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HOW ARBUSCULAR MYCORRHIZAL FUNGI INFLUENCE THE DEFENSE SYSTEM OF SUNFLOWER DURING DIFFERENT ABIOTIC STRESSES

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The association between terrestrial plants and arbuscular mycorrhizal (AM) fungi is one of the most common and widespread mutualistic plant-fungi interaction. AM fungi are of beneficial effects on the water and nutrient uptake of plants and increase plant defense mechanisms to alleviate different stresses. The aim of this study was to determine the level of polyphenol oxidase (PPO), guaiacol peroxidase (POX) and glutathione S-transferase (GST) enzyme activities and to track the expression of glutathione S-transferase (GST) gene in plant-arbuscular mycorrhizal system under temperature- and mechanical stress conditions. Our results suggest that induced tolerance of mycorrhizal sunflower to high temperature may be attributed to the induction of GST, POX and PPO enzyme activities as well as to the elevated expression of GST. However, the degree of tolerance of the plant is significantly influenced by the age which is probably justified by the energy considerations.

Keywords: Enzyme activity – GST gene-expression – arbuscular mycorrhizal fungi – stress – plant-defense system

INTRODUCTION

Plants are often faced to the combined effect of various biotic and abiotic stresses. Abiotic stresses such as drought, chilling are often the major limiting factors for crop productivity. Under stress conditions reactive oxygen species (ROS) are generated in plants which are highly reactive and toxic. Plants can detoxify these oxidative molecules through elevating antioxidant activity of ROS-scavenging enzymes such as catalase, superoxide dismutase and peroxidase and glutathione S-transferase [13, 15, 20, 26–28, 30], moreover enhancing non-enzymatic antioxidants [e.g., ascorbate, glutathione and tocopherols) in plants [11, 29].

Successful establishment, survival and productivity of a crop mainly depend on its adaptation to environmental conditions. There are some options how plants tolerate the extreme unfavourable conditions. One of the possibilities is the inoculation of crop by arbuscular mycorrhizal fungi. The association between terrestrial plants and

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arbuscular mycorrhizal (AM) fungi is one of the most widespread forms of mutual plant-fungal interaction in natural and agricultural systems. This symbiotic relationship has evolved more than 400 million years ago giving possibility for plants to previal on Earth [31, 46, 47, 50]. The beneficial effects of AM are well known, including enhanced uptake of water and – mostly phosphorus – nutrient, in addition to reduction of weeds and protection against pathogens [1, 4, 45, 48]. Several studies suggest that AM symbiosis can help plants to alleviate various biotic and abiotic stresses. For instance, mycorrhiza-induced resistance (MIR) provides systemic protection against a wide range of plant pathogens [10, 19, 35, 36]. Instead of constitutive activation of plant defense system, MIR is associated with priming for an efficient activation of JA-dependent system [9, 35]. Moreover, some works show clearly that the amelioration of stress resistance by AM symbiosis is often related to the enhancement of antioxidant levels or activities in mycorrhizal plants [2]. The regulations of these mechanisms are not well understood and only a few investigations have been carried out at early stages of plants concerning low level of mycorrhizal colonization. Therefore, the main objectives of current research were to study how the arbuscular mycorrhizal inoculation influence the activities of some plant defense enzymes together with the expression of GST gene under non-stress, mechanical and temperature stress conditions at early stage of plants.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of sunflower (*Helianthus annuus* L.) were surface sterilized by immersion into 70% ethanol for 5 min, rinsed four times with distilled water and placed on wet filter paper in Petri dishes at 28 °C for germination. After 3 days, the germinated seeds were transplanted into plastic pots containing 0.88 kg sterilized soil substrate (organic manure, soil and decomposed straw = 1:2:1). Half of the pots were inoculated with 15 g of a commercial mycorrhizal product, Symbivit® (mixture of *Rhizophagus irregularis*, *Funneliformis mosseae*, *Claroideoglomus etunicatum*, *Claroideoglomus claroideum*, *G. microaggregatum*, *Funneliformis geosporum*) produced by Symbiom Ltd. (Lanskroun, Czech Republic; www.symbiom.cz) and the rest was used for not AM-inoculated treatments. The seedlings of both treatments were grown in growth chamber under controlled conditions with 16 hours day length and at temperature of 24 °C ± 0.5 °C. The moisture of substrate was 60%.

