Effect of *Piriformospora indica* on antioxidant enzymes activity of tomato (*Lycopersicon esculentum* Mill) under lead stress

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**Abstract**

The endomycorrhizal fungus *Piriformospora indica* is well known for inducing disease resistance, elevation of salt tolerance and increasing biomass in symbiotic plants. The aim of the present study is to evaluate the effect of *P. indica* on tomato (*Lycopersicon esculentum* Mill) under lead stress (Concentrations of 1 and 2 mM Pb (NO3)2). Lead (Pb) is not an essential nutrient for plants and is known as a hazardous pollutant in the environment which originates from various sources. In this study, the effect of lead toxicity and *P. indica* on antioxidant enzyme activities in both mycorrhizal (MR) and non-mycorrhizal (NMR) tomato plants were investigated. The experiment was performed by using six treatments (mycorrhizal and non-mycorrhizal with and without lead stress) and two concentrations of Pb (1and 2 mM Pb (NO3)2 solution) and then antioxidant enzyme activities (CAT, SOD and APX) in roots and stems of plants were determined. The results demonstrated that lead could affect the activity of antioxidant enzymes in treating plants. As well, in most cases, the rates of the enzyme activities were higher in roots than those of stems, but the impact Pb treatment on enzyme activities in *P. indica* colonized plants was not significant.

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Introduction

Tomato (Lycopersicon esculentum Mill) is a eudicotyledonous plant that belongs to the family Solanaceae together with other economically important crops such as pepper, eggplant and potato. It is the most important grown fresh market vegetable worldwide with more than 5 million hectares harvested in China, United States of America, India, Turkey and Egypt as the five first producers, respectively (Peralta et al., 2008).

Plants are constantly exposed to a wide array of environmental stresses that cause major losses in productivity. Resistance and susceptibility to the biotic and abiotic stresses are complex phenomena, in part because stress may occur in multiple stages of plant development and often more than one stress simultaneously affects the plant. To handle with various environmental challenges, plants execute a number of physiological and metabolic responses (Bohnert et al., 1995). On the other hand, the effects of various environmental stresses are known to change the antioxidant content of tomato (Dumas et al., 2003).

With rapid development in industry all around the world since the twentieth century, the heavy metal concentrations in agricultural soil increased quickly in many areas around the world (Kabata and Pendas, 1984). Heavy metals are important environmental pollutants, and many of them are toxic even at very low concentrations. Lead (Pb) is not an essential nutrient for plants, and it is one of the hazardous heavy metal pollutants of the environment that originates from various sources like mining and smelting activities, burning of coal, effluents from storage battery industries, automobile exhausts, pesticides, and from additives in pigments and gasoline as well as from the disposal of municipal sewage sludge enriched with Pb (Eick et al., 1999). Pb pollution of the environment is a major ecological concern due to its impact on human health through the food chain and its high persistence in the environment (Piechalak et al., 2003). Toxic levels of heavy metal affect a variety of processes in plants. One of the major consequences is the enhanced production of reactive oxygen species (ROS) (Verma and Dubey 2003; Souguir et al., 2011). ROS can cause oxidative damage to the biomolecules when produced in larger amounts leading to cell membrane peroxidation, loss of ions, protein hydrolysis, and even DNA fragmentation. Plants’ defenses to metal toxicity may constitute different strategies. First is the avoidance of metal entry into the cell via exclusion or binding of metal in the cell wall. For lead, binding to cell wall is one of the major mechanisms of detoxification (Antosiewicz and Wierzbicka 1999). Second, plants have developed an anti-oxidative system, including low-molecular-mass antioxidants as well as anti-oxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbic acid peroxidase (APX) (Cakmak and Horst 1991).

The beneficial fungi play a crucial role in improving plant growth and yield, and also take part in stress tolerance, bioremediation, food safety and sustainable crop production (Amatussalam et al., 2011; Borde et al., 2009; Singh et al., 2011). In general, an endophyte can be defined as a microorganism, typically bacterial or fungal, that lives symptomatically within a plant, causing no signs of harm to the host (Clay and Schardl, 2002; Porras-Alfaro and Bayman, 2011). The endophytic fungus Piriformospora indica interacts with many plant species. It colonizes the roots, grows inter- and intracellularly, forms pear-shaped spores within the cortex and extramatrically, and does not invade the endodermis and the aerial parts of the plants. The endophyte promotes nutrient uptake, allows plants to survive underwater, temperature and salt stress, confers (systemic) resistance to toxins, heavy metal ions and pathogenic organisms and stimulates growth and yield (Varma et al., 2001). A symbiotic association of fungi leads to stress tolerance in plants which involves two mechanisms: (1) trigger-action of host stress responsive systems while exposed to a particular stress, directing the plants to either keep
away from or mitigate that stress; and (2) biosynthesis of anti-stress biochemical by endophytes (Singh et al., 2011).

