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Arbuscular mycorrhizal fungi alter thymol derivative contents of *Inula ensifolia* L.

Szymon Zubek · Anna Stojakowska · Teresa Anielska · Katarzyna Turnau

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Abstract Individuals of *Inula ensifolia* L. (Asteraceae), a valuable xerothermic plant species with potential therapeutic value, were inoculated under laboratory conditions with different strains of arbuscular mycorrhizal fungi (AMF): (1) *Glomus intraradices* UNIJAG PL-Bot, (2) *G. intraradices* UNIJAG PL-Kap, (3) *Glomus clarum* UNIJAG PL13-2, and (4) AMF crude inoculum from natural stands of *I. ensifolia*. We found AMF species specificity in the stimulation of thymol derivative production in the roots of *I. ensifolia*. There was an increase in thymol derivative contents in roots after *G. clarum* inoculation and at the same time the decreased production of these metabolites in the *G. intraradices* treatments. Moreover, no correlation between the extent of AMF colonization and the effects of the fungal symbionts on the plant was observed. A multilevel analysis of chlorophyll *a* fluorescence transients (JIP test) permitted an evaluation of plant vitality, expressed in photosynthetic performance index, influenced by the applied AMF strains, which was found to be in good agreement with the results concerning thymol derivative production. The mechanisms by which AMF trigger changes in phytochemical concentration in plant tissues and their consequences for practice are discussed.

Keywords Arbuscular mycorrhiza · AMF species specificity · JIP test · Narrow-leaved inula · Photosynthetic performance index · Thymol derivatives

S. Zubek (✉)
Mycology Unit, Institute of Botany, Jagiellonian University,
Lubicz 46,
31-512 Kraków, Poland
e-mail: szymon.zubek@uj.edu.pl

A. Stojakowska
Department of Phytochemistry, Institute of Pharmacology,
Polish Academy of Sciences,
Smętna 12,
31-343 Kraków, Poland

T. Anielska · K. Turnau
Institute of Environmental Sciences, Jagiellonian University,
Gronostajowa 7,
30-387 Kraków, Poland

Introduction

Inula ensifolia L. (narrow-leaved inula) (Asteraceae) is a Central European species which is characteristic to the plant community *Inuletum ensifoliae* Kozł. 1925 of seminatural xerothermic limestone grasslands. For several centuries, the grasslands occurring in southern Poland were regularly grazed, mostly by sheep, and their development and maintenance were dependent on this type of land use. Nevertheless, the traditional methods of management were discontinued, and the grasslands have been overgrown by shrubs and trees. In addition, they were isolated by fields and meadows, and due to the fact that most of grassland plant species have poor dispersal ability, they cannot re-colonize isolated sites nor colonize open sites without grazing animals acting as dispersal vectors (Dzwonko and Loster 2008). Therefore, the grasslands are threatened ecosystems and need active protection (Dzwonko and Loster 2008; Perzanowska and Grzegorczyk 2009). Several xerothermic grasslands in southern Poland were set aside as nature reserves and are also included in the European Union network Natura 2000. The aim of the network is to assure the long-term survival of Europe's most valuable and threatened species and their habitats (Perzanowska and Grzegorczyk 2009). Moreover, *I. ensifolia* belongs to the genus which includes several species of reputed medicinal values, e.g., *Inula britannica* L., *Inula helenium* L., and

Inula racemosa Hooker fil. Depending on the species, their roots contain, e.g., sesquiterpene lactones, thymol derivatives, and polysaccharide—inulin, as major secondary metabolites (reviewed in Stojakowska et al. 2006). Although *I. ensifolia* has no documented medicinal use, the extracts from this plant species have been shown to possess antiproliferative activity against human cancer cell lines in vitro (Réthy et al. 2007).

