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The effect of arbuscular mycorrhiza on physiological and biochemical parameters and capsaicinoid production in *Capsicum annuum* L.: A comparative study of extraction methods and solvents

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Abstract: This study aimed to determine the effect of two mycorrhizal fungi, *Funneliformis mosseae* (*Fm*) and *Rhizophagus intraradices* (*Ri*), on capsaicinoid production in *Capsicum annuum* L. by gas chromatography-mass spectrometry (GC-MS) via two different extraction approaches, magnetic stirring and ultrasound-assisted extraction with three different solvents, ethanol (EtOH), ethyl acetate (EtAce), and acetonitrile (AceN). The effect of mycorrhizal fungi on some physiological properties and biochemical activity, the content of total phenolic compounds, and antioxidant activity were also investigated. For all investigated parameters, the plants inoculated with mycorrhizal fungi showed significantly higher values than the non-mycorrhizal control plants, except for malondialdehyde (MDA), which was an indicator of lipid peroxidation due to damage that occurred in the cell membrane. It was concluded that inoculation with mycorrhizal fungi increased both capsaicin and dihydrocapsaicin production up to 4-fold in *C. annuum* compared to the control. Results also indicated that ultrasound-assisted extraction with EtAce was the most effective method for the determination of capsaicin by GC-MS.

Keywords: *Capsicum annuum*, *Funneliformis mosseae*, mycorrhiza, capsaicin, ultrasound-assisted extraction, gas chromatography-mass spectrometry (GC-MS)

INTRODUCTION

Peppers are a widely consumed food item globally. They possess diverse taste profiles, potential uses in cooking, and nutritional benefits [1]. Peppers belong to the *Capsicum* genus. Capsicum belongs to the Solanaceae family, which also includes other plants like tomato, potato, and tobacco. The *Capsicum* genus contains 22 wild and five domesticated species [2]; the five domestic species are *C. annuum* L., C. *baccatum* L., *C. chinense*, *C. frutescens* L., and *C. pubescens*. *C. annuum* L. is a major vegetable and spice crop grown worldwide [3]. China, Mexico, Turkey, and Indonesia are among the leading pepper producers globally. China accounts for 0.51 million of the total annual pepper crop, which is 31.1 million tons. Following China, Mexico produces 0.074, Turkey produces 0.069, and Indonesia produces 0.055 million tons of pepper [4].

Peppers are a great source of phytochemicals such as anthocyanins, vitamins, phenolic acids, flavonoids, and capsaicinoids. The attractive colors of peppers, which range from green to red, are attributed to alkaloids and carotenoid metabolites [5]. Pungency is a taste characteristic of capsaicinoids. Capsaicinoids are thought to have powerful pharmacological properties that could be used in pain relief, weight loss, cardiovascular disease prevention, cancer prevention, and relief from gastrointestinal diseases. Capsaicin and dihydrocapsaicin have been identified as major capsaicinoids, with 13 other homologs as important active components of chili peppers[1].

Plants associate with other organisms, including animals, bacteria, or fungi, to fight against pathogens or to thrive in adverse environments. Mycorrhiza is an excellent example of a symbiotic relationship between plants and fungi. The rhizosphere is the region of

mycorrhizal association that includes the plant root and its associated living organisms. Arbuscular mycorrhizal (AM) fungi are soil fungi that form a symbiotic relationship with most agricultural and ornamental plants [6]. Increased uptake of immobile mineral nutrients is one of the benefits of symbiosis for the host plant. The symbiotic relationship between AM fungi and plants induces physiological and biochemical alterations in the latter, triggering various metabolic pathways that may lead to the augmentation of secondary metabolites [7,8]. Hence, the use of AM fungi is a viable approach for achieving enhancements in specific target compounds. This symbiotic association is compatible with nearly 90% of vascular plant species, impacting plant growth, nutrient cycling, carbon sequestration, disease and stress tolerance, and decreased reliance on fertilizers. *Funneliformis mosseae* and *Rhizophagus intraradices* are symbiotic fungi that can be found in agricultural fields. They are used in research to resist soil-borne plant diseases, improve yield and crop quality, and develop plant species that are more resistant to abiotic stress conditions.

AM fungi represent a potentially effective alternative to existing agricultural methods that aim to achieve sustainability. Similar to other plant species that can form a symbiotic relationship with soil fungi, *Capsicum* spp. roots tend to establish AM in response to low nutrient availability in the soil [9]. Hirrel and Gerdemann [10] were the first to show the benefits of AM fungi on plants of the genus *Capsicum*, observing that bell pepper plants inoculated with AM fungi could be more salt-tolerant than non-inoculated plants. The benefits of pre-inoculation with AM fungus were validated in terms of the height, dry weight, and yield of hot peppers transplanted into the field two years later [11]. Thus, the potential significance of this symbiotic relationship with *Capsicum* spp. is immeasurable. Due to the nature of the symbiosis, which needs the presence of a host for production, large-scale use of AM fungi in economically important crops such as pepper remains a challenge.

Studies have investigated the effects of the relationship between the pepper plant and various mycorrhizal fungal species on plant development, yield, resistance to various abiotic factors, stress conditions, and various plant diseases [4]. To the best of the authors' knowledge, no study has investigated the effect of mycorrhizal fungi on capsaicin production in pepper plants.

Extraction is one of the basic operations in organic chemistry since it is one of the most popular methods for separating and purifying organic substances [12]. There are several ways to measure capsaicinoids in peppers and pepper products, such as high-performance liquid chromatography [13,14], spectrophotometry [15], and gas chromatography [16]. However, it is important to compare extraction methods to ensure that all capsaicinoids are fully extracted from the samples.