Healthy 9, 15 and 42 days-old seedlings (mycorrhizal and non-mycorrhizal plants) were selected and exposed to different stress conditions, including three stress treatments. Plants were exposed to 38 °C for 24 h (High Temperature stress, HT) or were incubated for 24 h at 4 °C (Chilling stress, LT). Moreover, hypocotyls were injured in sunflower seedlings by nicking them with a striped-tip forceps, after 24 h the leaves were collected and analyzed (Mechanical Wounding stress, MW). All treatments (HT, LT, MW and non-stressed control) were replicated five times.

RNA isolation and cDNA synthesis

To detect the expression profiles of glutathione S-transferase gene, total RNA was extracted from 15- and 42-day-old sunflower leaves using Vantage Total RNA Purification Kit (Origene, USA) according to the manufacturer's instructions, followed by DNase I (Fermentas) digestion to remove residual genomic DNA contamination. For qRT-PCR analysis, first-strand cDNA was synthesized from varieties of all treatments using First-strand cDNA Synthesis for Quantitative RT-PCR kit (Origene, USA). QRT-PCR was performed on Stratagene Mx3000P QPCR System (Agilent Technologies).

Each reaction was performed in a final volume of 25 µl containing 12.5 µl SYBR Green Master Mix reagent (Applied Biosystems), 1 µl of diluted cDNA sample (equal concentration in final volume, 4 ng/µl), 70 nM gene-specific primers and 8 µl nuclease-free water. The gene-specific primers GST-f (5'-GAGAAGGCTCAGGCTCGATT-3') and GST-r (5'-GCAACAGCTTGCTTCTCTCC-3') were designed according to Radwan et al. [37]. The thermal cycle was used as follows: 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 sec, 57 °C for 30 sec and 72 °C for 16 sec, finally 1 cycle at 95 °C 60 sec, 57 °C 30 sec and 95 °C 30 sec. The real-time PCR experiment was carried out at least three times under identical conditions. Amplification of actin gene was used as an internal reference. The gene expression of glutathione S-transferase was calculated using the $2^{-\Delta\Delta CT}$ method.

Enzyme assays

Half gram of fresh leaf and hypocotyl segment from 9-, 15- and 42-day-old sunflower leaves was homogenized in Tris-HCl (3 ml; 50 mM; pH 7.8); polyvinylpyrrolidone-K 25 (7.5% (w/v) and EDTA-Na_2 (1mM) buffer. The homogenate was centrifuged at 10,000 *g* for 20 min at 4 °C and the supernatant was used for further measurements. The level of guaiacol peroxidase activity was tested according to Rathmell and Sequera [39]. Sodium phosphate buffer (2.2 ml; 0.1 M; pH 6.0), guaiacol (100 µl; 50 mM) and H_2O_2 (100 µl; 12 mM) were added to the plant extract (10 µl). The absorbance was recorded at 436 nm using Nanophotometer 2210 (Implen, Germany). The activity of polyphenol oxidase in 9, 15 and 42 days-old sunflower was determined by adding 200 µl of plant extract to sodium phosphate buffer (2,2 ml; 0.1 M; pH 6.0), EDTA Na_2 (1 mM) and catechol (20 mM) as described by Fehrmann and Dimond [16]. The polyphenol oxidase activity was measured by the increase in the absorbance at 400 nm using Nanophotometer 2210 (Implen, Germany). Changes of absorbance per protein concentration per unit time were estimated. Glutathione S-transferase enzyme activity in 15- and 42-day-old sunflower plants was assayed according to the method of Habig et al. (1974) [18]. The absorbance was followed at 340 nm (Nanophotometer 2210, Implen, Germany) using 0.1 ml GSH (30 mM), 0.1 ml CDNB (30 mM) and 2.7 ml (100 mM, pH 6.5) phosphate buffer were added to 0.1 ml plant extract. Bradford reagent was used to determine the concentration of total

soluble protein in plant extract solutions using bovine serum albumin as a standard [5]. Statistics were performed with R Statistical Sofwer 3.3.1 (R Development Core Team 2011). Differences between treatments were determined by one-way analysis of variance (ANOVA).

