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Antioxidant activity and gene expression associated with cadmium toxicity in wheat affected by mycorrhizal fungus

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Abstract

In this study, the effects of *Funelliformis mosseae* and *Piriformospora indica* on wheat growth, enzyme activity and gene expression of catalase (CAT), ascorbate peroxidase (APX) and glutathione S-transferase (GST) in wheat leaves under different cadmium (Cd) toxicity were investigated. Cd exposure reduced plant dry mass and enzyme activities of CAT and APX, but it increased GST activity. The presence of *F. mosseae* and *P. indica* (alone or together) caused an increase in plant dry mass and activities of CAT, APX and GST. In response to Cd exposure, transcriptional level of CAT was decreased but, transcription of APX and GST was up-regulated. Although *F. mosseae* induced the expression of the antioxidant genes, *P. indica* was not detected to have such a strong induction in the genes expression. The results obtained from this study suggest that *F. mosseae* and *P. indica* can be used to alleviate Cd stress in wheat plants in contaminated soil. Also, no correlation was observed between mRNA and enzyme alterations in some cases, which indicate that post-transcriptional and post-translational regulations may play major roles.

Key words: cadmium, *Funelliformis mosseae*, gene expression, *Piriformospora indica*, wheat.

Introduction

Cadmium (Cd) is a toxic trace pollutant with high mobility for plants, animals and humans. It has been revealed that Cd as a toxic heavy metal causes growth inhibition and even plant death due to its interaction with photosynthesis, respiration and nitrogen assimilation. The main source of Cd is intake of food, primarily of cereals. Hence, it is necessary to decrease Cd accumulation in cereals aimed for food production, particularly in wheat which is one of the most frequently consumed cereals. Cd toxicity is also related to the production of reactive oxygen species (ROS) and alteration of the cellular antioxidant capacity (Smeets et al., 2008). However, the levels of oxidative stress and antioxidants vary with the plant species, and the dosage and period of Cd exposure. Smeets et al. (2008) showed that Cd induces oxidative stress and indicated that the antioxidative defense system is moderated by the activation of different antioxidant genes in different organs and cellular compartments.

Plants commonly employ various strategies to neutralize the inhibitory effect of Cd element. For instance, treatments of seedling with Cd element could induce a 20-fold increase in the normally spliced transcript, and a 50-fold increase in an intron-containing transcript. High level expression of the intron-retained transcript does not increase the activity of glutathione S-transferase, and may be used to sequester heavy metals (Kumar et al., 2013). In another study, late embryogenesis abundant (LEA) proteins have been linked with Cd tolerance. Gao et al. (2012) reported that the transgenic plants showed better growth than the wild plants did, indicating that TaLEA1 provides tolerance to Cd stress. These findings suggest that TaLEA1 confers tolerance to cadmium stress by enhancing reactive oxygen species (ROS)-scavenging ability and decreasing lipid peroxidation. Besides mentioned strategies, nutrient management (particularly the sulphur) is one of the possible ways to overcome Cd toxicity. Cd affects the S element assimilation pathway which leads to the activation of pathway responsible for the synthesis of cysteine (Cys), a precursor of glutathione (GSH) biosynthesis (Singh Gill, Tuteja, 2011). Further, it has been proven that mycorrizal has significant effect on alleviation of heavy metal toxicity. Studies have shown that about 80% of terrestrial plants, including most agricultural, horticultural, and hardwood crop species are able to establish the mutualistic association with arbuscular mycorrizal fungi (AMF). The benefits of the AMF symbiosis on plant fitness are largely known, such as providing mineral nutrients (especially phosphorus), and ability to overcome biotic and abiotic stresses (Pozo, Azcón-Aguilar, 2007). AMF plants may also express a variety of enzymes that contribute to the control of cellular ROS levels. Superoxide dismutase (SOD)

neutralizes superoxide anions (O_2^-), and catalase (CAT) and peroxidase (POD) have a role in quenching H_2O_2. The findings of Liu et al. (2011) suggest that antioxidant enzymes have a great influence on the biomass of marigold plants, and AMF can improve the capability of ROS scavenging and reduce Cd concentration in plants to alleviate Cd stress. *Piriformospora indica* belongs to the Sebacinales in Basidiomycota (Oelmüller et al., 2009) which has tremendous potential to be used as a biological agent for plant growth promotion, relieving stress conditions and control of plant diseases (Kumar et al., 2011). Because of the multiplicity of factors involved in heavy metal tolerance by AMF, interpretation of this tolerance is not easy.

So, as a follow-up to our earlier work that verified effectiveness of *Piriformospora indica* on Cd tolerance in wheat (Shahabivand et al., 2012), the present study was aimed to investigate the effects of two fungal species including *F. mosseae* (as AMF) and *P. indica* on alleviation of Cd-induced stress through alteration of antioxidative enzymatic activity and gene expression associated with cadmium toxicity in wheat.

Materials and methods

Plant materials. Seeds of wheat (*Triticum aestivum* L.) cv. 'Homa' were obtained from the Dryland Agricultural Research Institute, Maragheh, Iran. Seeds were surface sterilized for 20 minutes in 1% NaClO, then rinsed with distilled water five times and germinated on wet filter paper in Petri dishes at 25°C for 48 h.