In our previous investigations, we conducted the first detailed phytochemical analysis of *I. ensifolia*. We found several groups of biologically active secondary metabolites, i.e., terpenoid and phenolic compounds, including thymol derivatives (Stojakowska et al. 2010). Furthermore, our preliminary results from field investigations indicated that *I. ensifolia* is colonized by arbuscular mycorrhizal fungi (AMF; unpublished). In general, mycorrhizal plants may show larger biomass, faster growth rate, improved pathogen resistance, and more effective photosynthesis than non-mycorrhizal ones (reviewed in Smith and Read 2008). Mycorrhizal colonization induces many changes in plant physiology and was found to influence the level of secondary metabolites which may depend on root colonization by AMF (Morandi 1996; Abu-Zeyad et al. 1999; Fester et al. 1999; Strack et al. 2003; Copetta et al. 2006; Khaosaad et al. 2006; Kapoor et al. 2007). Therefore, the aim of our present study was to test four AMF inocula on *I. ensifolia* in order to show whether inoculation could influence plant vitality and secondary metabolite production, namely, thymol derivatives. The effect of the AMF inoculation on *I. ensifolia* was evaluated by physiological and phytochemical methods, namely, by shoot biomass, mycorrhizal colonization assessment, and the content of thymol derivatives as well as by biophysical methods, termed as JIP test. This test translates the polyphasic chlorophyll *a* fluorescence transient OJIP exhibited by plants upon illumination to biophysical parameters of the photosynthetic machinery, evaluating plants' vitality. The JIP test, which has been proven to be a very useful tool for the investigation of stress effects on plants (for reviews, see Strasser et al. 2000, 2004), has been also successfully used for the evaluation of the beneficial role of mycorrhization (Tsimilli-Michael et al. 2000; Pinior et al. 2005; Biró et al. 2006; Strasser et al. 2007; Tsimilli-Michael and Strasser 2008; Zubek et al. 2009; Jurkiewicz et al. 2010).

Materials and methods

AMF inocula preparation

Four kinds of inocula were applied in the experiment: (1) *Glomus intraradices* N.C. Schenck & G.S. Sm.UNIJAG PL-Bot, (2) *G. intraradices* UNIJAG PL-Kap, (3) *Glomus clarum* T.H. Nicolson & N.C. Schenck UNIJAG PL13-2, and (4) AMF crude inoculum from natural stands of *I. ensifolia*. In the case of crude inoculum, the soils were excavated from the immediate vicinity (within 20 cm) of several *I. ensifolia* individuals occurring in natural populations. The production of inocula and the quality control of produced material followed the procedure reported by Zubek et al. (2009). More than 90% of *Plantago lanceolata* L. root length was colonized by AMF in the case of all inocula; no other fungal endophytes were found in the material.

Plant material

The seeds of *I. ensifolia* were collected from natural habitats of the species—xerothermic grasslands in the Kalina-Lisiniec reserve which is included in the European Union Natura 2000 network (Wyżyna Miechowska upland, Małopolska Province, Poland; 50°33′94″N, 20°17′97″E). They were germinated on wet filter paper in Petri dishes.

Experiment design

Two-week-old seedlings were transferred into 200 ml pots (six plants for each treatment; three individuals in two pots) with the sterile substratum composed of soil collected from *I. ensifolia* natural habitats and expanded clay at the ratio 3:1 (*v*:*v*). The soil type was classified as rendzina according to FAO soil units. The chemical properties of the soil is reported in Table 1. The soil was sterilized in 90°C, three times for 1 h in 24-h intervals, and then it was sprayed with distilled water for 2 weeks before using in the experiment. The following treatments were prepared: (1) control—without inoculum, (2) *G. intraradices* UNIJAG PL-Bot, (3) *G. intraradices* UNIJAG PL-Kap, (4) *G. clarum* UNIJAG PL13-2, and (5) AMF crude inoculum from natural stands of *I. ensifolia*. The dried inocula (10 g/pot) were mixed with the substratum. The pots were kept in sealed Sunbags (Sigma-Aldrich) under greenhouse conditions at 20±2°C and the following light regime: 100–110 μmol PAR photons$\times m^{-2}\times s^{-1}$, 12/12 h. The cultures were watered one time per week.

After 8 months of *I. ensifolia* growth, the chlorophyll (Chl) *a* fluorescence measurements were conducted and then the plants were harvested. In the case of each treatment, the roots were divided into two portions. The smaller part, one fifth of the root fresh mass, was stained in order to visualize AMF mycelium for the mycorrhizal colonization assessment; the second part and also the shoots were dried (room temperature) and used for the evaluation of plant biomass and metabolite contents influenced by AMF. The material was weighed using electronic analytical balance (Radwag, WPA 60/c/1) with the precision of 0.0001 g.