This study investigated physiological and biochemical growth and development parameters after establishing a mycorrhizal relationship between the pepper plant and AM fungi. Furthermore, the objective was to investigate how these fungi affect capsaicin production, a valuable secondary metabolite with economic importance, and different extraction methods for major capsaicinoids from pepper fruits.

MATERIALS AND METHODS

Material

Seeds of a local hot chili pepper (*Capsicum annuum* L.) variety provided from Samandağ, Hatay (Türkiye) were used, and two different mycorrhizal fungi, *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler and *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler, provided by the Department of Soil Sciences, Faculty of Agriculture, Çukurova University (Adana, Türkiye), were used as the fungal material in this study.

Following surface sterilization with 5% commercial sodium hypochlorite, a 40-g fungal inoculum (bearing approximately 400 fungal spores) was applied to seeds planted in vials. The soil medium used in this study, consisting of soil and peat (1:1), was sterilized by autoclaving at 121°C, 1 atm for 30 min. The vials were kept in a plant growth chamber in 16 h light/8 h dark at 25±1°C with 45-50% relative humidity until germination. After 10 days of germination, the seedlings were transplanted into pots with the same fungal inoculum about 3 cm below the layer of the seedlings. Control plants did not receive any of the mycorrhizal inocula. Following the first day of pollination, the growth chamber temperature was set at 27±1°C until the harvest of the pepper fruits. Twenty-week-old plants were harvested after fully developed fruit formation.

The plants were not exposed to any natural or synthetic additives (fertilizer, plant growth regulators, soil supplements to support plant growth) from the day the seeds were planted into vials until the day the plants were harvested. Similarly, there was no use of natural or synthetic pesticides, including medicinal and aromatic plant extracts and fermented liquids that induce pH changes, such as vinegar or agricultural pesticides, approved by the Ministry of Food, Agriculture and Livestock.

Sampling of the plants for analysis

Root samples of the plants were washed with tap water to remove all soil debris, dried on blotting paper, and immediately used to determine fungal infection. Leaf samples were taken during plant growth as required by the planned biochemical analysis and kept at -80°C until the analysis was performed. Fruit samples were freeze-dried and kept in zipper bags away from direct sunlight at room temperature in the laboratory of the Biotechnology Department, Faculty of Arts and Sciences, Niğde Ömer Halisdemir University (Niğde, Türkiye) after the morphometric measurements were performed.

Determination of fungal infection

Root staining

To determine the mycorrhizal infection via light microscopy and to calculate the infection rate, the root samples were stained by the trypan blue method [17] with slight modifications. Briefly, the root samples were washed with tap water to remove all the soil debris, dried on blotting paper, immersed in 10% KOH solution, and incubated at room temperature overnight. The KOH solution then was discarded, and 1% HCl solution was added to the root samples, which were incubated at room temperature for 10 min. The samples were then transferred to the 0.05% Trypan Blue stain prepared in a lactic acid:glycerol:distilled water (1:1:1) mixture and incubated overnight. After the incubation, the samples were washed with distilled water and observed under a microscope.

The mycorrhizal infection rates were calculated using the Grid Line Intersection method [18]. The percentage of mycorrhizal root infection was calculated using the formula:

$$Infection\ \% = \frac{Total\ mycorrhizal\ root\ intercepts}{Total\ root\ intercepts} \times 100$$

Scanning electron microscopy (SEM)

To observe mycorrhizal infections via scanning electron microscopy (SEM), root samples were prepared according to a modified method influenced by various methods [19,20] used for biological sample preparation. Cleaned root samples were immersed in a mixture of 2.5% glutaraldehyde and 1% formaldehyde buffered in 0.1 M phosphate buffer saline (PBS) with a pH of 6.8 and incubated at room temperature for at least 3 h. The samples were washed with PBS for 5-6 cycles, each cycle lasting 15 min. The washed samples were then dehydrated using a seven-step ethanol series (20, 30, 40, 50, 70, 90, and 100%) prepared with freshly opened absolute ethanol, with each step lasting 15 min, and the last step was repeated 3 times. PBS was used instead of sodium cacodylate buffer, and the secondary fixation with osmium tetroxide was canceled [21] since these chemicals are highly hazardous. Following the dehydration step, the samples in absolute ethanol were freeze-dried at -85°C for 12 h (Scanvac Coolsafe 95-15, Labogene, Denmark). The dried samples were then mounted on aluminum stubs with carbon tapes, sputter-coated with Au-Pd (SC7620, Quorum, UK) for 90 s, and observed with a Zeiss EVO-40 SEM at high vacuum at 15 kV.

Effect of mycorrhiza on plant physiological properties

The effect of AM fungi on plant physiological properties was determined by measuring the height (cm), fresh and dry weight (g), and dry matter content of the aerial parts (%), dry matter content of the roots (%), number of fruits per plant, average fruit length (mm), average diameter of fruit (mm), average fresh weight of fruit (g), and yield as the fresh weight of fruits (g plant^{-1}).

The height of the aerial parts of the plants was measured after separating from the crown using scissors. The aerial parts were divided into smaller parts approximately 10 cm in length, and fresh weight was determined. Fresh samples were placed in an oven at 70°C for 48 h to obtain the dry weight. The same application was performed for root samples with a

fixed initial weight (0.5 g) to determine the root dry matter content. The measurements of fruit length and fruit diameter were performed using a digital caliper.

