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The influence of *Rhizophagus irregularis* arbuscular mycorrhizal fungus on *Echinacea purpurea* root biomass and bioactive compounds

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Abstract

Echinacea purpurea is a widely used medicinal plant valued for its bioactive constituents; however, cultivation presents several challenges (poor and imbalanced germination, slow initial development). Arbuscular mycorrhizal fungi (AMF), especially *Rhizophagus irregularis*, are known to influence plant growth and metabolite accumulation, yet limited multi-year field data exist. This is the first study that evaluates the effect of *R. irregularis* inoculation on root biomass, phenolic acid content, essential oil yield and its composition in two-year-old *E. purpurea* under greenhouse (four substrate types) and open-field conditions. Chicoric acid was the predominant phenolic in all samples. Significant substrate- and environment-dependent effects were found for other compounds: AMF-treated plants showed increased caftaric (2.87-fold) and caffeic (2.15-fold) acid levels on sterile peat and elevated cynarin (2.13-fold) under field conditions, but decreased caffeic acid (3.39-fold) and echinacoside (1.98-fold) on gleyic calcaric Fluvisol. On stagnic Luvisol and gleyic calcaric Fluvisol, treated plants showed a higher proportion of oxygenated sesquiterpenes in oil composition, while controls contained fatty acids along with sesquiterpene hydrocarbons. A negative correlation was found between AMF colonization rate and both phenolic content and essential oil yield under open-field conditions. The results suggest that the effects of *R. irregularis* are strongly influenced by substrate type and environmental conditions, indicating the need for further research.

Keywords: GC-MS; mycorrhizae; phenolic constituents; plant-fungus interactions; volatile profile

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Introduction

Echinacea purpurea (L.) Moench, commonly known as purple coneflower, is a genus of the Asteraceae family, native to North America, but is currently cultivated worldwide (Galambosi, 2004); it has well-documented medicinal uses in its native habitat. In the early literature, there are records of its medicinal use in traditional medicine, mainly for the relief of common colds, coughs, infections, and headaches (Kindscher, 2016). In recent decades, as pharmaceutical research has advanced, the *Echinacea* genus has received increasing attention and has been supported by numerous pharmacologically proven medicinal effects. Scientific papers report immune system-enhancing (Bruni *et al*., 2018; Senica *et al*., 2019), anticancer (Tsai *et al*., 2012; Fonseca *et al*., 2014), antioxidant (Noorolahi *et al*., 2013; Pires *et al*., 2016), antiviral (Vimalanathan *et al*., 2005; Liu *et al*., 2020), and antibacterial (Barnes *et al*., 2005; Sharma *et al*., 2008; Sharma *et al*., 2010) effects due to the bioactive compounds it contains. These include caffeic acid derivatives (CAD's) and flavonoids belonging to the group of phenolic compounds, alkamides, essential oils, polysaccharides and glycoproteins (Burlou-Nagy *et al*., 2022).

Despite being grown primarily for its above-ground parts (Muntean *et al*., 2012), *E. purpurea* also has significant medicinal value related to the roots, which typically reach harvestable size in 2-3 years (Tansı *et al*., 2015; Muntean *et al*., 2016). The main phenolic compounds are present in both above and below ground parts of the plant, particularly the three most crucial caffeic acid derivatives: chicoric acid, caffeic acid, and chlorogenic acid, which are mainly responsible for antioxidant, antiviral, and anti-inflammatory activities (Barrett, 2003). In addition, alkamides, which are the main bioactive compounds specific to the roots, are responsible for immunostimulant, and anti-inflammatory properties (Clifford *et al*., 2002; Moazami *et al*., 2015; Aarland *et al*., 2017), although studies have highlighted that the biological activity of the plants cannot be reduced to a single group of active ingredients, but rather is due to the synergistic action of the compounds found in the genus (Chicca *et al*., 2009; Dosoky *et al*., 2023; Ávila-Gálvez *et al*., 2024). Essential oils, which are known to be responsible for antimicrobial activity (Coss *et al*., 2018; Dosoky *et al*., 2023), can be found in varying amounts and composition in different above-ground parts (leaves, stems, inflorescences) and roots, typically with germacrene-D, caryophyllene, caryophyllene-epoxide, and α-phellandrene as the dominant components (Barnes *et al*., 2005; Diraz *et al*., 2012; Bruni *et al*., 2018). These secondary metabolites contribute to the plant's pharmacological value; therefore, optimizing cultivation technology, especially with respect to factors affecting root development and the accumulation of active substances, is key to producing high-quality plant material (Pandey *et al*., 2018).

However, several challenges arise in the cultivation of *E. purpurea*. The poor and imbalanced germination capacity of the species, the slow initial development of young plants, and the susceptibility to unfavorable soil conditions often inhibit the establishment of uniform populations and cost-effective production (Fariman *et al*., 2011; Zadeh *et al*., 2015; Parsons *et al*., 2018). This justifies investigating biological approaches, such as arbuscular mycorrhizal fungi (AMF), which can enhance nutrient uptake, promote growth, and facilitate the synthesis of secondary metabolites (Smith and Read, 2008a; Smith *et al*., 2011). Mycorrhizal approaches may be particularly promising for environmentally friendly, input-reduced cultivation of medicinal plants (Pandey *et al*., 2018).

Although a few studies have already investigated the effect of AMFs on *E. purpurea*, the available research is still somewhat limited. Araim *et al.* (2009) conducted a study *on E. purpurea*, in which plants were grown in pots filled with a sand-soil mixture (1:1, v/v) for 13 weeks. In their research, they observed that mycorrhizal colonization significantly improved root fresh and dry biomass, mineral nutrient uptake, and increased considerably concentrations of chicoric acid (1.5-fold), caffeic acid (1.7-fold), chlorogenic acid (2.6-fold), and cynarin (1.3-fold) in roots. Gualandi *et al.* (2014) investigated the effects of *Rhizophagus irregularis*, *Gigaspora margarita* and *Beauveria bassiana* species on growth parameters, phenolic compounds, and

sesquiterpenes of *E. purpurea* grown under nutrient-deficient stress conditions in Turface medium (calcined clay product) under greenhouse conditions. At the end of the 12-week experiment, it was found that inoculation with the fungus *R. irregularis* significantly increased the content of β-carophyllene, α-humulene, and germacrene D in leaves and positively affected the accumulation of chicoric and caffeic acids in leaves of plants grown under nutrient-deficient conditions. In another field study, Hajagha *et al.* (2017) investigated the effect of combining AMF *R. irregularis* with growth-promoting bacteria (*Azospirillum lipoferum*, *Azotobacter chroococcum* and *Pseudomonas fluorescens*) using different phosphorus supplementation rates. Their research revealed that combining these species increased root and shoot biomass, branch number, shoot length, and inflorescence number in *E. purpurea* plants grown under phosphorus-deficient conditions. In a recent study conducted by Sharma *et al.* (2021) investigated the effect of the consortium formed by *Glomus mossae, Gigaspora gigantea*, *P. fluorescens*, and *Bacillus subtilis* on three different *Echinacea* species: *E. angustifolia*, *E. pallida*, and *E. purpurea*. Their study indicate that inoculated plants had higher proportion of root essential oil compared to control, with *E. purpurea* presenting greater results.