Table 1 Chemical properties of soil used in the experiment for cultivation of *Inula ensifolia* (see "Materials and Methods" section)

pH (H_2O)	N%	C%	Organic matter%	C/N	Total content mg/100g of dry soil			Exchangeable cations mg/100g of dry soil			
					K_2O	P_2O_5	CaO	K	Na	Mg	Ca
7.0	0.2	2.8	4.8	13.2	53.4	6.6	1,411.2	46.0	10.6	11.9	1,090.0

Evaluation of plants' vitality

Measurement of Chl a fluorescence transient OJIP

Chl *a* fluorescence transients OJIP were measured with a HandyPEA-fluorimeter (Hansatech Instruments Ltd., King's Lynn Norfolk, PE 30 4NE, UK). The transients were induced by red light (peak at 650 nm) of 3,000 μmol photons $m^{-2}s^{-1}$ provided by an array of three light-emitting diodes and recorded for 1 s with 12 bit resolution. The data acquisition was every 10 μs (in the interval from 10 μs to 0.3 ms), every 0.1 ms (0.3–3 ms), every 1 ms (3–30 ms), every 10 ms (30–300 ms), and every 100 ms (300 ms to 1 s). The measurements were conducted on fully expanded leaves, still attached on the plants (ten to 15 replicates for each treatment), which were dark-adapted for 30 min prior to measuring.

The JIP test

For each treatment, the average OJIP fluorescence transient was analyzed according to the JIP test (Strasser et al. 2004), with the "Biolyzer" software (Laboratory of Bioenergetics, University of Geneva, Switzerland). The parameter chosen to be calculated in the present study was the performance index (PI_{total}), which evaluates the overall photosynthetic performance (for the analytical description, see Tsimilli-Michael and Strasser 2008).

AMF colonization assessment

The roots were prepared according to the modified Phillips and Hayman (1970) technique. After washing in running tap water, the roots were cleared in 10% KOH for 24 h and subsequently rinsed in water. The material was acidified in 5% lactic acid in water for 24 h, then stained with 0.05% aniline blue in 80% lactic acid for 24 h, and finally stored in 80% lactic acid. The roots were cut into 1-cm fragments, then mounted on slides in glycerol:lactic acid (5:1), and analyzed using Nikon Eclipse 800 microscope with Nomarski interference contrast optics. Mycorrhizal colonization assessment was carried out according to the Trouvelot method (Trouvelot et al. 1986). The parameters analyzed were mycorrhizal frequency (*F*), relative mycorrhizal root length (*M*), and relative arbuscular richness (*A*).

Analysis of secondary metabolite contents

The plant material for the assessment of secondary metabolite content was divided into roots and shoots. The dried material was stored in the dark at room temperature until required for analysis. The sample preparation and quantification of thymol derivatives were carried out according to the protocol reported by Stojakowska et al. (2006) using reversed-phase high-performance liquid chromatography. The content of following compounds was measured: (**1**) 10-isobutyryloxy-8,9-epoxythymol isobutyrate, (**2**) mixture of 10-(2-methylbutyryloxy)-8,9-epoxythymol isobutyrate and 10-isovaleroyloxy-8,9-epoxythymol isobutyrate, and (**3**) 7-isobutyryloxythymol methyl ether. The identification of the analyzed compounds was based on nuclear magnetic resonance and mass spectrometry data (Stojakowska et al. 2010).

Statistical analysis

The data from biomass and thymol derivative content assessments were evaluated by analysis of variance. Significance of differences between treatments was tested after Tukey. Analysis of the photosynthetic and mycorrhizal parameters was conducted with nonparametric Kruskal–Wallis test (for both, $p<0.05$). The analyses were carried out using Statistica ver. 7.1 (Statsoft).

Results

AMF colonization

Arbuscular mycorrhizae with arbuscules, which are the structural and functional criterion of the symbiosis, were observed in the case of all inoculated plants. Other root endophytes were not detected in the investigated material. No fungi were observed in the control material.

The applied AMF strains differed in the effectiveness of colonization of *I. ensifolia* root system (Table 2). Native AMF from the crude inoculum proved to be the most effective in root colonization (M=90%), whereas *G. clarum* was the least efficient AM fungus concerning mycorrhization (M=34%). However, statistically significant differences in mycorrhizal parameters (*M*, *A*) were found only in

Table 2 Mycorrhizal colonization (mean) of *Inula ensifolia* roots treated with four arbuscular mycorrhizal fungi inocula

Treatment	Mycorrhizal parameters [%]		
	F	M	A
Glomus intraradices UNIJAG PL-Bot	83 a	46 b	24 b
Glomus intraradices UNIJAG PL-Kap	90 a	70 ab	36 ab
Glomus clarum UNIJAG PL13-2	87 a	34 ab	10 ab
Crude inoculum	100 a	90 a	43 a

Different letters after values indicate statistically significant differences ($p<0.05$)

Mycorrhizal parameters: *F* mycorrhizal frequency, *M* relative mycorrhizal root length, *A* relative arbuscular richness

the case of application of the crude inoculum and *G. intraradices* UNIJAG PL-Bot (Table 2).