Soil preparation. The experiment soil was collected from the surface horizon of Maragheh University Campus farm. It contained 65% sand, 23% silt, 12% clay, 1.2% organic matter, 0.05% total N, 7 mg kg^{-1} available P, 35 mg kg^{-1} available K, 1.8 mg kg^{-1} total Cd, having pH of 7.3 and 1.3 ds m^{-1} electrical conductivity (EC). The soil samples were air-dried, sieved to pass 2 mm and were steam sterilized (100°C for 1 h, three consecutive days) by autoclaving to eliminate native arbuscular mycorrhizal (AM) fungal propagules as well as other microorganisms. After sterilization, four Cd concentrations (0, 0.3 mM or 33.7 mg kg^{-1} Cd soil, 0.6 mM or 67.4 mg kg^{-1} Cd soil and 0.9 mM or 101.1 mg kg^{-1} Cd soil) were added to the soil (as $CdCl_2$). The samples then were incubated at 20°C for one month allowing metal to distribute into various fractions and equilibrating with soil solid phase. Deionized water was added to the soils to achieve a moisture content of 70% field capacity at the incubation period.

Fungal materials. Piriformospora indica (received from professor Goltapeh, Department of Plant Pathology, Tarbiat Modares University, Tehran, Iran) was cultured in Petri dishes on a modified Kaefer medium with the following composition: 20.0 g L^{-1} glucose, 3.0 g L^{-1} peptone, 3.0 g L^{-1} yeast extract, 1.83 g L^{-1} KH_2PO_4, 0.65 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2.5 ml L^{-1} micro-elements stock solution, 1.0 ml L^{-1} vitamin stock solution, 1.0 ml L^{-1} of 0.1 M $CaCl_2$ solution, 1.0 ml L^{-1} of 0.1 M $FeCl_3$ solution, 2.5 ml L^{-1} of 5.0 g L^{-1} $FeSO_4 \cdot 7H_2O$ and 15 g L^{-1} agar. The micro-elements stock solution contained (g L^{-1}): $ZnSO_4 \cdot 7H_2O$, 22.4; H_3BO_3, 11.0; $MnCl_2 \cdot 4H2O$, 5.0; $CoCl_2 \cdot 6H_2O$, 1.6; $CuSO_4 \cdot 5H_2O$, 1.6; $(NH_4)_6 Mo_7O_{27} \cdot 4H_2O$, 1.1; Na_2EDTA, 50.0. The vitamin stock solution consisted of 0.5 g L^{-1} of biotin, 1.0 g L^{-1} of p-aminobenzoic acid, 5.0 g L^{-1} of nicotinamide, 1.0 g L^{-1} of pyridoxal phosphate solution and 2.5 g L^{-1} riboflavin solution (Kumar et al., 2011). *P. indica* was prepared as follows: circular agar discs inoculated with spores of *P. indica* were placed onto Petri dishes containing modified Kaefer medium and then incubated in an inverted position for 2 weeks at 28°C in a temperature-controlled growth chamber. Next, ten fully-grown fungi agar discs (5 mm in diameter) were inoculated into individual 500 mL Erlenmeyer flasks containing 250 mL of Kaefer broth (with content and density mentioned above except agar). The flasks (the liquid culture) were then incubated with constant shaking at 100 rpm on a rotary shaker for 15 d at room temperature in the dark. The amount of 50 ml liquid culture was added to pots that were treated with *P. indica*. *F. mosseae* inoculum consisted of spores, soil, hyphae and infected maize root fragments supplied from the Department of Plant Pathology, School of Agriculture, Tarbiat Modares University, Tehran, Iran and was produced over a four-month period on maize plants under greenhouse conditions using sterilized sand. The inoculated dosage was 50 g of inoculum per pot containing approximately 20 spores g^{-1} soil.

Planting and growth conditions. The experiment was carried out under growth chamber conditions and consisted of a completely randomized 4 × 4 factorial design with four replications at University of Maragheh, Iran during years of 2013 and 2014. Pots were filled with 5 kg of sterilized sandy soil that contained four added Cd (as $CdCl_2$) concentrations (0, 0.3, 0.6 and 0.9 mM). The fungal treatments were: 1) inoculation of *Piriformospora indica* (50 ml of liquid culture), 2) inoculation of *Funelliformis mosseae* (50 g of inoculated soil), 3) co-inoculation of *F. mosseae* and *P. indica* (50 ml of liquid culture + 50 g of inoculated soil) and 4) non-inoculated (control). *P. indica* and *F. mosseae* inoculums were placed 2 cm below the wheat seedlings at transferring (from filter paper into soil) time (72 h after first germination). The experimental pots were placed in a growth chamber under conditions of 14 h of light, 10 h darkness, 28/20°C day/night temperature, relative humidity of 50–65% and photosynthetic photon flux density of 200 μmol m^{-2} s^{-1}. Watering was done at 72 h intervals throughout the growth period using deionized water to near field capacity. Plants were harvested after 45 d. (at the beginning of flowering stage) for growth and biochemical analysis. Roots and shoots of the harvested wheat samples were rinsed with tap water to remove soil particles and then were carefully washed with deionized water. The samples were dried in an oven at 70°C for 48 h (to measure the dry weight and Cd determination).