Overall, these studies indicate that AMF inoculation - alone or in combination with growth-promoting bacteria - can enhance root and shoot biomass, improve nutrient uptake, and increase the accumulation of key bioactive compounds, including chicoric, caffeic, chlorogenic acids, and essential oils. However, most experiments have been conducted in sterile or inert media (e.g., sand, calcined clay) over short cultivation periods, which do not adequately reflect the complexity of soil-plant-microbe interactions in natural, microbially diverse field environments. Collectively, these findings suggest that while AMF and microbial consortia can positively influence both morphological traits and secondary metabolite production, there remains a need for comprehensive, multi-year studies covering 2-4 cultivation cycles of *E. purpurea* under varying environmental conditions. This need is highlighted by the fact that roots are usually harvested in the second or third year. The integration of soil types with distinct microbial communities would enable a more realistic assessment of AMF's efficacy and yield results of greater agronomic relevance, especially in the context of sustainable, resource-efficient medicinal plant cultivation.

Therefore, this research aims to investigate the effects of the arbuscular mycorrhizal fungus *R. irregularis* on root biomass, bioactive compound content, and the composition of *E. purpurea* plants in their second growing season under greenhouse (pots: sterile peat and different soil types) and field conditions.

Materials and Methods

Cultivation conditions and experimental layout

The study involved two-year-old *Echinacea purpurea* plants that were grown from seeds obtained from the Botanical Garden of George Emil Palade University of Medicine, Pharmacy, Science, and Technology in Târgu Mureș during the 2022 growing season. After undergoing 14 days of cold stratification at 4 °C (Li *et al*., 2007; Fariman *et al*., 2011), the seeds were sown in germination trays filled with peat (Blondy Romania SRL, Târgu Mureș, Romania) and maintained in a growth chamber.

Once the seedlings reached the 5-6 leaf stage, they were inoculated with 0.5 g of concentrated arbuscular mycorrhizal fungi (AMF) powder (1400 spores g^{-1}) containing *Rhizophagus irregularis* AMF spores (Italpollina SPA, Italy), dissolved in 400 mL of distilled water (Domokos *et al*., 2018). Two days after inoculation, the plants were transferred to a greenhouse, where they were acclimatized for 2 weeks with shading and increased humidity. Following acclimatization, the plants were distributed across two growing conditions: open field and greenhouse. The plants used in the greenhouse experiment were potted in 7.5 L pots filled with peat (Danmuld peat moss: pH 5.5-6, EC 1 mS cm^{-1}, density 300 kg m^{-3}, N 70 mg L^{-1}, P_2O_5 140 mg L^{-1}, K_2O 240 mg L^{-1}, moisture 40-60%, particle size 0-20 mm), as well as three different soil types collected from the most representative

habitats in the area: gleyic calcaric Fluvisol, calcaric Regosol, and stagnic Luvisol. The physicochemical characteristics of the soil types can be found in our previous studies (Iakab *et al.*, 2024; Iakab *et al.*, 2025).

For the open-field experiment, AMF-treated and control plants, which were already adapted to field conditions by mid-May, were planted at the Experimental Base of Medicinal and Aromatic Plants at Sapientia University, Faculty of Târgu Mureș (46°31'19.6"N, 24°35'56.5"E), where preliminary studies had shown that the soil was stagnic Luvisol (Iakab *et al.*, 2024).

Regarding the experimental layout (Figure 1), the greenhouse experiment consisted of 16 randomized blocks, with eight blocks assigned to control plants and eight blocks to AMF-treated plants. In the first year of the experiment (Iakab *et al.*, 2024) each block consisted of 20 plant across soil types. In the second year, which is the focus of the present study, the remaining 14 plants/block were used for further measurements. To prevent spores from spreading to control plants, the AMF-treated and control plants were placed in two separate greenhouses. The spacing between the pots within each block was 30 cm, and the distance between blocks was 1 meter. The field experiment was organized into four blocks: two for control plants and two for AMF-treated plants, each containing 32 plants. To prevent spore transfer from AMF-inoculated plants to the control plants, a 10-meter safety distance was maintained between them, with 40 cm spacing between plants and 50 cm between rows within each block (Iakab *et al.*, 2024).

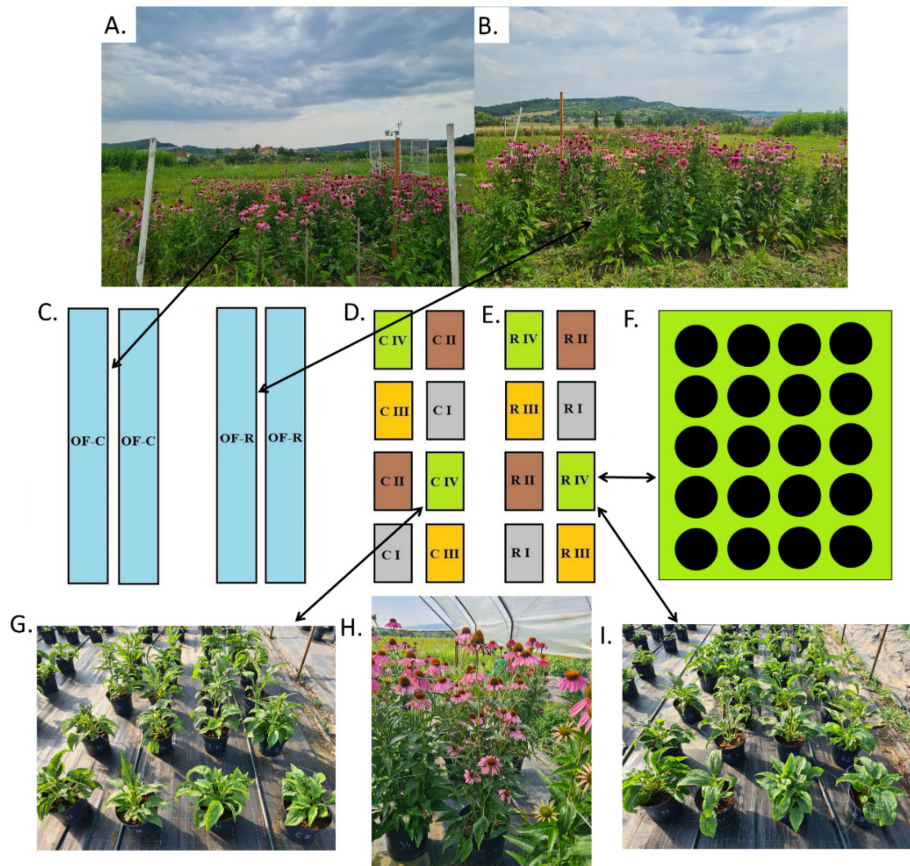


Figure 1. Experimental layout under greenhouse and open field conditions. Open field: block arrangement (C) and aspect of control (A) and *Rhizophagus irregularis* AMF-treated plants (B) at harvest; greenhouse: block arrangement of control (D) and AMF-treated (E) plants, single-block arrangement (F) of control (G) and treated plants (I), aspect of plants at harvest (H)

Abbreviations: C-control plants; R-*Rhizophagus irregularis* AMF inoculated plants; I-gleyic calcaric Fluvisol; II-calcaric Regosol; III-stagnic Luvisol; IV-sterile peat; OF-open field

The plants used in the greenhouse experiment were watered using a drip irrigation system equipped with a precision fertilizer dosing system (Dosatron) for nutrient supply. The watering rate was 400 ml/plant/day in May–June and 800 ml/plant/day in July–September. Fertilization was performed weekly using Universol water-soluble fertilizer (produced by ICL Fertilizers; provided from Blondy Romania SRL, Târgu Mureș) applied with the irrigation water. Initially, a 1.5 mS cm^{-1} EC nutrient solution (Universol Green: 23-06-10+2.7MgO) was applied, followed by a 2.5 mS cm^{-1} EC solution (Universol Blue: 18-11-18 + 2.5 MgO + TE) when the plants reached greater biomass. Based on the literature (Galambosi, 2004), the climatic conditions ensured sufficient water supply (500-600 mm); therefore, in the field experiment, the plants received water solely from natural precipitation.

