

Araştırma Makalesi/Research Article (Original Paper)

## ***Piriformospora indica* Promotes Growth and Antioxidant Activities of Wheat Plant under Cadmium Stress**

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**Abstract :** The influence of root endophytic fungus *Piriformospora indica* on growth, proline content, photosynthetic pigments and activity of antioxidant enzymes in wheat plant under 0.3 mM cadmium stress (Cd) was investigated. Cd exposure significantly reduced stem diameter, leaf area, chlorophyll *a*, carotenoid contents, activity of catalase (CAT) and ascorbate peroxidase (APX) in control plants, however, it notably increased malondialdehyde (MDA), proline and superoxide dismutase (SOD) activity. Inoculation of *P. indica* significantly increased the plant growth parameters, all photosynthetic pigments and proline contents. Also, the activity of CAT, APX and glutathione reductase (GR) were positively influenced by fungus treatment. Under the stress, Cd contents markedly increased in stem and root organs (particularly at roots) but its accumulation was significantly decreased at both organs by *P. indica*. Our results suggest that the increased concentrations of antioxidant enzymes, and pigments and proline contents found in *P. indica*-inoculated plants may serve to protect wheat plants against oxidative damage, enhancing Cd tolerance.

**Keywords:** Arbuscular mycorrhizal fungi, Cadmium stress, Wheat

### ***Piriformospora indica*'nın Kadmiyum Stresi altındaki Buğday Bitkisinin Büyüme ve Antioksidan Etkinliklerini Teşviki**

**Özet:** Bir kök endofitik fungusu olan *Piriformospora indica*'nın 0.3 mM kadmiyum (Cd) stresi altındaki buğday bitkisinin büyüme, prolin içeriği, fotosentetik pigmentler ve antioksidan enzim aktiviteleri üzerindeki etkisi araştırılmıştır. Cd'ye maruz bırakılma, kontrol bitkilerinde önemli ölçüde gövde çapı, yaprak alanı, klorofil *a*, karotenoid içeriği, katalaz (CAT) ve askorbat peroksidaz (APX) aktivitesini azaltmış; bununla birlikte, özellikle de malondialdehit (MDA), prolin ve süperoksit dismutaz (SOD) aktivitelerini de artırmıştır. *P. indica* inokülasyonu, önemli ölçüde bitki büyüme parametreleri, tüm fotosentetik pigmentleri ve prolin içeriğini artırmıştır. Ayrıca, KAT APX ve glutatyon redüktaz (GR) aktiviteleri de fungus uygulamasında pozitif şekilde etkilenmişlerdir. Stres altında Cd içerikleri, belirgin bir şekilde kök ve kök organlarda artmış (özellikle köklerde), ancak Her iki organda da Cd birikimi, *P. indica* tarafından önemli ölçüde azaltılmıştır. Elde edilen sonuçlar, *P. indica*-inoküle edilmiş bitkilerde bulunan antioksidan enzimler, pigmentler ve prolin içeriklerinin artan konsantrasyonlarının Cd toleransının güçlendirilmesinde, oksidatif hasara karşı buğday bitkileri korumak için hizmet edebileceğini düşündürmektedir.

**Anahtar kelimeler:** Arbusküler mikoriza fungusları, Kadmiyum stresi, Buğday

### **Introduction**

Cadmium (Cd) is a highly toxic trace pollutant for many organisms including plants (Pan et al. 2010). In plants, Cd interrupts numerous physiological and biochemical processes which lead to inhibition or reduction of growth (Xu et al. 2009; Dias et al. 2013). Cd stress, commonly induces the production of reactive oxygen species (ROS) in plant (Popova et al. 2009; Tuan et al. 2013) that are very toxic to the plant cells. These products (i.e ROS) easily interfere with many biomolecules including proteins, lipids, DNA and carbohydrates (Guo et al. 2009; Andresen & Küpper, 2013). In plants antioxidant activities such as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione

reductase (GR), glutathione, ascorbate and proline protect their cells against the damaging influence of ROS (Choudhury et al. 2013). Cd could bilateral effect on antioxidant activity and inhibit or stimulate influences have been usually reported from studies (Smeets et al. 2008; Islam et al. 2009).

*Piriformospora indica* is a root endophytic fungus that colonizes the roots of a wide variety of plant species and promotes their growth and tolerance against abiotic and biotic stresses (Sun et al. 2010; Ye et al. 2014). The activation of the antioxidant enzyme systems is the main target of *P. indica* in leaves of barley and Arabidopsis (Baltruschat et al. 2008; Vadassery et al. 2009; Murphy et al. 2014; Mandyam & Jumpponen 2014). Also in maize plants, growth and antioxidant capacity have been increased by *P. indica* (Kumar et al. 2009). The studies of Sun et al (2010) indicated that the *P. indica* induced drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes and reduction in MDA content (Sun et al. 2010).

