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Root Colonization by an Arbuscular Mycorrhizal (AM) Fungus Increases Growth and Secondary Metabolism of Purple Coneflower, *Echinacea purpurea* (L.) Moench

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Purple coneflower, *Echinacea purpurea* (L.) Moench, is an important phytomedicinal species that contains phenolics and alkamides with antipathogenic properties. This study aimed to examine the effect of arbuscular mycorrhizal (AM) colonization on the physiology and biochemistry of *E. purpurea*. It was hypothesized that AM colonization enhances the growth and secondary metabolism in *E. purpurea*. In this regard, a 13-week factorial greenhouse experiment was performed with *E. purpurea*, inoculated (or not) with the AM fungus *Glomus intraradices* Schenck & Smith. Overall, the results indicated that AM colonization significantly increased the mass of shoots and roots and the concentrations of proteins and most of the phenolics in the roots. Hence, the selected trait of mycorrhiza could play an important role in optimizing the growth of *E. purpurea* by inducing the production of secondary phytomedicinal metabolites.

KEYWORDS: *Echinacea purpurea*; *Glomus intraradices*; alkamides; phenolic acids; phytomedicine

INTRODUCTION

Echinacea purpurea (L.) Moench, Asteraceae, is a perennial plant species commonly known as purple coneflower. Of the nine species of *Echinacea*, described in the McGregor taxonomy (*1*), three are commonly used medicinally for their ability to enhance the activity of various immune cells, stimulate lymphocytes, and treat acute upper respiratory infection: *E. purpurea*, *Echinacea pallida*, and *Echinacea angustifolia* (*2*). These medicinal properties are attributed to three major groups of secondary compounds: caffeic acid derivatives, alkamides, and polysaccharides (*3, 4*). Caffeic acid derivatives, an important group of bioactive compounds, include caftaric, caffeic, chlorogenic, and cichoric acids, as well as cynarin and echinacoside. The major alkamides in *E. purpurea* are two tetraenes known as dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (*5*), and the polysaccharide fraction mainly contains arabinogalactans (*6*). Studies have shown that the chemical composition of *Echinacea* is complex and of beneficial pharmacological activity (*7*), including immunostimulatory effects (*8*).

Root colonization by arbuscular mycorrhizal (AM) fungi, which are microscopic soilborne glomeromycotan fungi, has been shown to increase the productivity of several crops, especially important in the context of sustainable agriculture and development (*9, 10*). Beneficial effects of AM symbiosis on plant growth, nutrient uptake, and tolerance to environmental stressors have been extensively reported (*11, 12*). However, relatively little is still known about the potential of this AM symbiosis to affect plant secondary metabolic pathways. Thus, the present study investigated the effect of AM colonization on plant growth status and phytochemical production in purple coneflower, a natural mycotrophic plant species.

MATERIALS AND METHODS

A factorial (1 plant species × 2 M × 12 replicates) greenhouse experimental design was performed including *E. purpurea* (L.) Moench, grown with or without the AM fungus, *Glomus intraradices* Schenck & Smith. *Echinacea* seeds (Florabanda Farm, ON, Canada) and pots (12.5 × 12.5 × 15 cm) were surface sterilized with 5% NaOCl before sowing. The soil, consisting of a sand/soil mixture (1:1, v/v), had been autoclaved for 20 min at 121 °C and 15 psi. Pots were filled with 1 L of this soil mixture and 500 mL of the inoculum or control substrate and topped with the soil mixture. Eight seeds were sown ~1 cm deep in the soil mixture and thinned after 2 weeks to one plant per pot, for a total of 24 pots (12 pots per treatment with one plant per pot). The AM inoculum consisted of *G. intraradices* (DAOM 181602) or a non-AM control substrate that contained filtrates of the rhizosphere microflora while excluding fungal spores (Mycorhize Pro, Premier Tech, Rivièredu-Loup, QC, Canada). Plants were grown for 13 weeks at 27/23 °C (day/night), 40% relative humidity, and a 16 h photoperiod, under natural light and high-pressure sodium lamps (PL Light Systems, Beamsville, ON, Canada). The average light intensity (408 μmol s^{-1} m^{-2}) was measured using a light meter with a quantum sensor (LiCor LI-250A and LI-190SA, Lincoln, NE). Plants were watered with distilled H_2O as required and fertilized after the third week following germination with 20 mL of Long Ashton Nutrient Solution (LANS) (*13*), once a week for 2 weeks (the fourth and fifth weeks) with 50% reduced ammonium nitrate. Then the plants were fertilized with the

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full LANS with 50 mL at the sixth week and with 100 mL until harvest. All of the chemicals used for LANS fertilizer preparation were purchased from Sigma-Aldrich Inc., St. Louis, MO.