At the end of the first growing season, the plants used in the greenhouse experiment were stored at 3 °C during the winter period to prevent frost damage. The plants used in the open-field experiment were covered with soil in late autumn (2022) for frost protection and were uncovered at the end of March 2023. The same cultivation techniques were used in the second year, taking into account the plants' higher water and nutrient requirements.

Root staining and evaluation of mycorrhizal colonization

At the end of the second growing season, AMF colonization was evaluated for both the greenhouse and field experiments. Six randomly selected plants/treatment/block were used for evaluation, with 24 plants per soil type and growing condition (a total of 120 plants). From each plant, 170 root fragments of about 1 cm in length were randomly collected, consisting mainly of root tips. The root fragments were stained with 0.05% of trypan blue (Sigma-Aldrich, Romania) according to Koske and Gemma (1989). Thirty 1-cm stained root fragments per plant (McGonigle *et al*., 1990) were analyzed in triplicate under a light microscope (Ceti Topic-T, B 2421.000, Belgium) at 200x magnification. Mycorrhizal colonization was estimated by determining the percentage frequency as the cumulative frequency of root segments containing at least one of the fungal structures (hyphae, vesicles, arbuscules, or spores) (Araim *et al*., 2009; Iakab *et al*., 2024).

Root biomass measurements

To measure root biomass, two-year-old *E. purpurea* plants were harvested in August, when leaves were starting to turn brown, in accordance with literature recommendations (Tansı *et al*., 2015; Muntean *et al*., 2016). The roots were harvested manually using a hand shovel. Each plant was removed together with an intact soil block, which was then broken apart and examined to ensure that no root fragments were left behind. Six plants were randomly selected from each treatment/substrate/block for the greenhouse plants, and eight plants/treatment/block for the field plants. After harvesting, the fresh root biomass was measured. The roots were then dried for two weeks at 21 °C in shaded conditions (following scientific recommendations for low-temperature drying to preserve the plant's active constituents (Poós and Varju, 2017; Burlou-Nagy *et al*., 2022)) and the dry biomass was subsequently measured.

Phenolic acid extraction and HPLC analysis

To obtain homogeneous plant material, the roots were finely chopped and ground at 6000 rpm in a ZM 200 Ultra Centrifugal Mill (Retsch GmbH, Germany). For the extraction of phenolic acids, 0.25 g of each sample was ultrasonicated in 70% methanol (v/v) for 10 minutes. The supernatant obtained by centrifugation at 6000 rpm for 10 minutes was collected, and the residue was subjected to further centrifugation, and the combined supernatants were filtered through a 45 μm pore diameter filter before analysis (Magwaza *et al*., 2016).

Samples were injected into an Agilent 1260 Infinity HPLC system equipped with a variable-wavelength UV detector. The chromatographic separation was performed on a Hypersil BDS C18 column (150×4.6 mm i.d., 5.0 μm) (Thermo Fisher Scientific, USA), according to Brown *et al.* (2011).

The chemical components were identified and quantified by comparing them to standard reference samples of caftaric acid, chicoric acid, caffeic acid, chlorogenic acid, cynarin, and echinacoside (Sigma–Aldrich, Germany). Quantification was performed using the standard external method, based on calibration curves (1, 2, 4, 8, 16, 32 μg mL^{-1} for chlorogenic acid, caffeic acid, cynarin, and echinacoside, and 0.25, 0.5, 1, 2, 4 mg mL^{-1} for caftaric and chicoric acid). The results are presented as milligrams of each caffeic acid derivative per gram of root dry material (Iakab *et al*., 2024).

Extraction of essential oils and GC-MS analysis

To extract the essential oil from the roots, the dried root material was finely chopped and 100 g of root material was placed in a 2 L round-bottomed flask with 1000 mL of water in a 1:10 ratio followed by distillation for 4 hours using a Clevenger-type apparatus (Nyalambisa *et al*., 2017). The essential oils were collected and stored in separate airtight containers at 4 °C until GC-MS analysis. For the extraction of root essential oils, three replicates were performed for each treatment and substrate in potted plants, and five in open-field cultivated plants. The oil yield (μL) obtained from 100 g of dried root was used to calculate the essential oil yield.

Essential oils were examined using an Agilent 7890B gas chromatograph paired with an Agilent 5977A mass spectrometer. Compound separation was performed using an HP-5 MS fused silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) coated with a 5% phenylmethyl siloxane stationary phase. The MS scanning range ($m\ z^{-1}$) was 50–550 atomic mass units (AMU) with electron impact (EI) ionization at 70 eV (Nyalambisa *et al*., 2017; Soltanbeigi and Maral, 2022).

Data processing and statistical evaluation

The collected data were organized using Microsoft Excel. One-way MANOVA followed by pairwise Hotelling's tests were performed to compare results from edge blocks with those from other blocks—first for the control plants, then for the treated plants—to evaluate potential block or border effects. Statistical evaluation was performed using Past 4.03 software. Because the data were non-normally distributed, they were analyzed using the Kruskal-Wallis test, and pairwise comparisons were performed using the Mann-Whitney post hoc test (for root colonization, biomass, and phenolic acid content). A two-sample *t*-test was used to compare the root essential oil yields of open-field cultivated control and inoculated plants. Due to the small sample size (N=3), no statistical analyses were performed on the root essential oil yields obtained from potted control and AMF-treated plants.

The effect of AMF on the composition of root essential oils was investigated under various soil types and cultivation conditions (greenhouse and field). For this purpose, principal component analysis (PCA) was applied to the data on the relative abundances of chemical constituents in the root essential oil. A second PCA with only treated plants was conducted to correlate the AMF colonization rate with the following parameters: root fresh biomass, root dry biomass, essential oil yield, caftaric acid concentration, caffeic acid concentration, echinachoside concentration, chicoric acid concentration, cynarin concentration.

Results

Block and edge effect

While the MANOVA indicated overall group differences under greenhouse conditions (control plants: $F = 1.45$, Wilk's $\Lambda = 0.122$, $p = 0.069$, treated plants: $F = 2.28$, Wilk's $\Lambda = 0.207$, $p < 0.001$), the post-hoc analysis revealed no significant variation in the measured parameters based on block position (Hotelling's $p > 0.05$ in all comparisons). In the open field, no block or edge effects were detected (control plants: Hotelling's $T2 = 82.68$, $F = 1.47$, $p = 0.41$; treated plants: Hotelling's $T2 = 80.84$, $F = 1.44$, $p = 0.42$).

Root colonization

Considering the root colonization rate in control plants, no fungal structures were found in the case of sterile peat. The colonization rate of control plants across different soil types was not evaluated due to the native microbiome in these substrates. Regarding plants treated with arbuscular mycorrhizal fungi (AMF), significant differences in colonization rate were observed between potted stagnic Luvisol and the other substrates (Table 1).