Effect of mycorrhiza on biochemical properties

Photosynthetic pigment analysis

The effect of AM fungi on photosynthetic pigment content was investigated by the colorimetric method [22]. For this purpose, 50 mg of fresh leaf samples were homogenized with 2 mL 95% ethanol and incubated overnight in the dark at room temperature. Following incubation, the samples were centrifuged at 10778 ×g for 5 min at 4°C. The supernatants were diluted with ethanol to a ratio of 1:9. The absorbance of the samples was measured at 470, 649, and 664 nm by a Thermo, MultiSkan Go spectrophotometer. The content of chlorophyll-a, chlorophyll-b, and carotenoids was calculated and expressed as µg/mL according to the formula below [23]:

$$\mathrm{Cha} = (13.36 \times \mathrm{A664}) - (5.19 \times \mathrm{A649})$$
$$\mathrm{Chb} = (27.43 \times \mathrm{A649}) - (8.12 \times \mathrm{A664})$$
$$\mathrm{Cx+c} = \frac{(1000 \times \mathrm{A470}) - (2.13 \times \mathrm{Cha}) - (97.63 \times Chb)}{209}$$

Malondialdehyde (MDA) content

Lipid peroxidation in the cell membrane due to biotic/abiotic stress conditions is an essential indicator for plants. The effect of mycorrhizal fungi infection on lipid peroxidation was investigated according to the accumulation of MDA in the plant leaves [24]. For this purpose, a 0.5-g fresh leaf sample was homogenized with 5 mL 0.1% trichloroacetic acid (TCA) using a Daihan HG-15D homogenizer at 8000 rpm for 5 min and centrifuged at 10778 ×g for 10 min at 4°C. One mL of the supernatant was added to 4 mL 0.5% thiobarbituric acid solution in 20% TCA and incubated at 95°C for 30 min in a water bath. Following the incubation, the sample was immediately put in an ice bath for 15 min to end the reaction and centrifuged at 10778 ×g for 5 min. The absorbance of the supernatant was measured at 532 and 600 nm by a Thermo, MultiSkan Go spectrophotometer. The MDA content was calculated and expressed as nmol mg fresh weight^{-1} using the formula:

$$MDA = \frac{(A532 - A600) \times Extract\ volume}{155 \times Sample\ mass}$$

Phenylalanine ammonia-lyase (PAL; EC: 4.3.1.24) activity

The effect of AM fungi on phenylalanine ammonia-lyase (PAL; EC. 4.3.1.24) activity was investigated [25]: 0.1 g of fresh leaf sample was homogenized with 1 mL 50 mM PBS containing 1% polyvinylpyrrolidone (PVP) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (pH 6.5). The homogenate was centrifuged at 18214 ×g for 25 min at 4°C. The supernatant obtained was used as the extract for PAL activity analysis. The PAL enzyme reacts with L-phenylalanine to produce trans-cinnamic acid. Thus, a 10-mM L-phenylalanine solution was prepared with 50 mM PBS (pH 6.5) as a substrate for the reaction. The reaction mixture was prepared by mixing 400 µL of extract with 500 µL 10 mM L-phenylalanine solution and 500 µL PBS. This mixture was then incubated at 30°C for 60 min. The absorbance of the sample was measured by a Thermo, MultiSkan Go spectrophotometer at 290 nm, and the result was expressed as µmol g cinnamic acid^{-1}.

Total phenolic content

The content of total phenolic compounds was determined using the Folin-Ciocalteu reagent [26,27]. The freeze-dried pepper samples were ground into a powder with a mortar and pestle, and 0.5 g of powdered material was mixed with 30 mL 80% methanol and homogenized by a Daihan HG-15D homogenizer at 8000 rpm for 5 min. The mixture was then incubated overnight at room temperature and for 15 min in an ultrasonic bath (Sonorex, Bandelin, Germany). The homogenate was filtered through Whatman No:1 filter paper and evaporated to dryness by a rotary evaporator (Hei-Vap Value, Heidolph, Germany). The residue was resuspended with methanol to obtain an extract with a 200-mg/mL concentration. One hundred µL of the extract was added to 1 mL of 1:10 diluted Folin-Ciocalteu reagent, shaken vigorously, and incubated for 5 min at room temperature. Then 1 mL of 7.5 % Na_2CO_3 solution was added to the mixture and incubated at room temperature in the dark for 90 min. The absorbance of the sample and blank was measured at 765 nm by a Thermo MultiSkan Go spectrophotometer. The results were expressed as µg GAE/mL extract.

Antioxidant activity

To determine the antioxidant activity of the pepper methanolic extract, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method was used [28]. The methanolic extract prepared to determine the content of total phenolic compounds was used for the DPPH assay as follows: 0.1 mM DPPH solution and a 5-step serial dilution of pepper methanolic extract were prepared with methanol, and 100 µL of the extract was added to 2.9 mL of DPPH solution, shaken, and incubated in the dark for 15 min. The absorbance of the mixture and blank DPPH solution was measured by a Thermo MultiSkan Go spectrophotometer at 517 nm. The DPPH scavenging activity of the pepper extract was evaluated using the formula:

$$SA\% = \frac{\mathrm{ADPPH} - \mathrm{ASample}}{\mathrm{ADPPH}} \times 100$$

where SA% is the DPPH scavenging activity of the extracts, ADPPH is the absorbance of 0.1 mM DPPH solution, and ASample is the absorbance of the sample at 517 nm.