Cadmium determination. First, the dried plant samples were finely ground (0.1 g) then, were digested with a mixture (7:1, v/v) of HNO_3 and $HClO_4$. Cd concentrations in digested solutions were determined using an atomic absorption spectrophotometer ("Shimadzu", Japan).

Root colonization. A small fraction of the root system was carefully washed in tap water, cut into 1 cm root pieces then cleaned with a 10% (w/v) KOH solution and finally stained with 0.05% (v/v) Trypan blue in lactic acid. The percentage of fungal root length infection, either by hyphae, arbuscules or vesicles of *F. mosseae* microscopically was assessed by the grid-line intersect technique. In *P. indica*-inoculated roots, the distribution of chlamydospores within the root was taken as an index of colonization (Oelmüller et al., 2009).

Enzyme extraction and activity assay, and H_2O_2 determination. For catalase (CAT) extraction, leaf samples (0.5 g) were homogenized in ice cold 0.1 M phosphate buffer (pH 7.5) containing 0.5 mM EDTA with pre-chilled mortar and pestle. Each homogenate was transferred to centrifuge tubes and was centrifuged at 4°C for 15 min at 15000 g. For APX extraction, 2 mM ascorbate and 5% polyvinylpyrrolidone were added to the above enzyme extraction solution. The supernatant was used for enzyme activity assay. APX and CAT activities were measured according to Yoshimura et al. (2000). For glutathione S-transferase (GST) extraction, leaf samples (0.5 g) were homogenized in ice cold 0.1 M phosphate buffer (pH 6.8) containing 0.4 mM EDTA, 0.5% polyvinylpyrolidone (PVP) and 1 mM sodium metabisulfite with pre-chilled pestle and mortar. Each homogenate was transferred to centrifuge tubes and was centrifuged at 4°C for 15 min at 21000 g. The supernatant was used for enzyme activity assay. The enzyme activities were expressed as units mg^{-1} protein per minute (U mg^{-1} protein min^{-1}). The protein content was determined according to Bradford method using bovine serum albumin as a standard. The concentration of H_2O_2 was measured by the method of (Loreto, Velikova, 2001). Leaves were homogenized in an ice bath with 0.1% (w/v) trichloroacetic acid (TCA). The extract was centrifuged at 12000 g for 15 min, and 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1.0 ml of 1.0 M potassium iodide (KI) were added to 0.5 ml of the supernatant, and the absorbance was read at 390 nm. The concentration of H_2O_2 was given on a standard curve.

Gene expression. 100 mg of leaf tissue was ground thoroughly in liquid nitrogen using a pre-chilled mortar and pestle. Total RNA was extracted by the kit *RNX-Plus* using guanidine/phenol solution according to the manufacturer's instructions ("Cinnagen", Iran). The concentration of the RNA was determined using spectrophotometer NanoDrop (Thermo Fisher Scientific, USA) at 260 nm. Quality of the RNA was checked by both gel and NanoDrop at the 260/280 ratio.

The expression of genes was analyzed using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). First strand cDNA synthesis was created from preheated and snap cold treated 1µl of total RNA using an oligo $(dT)_{16}$ primer in a 20 µl reaction containing: 40 U M-MuLV reverse transcriptase, 5x reverse transcription buffer, 20 U inhibitor RiboLock™ RNase ("Cinnagen", Iran) 10 mM each dNTPs. The reaction was carried out at 42°C for 60 min, followed by a 10-min step at 70°C and then by cooling to 4°C.

The specific primers (Table 1) were designed according to the gene bank, by software *primer3* and IDT website (http://eu.idtdna.com). All the used primers are listed in the supplementary file. The PCR reaction by specific primers for each gene was carried out as follows: 5 min at 94°C then 25 cycles of 30 s at 95°C, 30 s at 57–60°C (depending on the gene), and 30 min at 72°C, and a final extension of 10 min at 72°C. A no reverse transcriptase control (No-RT) was performed for all samples to monitor DNA contamination. Another control was amplification of a housekeeping gene, elongation factor 1-alpha, which was done to correct for quantity and quality differences (normalize) between samples. Each experiment was repeated at least three times in order to ensure reproducibility. Quantification of the amplified bands was done by the software *GelQuant.NET*. Each cDNA band density was normalized by dividing it by the density of the elongation factor 1-alpha band in the same lane. The relative gene expression was calculated by dividing the band density of the gene from the treated leaves by that of the same gene from the control leaves. PCR products were detected on 1% agarose gels by ethidium bromide staining.

