61 bc	8.63 $\pm$ 2 c	19.81 $\pm$ 11.61 def	1165.19 $\pm$ 99.3 cde	7.87 $\pm$ 2.34 bc
Growing media (GM)		***	***	***	***	***
Mycorrhizae (M)		***	***	***	NS	NS
M $\times$ GM		***	***	NS	NS	**

^zAMF+ represents the mycorrhizal; AMF- represents the nonmycorrhizal plants.^yDifferent letters in each parameter indicate significant difference according to the Tukey test ($P < 0.05$) among sand and peat ration.^xNS = contrast is nonsignificant, ***significant at $P < 0.001$. UDL = under detection limit. Fig. 4. HPLC profile of effective polyphenols extracted from *E. prostrata* leaves. Different numbers represent polyphenols: 1: Protocatechuic acid; 2: 5-*o*-caffeoylquinic acid; 3: Demethyl-wedelolactone; 4: 4-*o*-caffeoylquinic acid; 5: 3,5-dicaffeoylquinic acid; 6: 4,5-dicaffeoylquinic acid; 7: Quercetin-3-arabinoxide; 8: Luteolin; 9: Wedelolactone.

from stress more rapidly (Hossain et al., 2014).

The increased weight of roots has special importance not only in increasing the absorption surface but also in enhancing the sec-

ondary metabolites such as phenolic content from *Echinacea purpurea* (Araim et al., 2009) and stigmasterol, ecliptal, and heptacosanol from *E. prostrata* (Chung et al., 2017).

Plants protect themselves from different biotic and abiotic stress factors by synthesizing phenolic compounds, which act as a screen inside the epidermal cell layer, and by adjusting the antioxidant systems at both the cell and whole organism level (Bhattacharya et al., 2010; Lattanzio et al., 2006; Sirvent and Gibson, 2002). The importance of flavonoids in ultraviolet protection has also been proved using mutants ultraviolet-hypersensitive phenotypes of *Arabidopsis* (Ryan et al., 2001). Pre-formed antibiotic compounds such as phenolic and polyphenolic compounds are ubiquitous in plants and play an important role in nonhost resistance to pathogens (Lattanzio et al., 2006).

Furthermore, Stom (1982) showed that the polyphenolic compounds have a crucial role in regulating the growth and development of plants. The phenolic compounds have antioxidant properties also, which can reduce the peroxidation of membrane lipids by decreasing their fluidity in consequence, limiting the diffusion of free radicals that has been proved using *Allium sativum* L. (Bozin et al., 2008; Foti, 2007). Moreover, there is another valuable property of polyphenolic

Fig. 5. Principal component analysis of polyphenolic data of 12 different treatments. Depicts a scatter plot (PC1 versus PC2). (A), A zoomed-in scatterplot on the cluster containing treatments (B). A: AMF+ 100/0 sand/peat % (v/v); B: AMF+ 80/20 sand/peat % (v/v); C: AMF+ 60/40 sand/peat % (v/v); D: AMF+ 40/60 sand/peat % (v/v); E: AMF+ 20/80 sand/peat % (v/v); F: AMF+ 0/100 sand/peat % (v/v); CA: 100/0 sand/peat % (v/v); CB: 80/20 sand/peat % (v/v); CC: 60/40 sand/peat % (v/v); CD: 40/60 sand/peat % (v/v); CE: 20/80 sand/peat % (v/v); CF: 0/100 sand/peat % (v/v).

compounds: having the ability and capacity to chelate heavy metals ions, as demonstrated in a study with *Nymphaea* sp. (Lavid et al., 2001).

The polyphenol profiles of *Eclipta prostrata* measured in aerial parts showed the same tendency to the previous finding wedelolactone and demethyl-wedelolactone were

the main components (Fang et al., 2015; Mendes et al., 2014; Murali et al., 2002). Additionally, Fang et al. (2014) determined luteolin-glucoside and 4,5-dicaffeoylquinic acid as polyphenols in the extract of *E. prostrata*.