Effect of mycorrhiza on capsaicin production

Capsaicin extraction

Three different extractions with two methods were investigated to determine the effect of AM fungi on capsaicin production in pepper plants, namely magnetic stirring extraction and ultrasound-assisted extraction by an ultrasonic bath and an ultrasonic probe. Also, three different solvents were used to determine the effect of solvent on capsaicin extractions. For magnetic stirring extraction, 0.5 g of powdered pepper fruit sample was mixed with 40 mL of ethanol (EtOH), ethyl acetate (EtAce), or acetonitrile (AceN) in separate flasks, homogenized by a Daihan HG-15D homogenizer at 8000 rpm for 5 min, and stirred overnight. For ultrasonic bath extraction, 0.5 g of powdered sample was mixed with 40 mL of solvent, homogenized, and incubated in an ultrasonic bath (Sonorex, Bandelin, Germany) at 50°C for 30 min. For ultrasonic probe extraction, 0.5 g of powdered sample was mixed with 40 mL of solvent, homogenized, and treated with an ultrasonic probe (Sonoplus, Bandelin, Germany) at room temperature for 15 min with 60% amplitude. The samples were filtered through Whatman No:1 filter papers and evaporated to dryness. The residue was resuspended with dichloromethane for EtOH and EtAce extracts, and AceN in a volume of 5 mL, dried with anhydrous Na_2SO_4, and filtered through a nylon filter with a pore diameter of 0.45 µm.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analyses were performed by a QP2010 Ultra GC-MS system (Shimadzu, Japan) equipped with a Rxi-5MS column (30 m * 0.25 mmID * 0.25 µm df; Restek, Bellefonte, PA, USA). One µL of each sample was injected using an AOC-20i (Shimadzu, Japan) autosampler in splitless mode. Helium was used as carrier gas with a flow rate of 1.28 mL/min. The GC-MS program was as follows: the temperature of the injection port was 260°C, initial oven temperature was set to 40°C and held for 1 min., then the temperature was ramped to 300°C at a rate of 10°C/min and held for 10 min. The ion source and interface temperatures were 230°C and 270°C, respectively [29]. All spectra were acquired with Electron Impact (EI) mode. The mass ranged between 50-550 *m/z* in full scan mode. The capsaicin and dihydrocapsaicin extracted from *C. annuum* samples were characterized by comparing the mass spectra with those from the Wiley mass spectra library (W9N11). The total ion chromatogram indicating the retention times of the capsaicin and the dihydrocapsaicin is shown in Supplementary Fig. S1.

Establishment of the calibration curves

For the quantitative analyses in GC-MS, capsaicin and dihydrocapsaicin calibration curves were established using standard solutions. A stock solution of a natural capsaicin standard (Tokyo Chemical Industry, Tokyo, Japan) was prepared at a concentration of 1 mg/mL with dichloromethane, then diluted with dichloromethane to 10, 25, 50, 100, and 200 ppm concentrations. The prepared standard solutions with different concentrations were analyzed in GC-MS under the conditions mentioned above. The calibration curves were established for capsaicin and dihydrocapsaicin using the GC-MS LabSolutions software (Shimadzu, Japan) with regression coefficients of 0.9985 and 0.9975, respectively.

Statistical analysis

One-way analysis of variance (ANOVA) and Duncan's multiple range test were used for comparing the means between groups at a significance level of P<0.05. IBM Statistical Package for the Social Science (SPSS) software (Version 24.0, SPSS Inc., Chicago, IL, USA) was used for data evaluation.

RESULTS

Effect of mycorrhiza on plant physiological properties

The association of the plant roots and the mycorrhizal fungi increased in terms of height, fresh weight, dry weight, dry matter content of aerial parts, dry matter content of roots, number of fruits per plant, average fresh weight of fruits, average fruit length, average fruit diameter, and fruit yield as regards the infection rate. The alteration of the plant roots with AM fungi is shown in Fig. 1 by both root staining and SEM imaging techniques.

Table 1 shows the significant differences between the plants inoculated with AM fungi and control (P<0.05). The root infection rates were 72.49±0.85% for *Fm* and 73.47±1.19% for *Ri*. The plants in a mycorrhizal association showed better values for all parameters investigated than the control plants. The highest value of the plant aerial parts was measured as 57.03±1.31 cm for plants inoculated with *Fm* while plants with *Ri* and the control plants were 56.8±3.84 and 49.37±0.8 cm, respectively, in height. The fresh and dry weights of the aerial parts were found to be highest in the plants that had been inoculated with *Ri*, with respective values of 53.71±3.78 and 13.76±0.32 g. The aerial parts of the plants associated with *Fm* had a fresh weight of 52.68±1.66 g and a dry weight

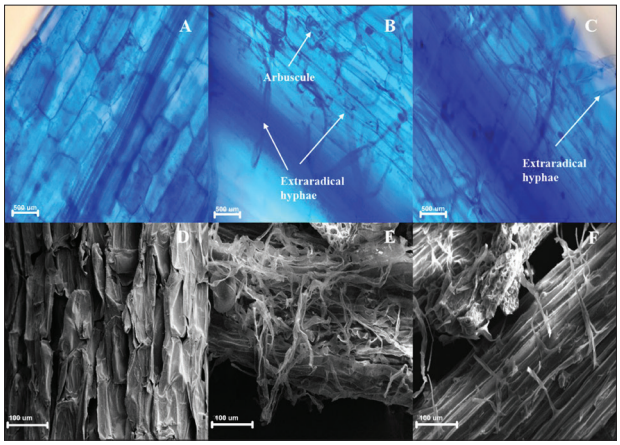

Fig. 1. Root images showing the mycorrhizal infections by light microscopy and SEM. The arrows indicate the extraradical hyphae, arbuscule, and vesicle. Letter A, B, and C refer to the light microscope images and D, E, and F refer to the SEM micrographs of control, *Fm,* and *Ri* plants, respectively. Bars indicate 500 µm for light microscope images and 100 µm for SEM micrographs.

of 13.15±0.85 g, while the control plants had values of 25.9±3.85 g and 5.50±0.68 g, respectively. Similarly, for the dry matter content of aerial parts and roots, the plants inoculated with *Ri* also showed the highest values with 24.98±1.77 and 15.89±0.56%. The plants with *Fm* had 21.32±1.57% dry matter content in plant aerial parts and 13.30±0.48% in roots, while the control plants had values of 10.8±4.03 and 10.43±0.46%, respectively (Table 1).