Table 1. Name and sequence of the used primers

Primer	Sequence (reverse/forward)
F-EF1	CAGATTGGCAACGGCTACG
R-EF1	CGGACAGCAAAACGACCAAG
F-CAT	CCTTCCTCTTCGACGACATC
R-CAT	CCGTCGTAGTGGTTGTTGTG
F-APX	GGAGCGAATGCTGGTCTTAC
R-APX	CCTGGTCCTCTGCGTACTTC
F-GST	AGCTCGTCCCCATCAACTTC
R-GST	ACATGGTTAAGGTCCGCAAG

Statistical analysis. The analysis of variance (*ANOVA*) was performed on all experimental data using software *GenStat 12*. The differences between means were determined using Duncan's multiple range test at 0.05 and 0.01 probability level.

Results and discussion

Effects of fungal treatments on root colonization, dry mass and Cd content. In *P. indica*-inoculated and *F. mosseae*-inoculated plants, root colonization was significantly decreased at 0.6 and 0.9 mM Cd compared to 0 mM Cd (Table 2). However, the sensitivity of *F. mosseae* was more than *P. indica* to Cd treatment at 0.6 and 0.9 mM Cd. This finding is in line with results of Long et al. (2010) who reported that sensitivity of AMF symbiosis to heavy metal contaminated soil is expressed as a reduction in spore germination, hyphal growth and root colonization. In co-inoculated plants, root colonization was significantly decreased with excess of Cd in the soil from 0 to 0.3 and from 0.6 to 0.9 mM Cd. Co-inoculated plants had less root colonization than *P. indica*-inoculated ones under 0.3, 0.6 and 0.9 mM Cd. AMF symbiosis is known to change physiological and biochemical properties of the host and these changes may alter the composition of root exudates which play a role in the modification of the microbial population in the mycorrhizosphere. It is likely that Cd treatment affects the composition of root exudates by *F. mosseae* (Shahabivand et al., 2012).

Cd is known to reduce or inhibit plant growth. In the present study and in non-inoculated plants, by increasing Cd concentrations in the soil, total plant dry mass was reduced; however, this reduction was significant only between 0 and 0.9 mM Cd (Table 2). Data from our work are in agreement with by Hassan et al. (2013) in sunflower. The colonization by *F. mosseae* increased total plant dry matter at different soil Cd concentrations and this increase was significant under 0 and 0.3 mM Cd, in comparison with non-colonized plants. It is well known that the association of plants with mycorrhizal fungi modifies plant responses to metal-induced stress which leads to increasing tolerance in metal-contaminated

Table 2. The effects of *Funelliformis mosseae* and *Piriformospora indica* on root colonization, biomass, and shoot and root cadmium (Cd) contents in wheat under increasing cadmium (Cd) in the soil

Cd level mM	Fungal treatment	Root colonization %	Total plant g $plant^{-1}$ dry mass	Shoot mg kg^{-1} Cd dry weight	Root mg kg^{-1} Cd dry weight
0.0	nF, nP	0.0 h	0.502 bcd	0.0 f	42 i
0.0	F, nP	45.0 f	0.692 a	0.0 f	51 i
0.0	nF, P	61.6 a	0.780 a	0.0 f	33 i
0.0	F, P	62.0 a	0.765 a	0.0 f	50 i
0.3	nF, nP	0.0 h	0.367 de	81 e	4770 g
0.3	F, nP	43.3 f	0.697 a	70 e	5078 g
0.3	nF, P	61.3 ab	0.705 a	74 e	3720 h
0.3	F, P	53.3 de	0.620 abc	77 e	4986 g
0.6	nF, nP	0.0 h	0.357 de	181 b	7506 ef
0.6	F, nP	25.0 g	0.380 de	154 d	8110 d
0.6	nF, P	58.0 bc	0.617 abc	171 bc	7148 f
0.6	F, P	54.6 cd	0.645 ab	166 c	7868 de
0.9	nF, nP	0.0 h	0.293 e	205 a	9522 c
0.9	F, nP	22.6 g	0.372 de	171 bc	10764 a
0.9	nF, P	55.3 cd	0.482 bcd	199 a	9496 c
0.9	F, P	50.0 e	0.470 cd	179 b	9960 b

Notes. F – *F. mosseae*, P – *P. indica*, nF – non-*F. mosseae*, nP – non-*P. indica*; values are mean: n = 4. The same letter within each column indicates no significant difference among treatments ($P < 0.05$) using Duncan's multiple range test.

soils (Göhre, Paszkowski, 2006). The uptake of metals by mycorrhizal plants and toxicity amelioration are major aspects to be considered by those interested in phytoremediation technologies (Ali et al., 2013). The positive effect of *F. mosseae* on growth is probably related to the improvement of mineral nutrition such as P, the uptake of water by hyphae and the increase of root length density. Presence of *P. indica* (alone or together with *F. mosseae*) significantly increased total plant dry matter under all Cd concentrations in the soil (Table 2). Many researchers have shown that *P. indica* colonizes the roots of a wide variety of plant species and promotes their growth (Varma et al., 2012). However, the mechanisms behind the growth-promoting effects of *P. indica* are a matter of debate.