Assessment of mycorrhizal colonization of AMF

Five plants both from mycorrhizal and non-mycorrhizal treatments were used for estimating root colonization. Approximately, 500 mg of fine roots from each plant were transferred to separate tubes and were subjected to the staining technique of Vierheilig et al. [51]. Internal fungal structures (hypae, arbuscules, vesicules) were examined under a stereomicroscope at ×100 magnification and the percentage of root length colonized calculated using the gridline intersect method [17].

RESULTS

During the 42 days of growth, no growth responses could be observed between mycorrhizal and non-mycorrhizal treatments (data not shown). No colonization was recognized in not-inoculated control plants and the highest level of mycorrhizal colo-

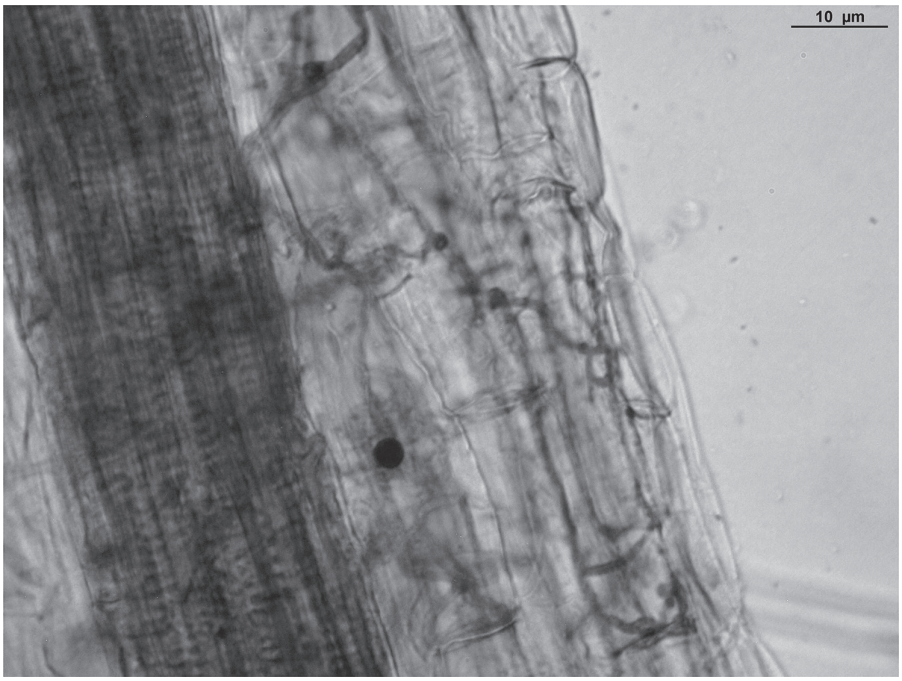

Fig. 1. Internal hyphae and arbusculum in 42-day-old plant

nization (58%) was reached by 6 weeks. We did not find any differences in mycorrhizal colonization among the plants used for the same stress situations (data not shown).

Nine days after mycorrhizal inoculation, at early stage of symbioses, appressorium and external hyphae together with germinated spores of AMF were only detected, however, internal hyphae (Fig. 1) and arbusculum were recorded after 42 days of growth. In general, mycorrhizal inoculation caused changes in all measured enzymes activities but significant differences detected only in POX activity. During control condition (without stress) the mycorrhizal plants showed a 11.5 times higher guaiacol-peroxidase activity than non-mycorrhizal one (AM-) (Fig. 2b). Mechanical wounding occurred enhancing activities of both POX and PPO in not inoculated plants, however mycorrhizal plants showed significantly lower enzymes activities compared to non-mycorrhizal ones (Fig. 2a, 2b).

Fifteen days after inoculation no significant differences could be detected regarding all measured enzyme activities (PPO, POX, GST) between mycorrhizal and non-mycorrhizal plants. Various stresses caused different effects. Chilling increased the PPO in both AM+ and AM– treatments (Fig. 2c). The short high temperature stress had significant influence only on GST activity, increasing the glutathione S-transferase level both in mycorrhizal and non mycorrhizal plants compared with nonstressed condition (Fig. 2g). Mechanical wounding increased the activity of POX enzyme, showing significant level only in non-mycorrhizal plants compared with control condition (Fig. 2d).