The plants inoculated with *Ri* showed the maximum fruit production with 5.67±1.53 fruits, 21.79±3.48 g of average fresh weight of fruits, 144.22±8.2 mm of average fruit length, 22.52±2.25 mm of average fruit diameter and 121.3±27.22 g of fresh fruit yield. These values were 4.67±0.58 fruits, 19.9±3.81 g, 142.31±10.25 mm, 21.41±1.58 mm, and 92.38±18.79 for the plants inoculated with *Fm* and 3.33±0.58 fruits, 13.89±1.42 g, 111.69±5.69 mm, 19.27±0.36 mm, and 46.74±12.47 g for the control plants, respectively.

Table 1. Effect of AM fungi on plant physiological properties in *Capsicum annuum*

Sample	IR	H-AP	FW-AP	DW-AP	DM-AP	DM-R	NF	Av-FrW	Av-FrL	Av-FrD	Yield
Control	none	49.37±0.8a	25.9±3.85a	5.50±0.68a	10.8±4.03a	10.43±0.46a	3.33±0.58a	13.89±1.42a	111.69±5.69a	19.27±0.36a	46.74±12.47a
F. mosseae	72.49±0.85	57.03±1.31b	52.68±1.66b	13.15±0.85b	21.32±1.57b	13.30±0.48b	4.67±0.58ab	19.9±3.81ab	142.31±10.25b	21.41±1.58b	92.38±18.79b
R.intraradices	73.47±1.19	56.8±3.84b	53.71±3.78b	13.76±0.32b	24.98±1.77b	15.89±0.56c	5.67±1.53b	21.79±3.48b	144.22±8.2b	22.52±2.25b	121.3±27.22b

*IR: Infection rate (%); H-AP: height of aerial parts (cm); FW-AP: fresh weight of aerial parts (g); DW-AP: dry weight of aerial parts (g); DM-AP: dry matter of aerial parts (%); DM-R: dry matter of roots (%); NF: number of fruits per plant; Av-FrW: average fruit weight (g); Av-FrL: average fruit length (mm); Av-FrD: average fruit diameter (mm); Yield: fresh weight of fruits per plant (g) ** All data shown in the table are expressed as means and standard deviations of three independent measurements. *** Lowercase letters indicate a significant difference between groups at P<0.05.

Effect of mycorrhiza on plant biochemical properties

Photosynthetic pigments

The results of photosynthetic pigment contents of pepper plants with and without AM fungi inoculation are shown in Table 2. AM fungi treatment had positive effects on the concentration of chlorophyll-a (Ch-a), chlorophyll-b (Ch-b), and carotenoid (Cx+c) pigments. The highest values in Ch-a, Ch-b, and Cx+c were determined in peppers inoculated with *Fm* (Table 2).

MDA content

The amount of MDA, which indicated the lipid peroxidation level for the plants inoculated with AM fungi and the control plants, is shown in Table 2. The highest MDA content was observed in the control plants with 5.2±0.19 nmol/mg FW while the plants inoculated with *Fm* and *Ri* had significantly different MDA values of 4.04±0.37 and 3.87±0.06 nmol/mg FW, respectively, with P<0.05 (Table 2).

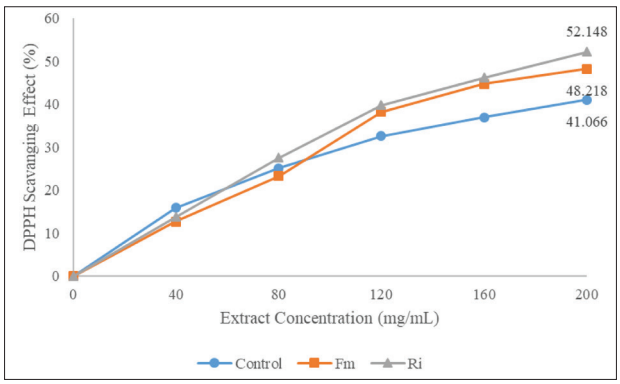

Fig. 2. DPPH scavenging activity of fruit methanolic extracts of *Capsicum annuum* inoculated with different AM fungi.

PAL activity

As can be seen in Table 2, PAL activity significantly increased among test groups (P<0.05). The highest PAL activity was determined in plants inoculated with *Ri* with 12.59±0.32 µmol/g cinnamic acid, while those inoculated with *Fm* had 11.34±0.36 µmol/g cinnamic acid. The lowest PAL activity was determined in the control plants with a 6.71±0.43 µmol/g cinnamic acid. These results suggest that the increased PAL activity due to mycorrhizal infections induces phenylpropanoid biosynthesis.

Total phenolic compounds and antioxidant activity

Total phenolic compounds (TPC) were quantified in fruit methanolic extracts using the spectrophotometric Folin-Ciocalteu's reagent method. The peppers were obtained from plants that were subjected to inoculation with AM fungi. The results are shown in Table 2. TPC was 386.49±28.64 in control samples. *Fm* treatment was 455.65±9.05, and *Ri* treatment was 463.78±13.06. This difference between AM fungi treatments is not statistically significant at P<0.05 (Table 2).