Table 1. Colonization rate in the roots of *Echinacea purpurea* in the second vegetation period

Substrate type	Mean (%)	Standard deviation
Gleyic calcaric Fluvisol (RI)	55.33[c]	13.421
Calcaric Regosol (RII)	61.06[bc]	11.725
Stagnic Luvisol (RIII)	77.78[a]	13.221
Sterile peat (RIV)	64.67[b]	11.962
Open-field stagnic Luvisol (OF-R)	49.94[d]	9.580

*Notes (legend): Kruskal–Wallis tests were performed, followed by pairwise Mann–Whitney tests for comparisons. The different letters beside means indicate statistically significant differences at $p < 0.05$ level

Root biomass measurements

No significant differences were found within the same substrate between fresh and dry root biomass for control and AMF-treated plants (Figure 2. A-B). The highest values for root fresh biomass were observed in control plants cultivated in open-field stagnic Luvisol (229.06 g, SD: ± 77.249), while the lowest were in control plants cultivated in stagnic Luvisol (120.625 g, SD: ± 56.470). For root dry biomass, the highest values were observed in control plants cultivated in open-field stagnic Luvisol (89.25 g, SD: ± 32.504), and the lowest in control plants cultivated in stagnic Luvisol (49.56 g, SD: ± 23.523) and sterile peat (55.66 g, SD: ± 29.572).

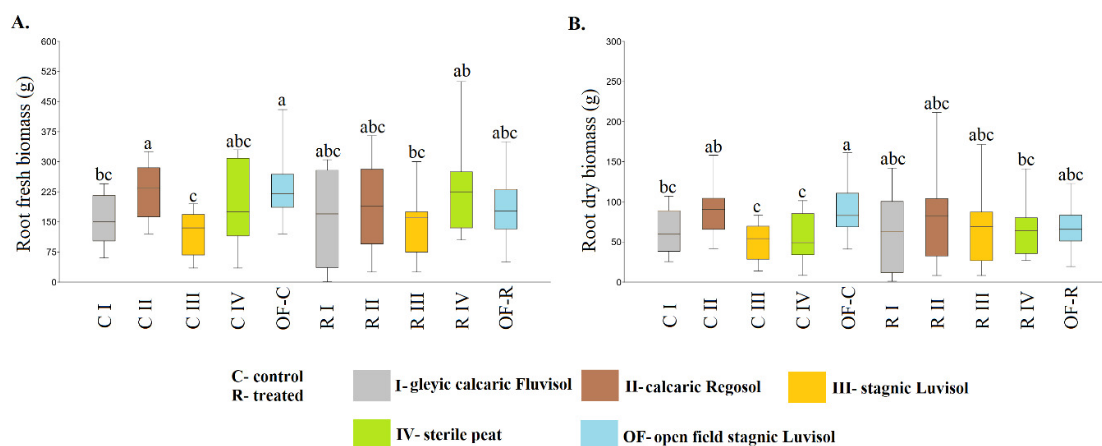


Figure 2. Fresh (A) and dry (B) biomass of two-year-old *Echinacea purpurea* roots under different growing conditions

Kruskal-Wallis tests were performed, followed by pairwise Mann-Whitney tests for comparisons. The different letters above the bar charts indicate statistically significant differences at $p < 0.05$ level

Phenolic acids content

Regarding the phenolic acid content of the roots of two-year-old *Echinacea purpurea* plants, HPLC analysis detected five phenolic acids: caftaric acid, caffeic acid, cynarin, echinacoside, and chicoric acid.

Caftaric acid content was significantly higher in treated plants compared to controls on sterile peat, with a 2.87-fold increase observed (Kruskal-Wallis $p < 0.001$, Mann-Whitney $p < 0.001$). The highest caftaric acid

concentrations were detected in plants cultivated under field conditions (AMF-treated plants: 54.06 mg g^{-1}, SD: ± 26.829; control plants: 50.75 mg g^{-1}, SD: ± 20.704), whereas the lowest levels were recorded in plants grown in stagnic Luvisol (AMF-treated plants: 10.77 mg g^{-1}, SD: ± 11.123; control plants: 11.79 mg g^{-1}, SD: ± 3.057) (Figure 3. A).

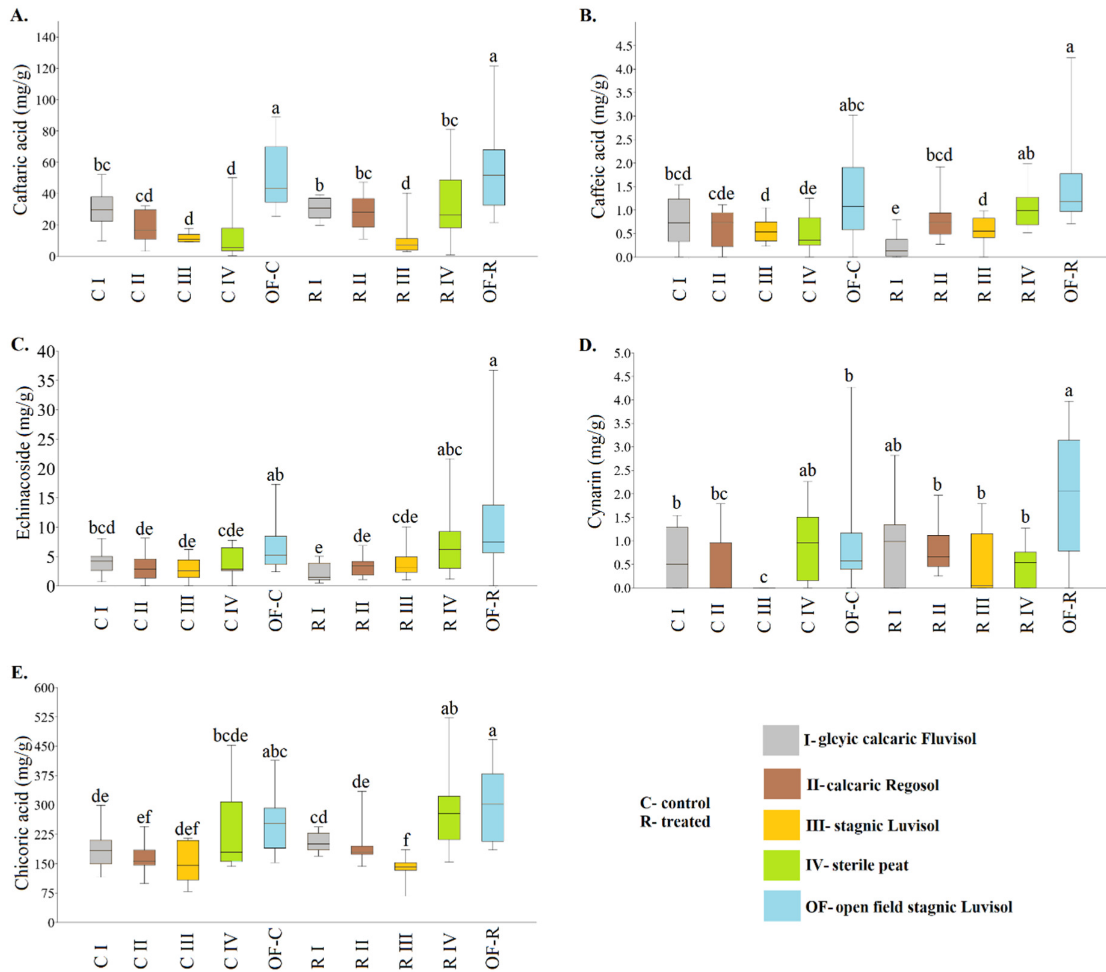


Figure 3. Caftaric acid (A), caffeic acid (B), echinacoside (C), cynarin (D) and chicoric acid (E) content of two-year-old *Echinacea purpurea* roots under different growing conditions

Kruskal-Wallis tests were performed, followed by pairwise Mann-Whitney tests for comparisons. The different letters above the bar charts indicate statistically significant differences at $p < 0.05$ level

Significant differences in root caffeic acid content (Kruskal-Wallis $p < 0.001$) were observed on gleyic calcaric Fluvisol, where a 3.39-fold decrease was found in AMF-treated plants compared to controls (Mann-Whintey $p = 0.016$) and on sterile peat, where AMF-treated plants showed a 2.15-fold increase in caffeic acid content compared to controls (Mann-Whintey $p < 0.001$) (Figure 3. B**).** The highest caffeic acid concentration was measured in AMF-treated plants grown under field conditions in stagnic Luvisol (1.49 mg g^{-1}, SD: ± 0.869). In comparison, the lowest was recorded in AMF-treated plants on gleyic calcaric Fluvisol (0.21 mg g^{-1}, SD: ± 0.270).