Harvesting. Plants, harvested after 13 weeks of growth, were separated into shoots and roots at the basal knot from which the shoot height was measured. Roots were rinsed in water to remove excess substrate, and three fresh root samples per treatment were put apart for the AM colonization determination. After the fresh mass was taken, fresh roots and shoots were frozen with liquid nitrogen and stored at −80 °C until freeze-drying for 72 h, at −50 °C, under vapor pressure at 3.7×10^{-1} mbar (Unitrap model 10-100, Virtis Inc., Gardiner, NY), followed by measurement of the dry mass.

Root Colonization. The fresh roots were washed, patted dry, and stained (*14*) using a 0.02% aniline blue dye solution containing 0.5 g of aniline blue, 500 mL of glycerol, 450 mL of dH_2O, and 50 mL of 1% HCl. The aniline blue was purchased from Aldrich, Milwaukee, WI; all other chemicals were from Fisher Scientific, Ottawa, ON, Canada. Fifty 1-cm root segments (10/slide) were observed for each of the three replicates per treatment. Root segments were examined at 100× and 400× magnification under a compound microscope (CX41, Olympus Inc., Canada). Mycorrhizal colonization was estimated by determining the percentage frequency as the total of root segments containing at least one of the fungal structures (hyphae, vesicles, arbuscules, or spores).

Mineral Analyses. For the P, K, Ca, Mg, Mn, and Cu analyses, 2 g per sample of dry shoot or root material was ground and submitted for analyses at the Laboratoire de chimie organique et inorganique, Direction de la recherche forestière, MRN, Québec, QC, Canada. A sulfuric acid digestion on hot plate was carried out, followed by the analysis of these elements using atomic plasma emission spectroscopy.

Protein Analysis. From each root or shoot sample (200 mg of freeze-dried tissue), soluble proteins were extracted on ice using 10 mL of Tris-HCl (Sigma-Aldrich) buffer (25 mM, pH 8.0), centrifuged at 11500*g* for 25 min at 4 °C, and determined according to the micromethod of Bradford (*15*). For each extract, the OD was read at 595 nm and the protein concentration measured from a bovine serum albumin (Sigma-Aldrich) standard curve ($0-1.2$ mg mL^{-1}).

Phytochemical Analyses. *Sample Preparation.* Phenolics and alkamides were quantified by using a method adapted from Bergeron et al. (*16*). Each freeze-dried ground sample (100 mg) was placed in a centrifuge tube and extracted with 2 mL of 70% ethanol by sonication for 15 min followed by centrifugation at 180*g* for 5 min at 21 °C. The pellet was extracted twice, and the volume of pooled supernatants was adjusted to 4 mL with 70% ethanol. Each extract (1 mL) was filtered through a 0.2 μm polytetrafluoroethylene (PTFE) membrane filter (Chromatographic Specialties, Brockville, ON, Canada), prior to injection of 1 μL into the HPLC system.

HPLC-DAD Analysis. The extracts were analyzed using an 1100 series HPLC-DAD system (Agilent Technologies, Santa Clara, CA), consisting of an autosampler with a 100 μL loop, a quaternary pump, a photodiode array detector, a column thermostat, and Chemstation software (version B.03.01). Separations of target phytochemicals were achieved on a narrow-bore YMC ODS-AM column (2 × 100 mm, particle size = 3 μm) (Waters, Mississauga, ON, Canada), at a column temperature of 47 °C. A linear solvent gradient system based on solvent A [0.05% trifluoroacetic acid (TFA) in HPLC grade water] and solvent B [0.05% TFA in HPLC grade acetonitrile (MeCN)], at a flow rate of 0.4 mL min^{-1}, permitted the separation and quantification of phenolics and alkamides. Calibration curves were generated by injecting known concentrations of pure standards (ChromaDex Inc., Santa Ana, CA). The elution times for the phenolics ranged from 3.3 to 8.8 min and those for alkamides from 14.1 to 15.2 min. The identification of the compounds present in the extracts of selected plant organs of *E. purpurea* were confirmed by matching the UV spectra of the unknowns with the entries in the library of the phenolic standards and further confirming their identity by injecting commercially available standards with the extracts under similar chromatographic conditions. Quantification, based on the area under the peak, was done at monitoring wavelengths of 260 nm for the alkamides and 326 nm for the phenolic acids. Each extract was injected with authentic standard mixture, and the calibration curve was updated at regular intervals. All standards for HPLC analyses were purchased from Chromadex Inc., and all solvents were of HPLC grade purchased from Fisher Scientific (Ottawa, ON, Canada).