Mycorrhizal inoculation did not cause any differences in measured enzyme activities during control, non-stressed condition after 42 days of growth. However, high temperature increased significantly all tested enzyme activities (PPO, POX, GST) in both mycorrhizal and non-mycorrhizal plants. Moreover, differences in enzyme levels between inoculated and non-inoculated plants were also detected. Mycorrhizal plants showed 3.58, 1.65 and 1.6 times higher activity of PPO, POX and GST, respectively, after short high temperature stress compared with non-inoculated ones (Fig. 2e, 2f, 2h). Mechanical wounding caused a similar effect, however it was detected only in POX activity (Fig. 2f). We did not find any significant difference between mycorrhizal and non-mycorrhizal plants in PPO, but mechanical stress caused increased enzyme activity in plants compared with control ones. Chilling stress enhanced PPO activities in both AM+ and AM– treatments compared with non-stressed condition, however, no significant difference was found between inoculation treatments (Fig. 2e).

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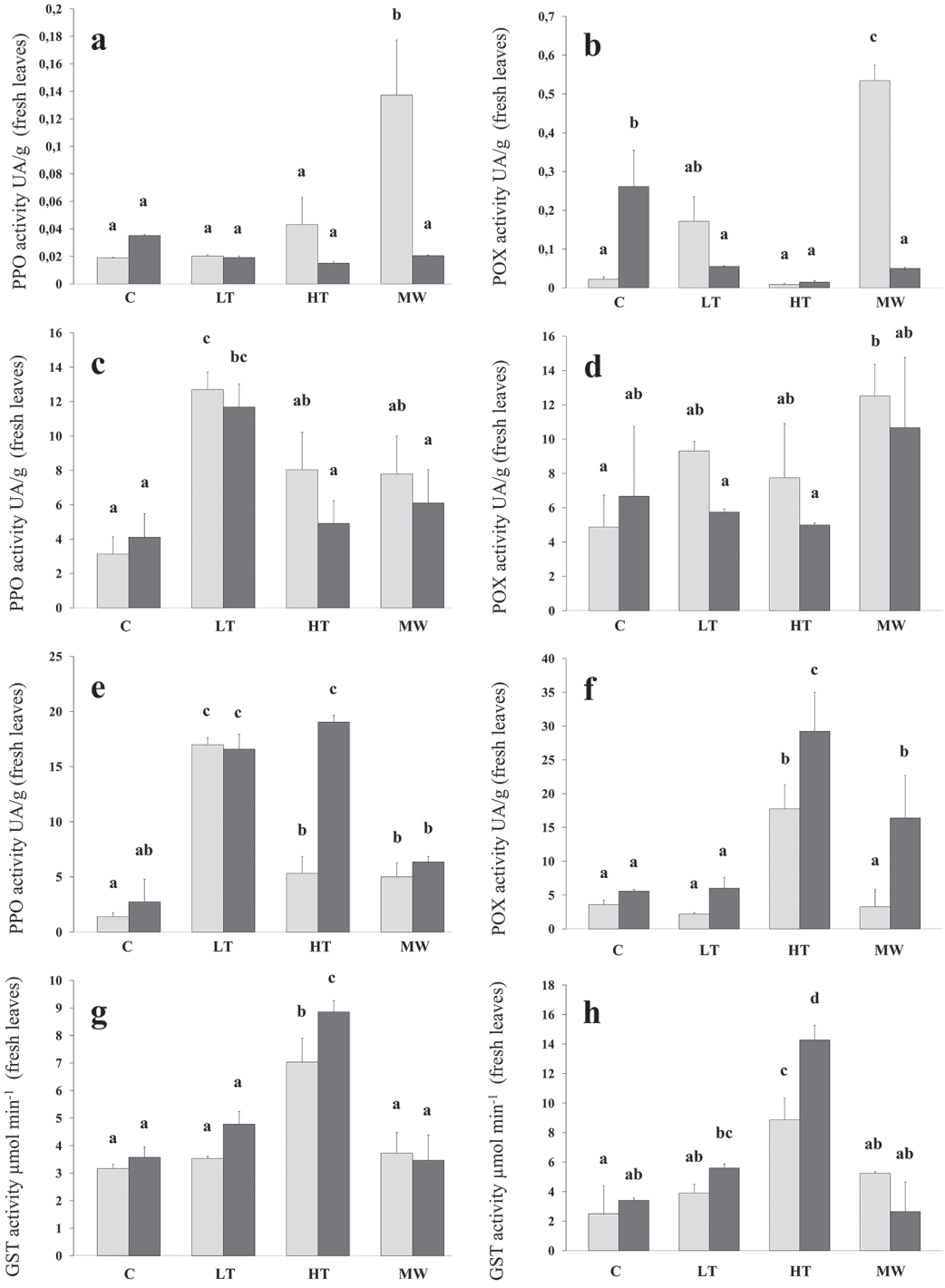
Fig. 2. Polyphenol oxidase, guaiacol peroxidase and glutathione S-transferase enzyme activity. (a) PPO activity in 9-day-old plant; (b) POX activity in 9-day-old plant; (c) PPO activity in 15-day-old plant; (d) POX activity in 15-day-old plant; (e) PPO activity in 42-day-old plant; (f) POX activity in 42-day-old plant; (g) GST activity in 15-day-old plant; (h) GST activity in 42-day-old plant; C – Control; LT – Low Temperature stress; HT – High Temperature stress; MW – Mechanical Wounding stress; Light grey – represents the non-inoculated plants; Dark grey – represents the inoculated plants with mycorrhizal fungi. Different letters represent significantly different ($P < 0.05$) values