Free radical scavenging activity was investigated by the DPPH assay. The DPPH method, widely applied to examine antioxidant activity, is considered one of the most reliable methods, especially for fruits, vegetable juices, and plant extracts [30]. The DPPH radical scavenging activity results are presented in Fig. 2. The ability of AM fungi extracts to scavenge DPPH radicals showed a growing trend with increasing sample concentration. The percentage of DPPH radical scavenging activity of *Ri* extracts was 52.148%, while it was 48.218% for *Fm*. This increase is 26.98% and 17.42%, respectively, when compared to the control (P<0.05).

Table 2. Effect of AM fungi on biological activities in *Capsicum annuum*

	IR	Ch-a	Ch-b	Cx+c	MDA	PAL	TPC
Control	none	3.3±0.14a	2.43±0.25a	0.16±0.07a	5.2±0.19b	6.71±0.43a	386.49±28.64a
F. mosseae	72.49±0.85	5.04±0.16b	3.00±0.11b	0.75±0.03c	4.04±0.37a	11.34±0.36b	455.65±9.05b
R. intraradices	73.47±1.19	4.52±0.41b	2.72±0.22ab	0.63±0.07b	3.87±0.06a	12.59±0.32c	463.78±13.06b

*IR: Infection rate (%); Ch-a: chlorophyll-a (µg/mL); Ch-b: chlorophyll-b (µg/mL); Cx+c: carotenoid (µg/mL); MDA: malondialdehyde nmol/mg FW; PAL: phenylalanine ammonia-lyase (µmol/g cinnamic acid); TPC: total phenolic compounds (µg GAE/mL) ** All data shown in the table are expressed as means and standard deviations of three independent measurements. *** Lowercase letters indicate a significant difference between groups at P<0.05.

Effect of mycorrhiza on capsaicin production

The contents of capsaicin and dihydrocapsaicin in extracts obtained from fruits harvested from pepper plants inoculated with AM fungi are presented in Table 3. The fruit extracts obtained by ultrasonic probe extraction with EtAce from the plants inoculated with *Fm* exhibited the highest capsaicin and dihydrocapsaicin content of 6.811±0.103 and 4.041±0.059 mg/g DW, respectively. Interestingly, the highest capsaicin content of the fruits of plants inoculated with *Ri* was determined as 5.789±0.081 mg/g DW by ultrasonic probe extraction with EtAce. In contrast, the highest dihydrocapsaicin content was found by magnetic stirring extraction with EtAce as 3.923±0.099 mg/g DW. For control plants, the highest capsaicin content was 1.834±0.032 mg/g DW by ultrasonic bath extraction with EtAce, while it was 2.019 mg/g DW for dihydrocapsaicin by magnetic stirring extraction with EtAce. Our results, with 1.099±0.016 DW capsaicin and 0.92±0.02 mg/g DW dihydrocapsaicin content, reveal that the lowest values were detected in the control groups, showing that both mycorrhizal fungi species increased the capsaicin and dihydrocapsaicin amounts produced in pepper plants with $P<0.05$ (Table 3).

DISCUSSION

Many kinds of microorganisms live in the rhizosphere of plants, and many of them naturally interact with plant roots. There has been much interest in using good microorganisms to grow crops and keep the soil healthy in recent years. In general, using AM fungi has been shown to stimulate plant growth and improve yields and quality in different types of horticultural crops. We also observed these benefits in our research, which we will discuss in detail below.

It is commonly accepted that AM fungi have the potential to enhance the growth of plants, according to numerous documented sources [31-33]. Research has shown that when pepper plants are inoculated with AM fungi, their root and shoot dry weight and fruit yield increase significantly. The data we obtained from this study align with the research results, which have been confirmed by various studies on biomass and fruit yield production [34,35]. However, there are also instances where there is no difference in pepper fruit yield between infected and uninoculated plants [36]. It has been found that the response of plants to AM fungi inoculation can vary greatly depending on the cultivar, as demonstrated by previous research

Table 3. Effect of method and solvent on capsaicin and dihydrocapsaicin extraction from *Capsicum annuum* plants inoculated with different AM fungi

	Magnetic Stirring					
	EtOH		**EtAce**		**AceN**	
	Cap	**Dcap**	**Cap**	**Dcap**	**Cap**	**Dcap**
Control	1.099±0.02a	1.901±0.06a	1.757±0.55a	2.019±0.01a	1.136±0.06a	1.067±0.04a
F. mosseae	5.103±0.12b	2.915±0.09b	5.897±0.07c	3.871±0.07b	5.607±0.16c	3.450±0.04b
R. intraradices	4.964±0.06b	3.552±0.14c	5.640±0.14b	3.923±0.10b	5.172±0.17b	3.764±0.12c
	Ultrasonic Bath					
	EtOH		**EtAce**		**AceN**	
	Cap	**Dcap**	**Cap**	**Dcap**	**Cap**	**Dcap**
Control	1.141±0.06a	0.920±0.02a	1.834±0.03a	1.768±0.04a	1.818±0.02a	1.615±0.02a
F. mosseae	5.614±0.11c	3.575±0.09c	6.222±0.19c	4.032±0.05c	6.020±0.14c	3.777±0.22c
R. intraradices	4.244±0.27b	3.029±0.08b	5.205±0.19b	3.796±0.12b	5.034±0.22b	3.621±0.10b
	Ultrasonic Probe					
	EtOH		**EtAce**		**AceN**	
	Cap	**Dcap**	**Cap**	**Dcap**	**Cap**	**Dcap**
Control	1.151±0.04a	1.021±0.03a	1.739±0.02a	1.636±0.03a	1.506±0.04a	1.449±0.04a
F. mosseae	5.511±0.11b	3.596±0.15b	6.811±0.10c	4.041±0.06c	5.748±0.17c	3.863±0.11c
R. intraradices	5.683±0.07b	3.523±0.05b	5.789±0.08b	3.744±0.08b	5.553±0.07b	3.691±0.09b

*EtOH: Ethanol; EtAce: ethyl acetate; AceN: acetonitrile; Cap: capsaicin (mg/g DW); Dcap: dihydrocapsaicin (mg/g DW) ** All data shown in the table are expressed as means and standard deviations of three independent measurements. *** Lowercase letters indicate a significant difference between groups at $P<0.05$.