The relevance of tomato in human nutrition is increasing, since it is generally considered as a healthy food because of the high content of lycopene and other health promoting natural compounds. A large part of this crop is grown in greenhouses, using special substrates and fertilization techniques involving utilization of water, therefore implying an increased risk of heavy metal concentration increases (Gil et al., 2004). Some research was done on the effects of mycorrhiza fungi including Piriformospora indica on plants growth and accumulation of heavy metals (Sun et al., 2010; Shahabivand et al., 2012) and lead stress effects on antioxidant enzyme activities in different plants (Verma and Dubey, 2003; Gupta et al., 2009; Wang et al., 2012), which often reflects the positive impact of fungi on plant growth and increasing effect of lead on activities of antioxidant enzymes. However, no comprehensive study has been done on the simultaneous effects of lead stress and Piriformospora indica on antioxidant enzyme activities as an indicator of oxidative stress in the tomato plant in response to heavy metals (especially lead) as one of the most widely consumed crops and exposed to lead contamination in many parts of the world. Therefore, research in this area may be a new way for the diagnosis and management of heavy metal stress, with regard to changes in the antioxidant enzyme activities and the ability of the Piriformospora indica to reducing lead stress in tomato. Therefore, inspection of plants such as tomato mechanisms in response to Pb toxicity is worthy to known. Thus, the aim of this work is to evaluate antioxidant enzyme activities in roots and stems of tomato plant inoculated with P. indica under Pb stress.

**Materials and methods**

**Preparation and planting of tomato seeds**

Healthy and improved seeds of tomato (Lycopersicon esculentum Mill.) obtained from seed and Plant Improvement Institute, Karaj, Iran. To sterilize, the seeds placed in 50% ethanol for 1 minute, then immersed for 15 minutes in 1.5 % of the active chlorine solution. Treated seeds washed several times with sterilized distilled water. For germination, the disinfected seeds were sown on sterilized sand and perlite (50:50) substrate.

**Preparation of P. indica culture and inoculation of tomato seedlings**

The fungus culture was grown on a complex medium (CM) at the 27 °C for one month. The root of 2 weeks old seedlings immersed in 10^6 ml^-1 of P. indica chlamydomspores suspension for 12 hours and shook. Infected seedlings transferred into pots containing sterilized mixture of sand and perlite substrate. After planting, the pots were transferred to growth chamber with 16 h light period and 22 to 24 °C, 60% relative humidity, for 4 weeks. To detect P. indica root samples, stained with ink following by Vierheilig et al. (2005) method with minor changes. Molecular detection of P. indica in plants carried out using Tef specific primer pairs (accession no. AJ249911).

**Treatments conditions**

In this research, experiment was arranged in six treatments with three replicates. treatments were as follows: A (tomato inoculated with p.indica), B (tomato), A1 (tomato inoculated with p. indica under lead stress (1 mm pb (no3)2), B1 (tomato under lead stress (1 mm pb (no3)2), A2 (tomato inoculated with p. indica under lead stress (2mm pb (no3)2), B2 (tomato under lead stress (2 mm pb (no3)2).

**Antioxidant enzyme activities in tomato plants**

In order to study the impact of colonization with P. indica on the antioxidant enzyme activity of the plant, the antioxidant enzyme activities were checked in the presence and absence of P. indica. For this purpose, all experiments were carried out 28 days after the colonization under the conditions employed. The antioxidant enzyme activities were measured in roots and stems of tomato plants.