a
PPO activity UA/g (fresh leaves)
C
LT
HT
MW
b
POX activity UA/g (fresh leaves)
c
PPO activity UA/g (fresh leaves)
d
POX activity UA/g (fresh leaves)
e
PPO activity UA/g (fresh leaves)
f
POX activity UA/g (fresh leaves)
g
GST activity µmol min-1 (fresh leaves)
h
GST activity µmol min-1 (fresh leaves)

Mycorrhizal inoculation increased GST gene expression both after 15 and 42 days of growth. From tested stresses the high temperature caused the most significant differences in glutathione S-transferase gene expression between mycorrhizal and non-mycorrhizal treatments at both sampling times. However, the two-fold higher gene expression in AM+ plants measured after 15 days of inoculation decreased to a 1.4-fold level at 42 days (Fig. 3a, 3b). In general, stress condition enhanced GST gene expression compared to the control one; only mechanical wounding had negative effect on expression level of mycorrhizal plant.

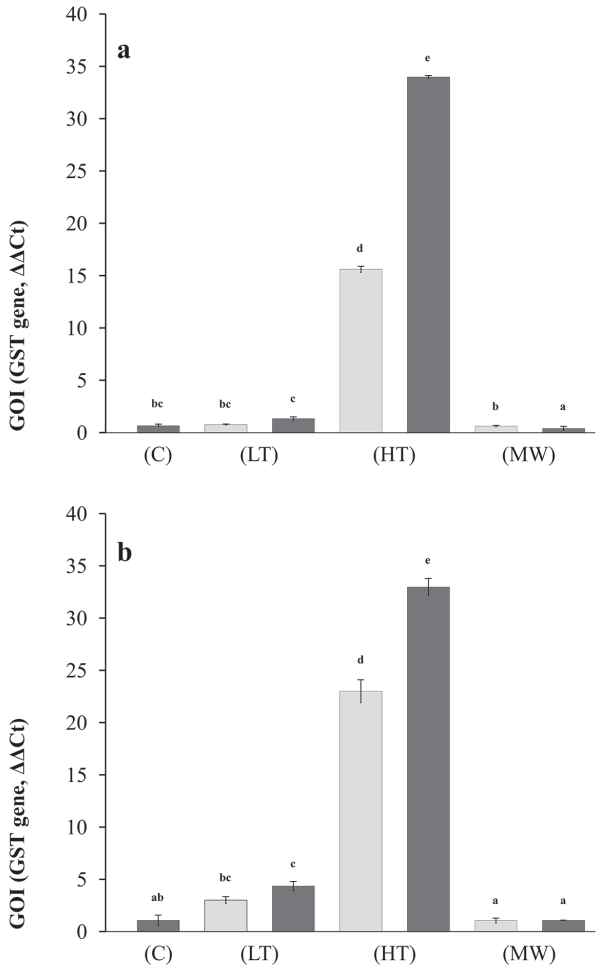


Fig. 3. Glutathione S-transferase gene expression. (a) GST gene expression in 15-day-old plant; (b) GST gene expression in 42-day-old plant; C – Control, inoculated with mycorrhizal fungi; LT – Low Temperature stress; HT – High Temperature stress; MW – Mechanical Wounding stress; Light grey – represents the non-inoculated plants; Dark grey – represents the inoculated plants with mycorrhizal fungi. Different letters represent significantly different ($P < 0.05$) values