[37]. To maximize the benefits of the inoculum and host, it is important to study the ideal application form based on the specific cultivar, soil conditions, and environmental factors [38].

Several studies have reported that AM fungi can enhance photosynthetic pigments and parameters [39,40]. Our study also found that Ch-a, Ch-b, and Cx+c concentrations increased after being inoculated with each AM fungi strain (Table 2). The increase in photosynthetic pigment contents in plants grown under AM fungi treatment could be due to the stimulation of root growth and enhanced nutrient uptake. As a result, higher concentrations of photosynthetic pigments led to improved plant photosynthetic rates, resulting in increased biomass accumulation.

A study investigating the biochemical changes and antioxidant enzyme activities in pepper plants inoculated with *Ri* and *Fm* mycorrhizal fungi exposed to long-term salt stress determined that mycorrhizal plants had lower MDA content than control plants [41]. In the experiments conducted with pepper plants inoculated with *Fm* fungus against salt stress, it was also revealed that plants treated with mycorrhiza were exposed to less lipid peroxidation at varying NaCl concentrations and therefore had lower MDA content compared to plants in the control group [42]. On the other hand, it was reported that there is a direct correlation between the amount of increased pathogenic microorganism inoculum and the amount of MDA detected in the plant leaves in the study investigating the effect of the pathogenic fungus *Phytophthora capsici* on the amount of MDA in pepper plants [43].

Although AM fungi species, which establish a symbiotic relationship with plant roots, have a protective effect on the plant against environmental stress factors, they cause a certain amount of damage to the root cell membranes during the establishment of symbiosis. When the results were compared with the mycorrhizal infection rates in pepper plants, it was suggested that there might be a relationship between the MDA contents of the plant groups and the mycorrhizal infection rates detected in the roots.

The results of our research showed that the phenylalanine ammonium lyase (PAL, EC. 4.3.1.24) activity was significantly amplified by the use of AM fungi. PAL is responsible for the catalysis of L-phenylalanine to cinnamic acid as the first enzyme involved in phenylpropanoid biosynthesis in plants. The synthesis of many metabolites (flavonoids, tannins, coumarins, etc.) produced by the plant defense mechanism under various biotic and abiotic stress conditions (UV, drought, pathogen infection, etc.) is carried out by phenylpropanoid biosynthesis, in which the PAL enzyme takes part. The significant increase in PAL enzyme activity in AM fungi inoculated plants may be because of the fungi during the establishment of symbiosis. Many studies have reported increased PAL enzyme activity in plants under different stress conditions [44-46]. Combined inoculation of AM fungi and *P. capsici* caused an increase in TPC in plants [47]. The present study shows that the TPC increases in plants inoculated with AM fungi are in line with the existing literature when compared to the control. In one study, it was reported that phenolic compound concentrations increased especially with AM symbiosis [48].

AM fungi can interact with the host plant metabolism, causing the accumulation of health-promoting phytochemicals and antioxidant molecules [49]. The TPC content was higher in fruits from inoculated plants than those from non-inoculated plants. The research investigating the efficacy of *Fm* and *Ri* mycorrhizal fungi in controlling the *Nacobbus aberrans* (Thorne & Allen) nematode in pepper plant roots revealed a significant reduction in the total phenolic compound levels in plants treated with mycorrhiza [50]. There was no significant difference in TPC content between control and experimental group plants in two different pepper cultivars grown under organic farming practices in different seasons and soil conditions with AM fungi treatment [38]. According to the researchers, the levels of TPC in pepper fruits exhibited variability based on the variety of the plant and the growing season in both inoculated and non-inoculated plants.

The DPPH scavenging activity of pepper plants was also significantly affected by AM fungi inoculation. As with our results, several previous experiments have recorded the positive effects of AM fungi on antioxidant activity in different plants. Some researchers have reported that mycorrhizal inoculation can increase antioxidant activity and phenolic compounds [51,52]. Mycorrhizal association caused an approximately six-fold increase in radical scavenging capacity in leaf extracts when the effect of mycorrhizal fungi on the essential oil composition and antioxidant activity of black pepper (*Piper nigrum* L.) was investigated [53].

The pungency level in peppers is determined by the amount of capsaicinoids, which are alkaloids found in varying proportions [54]. Capsaicin and dihydrocapsaicin make up the majority of capsaicinoids in most spicy pepper varieties, accounting for over 80% of the total [55]. Our study thoroughly examined the effects of different mycorrhizal fungi on capsaicin production in pepper plants. We utilized two extraction methods, magnetic stirring and ultrasound-assisted extraction, in an ultrasonic bath and probed and tested three different solvents to determine their impact on capsaicin extraction. The capsaicin content of *C. annuum* was determined based on compliance with the standard of capsaicin using GC-MS. The results showed that the *Fm* treatment had the highest amount of capsaicin. No research has investigated the impact of AM fungi colonization on capsaicinoid levels in pepper in terms of enhanced quality attributes. Beneficial fungi stimulate plant growth by exchanging signals with the host plants and affecting their hormone balance and metabolism. These treatments promote the accumulation of various classes of secondary metabolites and phospholipids, which are related to secondary plant metabolism [4,7,8]. Both capsaicin and dihydrocapsaicin exhibit a similar pattern in capsaicinoid levels. In our study, capsaicin was detected in higher amounts than dihydrocapsaicin in all analyzed samples.