In our previous work a significant increase in Cd concentration of both wheat shoot and root was observed by increasing soil Cd concentration compared to control treatment (Shahabivand et al., 2012) (Table 2). The results from Table 2 showed that the Cd concentration in roots was more than that of soil Cd, indicating that the Cd absorption mechanism for roots is an active process in wheat. It is suggested that the mechanisms of Cd absorption in roots and xylem loading are related to an energy-dependent active process (Mori et al., 2009). Based on Table 2, root accumulated more Cd than shoot. It could be calculated that only 3–4% of the total Cd accumulated in roots reached the shoot cells. Accumulation of large amounts of Cd in the roots may limit the accumulation of Cd in above-ground portions of wheat plants. The presence of *P. indica* (alone or together with *F. mosseae*) increased Cd concentration in root but decreased Cd content in shoot with the excess of Cd concentrations in the soil (Table 2). These results showed that chelation of Cd inside the fungus or adsorption of Cd to chitin in the fungal cell wall caused accumulation of Cd in root and prevented the Cd translocation from root to shoot. Similarly, in AMF, there is evidence suggesting that fungal hyphae components may provide additional detoxification mechanisms by storing toxic compounds (Göhre, Paszkowski, 2006). *F. mosseae* inoculation reduced root and shoot Cd at all Cd levels in the soil. The reduction in root Cd content under *F. mosseae* inoculation might be related to the adsorptive capability for metals of the relatively large fungal biomass (especially extra-radical hyphal cell wall) associated with the host plant roots, which may physically minimize or exclude the entry of metals into host plant (Shahabivand et al., 2012).

Effects of fungal treatments on H_2O_2 content and enzyme activities. In non-inoculated plants, increasing Cd concentration in the soil significantly ($p < 0.01$) increased H_2O_2 contents (1.5–2.5 fold) in wheat leaves (Table 3). Increased levels of H_2O_2 indicated that Cd exposure results in generation of ROS, which are highly toxic molecules and cause cellular damage in plants. In this work, we observed decreased H_2O_2 content in leaves of wheat plants colonized with *P. indica* and *F. mosseae* (alone or together, except for *F. mosseae*-inoculated plants at 0.6 and 0.9 mM Cd), indicating a lower accumulation of H_2O_2 in fungi-inoculated plants. The root cells of AMF treated plants may locally induce accumulation of H_2O_2 (in the intra-cellular AM hyphae or at the intercellular hyphal surface).

In non-inoculated wheat plants, the Cd concentration of 0.3 mM in the soil, significantly reduced CAT and APX activities compared to 0 mM Cd, whereas concentrations of 0.6 and 0.9 mM had no further influence on CAT and APX activities compared to 0.3 mM Cd (Table 3). Cd stress elicits biochemical responses in higher plants that minimize its deleterious effects. One important component of protective systems is enzymatic defense. CAT dismutates reduces H_2O_2 to oxygen and water and APX reduces H_2O_2 to water by ascorbate as specific electron donor. A significant reduction in CAT and APX activities of wheat plants was very likely due to the harmful effect of overproduction of H_2O_2 or its poisonous ROS derivatives such as MDA (malondialdehyde) as a biomarker for oxidative stress induced under Cd stress.

GSTs are one of the major cellular detoxification enzymes protecting plants from oxidative stress. GST catalyzes the conjugation of various electrophiles with

Table 3. The effects of *Funelliformis mosseae* and *Piriformospora indica* on H_2O_2 and activities of catalase (CAT), ascorbate peroxidase (APX) and glutathione S-transferase (GST) of leaves in wheat under increasing cadmium (Cd) in the soil

Cd level mM	Fungal treatment	H_2O_2 mmol g^{-1} fresh weight	CAT U mg^{-1} protein min^{-1}	APX U mg^{-1} protein min^{-1}	GST U mg^{-1} protein min^{-1}
0.0	nF, nP	0.068 f	0.094 cd	0.275 c	0.129 f
0.0	F, nP	0.032 g	0.090 d	0.428 a	0.205 e
0.0	nF, P	0.040 g	0.118 a	0.403 a	0.218 e
0.0	F, P	0.033 g	0.116 a	0.453 a	0.211 e
0.3	nF, nP	0.105 c	0.058 i	0.167 d	0.292 c
0.3	F, nP	0.059 f	0.076 fg	0.244 c	0.274 cd
0.3	nF, P	0.067 f	0.100 b	0.340 b	0.273 cd
0.3	F, P	0.059 f	0.098 bc	0.346 b	0.264 d
0.6	nF, nP	0.127 b	0.055 i	0.157 d	0.340 b
0.6	F, nP	0.080 e	0.072 gh	0.235 c	0.330 b
0.6	nF, P	0.120 b	0.082 e	0.260 c	0.321 b
0.6	F, P	0.085 de	0.084 e	0.269 c	0.332 b
0.9	nF, nP	0.174 a	0.054 i	0.181 d	0.396 a
0.9	F, nP	0.090 de	0.067 h	0.252 c	0.388 a
0.9	nF, P	0.164 a	0.079 ef	0.279 c	0.387 a
0.9	F, P	0.094 cd	0.081 ef	0.267 c	0.385 a

Notes. F – *F. mosseae*, P – *P. indica*, nF – non-*F. mosseae*, nP – non-*P. indica*; values are mean; n = 4. The same letter within each column indicates no significant difference among treatments ($P < 0.05$) using Duncan's multiple range test.