The reports on the influence of *P. indica* in biochemical and physiological changes of various plants are scarce or null under Cd stress. Therefore, the present study focuses on the interaction of *P. indica* with wheat plants and its impact on growth, Cd contents of root and shoot, pigments and proline concentrations, and activity of antioxidant enzymes in *Triticum aestivum* cv. Sardari39 under Cd stress

## Material and Methods

### *Plant and fungal materials*

Wheat seeds (*Triticum aestivum* cv. Sardari39) were obtained from the Dryland Agricultural Research Institute, Maragheh, Iran, and were surface sterilized for 20 minutes in 1% NaClO, rinsed with distilled water five times, and germinated on wet filter paper in Petri dishes for 48 hours at 25 °C. *Piriformospora indica* was cultured in Petri dishes on a modified Kafer medium (Sherameti et al. 2005). The plates were placed in a temperature-controlled growth chamber at 25 °C for 2 weeks. The liquid culture was kept in shaker incubator at 100 rpm for 15 days at room temperature.

### *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 available P, 35 mg/kg available K, 1.8 mg/kg total Cd, having pH of 7.3 and 1.3ds/m EC. The soil samples were air-dried, sieved to pass 2 mm and was steam sterilized (100 °C for 1 h, three consecutive days) by autoclaving to eliminate native AM fungus propagules as well as other microorganisms. After sterilization, experiment soil (control) as well as experiment soil fortified with 0.3 mM Cd (as CdCl<sub>2</sub>) were incubated at 20 °C for one month allowing metal to distribute into various fractions and equilibrating with soil solid phase.

### *Planting and growth conditions*

The experiment was carried out under growth chamber conditions and consisted of a completely randomized 2 × 2 factorial design. Four replicate pots were filled with either 5 kg of one month old sterilized experiment soil (control) or fortified experiment soil with 0.3 mM Cd. The fungal treatments were: (1) inoculation of *Piriformospora indica* (50 ml of liquid culture), (2) non-inoculation. Non-endophytic fungus treatments received the same weight of autoclaved growth mixture. Fungal inoculum was placed 2 cm below wheat seeds at sowing time. 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<sup>-2</sup> s<sup>-1</sup>. Watering was done at 72 h intervals throughout the growth period using deionized water to near field capacity. Plants were harvested after 45 days. Roots and shoots of the harvested wheat samples were rinsed with tap water to remove soil particles and then carefully washed with deionized water. The samples then were dried by filter paper for growth analysis and then were dried in an oven at 70 °C for 48 h (to measure the shoot dry weights and Cd contents of roots and shoots). The root samples were stored in water for 1 h to study colonization and for biochemical analysis leaves samples were stored in liquid N<sub>2</sub> immediately.

#### *Cd determination*

The dried samples of finely ground (0.1 g) plant tissue were digested with a mixture (7:1, v/v) of HNO<sub>3</sub> and HClO<sub>4</sub> (Zhao et al. 1994). Cd concentrations in digested solutions were determined using an atomic absorption spectrophotometer (Shimadzu, Japan).

#### *Root colonization*

The percentage of fungal root length infection was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% Trypan blue in lactic acid (Philips and Hayman, 1970). The distribution of chlamydospores within the root was taken as an index of colonization (Sun et al. 2010).

#### *MDA, proline and pigments determination*

Malondialdehyde (MDA) was measured by the colorimetric method (Stewart et al. 1980). Proline was measured according to the method of Bates et al. (1973). Proline content was calculated from a standard curve. Photosynthetic pigments were determined according to photometric method given by Wellburn (1994).

#### *Enzyme extraction and activity assay*

For CAT, SOD and GR 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 pestle and mortar. Each homogenate was transferred to centrifuge tubes and was centrifuged at 4 °C for 15 min at 15000 g. For APX extraction, 2mM ascorbate and 5% polyvinylpyrrolidin added to enzyme extraction solution. The supernatant was used for enzyme activity assay (Esfandiari et al. 2007). SOD activity was estimated by recording the decrease in absorbance of superoxide-nitro blue tetrazolium complex by the enzyme (Sen Gupta et al. 1993). CAT activity was measured according to Aebi (1984). APX and GR activities were measured according to Yoshimura et al. (2000) and Sairam et al. (2002), respectively. The protein content was determined according to Bradford (1976) using bovine serum albumin as a standard.

#### *Statistical analysis*

The analysis of variance (ANOVA) was performed on all experimental data using SAS and GenStat 12 software. The differences between means were determined using Duncan's Multiple Range Test at 0.05 probability level.