Consistent with the findings for caffeic acid, echinacoside levels were significantly lower in AMF-treated plants compared to control plants grown on gleyic calcaric Fluvisol (Kruskal-Wallis $p < 0.001$, Mann-Whitney

p = 0.040), representing a 1.98-fold decrease (Figure 3. C). The highest concentration of echinacoside was detected in AMF-treated plants grown on open-field stagnic Luvisol (10.76 mg g^{-1}, SD: ± 9.082), while the lowest quantity was found in AMF-treated plants grown on gleyic calcaric Regosol (2.11 mg g^{-1}, SD: ± 1.697).

Within the same substrate, significant differences in cynarin content were detected between control and AMF-treated *E. purpurea* roots (Kruskal-Wallis $p < 0.001$). On stagnic Luvisol (Mann-Whitney $p = 0.018$), cynarin was not detected in measurable quantities in control plants. In contrast in open-field stagnic Luvisol (Mann-Whitney $p = 0.020$), AMF-treated plants showed a significantly higher cynarin content than controls, with a 2.13-fold increase (Figure 3. D). The highest cynarin concentration was observed in AMF-treated plants grown under field conditions (1.96 mg g^{-1}, SD: ± 1.311).

Chicoric acid was the predominant phenolic acid in the roots. No significant differences were observed between control and AMF-treated plants grown under the same conditions (Figure 3. E). The highest concentration of chicoric acid was recorded in AMF-treated plants cultivated under field conditions (303.29 mg g^{-1}, SD: ± 92.637). In comparison, the lowest concentration was observed in AMF-treated plants grown on stagnic Luvisol (139.30 mg g^{-1}, SD: ± 29.834).

Essential oil yield and composition

Regarding the average essential oil yield (μL 100 g^{-1}) of the *radix*, in the case of open-field cultivated control and inoculated plants no significant differences were detected (OF-C: 20, N=5, SD: ±6.666; OF-R: 20.53, N=5, SD: ±7.865; $t = 0.11534$, p=0.91102). The average yields and standard deviations of essential oils for potted control and treated plants are presented in Table 2. The highest essential oil yields were obtained from control plants cultivated on sterile peat (22.22 μL 100 g^{-1}, N=3, SD: ± 7.698), while the lowest were found in AMF-treated plants cultivated on gleyic calcaric Fluvisol (6.67 μL 100 g^{-1}, N=3, SD: ± 2.886). However, no statistical analysis could be conducted, the following tendencies were observed in the case of potted plants: on gleyic calcaric Fluvisol, calcaric Regosol, and sterile peat inoculation with *Rhizophagus irregularis* resulted in lower essential oil yields compared to the control plants, while on potted stagnic Luvisol, AMF-treated plants showed an increased essential oil yield in comparison with control plants.

Table 2. Average essential oil yields of two-year-old *Echinacea purpurea* roots

Substrate and treatment type	Average yield (μL 100 g^{-1})
C I	16.74 (N=3, SD: ± 10.819)
C II	13.33 (N=3, SD: ± 6.665)
C III	8.33 (N=3, SD: ± 3.608)
C IV	22.22 (N=3, SD: ± 7.698)
OF-C	20 (N=5, SD: ± 6.666)
R I	6.67 (N=3, SD: ± 2.886)
R II	8.89 (N=3, SD: ± 3.845)
R III	14.81 (N=3, SD: ± 6.415)
R IV	15.56 (N=3, SD: ± 10.183)
OF-R	20.53 (N=5, SD: ± 7,865)

*Notes: The average essential oil yields are expressed as μL of essential oil extracted from 100 grams of dry root. Bold numbers indicate the highest yield. N denotes the number of samples, and SD indicates the standard deviation from the mean. Abbreviations: C-control; R-treated with *Rhizophagus irregularis*; I-gleyic calcaric Fluvisol; II-calcaric Regosol; III-stagnic Luvisol; IV-sterile peat; OF-open-field stagnic Luvisol

The most abundant components of the essential oil were τ-cadinol (6.42-19.28%), followed by n-hexadecanoic acid (0.89-34.14%), and caryophyllene oxide (0-10.30%). The compositional profiles of essential oils extracted from the roots of control and AMF-treated *E. purpurea* plants, cultivated under different growing conditions, are included in Table 3. A representative chromatogram is presented in Figure 4 to illustrate the method's separation and detection characteristics.

Table 3. The essential oil profiles of the two-year-old *Echinacea purpurea* roots under different growing conditions