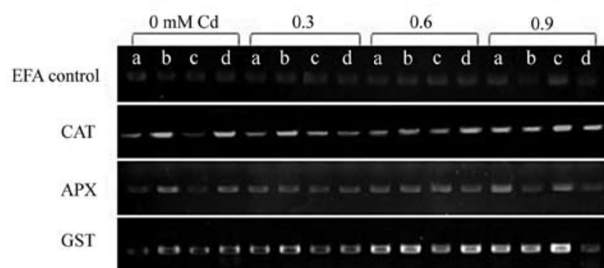
reduced glutathione, detoxifying both exogenously (such as Cd) and endogenously derived toxic compounds (Dixit et al., 2011). By increasing soil Cd concentrations, GST activity was significantly increased, in non-inoculated wheat (Table 3). Similarly, the activity of GST in barley roots treated with Cd was significantly stimulated (Tamas et al., 2008). GST has also been suggested as main enzyme in detoxification processes in *Phragmites australis* exposed to high concentration of Cd. In addition, certain plant GSTs have secondary glutathione peroxidase activity capable of reducing organic hydroperoxides protecting cells during oxidative stress (Flohé, 2012). High GST activity is a common characteristic of the most widespread *Triticum aestivum* cultivars, and several studies have revealed a high correlation between their GST activity and stress tolerance (Jiang, Yang, 2009).

Inoculation of *F. mosseae* increased CAT activity under 0.3, 0.6 and 0.9 mM, APX activity under all Cd concentrations, and GST activity under 0 mM Cd in comparison with un-inoculated plants (Table 3). Inoculation of *P. indica* (alone or together with *F. mosseae*) increased CAT and APX activities under all Cd concentrations, and GST activity in 0 mM Cd compared to non-inoculated plants. GSTs have an important role in the detoxification of wide variety of toxic compounds (such as cadmium) through their conjugation to glutathione. Thus, decline in leaf GST activity under fungal inoculation at different Cd levels was probably due to reducing Cd accumulation in leaves of wheat plants after fungal inoculation (Table 2). CAT activity in leaves of AM plants has been shown to double in relation to control plants under heavy metal stress in the soil (Andrade et al., 2010). The CAT response is associated with AM colonization, and the induced activity of this enzyme by AMF can contribute to the enhanced plant growth and Cd tolerance of AM plants under Cd stress (Liu et al., 2011). In the case of plants colonized with *P. indica*, a 23-fold and a 3.8-fold increased activity was found for CAT and GST, respectively, as compared to non-colonized plants (Kumar et al., 2009). The data from our study are consistent with previous observations in which activation of the antioxidant enzyme systems is a major target of *P. indica* in leaves (Sun et al., 2010). In our study, the higher CAT, APX and GST activities in AMF and endophyte fungus seedling would partly explain the lower H_2O_2 concentration (Table 3) in these seedlings, protecting the organism against oxidative damage, which in turn enhances Cd tolerance.

Gene expression in response to fungi. The analysis of transcriptional level of the three antioxidant genes in leaves was examined by semi-quantitative RT-PCR. Transcript levels of CAT, APX and GST genes were altered in response to different Cd concentrations in uncolonized plants (Fig. 1).

Expression levels of CAT were decreased under 0.3, 0.6 and 0.9 mM Cd compared to 0 mM Cd (Fig. 2). However, CAT expression level was higher at 0.9 mM than 0.3 and 0.6 mM Cd. The decrease in CAT transcription was consistent with the change in CAT enzyme activity. In pea plants, it was found that Cd caused a reduction in the enzyme activities and transcription of two isoforms of superoxide dismutase (CuZn-SODs and Mn-SOD) but up-regulated the expression of Fe-SOD isoform (Rodriguez-Serrano et al., 2006). In the case of APX, in contrast to the overall changes in enzyme activity, an induction was observed after application of 0.3, 0.6 and 0.9 mM Cd (Fig. 2 B). Wu et al. (2009) reported that transcriptional level of CAT and APX declined in response to Cd accumulations in *Ulva fasciata* while an increase in enzyme activity was observed in higher concentrations. In contrast, CAT and APX were up-regulated transcriptionally in leaves of *Arabidopsis thaliana* under Cd stress (Smeets et al., 2008) whereas CAT enzymatic activity did not change significantly. They also showed that Cd caused accumulation of glutathione reductase (GR) transcripts, on the other hand, GR enzymatic activity decreased in leaves. It is likely that Cd interacts with the translation complex, reducing the activity of APX, or may be Cd alters the turnover of the enzyme. As shown by Cuypers et al. (2010), an oxidative modification could be induced by Cd exposure which possibly led to higher proteolytic degradation.