**Superoxide dismutase assay**
The activity of SOD was analyzed by measuring its ability to inhibit the photochemical reduction of Nitro blue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). Three milliliters of reaction mixture containing 50 mM phosphate buffer at pH 7.8, 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 1.0 mM EDTA and 20 μl enzyme extraction. Riboflavin was added last and the reaction was initiated by placing the tubes 30 cm below 15 W fluorescent lamps. The reaction was started by switching on the light and was allowed to run for 10 min. Switching off the light stopped the reaction and the tubes were covered with black cloth. Non-illuminated tubes served as control. The absorbance at 560 nm was read. One unit of SOD is the amount of extracts that gives 50% inhibition of the rate of NBT reduction (El-Beltagi et al., 2012).

Catalase activity assay
Catalase activity (CAT; EC 1.11.1.6) was determined by consumption of H$_2$O$_2$ using the method of Dhindsa et al. (1981). The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer at pH 7.0, 15 mM H$_2$O$_2$ and 50 ml enzyme extraction. The reaction was initiated by adding the H$_2$O$_2$. The consumption of H$_2$O$_2$ was monitored spectrophotometrically at 240 nm (extinction coefficient 39.4 mM$^{-1}$ cm$^{-1}$) for 3 min (El-Beltagi et al., 2012).

Ascorbate peroxidase assay
Ascorbate peroxidase activity (APOX; E.C. 1.11.1.11) was estimated according to the method of Nakano and Asada (1981). Enzyme activity was determined by the decrease in absorbance of ascorbate at 290 nm. The reaction mixture consisted of enzymatic extract, 50 mM sodium phosphate buffer (cold), pH 7, 0.5 mM ascorbate, 0.5 mM H$_2$O$_2$ and 0.1 mM EDTA, in a 0.3 ml final volume. The reaction started after the hydrogen peroxide addition. The molar extinction coefficient 2.8 mM$^{-1}$ cm$^{-1}$ was used to calculate ascorbate peroxidase activity. Enzyme activity was expressed as units mg$^{-1}$ protein. One unit of enzyme was the amount necessary to decompose 1μmol of substrate per minute at 25°C (El-Beltagi et al., 2012).

Statistical analyses
Results of all experiments were expressed as means ± SE. Analysis of variance performed by ANOVA procedures using SPSS 17.0 and means was compared with Duncan test at the 0.05 level of confidence.

Results and discussion
Microscopic images show the proper growth of fungus P. indica in tomato plant root cells, and this phenomenon has a considerable importance in the study (Fig. 1). Then the visual appearance of plants in different treatments was studied, the results showed that the apparent plant growth rate and root growth in mycorrhiza plants relative to non-mycorrhiza plants was significantly higher. Heavy metals such as lead induce oxidative stress by generation of superoxide radical, hydrogen peroxide, hydroxyl radical and single oxygen that called ROS (Devi and Prasad, 1998). ROS can rapidly attack all types of biomolecules such as nucleic acids, proteins, lipids and amino acids (Luna et al., 1994), leading to irreparable metabolism and cell death. To cope and repair the damage caused by ROS, plants have evolved complex antioxidant systems, such as antioxidant enzymes (CAT, SOD, APX, and POX). The results presented in Figures 2-7 reveal the antioxidant enzymes responses of tomato plants grown under different Pb concentrations (1 and 2 mM Pb(NO$_3$)$_2$) in the presence and in the absence of endomycorrhizal fungus P. indica.

Fig. 1. The fungus P. indica into tomato root cells.

Superoxide dismutase (SOD) activity
In the case of roots, highest and lowest SOD activity belong to B2 (6.803 ± 0.612) and A (3.534 ± 0.537)
treatments respectively (Fig. 2). Results showed significant effects of lead stress on SOD activity in roots of Pb treated plants as compared to that of untreated one (P<0.05). Moreover, SOD activity was increased with increasing lead concentration but there is not difference between two concentrations of Pb (1 and 2 mM). Also it was observed that tomato plants colonized with P. indica showed low SOD activity as compared to those of non-colonized plants, which was not statistically significant between treatments (P> 0.05) but was statistically significant between treatments (Fig. 2). These results showed that P. indica has not significant effect on SOD activity in tomato plant roots.