Statistical Analyses. A Student's *t* test was performed on each parameter, using S-Plus (*17*). All of the data were verified for the assumptions of normality and homoscedasticity. When needed, log or square-root transformations were performed. When statistical assumptions were not met, nonparametric Kruskall−Wallis or nonparametric Wilcoxon *t*-test analyses were done (*18*).

RESULTS

The AM root colonization in *E. purpurea* with *G. intraradices* reached almost 100% after 13 weeks of growth, and most of the fungal structures were present, for example, hyphae, arbuscules, and vesicles (data not shown). The AM plants had significant ($p \leq 0.01$) increases, by approximately 3 times higher, of fresh and dry shoot, root, and total masses compared to non-AM plants (**Table 1**). The AM plants also had significantly ($p \leq 0.05$) higher shoot and total plant lengths, by approximately 1.5 times, and higher root length ($p = 0.06$) than non-AM plants. The number of leaves of the AM plants was significantly ($p \leq 0.05$) higher, by 2.3 times, than that of non-AM plants (**Table 1**). For the mineral content in shoots (**Table 2**), the AM treatment significantly ($p \leq 0.05$) increased the content of P and Cu by 1.5 and 1.2 times, respectively, and increased Mg ($p = 0.06$) by 1.3 times. There were no significant differences for these mineral in roots as for all the other minerals analyzed (data not shown).

The protein concentrations were similar in shoots but increased marginally ($p = 0.06$) in the AM roots (**Table 3**). In roots also, the AM treatment significantly increased the concentrations of cichoric ($p \leq 0.05$), caftaric ($p \leq 0.001$), and chlorogenic ($p \leq 0.01$) acids and cynarin ($p \leq 0.05$) by 1.5,

Table 1. Physiological Parameters in the Roots, Shoots, and Whole Plants of *E. purpurea,* without (M−) or with (M+) AM Colonization[a]

plant organ	treatment	fresh mass (g)	dry mass (g)	length (cm)	no. of leaves
shoots	M−	8.1 (2.8)	1.5 (0.5)	23 (5)	5.6 (1.1)
	M+	23.8 (4.5)	4.6 (1.0)	35.6 (1.4)	12.8 (2.7)
	t test	**	*	*	*
roots	M-	4.9 (1.8)	0.6 (0.3)	18.6 (3.3)	
	M+	16.7 (4.4)	2.0 (0.6)	24.8 (1.3)	
	t test	**	*	MS	
whole plants	M−	13.0 (4)	2.1 (0.7)	41.6 (6.2)	
	M+	40.5 (8.9)	6.6 (1.5)	60.4 (1.4)	
	t test	**	*	*	

[a] Means ($n = 6$) (SEs) and *t*-test results are shown. *, $p \leq 0.05$; **, $p \leq 0.01$; MS, marginally not significant ($0.05 \leq p \leq 0.1$).

Table 2. Mineral Contents in Shoots (Milligrams per Shoot DM) and Roots (Milligrams per Root DM) of *E. purpurea*, without (M−) or with (M+) AM Colonization[a]

plant organ	treatment	P	Mg	Cu
shoots	M−	17.0 (1.7)	65.0 (5.6)	0.11 (0.0)
	M+	25.0 (1.7)	83.7 (4.0)	0.14 (0.0)
	t test	*	MS	*
roots	M−	22.6 (3.6)	47.3 (11.5)	0.11 (0.01)
	M+	21.1 (1.1)	40.1 (4.6)	0.10 (0.01)
	t test	NS	NS	NS

[a] Means ($n = 3$) (SEs) and *t*-test results are shown. *, $p \leq 0.05$; MS, marginally not significant ($0.05 \leq p \leq 0.1$); NS, not significant.

Table 3. Phytochemical and Soluble Protein Concentrations (Milligrams per Gram of DM) and Total Phenolic Contents (Milligrams per Organ DM) in Shoots and Roots of *E. purpurea*, without (M−) or with (M+) AM Colonization

plant organ	treatment	cichoric acid	caftaric acid	chlorogenic acid	caffeic acid	cynarin	echinacoside	alkamides[b]	protein	phenolics
shoots	M−	20.2 (2.3)	11.3 (0.8)	0.3 (0.1)	1.0 (0.3)	0.2 (0.1)	0.5 (0.3)		30.4 (2.9)	68.8 (17.0)
	M+	21.3 (1.8)	9.3 (0.7)	0.2 (0.1)	0.7 (0.1)	0.3 (0.1)	0.4 (0.2)		24.1 (2.4)	115.2 (39.5)
	t test	NS	MS	NS	NS	NS	NS		NS	*
roots	M−	11.3 (1.3)	2.9 (0.3)	0.16 (0.02)	0.6 (0.2)	0.13 (0.01)		1.8 (0.4)	23.9 (2.0)	17.9 (5.0)
	M+	16.6 (1.6)	4.9 (0.3)	0.42 (0.07)	0.7 (0.1)	0.17 (0.01)		2.2 (0.1)	31.5 (2.6)	55.1 (21.2)
	t test	*	***	**	NS	*		NS	MS	MS