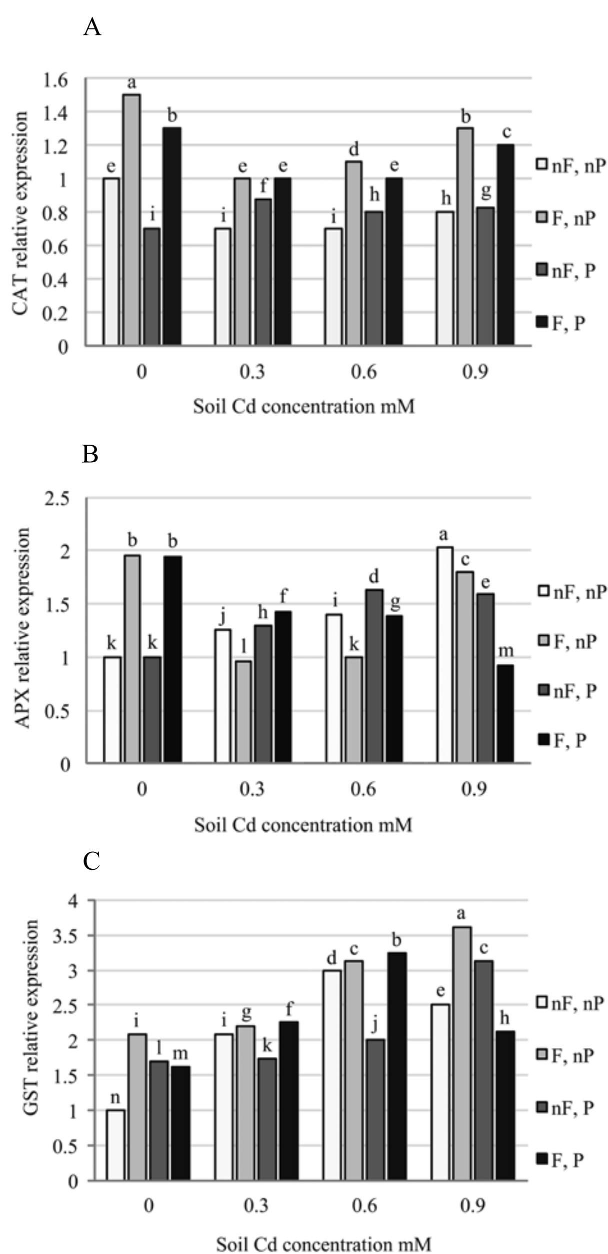
Also, it has been indicated that Cd stress can result in protein denaturation in *A. thaliana* (Semane et al., 2010). Similar to APX, transcriptional pattern of GST represented an increasing trend (Figs 1 and 2 C). As the Cd concentration rose from 0 to 0.3 mM, GST transcripts started to increase rapidly reaching the maximum at 0.6 mM Cd. This change agreed with the rise in GST activity. The coincidence between GST activity and its transcript level implies that the induction of activity of this enzyme by Cd is attributable to enhanced expression of GST.



Notes. The effects of *Funelliformis mosseae* and *Piriformospora indica* on gene expression in wheat leaves under increasing cadmium (Cd) in the soil are represented. Elongation factor 1-alpha was used as internal control gene to normalize different samples. a – control (non-inoculation), b – *F. mosseae*, c – *P. indica*, d – *F. mosseae* + *P. indica*. Experiments were repeated three times.

Figure 1. Analysis of mRNA expression of the antioxidant genes by semi-quantitative RT-PCR

Furthermore, the transcription levels of these genes were determined in the leaves after application of three fungal treatments. As shown in Figure 2 A, an increase in CAT transcript level was detectable in 0.3, 0.6 and 0.9 mM Cd concentrations in the colonized plants in comparison with uncolonized control; however, this increase was not significant for *P. indica* in 0 Mm Cd. Fungus *F. mosseae* and mix-colonization (*F. mosseae* + *P. indica*) caused induction in CAT transcription at all Cd levels, and *P. indica* caused induction in 0.3, 0.6 and 0.9 mM Cd in comparison with non-colonized plants (Fig. 2 A). In 0 Cd mM level, similar results were obtained for APX gene as transcription level were increased in colonized wheat with the exception of *P. indica* that could not induce APX transcription significantly (Fig. 2 B). Interaction effects between fungus and the Cd levels were slightly different from the main effects (0 mM Cd); *F. mosseae* caused induction of the gene only at 0 mM Cd and *P. indica* and co-colonization induced APX transcription at 0.3 and 0.6 mM Cd. But, APX was down-regulated in the all colonized plants at 0.9 mM Cd. These alternations were rather similar to those of enzyme activity where both *F. mosseae* and *P. indica* had the most impact on the enzyme activity in 0 mM Cd levels. Fungal treatments including *F. mosseae*, *P. indica* and mix-colonization were able to up-regulate GST expression notably in 0 mM Cd. Fungus *F. mosseae* and mix-colonization increased transcription level in all Cd concentrations except 0.9 mM in which mix-colonized plants reduced GST transcripts (Fig. 2 C). Also, *P. indica* induced GST transcription in 0 and 0.9 mM Cd but reduced its transcription in 0.3 and 0.6 mM Cd. On the other hand, the effects of three fungal treatments were not significant in APX, CAT and GST activity in 0.3, 0.6



Notes. F – *F. mosseae*, P – *P. indica*, nF – non-*F. mosseae*, nP – non-*P. indica.* Values with different letter are significantly different at $p < 0.01$; mean ± SE of three independent experiments.