In stems of tomato plants, highest and lowest SOD activity belong to B1 (5.747 ± 0.478) and A2 (1.189 ± 0.357) treatments respectively (Fig. 3). The results did not show significant effects of lead stress on SOD activity in stems of treatments (P>0.05). Moreover, SOD activity doesn’t show the same trend with increasing of lead concentration between treatments. In the case of plants colonized with P. indica under high Pb concentration (2 mM), significance decreased activity was found for SOD as compared to non-colonized plants. These results showed that P. indica has no significant effect on SOD activity in roots, but Pb concentration, especially in high concentration (2 mM) has a significant effect on SOD activity in stems (Fig. 3).

SOD is a metallo enzyme present in various cellular compartments, functioning at the first step of ROS generation, i.e., superoxide formation and superoxide radicals can act as a precursor to other ROS (Alscher et al., 2002). SOD dismutates two superoxide radicals to H2O2 and oxygen, thus maintains superoxide radicals in a steady state level. In this study, increasing SOD activity at two concentrations of Pb (1 and 2 mM) compared to non-lead stress treatment (A and B) may be indicating that the oxygen scavenging function of SOD was not impaired or response of plants to this stress. Some authors suggested that increasing of SOD activity could possibly be the result of both a direct effect of heavy metal ions and an indirect effect mediated via increasing in levels of O2 (Chongpraditnum et al., 1992).
Increased activity of antioxidant enzymes minimizes the chances of oxidative burst (excessive ROS production), and therefore *P. indica* might be protected by the oxidative defense system during colonization. Also, induction of antioxidant enzymes observed in shoots of plants colonized with *P. indica*. These data suggest the systemic induction of antioxidative defense in the case of colonization by *P. indica* (Kumar et al., 2009). Garg and Aggarwal (2012) reported that superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) increased in *Cajanus cajan* under cadmium and lead stress and increased activities of SOD, CAT, POX as well as GR were observed in all mycorrhizal (arbuscular mycorrhizal fungi) stressed plants.

Fig. 4. CAT activity in roots of tomato exposed to lead. A (tomato inoculated with *P.indica*), B (tomato), A1 (tomato inoculated with *P.indica* under lead stress (1 mM Pb (NO₃)₂), B1 (tomato under lead stress (1 mM Pb (NO₃)₂), A2 (tomato inoculated with *P.indica* under lead stress (2 mM Pb (NO₃)₂), B2 (tomato under lead stress (2 mM Pb (NO₃)₂)). MR: Mycorrhized tomato, NMR: Non Mycorrhized tomato. The means marked by different letters are significant differences according to the Duncan test (P<0.05).

Catalase (CAT) activity

Under similar conditions, the results showed that despite the changes in CAT activity in the roots have not observed significant differences between treatments (P>0.05). As seen in the figure 4, the highest CAT activity was observed in treatment B1 (0.1589 ± 0.0677) and the lowest rate in treatment A2 (0.0233 ± 0.0030), but these differences were not significant (P>0.05). Moreover, in this research did not find a significant difference in CAT activities of root plants colonized with *P. indica* (MR) and non-colonized with *P. indica* (NMR) (Fig. 3). Apparently, overall lead stress causes to increasing of CAT activity and in lead stress treatments, increasing the lead concentration led to reduced levels of CAT activity (2 mM to 1 mM) but not statistically significant to other treatments (P>0.05).

Fig. 5. CAT activity in stems of tomato exposed to lead. A (tomato inoculated with *P.indica*), B (tomato), A1 (tomato inoculated with *P.indica* under lead stress (1 mM Pb (NO₃)₂), B1 (tomato under lead stress (1 mM Pb (NO₃)₂), A2 (tomato inoculated with *P.indica* under lead stress (2 mM Pb (NO₃)₂), B2 (tomato under lead stress (2 mM Pb (NO₃)₂)). MR: Mycorrhized tomato, NMR: Non Mycorrhized tomato. The means marked by different letters are significant differences according to the Duncan test (P<0.05).

In the case of stems, plants colonized with *P. indica* did not show a significant increase in CAT activity (Fig. 5), but it seems, lead stress, decreased effect on CAT activity in stems. As seen in the figure 4, the highest CAT activity was observed in treatment B (0.7013 ± 0.0021) and the lowest in B1 (0.0282 ± 0.0036), and this difference was significant (P<0.05). Moreover, in this study did not find a significant difference in CAT activities of stems plants colonized with *P. indica* (MR) and non-colonized with *P. indica* (NMR) in the treatment (Fig. 5). The results showed that increasing the concentration of lead has no significant effect on CAT activity in stems.