[a] Means (shoots, $n = 5-6$; roots, $n = 3-5$) (SEs) and *t*-test results are shown. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; MS, marginally not significant ($0.05 \leq p \leq 0.1$); NS, not significant. [b] Total amount of the two isomers: dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides.

1.7, 2.6, and 1.3 times, respectively, without any significant differences in the concentration of alkamides. In shoots, the AM treatment decreased only marginally ($p = 0.06$) the concentration of caftaric acid. As a whole, the AM treatment significantly increased the total content of phenolics (**Table 3**), by approximately 3 times, in shoots ($p \leq 0.05$) and roots ($p = 0.07$) and marginally increased ($p = 0.07$), by 2.2 times (data not shown), the total content of alkamides in roots.

DISCUSSION

The high root colonization level in *E. purpurea* with *G. intraradices* is in agreement with a study (*19*) on *E. pallida*, cultivated in vitro and inoculated with four different AM fungal species, which showed increasing levels from *Scutellospora fulgida* (20%), *Entrophospora colombiana* (29%), and *Gigaspora ramisporophora* (30%) to *Glomus mosseae* (77%). This suggests a preference of these two *Echinacea* species for the genus *Glomus*. In a study with maize (*20*), the root colonization also ranged from 26 to 72% depending on the *Glomus* species inoculated. Our study showed a significant growth increase of *E. purpurea* when colonized with *G. intraradices*. This is in agreement with the enhanced growth of *E. pallida* (*19*) when colonized with AM fungi. We also showed a significant increase in the content of P and Cu in the shoots of AM plants. Increased plant growth with AM colonization is believed to result from a greater efficiency of nutrient uptake (*21*−*23*). These results correspond to a number of studies (*22*, *24*), which indicated that mycorrhizal benefits are not limited to improving P uptake, but to other mobile (e.g., N) or less mobile minerals (e.g., Zn and Cu). The increase of proteins in the AM-colonized roots of *E. purpurea* is in accordance with other studies on maize (*20*, *22*). We previously reported from in vivo (*23*) and in vitro (*25*) compartmental systems that the extraradical hyphae of *G. intraradices* play a direct role in the N uptake and its translocation to the roots, then contributing to its assimilation in nitrogenous compounds.

In the present study, the highest levels of phenolics in *E. purpurea* were detected in shoots and those of the alkamides in roots. These results are in agreement with those obtained for seven species of *Echinacea* in which the phenolics were also predominantly located in shoots and the alkamides in roots (*3*−*5*). In *E. purpurea*, the major constituents were the cichoric and caftaric acids, whereas caffeic acid, chlorogenic acid, and cynarin were the minor ones. The fact that echinacoside was absent in roots of *E. purpurea* is also in agreement with these studies. By contrast, we detected here the presence of cynarin, although at low concentrations, in shoots and roots. This phytochemical variability may be attributed to growth conditions or genetic adaptation as it was suggested (*3*) that different species of *Echinacea* produce offspring that varied in the composition of defensive phytochemicals according to their geographical origin and that the seed source, within a species, influences the overall levels.

The AM root colonization factor led in *E. purpurea* to significantly increased concentrations of most of the phenolics, especially cynarin and cichoric, caftaric, and chlorogenic acids. In addition, due to the enhanced biomass with the AM colonization, the content of these phenolics was significantly increased in the roots and shoots. These results correspond to other studies (*26*, *27*) which indicated that AM colonization induces the synthesis of secondary compounds (phenolics, cyclohexenone derivatives, and blumenin) in roots or shoots of barley and wheat and tobacco, respectively. Toussaint et al. (*28*), who investigated the effect of different AM fungal species on the phytochemicals in sweet basil, showed increased concentrations of rosmarinic and caffeic acids with *G. caledonium* and of caffeic acid with *G. mosseae*. Our study shows that AM colonization significantly increased the growth and most of the phenolics in *E. purpurea*. Improving the growth of medicinal plants under organic conditions is one of the most important requirements for natural health products.

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