Figure 2. The effects of *Funelliformis mosseae* and *Piriformospora indica* on the expression of three genes catalase (CAT) (A), ascorbate peroxidase (APX) (B) and glutathione S-transferase (GST) (C) in wheat under increasing cadmium (Cd) in the soil

and 0.9 mM Cd. Post-transcriptional regulation is likely to play a key role in this case. These differences might be due to the presence of multiple allo or isozymes. Alternatively, an enhanced breakdown of the proteins can be caused by Cd concentrations, which in turn also results in high level of transcription (Smeets et al., 2008). Fungus *F. mosseae* had a positive impact on transcript accumulation of stress genes. Interestingly, this positive effect was not detected in some of *P. indica*-colonized plants. In particular, the main effects of this fungus were not remarkable, resulting in no significant induction in transcription of the studied antioxidant genes. Therefore, influence of mix-colonization treatment, particularly in

main effects, mostly resulted from *F. mosseae* activity. Molitor et al. (2011) indicated that *P. indica* could induce transcription of a wide range of pathogenesis-related genes and they concluded that *P. indica* likely induced disease resistance through genes encoding heat-shock proteins.

It is believed that regulation of antioxidant enzymes is performed post-translationally under oxidative stress (Sun et al., 2010), therefore, it seems that measurement of enzymatic activity is more reliable for the evaluation of *P. indica* and *F. mosseae* effects than expression level. Increase in antioxidant activity was a major target for *P. indica* in barely leaves. However, it has been showed that *P. indica* is able to up-regulate drought-related genes in Arabidopsis leaves colonized by *P. indica* (Sherameti et al., 2008). Our findings suggest that *P. indica* may function more through enhancing enzyme activity than inducing transcription under cadmium toxicity. The alteration of antioxidative defence genes in plants colonized by mycorrhiza has previously been investigated. For example, in mycorrhiza-colonized lettuce, Mn-SOD2 transcripts accumulated under water stress which led to a higher resistance in plants (Gill, Tuteja, 2010). They also found that expression level of SOD genes were reduced in non-stress conditions. Bagheri et al. (2013) revealed that GST transcription and its protein synthesis were induced in mycorrhiza-colonized plants. Transcriptional induction in the studied antioxidant genes in the *F. mosseae*-colonized wheat, in present work, was consistent with the above-mentioned studies.

Conclusions

1. Cadmium (Cd) toxicity significantly altered enzyme activity and transcription level of catalase (CAT), ascorbate peroxidase (APX) and glutathione S-transferase (GST).

2. Inoculation of wheat plants with arbuscular mycorrhizal and root-endophytic fungi could be efficient to reduce Cd stress.

3. Both *Funelliformis mosseae* and *Piriformospora indica* confer tolerance to Cd stress in pot cultured wheat by increasing plant dry mass and antioxidant enzyme activities.

4. No coincidence was observed between enzyme activity and transcription in some treatments, showing that these enzymes are not under transcriptional regulation.

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Kviečių antioksidacinis aktyvumas ir genų raiška sąveikoje su mikoriziniais grybais kadmiu užterštame dirvožemyje

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Santrauka

Tirta *Funelliformis mosseae* ir *Piriformospora indica* įtaka kviečių augimui, fermentų aktyvumui, katalazės genų ekspresijai, askorbato peroksidazei ir glutationo S-transferazei kviečių lapuose, esant skirtingam kadmio (Cd) toksiškumui. Kadmis sumažino augalų sausąją masę ir kalatazės bei askorbato peroksidazės fermentų aktyvumą, bet padidino glutationo S-transferazės aktyvumą. *F. mosseae* ir *P. indica* (po vieną ir kartu) padidino augalų sausąją masę ir kalatazės, askorbato peroksidazės bei glutationo S-transferazės aktyvumą. Reaguojant į Cd poveikį, sumažėjo transkripcinis kalatazės lygis, tačiau askorbato peroksidazės ir glutationo S-transferazės transkripcija padidėjo. Nors *F. mosseae* sukėlė antioksidacinių genų ekspresiją, *P. indica* neturėjo tokio stipraus poveikio suaktyvinant genų ekspresiją. Tyrimo rezultatai rodo, kad *F. mosseae* ir *P. indica* naudotini siekiant sušvelninti Cd stresą kviečių augaluose, auginamuose Cd užterštoje dirvoje. Taip pat kai kuriais atvejais nebuvo nustatyta koreliacija tarp iRNA ir fermentų pokyčių; tai rodo, kad didelės įtakos gali turėti posttranskripcinė ir posttransliacinė genų raiškos reguliacija.

Reikšminiai žodžiai: *Funelliformis mosseae*, genų ekspresija, kadmis, kviečiai, *Piriformospora indica*.

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