Plant growth

There were no statistical differences between the treatments. However, a general tendency that mycorrhizal plants were characterized by increased biomass of roots and shoots in comparison to nonmycorrhizal control was found (Table 3).

Photosynthetic performance index

The AMF from the applied inocula were found to differ in the influence on photosynthetic activity of *I. ensifolia*. As shown in Fig. 1, the most efficient AMF strain in stimulating plant photosynthetic performance (expressed in PI_{total}) was *G. clarum*, a species that at the same time was the least efficient concerning mycorrhizal colonization. However, the statistically significant differences were found only between the plants treated with *G. clarum* and *G. intraradices* UNIJAG PL-Kap.

Content of thymol derivatives in plants

In the case of roots, the applied AMF had an impact on the content of all thymol derivatives (**1**–**3**, see "Materials and methods" section), which was especially pronounced in the differences found between the plants colonized with *G. clarum* and both *G. intraradices* treatments. The highest concentration of all analyzed compounds was found after *G. clarum* inoculation. Nevertheless, the amount of **1**–**3** in roots inoculated with *G. clarum* did not differ statistically from the nonmycorrhizal control (Fig. 2). The lowest concentration of **1**–**3** were detected in the case of both *G. intraradices* treatments, still with no statistical differences from the control (Fig. 2).

The mycorrhizal plants were characterized by lower concentration of **1** and **3** in shoots in comparison to control; however, statistically significant differences were detected only in the case of compound **3**. Moreover, no statistically important differences in the content of all analyzed thymol derivatives were found between the mycorrhizal treatments (Fig. 3).

Discussion

AMF have been found to enhance biomass, improve pathogen, heavy metal, salinity resistance, and stimulate photosynthesis as well as influence the level of secondary metabolites in plants (reviewed in Smith and Read 2008). Therefore, the biotechnological use of AMF was proposed for agricultural (Hamel 1996), endangered (Gemma et al. 2002; Zubek et al. 2008, 2009), medicinal plant species (Kapoor et al. 2002a, b, 2007; Copetta et al. 2006; Khaosaad et al. 2006; Toussaint 2007; Toussaint et al. 2007; Zubek and Błaszkowski 2009), as well as plants applied in restoration processes of destroyed habitats (Turnau and Haselwandter 2002). The knowledge of AMF interactions with plants is important, as not only the selection of appropriate plant species/cultivar/ecotype but also well-selected microbial consortia could be essential for

Table 3 *Inula ensifolia* dry biomass (grams; mean±SD) from different treatments

No statistically significant differences were found between treatments ($p>0.05$)

Treatment	Roots	Shoots
Control	0.1396±0.0970	0.1176±0.0626
Glomus intraradices UNIJAG PL-Bot	0.1495±0.0792	0.1734±0.1039
Glomus intraradices UNIJAG PL-Kap	0.1964±0.0810	0.1858±0.1211
Glomus clarum UNIJAG PL13-2	0.1704±0.1115	0.1615±0.1113
Crude inoculum	0.1773±0.0639	0.1506±0.0558

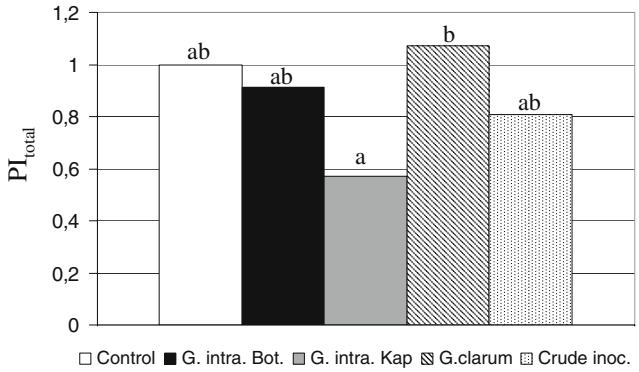

Fig. 1 The performance index PI_{total} (mean) of *Inula ensifolia* for the treatments presented in Table 2. *Different letters above the bars* indicate statistically significant differences ($p<0.05$)