Fig. 6. APX activity in roots of tomato exposed to lead. A (tomato inoculated with *P.indica*), B (tomato).
(tomato), \textbf{A1} (tomato inoculated with \textit{P.indica} under lead stress (1 mM Pb (NO$_3$)$_2$), \textbf{B1} (tomato under lead stress (1 mM Pb (NO$_3$)$_2$), \textbf{A2} (tomato inoculated with \textit{P.indica} under lead stress (2 mM Pb (NO$_3$)$_2$), \textbf{B2} (tomato under lead stress (2 mM Pb (NO$_3$)$_2$).\textbf{MR}: Mycorrhized tomato, \textbf{NMR}: Non Mycorrhized tomato. The means marked by different letters are significant differences according to the Duncan test (P<0.05).

CAT is a universally present oxidoreductase that decomposes H$_2$O$_2$ to water and molecular oxygen, and it is one of the key enzyme involved in the removal of toxic peroxides. The scavenging of H$_2$O$_2$ by CAT may be an efficient mechanism to attenuate the elicitation of plant defense responses, for example against heavy metal stress. In the present study, CAT activities in roots of non-inoculated tomatoes increased (but not significantly) at two Pb concentrations, while at inoculated tomatoes with lead stress decreased, however, not significant (P>0.05). Increase in CAT activity can be explained by an increase in its substrate, i.e., to maintain the level of H$_2$O$_2$ as an adaptive mechanism of the plants (Reddy et al., 2005). Decline observed at higher concentration of Pb (2 mM) in roots, might be attributed to inactivation of enzyme by ROS, decrease in synthesis of enzyme, or change in the assembly of its subunits (Verma and Dubey, 2003).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig7}
\caption{APX activity in stems of tomato exposed to lead. \textbf{A} (tomato inoculated with \textit{P.indica}), \textbf{B} (tomato), \textbf{A1} (tomato inoculated with \textit{P.indica} under lead stress (1 mM Pb (NO$_3$)$_2$), \textbf{B1} (tomato under lead stress (1 mM Pb (NO$_3$)$_2$), \textbf{A2} (tomato inoculated with \textit{P.indica} under lead stress (2 mM Pb (NO$_3$)$_2$), \textbf{B2} (tomato under lead stress (2 mM Pb (NO$_3$)$_2$).MR: Mycorrhized tomato, NMR: Non Mycorrhized tomato. The means marked by different letters are significant differences according to the Duncan test (P<0.05).}
\end{figure}

\textbf{Ascorbate peroxidase (APX) activity}

APX Activity results showed that despite the changes in APX activity in the roots and stems (Fig. 6 and Fig.7), but not observed significant difference between treatments (P>0.05). As seen in the figure 5, in roots, the highest APX activity was observed in treatment A (0.0630 ± 0.0091) and the lowest rate in treatment B (0.0553 ± 0.0002) and the lowest rate in treatment B (0.0105 ± 0.0003), but these differences were not significant (P>0.05). Moreover, in this research did not find a significant difference in APX activities of root or stems plants colonized with \textit{P. indica} (MR) and non- colonized with \textit{P. indica} (NMR) (Fig.6-7).

Ekmekci et al. (2009) showed APX activity significantly decreased as the highly toxic Pb level (8 mM Pb (NO$_3$)$_2$), 4H$_2$O in the one cultivar of maize while a significant increase observed in other cultivars, therefore, some cultivar of plants are more tolerant to Pb toxicity compared to other cultivars. The results of the present study suggested that APX not involved in the plant response to lead stress.

\textbf{Conclusions}

Some authors suggested that all the antioxidant enzyme activities (SOD, CAT, POX, and GR) in case of mycorrhizal plants were significantly higher than those of non-mycorrhizal plants at all concentrations of fungal inoculation could improve the antioxidant enzyme systems to alleviate destructive stress. A positive role of fungi in defense mechanisms under individual metal treatments has been reported (Bowler et al., 1992; Rivera-Becerrile et al., 2005). The results of our study showed that lead can affect the activity of antioxidant enzymes in tomato plant which most cases higher in roots compared to stems, but in this case, the impact of colonization by fungi was not significant and maybe additional researches are needed to understand effect of \textit{P. indica} in plant protection under Pb stress.
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