Demethyl-wedelolactone (DWL) is an important polyphenolic component in the profile of *E. prostrata* due to its antihepatotoxic properties. Using 60/40% sand/peat as a growth substrate, we found about ten times higher concentration of DWL than reported by Murali et al. (2002). Some minor polyphenols (such as 5-*o*-caffeoylquinic acid, quercetin-3-arabinoside, 4-*o*-caffeoylquinic acid, and protocatechuic) are detected for the first time in the extract of *E. prostrata* samples. Protocatechuic acid plays an important role in the tolerance of rice during anaerobic flooding germination, promotion of shoot elongation, and the increase in chlorophyll *b* (Khanh et al., 2018).

Our results indicate that growth medium, together with mycorrhizal inoculation, induced changes in tested secondary metabolites of *E. prostrata*. These findings confirm some previous work, where qualitative changes due to AMF were recognized in alkaloid, terpen, flavonoid, and phenolic acids on some medicinal plants (Zeng et al., 2013)—but not in *E. prostrata*. To the best of our knowledge, our study is the first to report how AMF symbiosis influences plant performance and active ingredients of *E. prostrata*.

The mechanism by which AMF triggered the quality and quantity of secondary metabolites products in medicinal plants can be multidirectional and not well understood (Toussaint, 2007). On one side, AMF symbiosis can significantly increase the contents of some secondary metabolites of medicinal plants due to increased nutrient availability to plants (Chandra et al., 2010). Enhanced N-uptake by AMF is well documented (Johansen et al., 1996), showing an increased synthesis of amino acids and specific metabolites in medicinal plants. The results showed higher phenolic content (Fig. 3) and improvement of main polyphenols (such as wedelolactone; luteolin; 4,5-dicaffeoylquinic acid; and quercetin) of mycorrhizae inoculated plants compared with the controls (Tables 3 and 4). These findings agree with other studies where the target plants *Cynara cardunculus* and *Ocimum basilicum* increased the phenolic contents (Zeng et al., 2013). The increased concentration in total phenols can be explained by the influence in the cycle of tricarboxylic acids, which results in sub-products used in the integration of phenolic compounds (Lohse et al., 2005). Moreover, Kapoor et al. (2002) showed that increased absorption of mineral nutrients (especially P and N), together with changed phytohormone level in the mycorrhizal plant (Toussaint, 2007), enhanced plant terpenoids and phenolic acids. Another reason could be that AMF can induce defense-related compounds (including the production of phenolic compounds) in plants (Volpin et al., 1994). However, AMF did not always increase the

Fig. 6. Dendrogram for 12 treatments with and without AMF obtained from the hierarchical cluster analysis. AMF+: mycorrhizal plants, AMF–: nonmycorrhizal plants; A: AMF+ 100/0 sand/peat % (v/v); B: AMF+ 80/20 sand/peat % (v/v); C: AMF+ 60/40 sand/peat % (v/v); D: AMF+ 40/60 sand/peat % (v/v); E: AMF+ 20/80 sand/peat % (v/v); F: AMF+ 0/100 sand/peat % (v/v); CA: 100/0 sand/peat % (v/v); CB: 80/20 sand/peat % (v/v); CC: 60/40 sand/peat % (v/v); CD: 40/60 sand/peat % (v/v); CE: 20/80 sand/peat % (v/v); CF: 0/100 sand/peat % (v/v).

phenolic content in *Ocimum basilicum* and *Salvia officinalis* (Lee and Scagel, 2009), and the different genotype of host plants also influenced it.

Conclusion

This study presents for the first time how arbuscular mycorrhizal fungi and different sand and peat proportions influence the growth rate and the polyphenol profile of *E. prostrata*. Our results showed that a 60/40% (v/v) sand and peat ration seemed to be the best and is thus recommended for a large-scale cultivation of *E. prostrata*, moreover supporting the highest total phenolic content of plants. The AMF inoculation successfully affected the growth, biomass, and polyphenol components of *E. prostrata*. Through an HPLC analytical method, polyphenol compositions were successfully assessed in *E. prostrata*, and nine individual phenolic components were quantified. Further research will be carried out under different types of abiotic and biotic stress conditions, focusing on single or mix arbuscular mycorrhizal fungi in an open field experiment.

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