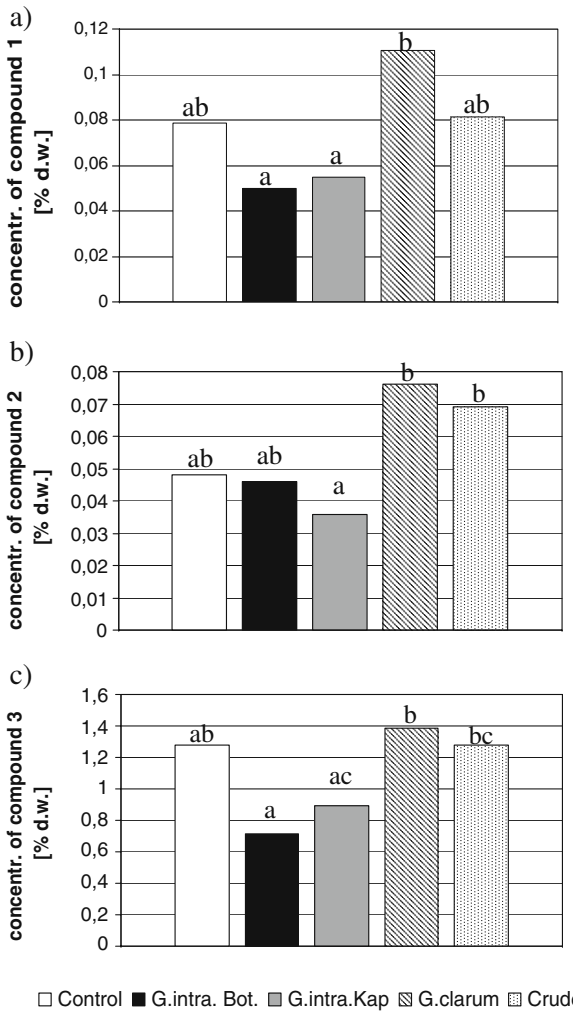

Fig. 2 Concentration of thymol derivatives (mean; percentage of dry weight) in *Inula ensifolia* roots from the treatments presented in Table 2. For the names of compounds **1** (**a**), **2** (**b**), and **3** (**c**), see the "Materials and methods" section. *Different letters above the bars* indicate statistically significant differences ($p<0.05$)

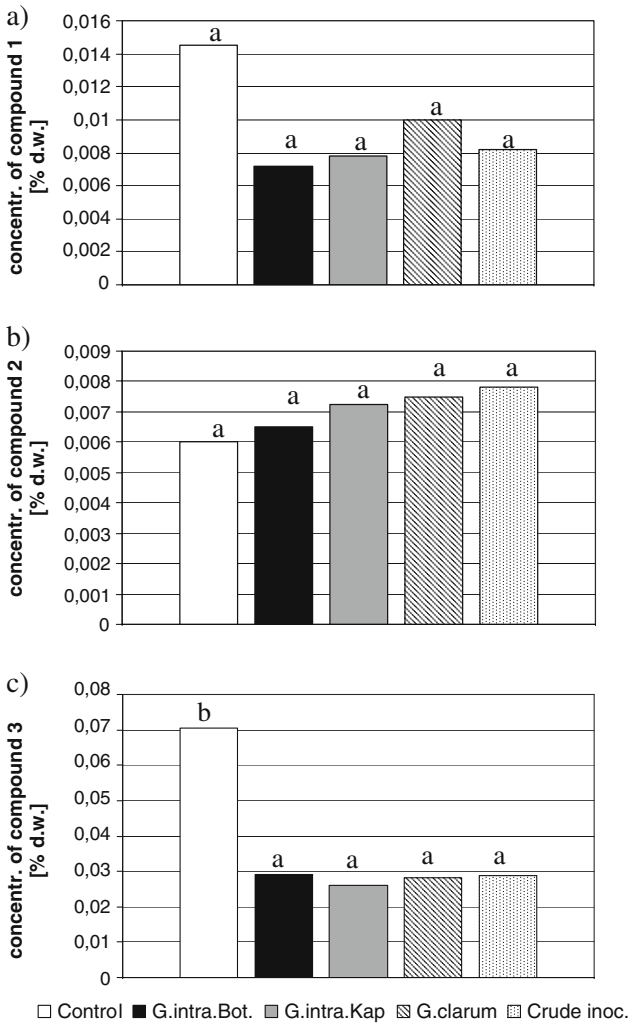

Fig. 3 Concentration of thymol derivatives (mean; percentage of dry weight) in *Inula ensifolia* shoots from the treatments presented in Table 2. For the names of compounds **1** (**a**), **2** (**b**), and **3** (**c**), see the "Materials and methods" section. *Different letters above the bars* indicate statistically significant differences ($p<0.05$)