The process of extracting capsaicinoids from *Capsicum* plants is affected by various factors such as their chemical composition, extraction method, particle size, and presence of other substances [56]. The process in question includes the utilization of a solvent, a liquid that can dissolve a separate chemical substance. This results in plant extracts containing different organic compounds that can dissolve in the solvents. Solvent extraction is a technique for different chemicals based on their specific solubility properties. The solid-liquid extraction process involves the combination of the solvent and dried pepper phases and the subsequent separation of the stated phases. The results of the GC-MS measurement of peppers treated with AM fungi showed that the capsaicin content decreased in the following order: ethyl acetate, acetonitrile, and ethyl alcohol. It was found that the seeds of peppers yielded approximately 16 mg of capsaicin per gram DW when tested with ethanol, acetone, and acetonitrile solvents. The recovery of capsaicin from whole peppers was 8 mg/g DW, while pepper shells yielded 5 mg/g DW [57].

The highest amounts of capsaicin and dihydrocapsaicin were determined in the extraction using an ultrasonic probe and using ethyl acetate solvent in peppers with both AM fungi applications. Capsaicin and other capsaicinoids are valuable secondary metabolites, and many studies have reported using ethyl acetate to extract them [58,59]. It was also reported that ethyl acetate was the most effective solvent for extracting capsaicinoids from *C. annuum*, followed by dichloromethane and acetone [58]. These findings are consistent with those of our research.

Ultrasound-assisted extraction has numerous advantages, such as reducing solvents and extraction time and temperature, which is essential for extracting thermolabile and unstable compounds. Ultrasonic waves passing through an organic solvent cause cavitation, which improves solvent mixing and penetration into the sample matrix, making the ultrasound-assisted extraction technique effective [60]. When the effect of the method on the extraction of capsaicin and dihydrocapsaicin from *C. annuum* samples inoculated with different mycorrhizal fungi was examined, using an ultrasonic probe was determined as the most effective method. A rapid and reproducible ultrasound-assisted extraction method for capsaicinoids was developed using 25 mL of methanol as a solvent at 50°C for 10 min [13]. According to researchers, the effectiveness of ultrasound-assisted extraction relies on the solvent's capability to absorb and transfer ultrasound energy and the solubility of capsaicinoids in the solvent.

CONCLUSIONS

Using AM fungi in agriculture could be a sustainable alternative to current practices. This makes the symbiosis with *Capsicum* spp. extremely valuable. AM fungi play a crucial role in agricultural production and can significantly enhance the sustainability of agricultural systems when appropriately managed. Moreover, advancing techniques that aim to comprehend the mechanisms that regulate AM fungi formation may lead to the successful widespread implementation of AM fungi in agriculture. Our research confirms that the hypothesis proposed is valid. Inoculation with mycorrhizal fungi positively impacted plant growth, antioxidant activity, photosynthetic activity, and capsaicin production. Capsaicin is the primary flavor and spice compound found in hot peppers. To

produce high-quality capsaicin as an aromatic flavor and medicinal ingredient, an efficient extraction method and solvent system is necessary to prevent degradation. Our study revealed that using the ultrasonic probe method and ethyl acetate solvent is successful in extracting capsaicin. Ultrasonic extraction is a mechanical extraction technique that provides high yields of capsaicin in a very brief period. It is suggested that future studies should aim to increase the bioavailability of capsaicinoids.

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Data availability: All data underlying the reported findings have been provided with the submitted article and are available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Canpolat%20and%20Islek_8756_Dataset.pdf

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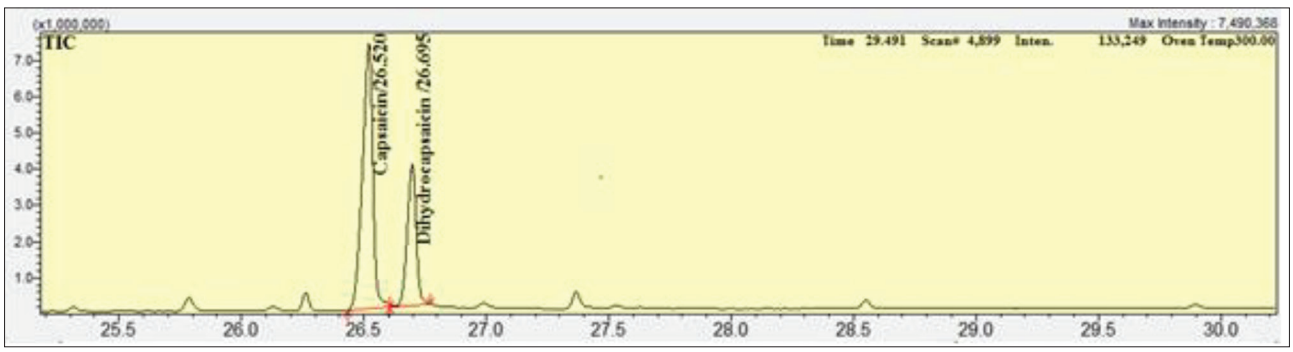
SUPPLEMENTARY MATERIAL



Supplementary Fig. S1. The total ion chromatogram showing capsaicin and dihydrocapsaicin with retention times.