Compounds	C I	C II	C III	C IV	OFC	R I	R II	R III	R IV	OFR
τ-Cadinol	12.91	17.84	16.37	12.36	6.42	8.62	19.28	16.24	11.95	8.28
n-Hexadecanoic acid	34.14	3.87	27.73	3.49	11.7	3.4	25.11	0.89	3.81	1.3
Caryophyllene oxide	0	5.11	3.86	7.89	5.72	3.06	10.3	8.92	2.7	7.59
τ-Muurolol	4.87	5.39	4.96	4.92	3.23	2.91	5.28	7.46	2.39	3.86
Longifolene	4.04	5.41	3.77	5.2	2.1	2.55	3.71	4.68	2.76	2.72
Germacrene D	3.57	1.99	2.05	4.18	4.52	1.7	4.02	0.77	1.6	6.86
Shyobunol	7.29	0	2.88	0	2.3	0.66	3.21	4.94	1.33	3.23
Caryophyllene	0	1.48	3.57	3.4	4.3	0.89	2.7	3.23	0.98	4.98
Spatulenol	0	2.79	2.45	2.25	1.51	1.18	2.51	2.15	1.25	2.04
Eudesma-1,4(15),11-triene	2.35	0	0	3.71	1.5	0	2.56	2.89	0	1.8
Isospathulenol	0	2	1.32	2.73	1.34	1.11	1.5	2.44	0	1.69
Salvial-4(14)-en-1-one	0	1.54	0	1.2	1.28	1.22	2.43	2.7	0	1.78
1,11-Dodecadiene	0	2.1	0	0.99	2.69	0.91	0	1.99	0	2.92
δ-Cadinene	0	0.73	0	0.9	1.87	0	0	2.32	0.45	3.18
Junenol	0	0	0	0	2.29	0	0	2.41	0	3.55
β-Calacorene	0	0	0	0	0	0	0	2.1	4.71	1.07
Humulene epoxide 2	0	0	0	0	0.93	0	1.66	2.82	0	1.71
Nerolidol	0	0	0	0.45	1.23	0.37	0	0.9	0	2.87
Cubebol	0	0	0	0	1.2	0	0	2.12	0	1.24
Aromandrene	0	0	4.09	0	0.22	0	0	0	0	0
m-Mentha-4,8-diene, (1S,3S)-(+)-	0	0	0	2.7	1.1	0	0	0	0	0
Ylangenol	0	0	0	0	0.82	0	0	1.4	0	1.27
α-Muurolene	0	0	0	0.17	0.42	0	1.73	0.7	0	0
Valerena-4.7(11)-diene	0	0	0	0	0.96	0.78	0	0.86	0	0
Humulene	0	0	0	0.51	0.8	0	0	0	0	0.86
γ-Muurolene	0	0	0	0	0.31	0	0	0	0	1.53
4,8-Epoxyazulene	0	0	0	0	0.38	0.27	0	1.16	0	0
α-Guaiene	0	0	0	0	0.32	0	0	1.03	0	0
2-Naphtalenemethanol	0	0	0	0	0.35	0	0	0	0	0.89
Valencene	0	0	0	0	0.7	0	0	0	0	0.49
0-Cymene	0	0	0	0.44	0	0	0	0	0.65	0
cis-Muurola-3,5-diene	0	0	0	0	0.44	0.42	0	0	0	0
Isoshyobunone	0	0	0	0.43	0.16	0	0	0	0	0
Eudesma-4,11-dien-2-ol	0	0	0	0	0.42	0	0	0	0	0
β-Bourbonene	0	0	0	0	0.34	0	0	0	0	0
β-Ionone	0	0	0	0	0	0.31	0	0	0	0
γ-Gurjunenepoxide	0	0	0	0	0.29	0	0	0	0	0
β-Elemene	0	0	0	0	0.28	0	0	0	0	0
α-Phellandrene	0	0	0	0.26	0	0	0	0	0	0
β-Guaiene	0	0	0	0	0.18	0	0	0	0	0
α-Copaene	0	0	0	0	0.14	0	0	0	0	0
Chemical classes	**C I**	**C II**	**C III**	**C IV**	**OFC**	**R I**	**R II**	**R III**	**R IV**	**OFR**
Oxygenated sesquiterpenes %	27.42	35.4	31.84	36.41	30.79	19.4	48.73	58.46	20.07	42.07
Sesquiterpene hydrocarbons %	7.61	10.98	13.48	14.45	17.95	7.25	12.16	13.26	5.34	18.83
Monoterpene hydrocarbons %	0	0	0	3.4	1.1	0	0	0	0.65	0
Other %	34.14	3.87	27.73	3.92	14.92	3.71	25.11	5.4	8.52	6.81

*Notes: The values are expressed as percentage (%), representing the mean of three replicates for each treatment and substrate. The essential oil components were listed according to their abundancy. Abbreviations: C-control, R-treated with *Rhizophagus irregularis*, I-gleyic calcaric Fluvisol, II-calcaric Regosol, III-stagnic Luvisol, IV-sterile peat, and OF-open field stagnic Luvisol

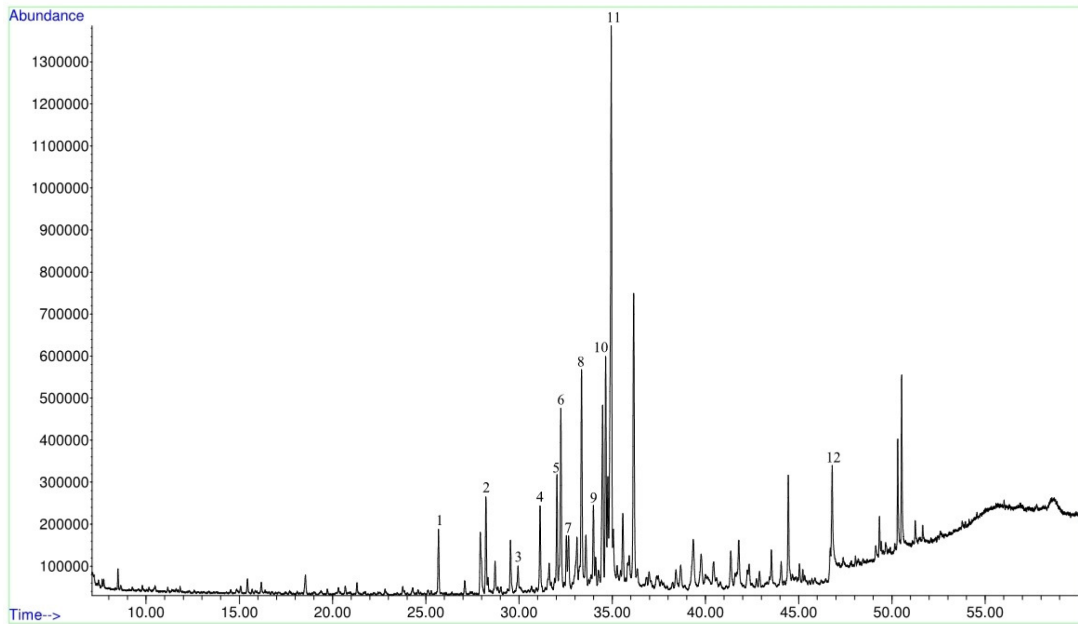
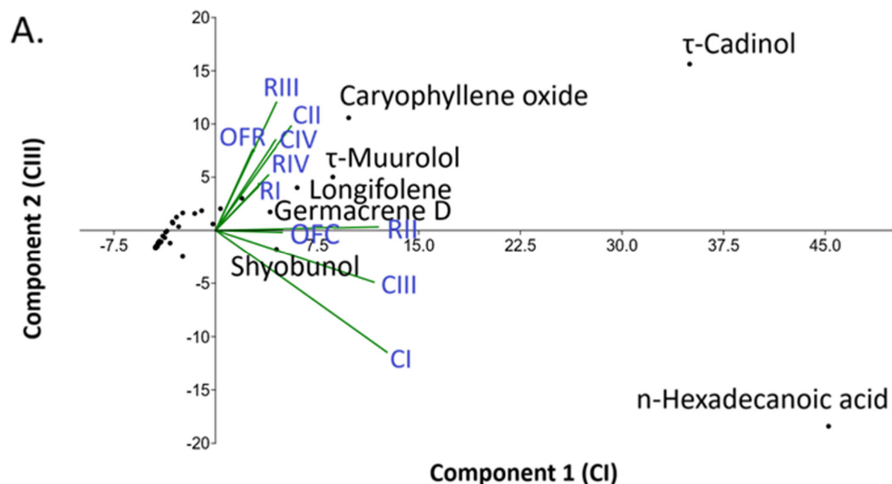


Figure 4. GC-MS analysis chromatogram of *Echinacea purpurea* root essential oil
The sample was obtained from control plants cultivated in pots (greenhouse) on calcaric Regosol. Peak numbering: 1. Caryophyllene; 2. 1,11-Dodecadiene; 3. δ-Cadinene; 4. Germacrene D; 5. Spatulenol; 6. Caryophyllene oxide; 7. Salvial-4(14)-en-1-one; 8. Longifolene; 9. Isospathulenol; 10. τ-Muurolol; 11. τ-Cadinol; 12. n-Hexadecanoic acid

The variation in the data was explained by 94.77% (PC1: 78.67% and PC2: 16.09%). On gleyic calcaric Fluvisol and stagnic Luvisol, the essential oil obtained from potted control plants presented a higher proportion of n-hexadecanoic acid and shyobunol. In contrast, the treated plants were abundant in τ-cadinol, caryophyllene oxide, τ-muurolol, longifolene, and germacrene D (Figure 5. A). When analyzing the principal chemical groups (PC1: 86.16% and PC2: 12.74%), it was found that on potted stagnic Luvisol AMF-treated plants were abundant in oxygenated sesquiterpenes, while control plants contained in higher proportions fatty acids (n-hexadecanoic acid) along with sesquiterpene hydrocarbons (Figure 5. B).