DISCUSSION

Pre-symbiotic stage of sunflower-mycorrhizal fungi interaction seems similar to pathogen attack (against plant) restarting defense system of the plant. One of the earliest responses of plants to different stresses, involving AMF colonization, is the rapid accumulation of reactive oxygen species (ROS). ROS are not only harmful to the plant cell but also to mycorrhizal fungi which could be one reason for higher POX activities found in mycorrhizal plants than in non mycorrhizal plants under non-stressed conditions. It is in agreement with the studies of Spanu and Bonfante-Fasolo [3] finding transient and weak increments of POX activity at early colonization stages of the AM symbiosis by *Allium* roots. Increased peroxidase activity in plants was evoked not only by mycorrhiza, but also by different abiotic stresses [33, 38, 53]. This enzyme plays a role in producing of the monomers, dimers and phenoxy-free radicals as well and regulating the detrimental accumulation of hydrogen peroxide (H_2O_2). Interestingly, neither high nor low temperatures caused any changes in this enzyme activity but only mechanical wounding as abiotic stress has significant effect on plant peroxidase after 9 days of growth. While decreased activity of POX in mycorrhizal plants was found compared to control ones, an opposite tendency was recognised in non-mycorrhizal plants under mechanical wounding and low temperature stress. At early stage of plant, mycorrhiza often seems to parasites, because the energy from plant photosynthesis does not cover the growth of mycorrhizal fungi together with plant requirement. It could after be one reason for lower enzyme activity of mycorrhizal plant than non-mycorrhizal one. Having bigger leaf surface, the plant has more energy for covering both the production of enzymes and the growth of fungi as detected during high temperature stress caused 42 days of growth compared to non-inoculated ones. Several authors [21, 32, 36] have reported various results (induction or suppression) of these enzymes during AM colonization indicating that the enzymes are somehow linked with the AM symbiotic process. Functional differences in interaction between AMF genera, species, even among strains within the same species have also been recognized [41–43]. Our result is in agreement with the work of Rodríguez et al. [40] who described in tomato roots that alterations of the antioxidant enzyme activities are not general characteristics of the colonization process by arbuscular mycorrhizal fungi, probably having the key role on those responses of the specific feature of each strain rather than colonization.

The change from pathogen to symbiotic processes are controlled by plant hormones such as strigolactone (SL) and salicylic acid (SA) in the roots [7, 19, 34, 35]. In a well-established mycorrhiza both SL and SA production are reduced while biosynthesis of jasmonates (JA) increased [23, 25, 49].

Glutathione S-transferases (GST) belong to the best known and most studied detoxification enzymes group [12]. They play a role in the inactivation of the products of the oxidative stress metabolites, which method defends the cell from the damages [44]. The glutathione S-transferases in plants not only neutralize the toxic substances, but also promote a number of defensive mechanisms in the plant's defense system against oxidative membrane damage and necrotic disease symptoms. Some of them

can facilitate the intracellular storage and transport of hydrophobic non-substrate compounds, such as metabolites.

Similar to our results, the expression of glutathione S-transferase gene showed different gene expression under various stress situations [52] and the age of the plants had influence on it. In the present study, GST gene expression and GST enzyme activity showed the same tendency under high temperature stresses, supporting the results of Cetinkaya et al. [8]. In addition there are many post-transcriptional as well as post translational events that are involved in the processing of defense system which may explain the discrepancy of results between enzymes and transcripts under low temperature and wounding stresses.

Low temperature caused significant increase only in PPO activity compared to control plants, mycorrhizal inoculation had not any effect on it. Our result proves that often found AMF-induced enhanced chilling tolerance of plants may be attributed not only to enhanced antioxidant enzyme activity, but also to increased osmolyte accumulation, reduced lipid peroxidation and permeability in the plasma membrane, photosynthesis and secondary metabolism [6, 9, 22, 55]. Moreover higher leaf water potential [14] increased calcium precipitates in the apoplast and vacuole of root cells are described [24] compared with the non AMF control.

CONCLUSION

Our results suggest that induced resistance of mycorrhizal sunflower to high temperature may be due to the induction of GST, POX and PPO enzyme activities as well as to the elevated expression of GST. However, the degree of tolerance of the plant is significantly influenced by the age which is probably justified by the energy considerations.

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