the success of restoration, plant maintenance, or cropping (Turnau and Haselwandter 2002; Orłowska et al. 2005; Copetta et al. 2006; Toussaint 2007; Zubek et al. 2009). In the present studies, we demonstrated that AMF influence vitality and thymol derivative contents of *I. ensifolia*. Our findings broaden the knowledge of biology and ecology of this valuable plant species. As there is a need to develop herb varieties and produce medicinal plants with better growth and increased levels of active compounds, the exploitation of the AM symbiosis could greatly benefit herbal industry; providing that attention is paid towards the understanding of the effects of AMF on biochemical pathways in the host plants (Copetta et al. 2006; Toussaint 2007; Zubek and Błaszkowski 2009).

AMF have been shown to influence concentration of several groups of plant metabolites in roots and/or aerial

parts of plants, e.g., alkaloids (Abu-Zeyad et al. 1999; Rojas-Andrade et al. 2003), terpenoids (Akiyama and Hayashi 2002; Kapoor et al. 2002a, b, 2007; Copetta et al. 2006; Jurkiewicz et al. 2010), carotenoids (Fester et al. 2002), flavonoids (Larose et al. 2002), glucosinolates (Vierheilig et al. 2000), and phenolic acids (Toussaint et al. 2007; Jurkiewicz et al. 2010). The mechanism by which AMF trigger changes in phytochemical concentration in plant tissues can be multidirectional and is not quite clear yet (Toussaint 2007). Firstly, the modification of compounds produced in roots may be the consequence of signaling mechanisms between symbionts and plant response to AMF colonization (Larose et al. 2002; Toussaint 2007). As it was found in the studies conducted by Larose et al. (2002), several flavonoids of both stimulating and depressing effect on the AMF development were produced in different quantities at different stages—before and during AMF colonization of *Medicago sativa* L. cv. Sitel roots. In addition, also an alkaloid, trigonelline is suggested to be a regulatory factor during early signal events in the establishment of AM. The concentration of this compound in the roots of *Prosopis laevigata* (Willd.) M.C. Johnst increased when an AM fungus was present (Rojas-Andrade et al. 2003). In general, the production of phenolic compounds and terpenoids, the components of essential oils, is considered as a defense response to fungal colonization/infection. Considering the fungicide properties of several constituents of essential oils, and the increased production of these metabolites in mycorrhizal plants, it was suggested that they could be synthesized as a defense response to AMF presence in roots (Copetta et al. 2006). Secondly, the enhanced production of secondary metabolites may involve several metabolic processes that could be mediated by improved P or N nutrition due to AMF symbiosis. Kapoor et al. (2002a, b) and Toussaint et al. (2007) suggested that the increased production of terpenoids and phenolic acids could be due to enhanced mineral nutrition, especially P and N, respectively. Finally, a possible mechanism may reside in the potential of AMF to induce changes in phytohormone levels in plants (Copetta et al. 2006; Kapoor et al. 2007; Toussaint 2007; Toussaint et al. 2007). Copetta et al. (2006) found that *Gigaspora rosea* increased biomass and the total amount of essential oil in the case of *Ocimum basilicum* L. var. *Genovese*. The increased essential oil yield was associated to a significantly larger number of peltate glandular trichomes (main sites of essential oil synthesis and accumulation) in the leaves of inoculated plants. They suggested that the greater number of trichomes could be related to alterations in the phytohormonal profile induced by AMF (Copetta et al. 2006). Moreover, in the studies by Kapoor et al. (2007), AMF were found to increase the number of glandular trichomes of *Artemisia annua* L. and, as a consequence, enhance the concentration of artemisinin in leaves. The authors also pointed out that this could be related to the changes in the hormonal balance of *A. annua* influenced by AMF.