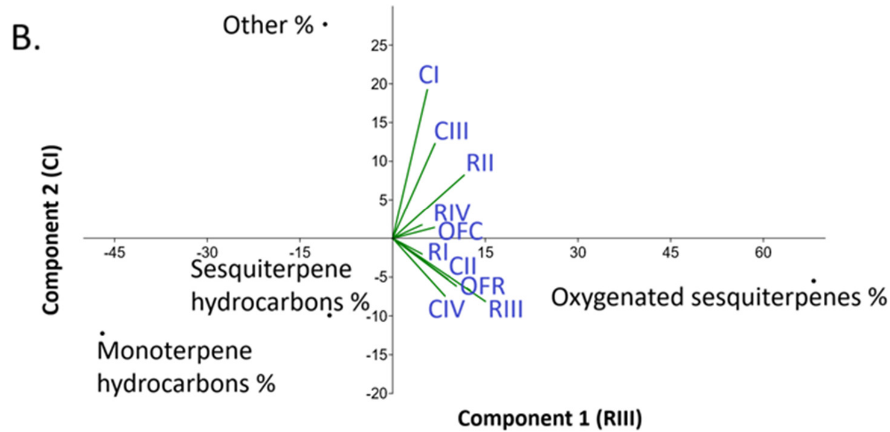


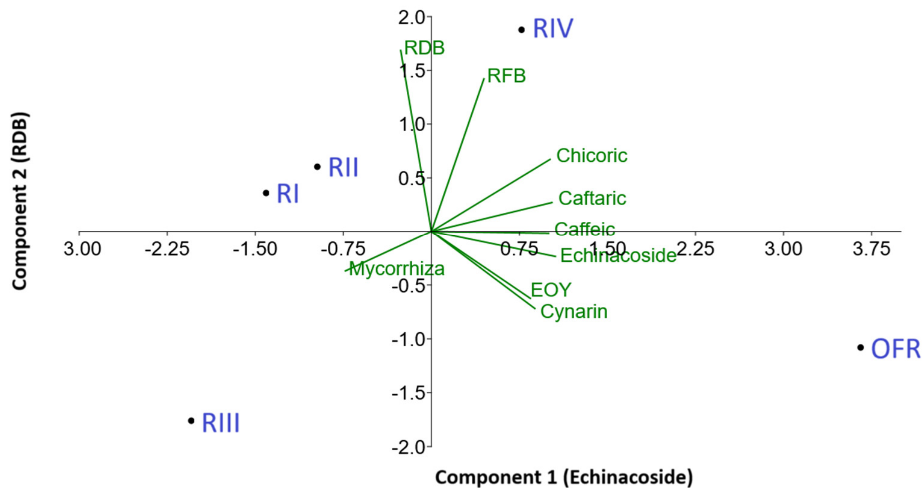
Figure 5. Principal component analysis of essential oil compounds (A.) and chemical classes (B.) in roots of two-year-old *Echinacea purpurea*

Variables are explained by treatments (C-control and R-AMF treated plants) and growing conditions (greenhouse conditions: I-gleyic calcaric Fluvisol, II-calcaric Regosol, III-stagnic Luvisol; open-field conditions: OF-open field stagnic Luvisol). On potted stagnic Luvisol essential oil composition differed between control and *Rhizophagus irregularis* AMF-treated plants. Treated plants presented higher proportions of oxygenated sesquiterpenes, while control plants essential oil contained also other chemical classes (fatty acids – n-hexadecanoic acid) in higher proportions

Effect of colonization rate on the assessed parameters

The highest levels of AMF colonization were obtained on potted plants cultivated in stagnic Luvisol. Echinacoside was the first principal component (PC1) and root dry biomass represented the second principal component (PC2). The PCA explained 81.52% of the correlations among the variables (PC1: 58.52% and PC2: 23%).

Plants from open-field stagnic Luvisol contained the highest echinacoside concentrations. There was a negative correlation between AMF colonization rate and echinacoside, caftaric acid concentration, chicoric acid concentration, caffeic acid concentration, cynarin, but also essential oil yield in case of plants grown in open-field conditions (Figure 6). No other correlations were found between AMF colonization rate and the assessed plant parameters.



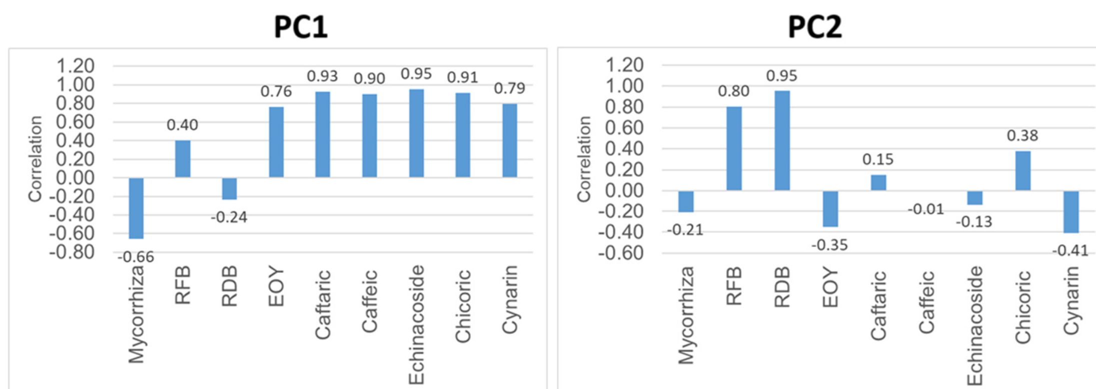


Figure 6. Principal components analysis (PCA) was performed to assess correlations between the AMF colonization rate (without control in this case) with the parameters assessed (root fresh and dry biomass, phenolic acid content and essential oil yield) during the second growing season of *Echinacea purpurea*
Before analysis, all data were first averaged and log_{10}-transformed, then PCA correlation analyses were performed. Abbrevations: R-treated, I-gleyic calcaric Fluvisol, II-calcaric Regosol, III-stagnic Luvisol. IV-sterile peat, OF-open-field stagnic Luvisol, RFB-root fresh biomass, RDB-root dry biomass, EOY-essential oil yield, Caftaric-caftaric acid concentration, Caffeic-caffeic acid concentration, Echinacoside-echinacoside concentration, Chicoric-chicoric acid concentration, Cynarin-cynarin concetration
However, colonization rate was lower in open-field conditions than in greenhouse, plants presented higher values for phenolic acids and also for essential oil yield than potted plants

Discussion

The present study investigated the influence of *Rhizophagus irregularis*, an arbuscular mycorrhizal fungus (AMF), on root biomass, phenolic acid content, and essential oil characteristics in two-year-old *Echinacea purpurea* plants grown under different soil types and conditions. Although AMF are often associated with increased growth and phytochemical content (Smith and Read, 2008b; Begum *et al.*, 2019) of medicinal plants, especially in *Echinacea* (Araim *et al.*, 2009; Gualandi *et al.*, 2014), the results of this study show that the effects of AMF inoculation may depend on several variables.