## **Results**

The roots of wheat plants inoculated with *P. indica* were infected and Cd exposure did not effect on root colonization (Table 1). Cd exposure reduced shoot dry weight in the absence of *P. indica* compared to control, but this reduction was not significant (Table 1). Cd treatment significantly decreased stem diameter in the absence of *P. indica* and leaf area in the presence and absence of *P. indica* in comparison with control. Inoculation of *P. indica* significantly increased shoot dry weight and stem diameter under 0 and 0.3 mM Cd treatments, and leaf area under 0.3 mM Cd treatment compared to non-inoculated plants (Table 1). Natural content of Cd in soil of control pots (0 mM Cd) showed up only in the roots of both *P. indica*- inoculated and non-inoculated wheat plants. Cd treatment, however, significantly increased Cd contents in roots and shoots (Table 2). Inoculation of *P. indica* significantly increased root Cd, but reduced shoot Cd of plants grown under 0.3 mM Cd treatment compared to non-inoculated wheat plants (Table 2).

Table 1. Root colonization, shoot dry weight, stem diameter and leaf area in wheat subjected to four different treatments.

Added Cd status (mM)	<i>P. indica</i> status	Root colonization (%)	Shoot dry weight (g/plant)	Stem diameter (mm)	Leaf area (cm <sup>2</sup> )
0	Non- <i>P. indica</i> (C)	00.0 ± 0.0 b	0.40 ± 0.06 bc	1.92 ± 0.07 b	18.1 ± 0.65 a
0	<i>P. indica</i>	61.7 ± 1.2 a	0.68 ± 0.05 a	2.32 ± 0.05 a	19.3 ± 0.64 a
0.3	Non- <i>P. indica</i>	00.0 ± 0.0 b	0.29 ± 0.02 c	1.62 ± 0.05 c	7.6 ± 0.32 c
0.3	<i>P. indica</i>	61.2 ± 1.3 a	0.55 ± 0.08 ab	1.95 ± 0.05 b	12.0 ± 0.48 b

(C): Control plants. Values are mean ± SE; n = 4. The same letter within each column indicates no significant difference among treatments (P<0.05) using Duncan's Multiple Range Test.

Table 2. Cd contents in wheat shoot and root subjected to four different treatments.

Added Cd status (mM)	<i>P. indica</i> status	Shoot Cd (mg/kg DW)	Root Cd (mg/kg DW)
0	Non- <i>P. indica</i> (C)	00.00 ± 00 c	42 ± 3.0 c
0	<i>P. indica</i>	00.00 ± 00 c	51 ± 1.5 c
0.3	Non- <i>P. indica</i>	81.25 ± 3.5 a	4770 ± 140 b
0.3	<i>P. indica</i>	70.02 ± 2.4 b	5078 ± 126 a

(C): Control plants. Values are mean ± SE; n = 4. The same letter within each column indicates no significant difference among treatments (P<0.05) using Duncan's Multiple Range Test.

Table 3. Chlorophyll a, chlorophyll b, carotenoid and proline concentrations in wheat subjected to four different treatments.

Added Cd status (mM)	<i>P. indica</i> status	Chl. a (mg/g FW)	Chl. b (mg/g FW)	Car. (mg/g FW)	Proline (mg/g FW)
0	Non- <i>P. indica</i> (C)	6.16 ± 0.08 c	2.62 ± 0.14 c	1.49 ± 0.05 b	0.51 ± 0.01 d
0	<i>P. indica</i>	8.17 ± 0.06 a	4.43 ± 0.03 a	1.79 ± 0.04 a	0.84 ± 0.02 c
0.3	Non- <i>P. indica</i>	4.48 ± 0.08 d	2.34 ± 0.13 c	1.15 ± 0.06 c	1.03 ± 0.01 b
0.3	<i>P. indica</i>	7.00 ± 0.11 b	4.09 ± 0.07 b	1.58 ± 0.03 b	1.29 ± 0.08 a

(C): Control plants. Values are mean ± SE; n = 4. The same letter within each column indicates no significant difference among treatments (P<0.05) using Duncan's Multiple Range Test.