In the present studies, we report, to the best of our knowledge for the first time, the influence of AMF on the production of thymol derivatives. Thymol and its derivatives are present in plant species belonging to, e.g., Lamiaceae and Asteraceae families. Thymol is produced by plants as a chemical defense mechanism against phytopathogenic microorganisms (Shimoda et al. 2006). Some data indicate also a similar antimicrobial activity of thymol derivatives (Stojakowska et al. 2005). Therefore, we could have expected increased amount of these compounds in the tissues of *I. ensifolia*, as a potential plant reaction to fungal colonization. In the present studies, however, the tested AMF species influenced the production of thymol derivatives differently; the highest concentration of all analyzed compounds in roots were found after *G. clarum* inoculation, and the lowest concentration of **1**–**3** were detected in the case of *G. intraradices* treatments (in both cases, there were no statistical differences from the control). In the case of shoots, the mycorrhizal plants were characterized by lower concentration of **1** and **3** in comparison to control. Nevertheless, our findings are in agreement with the observations of Copetta et al. (2006) concerning the production of metabolites in *O. basilicum* var. *Genovese* leaves and confirm earlier observations that functional diversity exists between fungal isolates, even belonging to the same genus. Copetta et al. (2006) showed that *G. rosea* increased concentration of camphor and alpha-terpineol, while plants treated with *Gigaspora margarita* significantly decreased eucalyptol, linalool, and eugenol contents relative to control. In addition, *Glomus mosseae* did not alter the proportion of the aforementioned compounds (Copetta et al. 2006). Kapoor et al. (2002b) showed increased concentration of geraniol in the fruits of *Coriandrum sativum* L. individuals inoculated with *Glomus macrocarpum*, while the content of linalool was higher in the material treated with *Glomus fasciculatum*. Also, the levels of limonene and carvone were enhanced in the essential oil obtained from the fruits of *Anethum graveolens* L. inoculated with *G. macrocarpum*, whereas *G. fasciculatum* inoculation resulted in a higher content of thymol in *Trachyspermum ammi* (Linn.) Sprague fruits (Kapoor et al. 2002a). Moreover, Khaosaad et al. (2006) observed that *G. mosseae* had no effect on the composition of essential oil in the three tested *Origanum vulgare* L. genotypes; however, AMF inoculation increased the total essential oil contents in two genotypes. Furthermore, AMF species specificity was also found in the case of flavonoid production in *M. sativa* (Larose et al. 2002) and phenolic acids in *O. basilicum* (Toussaint et al. 2007).

In our experimental approach, the increased amount of thymol derivatives in roots after *G. clarum* inoculation and, at the same time, good plant vitality indicated by high values of PI, and also the decreased production of these metabolites and the lowest PI in the *G. intraradices* treatments may suggest positive effect of *G. clarum*, e.g., by improved mineral nutrition on the production of these metabolites rather than a plant stress reaction to AMF colonization. Moreover, the second possible explanation could also be that the applied inoculants influenced differently the phytohormone content, thus changes in the concentration of thymol derivatives in roots and shoots of *I. ensifolia* occurred. However, in order to support these mechanisms, further studies are needed concerning the parallel analysis of AMF influence on several groups of secondary metabolites in *I. ensifolia*, including phytohormones, as well as P and N contents, which were not analyzed in our studies due to limited amount of plant material for the phytochemical analyses.

G. clarum was the least efficient AM fungus concerning mycorrhization but, at the same time, the highest content of thymol derivatives in roots and the highest value of photosynthetic PI were found in the case of plants inoculated with this fungus. Moreover, the content of analyzed metabolites in *G. clarum* treatment was comparable to the amounts detected after application of AMF from crude inoculum, which were found to be the most effective as root colonizers. As it was shown in earlier studies, the extent of AMF colonization is not necessarily correlated with the effects of the symbionts on plants (Kapoor et al. 2002a; Toussaint et al. 2007; Smith and Read 2008). Experimental and field data by Feldmann et al. (2009) showed that AMF symbiosis was the most effective, while root colonization ranged between 20% and 30%. It was also reported that the higher degree of colonization either did not make the symbiosis more efficient (Feldmann et al. 2008) or could be disadvantageous (Graham et al. 1991). However, a positive correlation was found between AMF colonization and the castanospermine (an alkaloid of the indolizidine type) content of seeds of medicinal plant—*Castanospermum australe* A. Cunn & C. Fraser. The results from field observations were confirmed by growing *C. australe* seedlings under greenhouse conditions and inoculating them with two AMF strains. The fungi increased the growth and the yield of castanospermine in leaves (Abu-Zeyad et al. 1999).

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