Contrary to expectations, AMF inoculation did not significantly enhance root biomass production in *E. purpurea* under any of the tested substrates or growing conditions. This is consistent with the findings of Attarzadeh *et al.* (2019), who noted that the growth benefits of AMF may be reduced or reversed under certain environmental or nutrient conditions. Negative or absent responses may occur if the mutualistic balance is altered under high nutrient supply, if fungal colonization has a high carbon cost to the host, or if the AMF strain is incompatible with the plant species or genotype (Johnson *et al.*, 1997; Hoeksema *et al.*, 2010). It is also possible that the specific soil microbiota present in some substrates limited the establishment or function of the symbiosis (van Der Heijden *et al.*, 2015). However, considering the climatic region, it can be stated that the results obtained by Muntean *et al.* (2016), where *E. purpurea* plants reached 98 g (SD: ± 3.1) of fresh root biomass in their second vegetation period under field conditions, were exceeded under all soil types and cultivation conditions in our study (highest: control plants cultivated in open-field stagnic Luvisol 229.06 g, SD: ± 77.249; control plants cultivated in stagnic Luvisol 120.625 g, SD: ± 56.470).

Chicoric acid was confirmed as the dominant phenolic compound (highest concentration in AMF-treated plants under field conditions, 303.29 mg g^{-1}, SD: ± 92.637; lowest concentration in AMF-treated plants on stagnic Luvisol, 139.30 mg g^{-1}, SD: ± 29.834) in the roots of *E. purpurea*, in agreement with previous research (Perry *et al.*, 2001; Pellati *et al.*, 2004; Matthias *et al.*, 2008). While AMF treatment did not

significantly affect chicoric acid levels within the same substrates, significant differences were observed for other phenolic acids, including caftaric acid, caffeic acid, and echinacoside. For instance, both caffeic acid (3.39-fold decrease) and echinacoside (1.98-fold decrease) showed significantly lower concentrations in AMF-treated plants grown on gleyic calcaric Fluvisol. On the other hand, higher levels of caftaric acid (2.87-fold increase) and caffeic acid (2.15-fold increase) were detected on sterile peat, and increased cynarin content (2.13-fold increase) was found in those cultivated under field conditions, in the case of AMF-treated plants. These results suggest that soil microbiota may significantly influence the mode of action of AMF on phenolic acid biosynthesis (Kaur and Suseela, 2020; Sharma *et al*., 2021). This is particularly observable in sterile peat, which lacks a native microbial community, where *R. irregularis* exerted its positive effects on root caftaric and caffeic acid content. Although a similar, two-year-long study under open-field conditions conducted by Attarzadeh *et al.* (2020) reported a positive impact of *R. irregularis* on root chlorogenic acid, caftaric acid, and echinacoside content, in our experiment, *R. irregularis* significantly increased only cynarin concentration under open-field conditions.

When the effect of AMF on essential oil yield was assessed under open-field conditions, no significant differences were found between control and treated plants. These results are consistent with those obtained by Hajagha *et al.* (2017) in their second year of the experiment, when *R. irregularis* was applied alone. Regarding the quality of the essential oil, *R. irregularis* induced changes on stagnic Luvisol and gleyic calcaric Fluvisol: the essential oil obtained from control plants contained higher proportions of n-hexadecanoic acid and shyobunol, while τ-cadinol, caryophyllene oxide, τ-muurolol, longifolene, and germacrene D were abundant in treated plants. Furthermore, on these substrates, the essential oil from control plants contained very few volatile compounds (7-11 substances). In the study conducted with *E. purpurea* roots, extracts obtained with hexane, chloroform, and ethyl acetate were rich in fatty acids (n-hexadecanoic acid, linoleic acid, and α-linolenic acid) (Petkova *et al*., 2023). Although n-hexadecanoic acid is not a volatile compound, it is a common phytochemical in *E. purpurea* roots, which may explain its detection in the hydrodistilled extract.

In our experiment, τ-cadinol, n-hexadecanoic acid, caryophyllene oxide, τ-muurolol, longifolene, and germacrene D were the dominant chemical compounds in root essential oil, whereas *E. purpurea* roots from South Africa (Nyalambisa *et al*., 2017) and Bulgaria (Dosoky *et al*., 2023) were dominated by germacrene D and α-phellandrene.

Although AMF colonization was significantly higher in potted plants than in field conditions, field-grown plants accumulated more phenolics and produced higher amounts of essential oils. This pattern is consistent with the carbon cost of symbiosis: maintaining a high level of AMF colonization requires a continuous allocation of assimilated carbon to the fungal partner, which may limit the carbon available for secondary metabolite biosynthesis (Johnson *et al*., 1997; Hoeksema *et al*., 2010). In contrast, under field conditions, colonization levels were lower, reducing the carbon costs of symbiosis and allowing more assimilates to be directed toward the production of phenols and essential oils. Environmental stressors and nutrient heterogeneity in the field may further stimulate secondary metabolism, contributing to the higher metabolite levels observed despite lower AMF colonization.

Despite the limited effect of *R. irregularis* inoculation in this study, AMF remains a promising approach for the sustainable cultivation of medicinal plants. Their effects are highly dependent on both taxa and environmental conditions, highlighting the need for further investigations. For *E. purpurea*, future studies should investigate other AMF species or mixed inocula, as well as longer-term effects under field conditions in different soil types, examining the impact of varying microbiome communities. A better understanding of the changes in gene expression, root development, and metabolite biosynthesis induced by AMFs would help to understand the mechanisms underlying their effects on the phenolic compounds and essential oils of *Echinacea* roots.

Conclusions

Overall, our experiment confirms the results of previous works, that *Rhizophagus irregularis* significantly increases the phenolic acid content of *Echinacea purpurea* roots under sterile conditions. Furthermore, in stagnic Luvisol and gleyic calcaric Fluvisol, changes in the chemical composition of the essential oils were observed between control and treated plants. Although the scientific literature has reported positive effects of the fungus on various phenolic acids, in our experiment *R. irregularis* significantly increased only the concentration of cynarin in field cultivation. Our results highlight the complexity of *R. irregularis*-host interactions and emphasize the strong influence of environmental and soil-related factors. Future research should involve multi-year field experiments to better understand how environmental variables influence the long-term effects of arbuscular mycorrhizal fungi (AMF) on *E. purpurea*. Testing multi-species AMF consortia could reveal whether functionally diverse fungal communities provide more consistent benefits than inoculation with a single species. In addition, extending chemical analysis beyond phenolic acids to broader metabolomic profiling would clarify how AMF influence multiple pathways of secondary metabolism. These approaches would help identify more reliable and field-relevant strategies for improving the cultivation and production of bioactive compounds from *E. purpurea*.

Authors' Contributions

Conceptualization: MI, ED, EB, and FVD; Data curation: MI and ZSI; Formal analysis MI, ED and ZSI; Investigation: MI, ED, CSA and ZSI; Methodology: MI, ED, EB, and FVD; Project administration: ED, EB and FVD; Resources: ED and FVD; Software: ED; Supervision: ED and FVD; Validation: ED, CSA EB and FVD; Visualization: MI and ED; Writing - original draft: MI; Writing - review and editing: ED, CSA, EB and FVD; MI, ED, CSA, EB, ZSI, and FVD contributed equally to this work.

All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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