Table 4. MDA concentration and CAT, APX, SOD and GR activities in wheat subjected to four different treatments

Added Cd Status(mM)	<i>P. indica</i> status	MDA (nM/g FW)	CAT (U/mg Prot. Min)	APX (U/mg Prot. Min)	SOD (U/mg Prot.)	GR (Δ <sub>412</sub> /mg Prot. Min)
0	Non- <i>P. indica</i> (C)	176.5 ± 5.3 c	0.095 ± 0.002 c	0.27 ± 0.02 c	35.28 ± 1.0 b	0.66 ± 0.07 bc
0	<i>P. indica</i>	149.6 ± 2.6 d	0.118 ± 0.002 a	0.40 ± 0.01 a	44.39 ± 1.2 a	0.96 ± 0.13 a
0.3	Non- <i>P. indica</i>	302.7 ± 3.6 a	0.059 ± 0.002 d	0.16 ± 0.01 d	44.99 ± 2.3 a	0.43 ± 0.05 c
0.3	<i>P. indica</i>	210.1 ± 1.2 b	0.100 ± 0.001 b	0.34 ± 0.01 b	45.48 ± 0.7 a	0.69 ± 0.03 b

(C): Control plants. Values are mean ± SE; n = 4. The same letter within each column indicates no significant difference among treatments (P<0.05) using Duncan's Multiple Range Test.

Cd stress significantly decreased chlorophyll a and carotenoid concentrations in the absence of *P. indica*, whereas it significantly increased proline concentration in the presence and absence of *P. indica* compared to control (Table 3). In *P. indica*-inoculated plants, chlorophyll a, chlorophyll b, carotenoid and proline concentrations were significantly increased under 0 and 0.3 mM Cd treatments compared to non-inoculated plants (Table 3). Addition of Cd into the soil significantly increased MDA content in the presence and absence of *P. indica* compared to control (Table 4). In *P. indica*-inoculated wheats, MDA

content was significantly decreased at 0 and 0.3 mM Cd treatments (Table 4). In response to addition of Cd to soil, CAT and APX activities significantly decreased in the absence of *P. indica*, whereas SOD activity increased in the presence and absence of *P. indica*. Cd exposure did not markedly effect on GR activity. *P. indica*-inoculated plants had higher CAT, APX and GR activities in 0 and 0.3 mM Cd treatments and SOD activity in 0 mM Cd treatment than corresponding non-*P. indica* plants (Table 4).

## Discussion

Cadmium exposure reduced growth parameters including shoot dry weight, stem diameter and leaf area in agreement with reports by Lopez-Millan et al. (2009). Inoculation of *P. indica* increased shoot dry weight, stem diameter and leaf area in comparison with non-inoculated plants. Phytohormones like ethylene, auxin and cytokinin play a vital role behind the growth-promoting effects of *P. indica* (Vadassery et al. 2008; Shrivastava & Varma 2014). *P. indica* produces low quantities of auxins and relatively high rates of cytokinins, and the cytokinin levels are higher in colonized roots compared to the un-colonized roots of *Arabidopsis* (Vadassery et al. 2008). Production of these phytohormones helps root growth and contributes to beneficial effect on its host plant (Sirrenberg et al. 2007) and induces a systemic resistance to abiotic stresses (Deshmukh et al. 2006; Pozo et al. 2015). In addition, the beneficial effects for the plant such as increased shoot dry matter, stem diameter and leaf area can be a result of an improved nutrient supply by the root endophytic fungus.

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 (Ueno et al. 2008; Mori et al. 2009). It seems that wheat roots could accumulate Cd naturally occurring in the soil (Table 2). However, in the presence of *P. indica*, the Cd concentration was increased in root (about 10%), whereas it was decreased in shoot (about 10%). These results showed that chelation of Cd inside the fungus or adsorption of Cd to chitin in the fungal cell wall causes accumulation of Cd in root and prevents the Cd translocation from roots to shoots. It has been shown that entophytes possessing suitable degradation pathways or metal sequestration or chelation systems are able to increase host plant tolerance to presence of heavy metal, thereby helping their hosts to endure in contaminated soil (Weyens et al. 2009; Sharma et al. 2015). This finding suggests that *P. indica* has a positive influence in reducing shoots Cd contents. We have not found reports of increased or reduced Cd concentrations in plant tissues as a response to excessive soil Cd concentrations in *P. indica*.

The pigments concentrations were negatively affected by Cd exposure. Similar results have been obtained in other studies (Jiang et al. 2007; Chen et al. 2011; Yan et al. 2015). Toxic concentrations of heavy metals can degrade the activities of photosynthetic processes (enzymes and electron transport chain), resulting in decline of chlorophyll concentration (Thapar et al. 2008; Kataria et al. 2015). It was reported that cadmium caused a decline in carotenoid content (Thapar et al. 2008; Chen et al. 2011). In this study, the carotenoid content significantly decreased when wheat plants were exposed to Cd. Similar to Sun et al. (2010), our wheat plants inoculated with *P. indica* had higher chlorophyll a, chlorophyll b and carotenoid concentrations compared to plants without *P. indica*.

Proline acts as a source of carbon and nitrogen for rapid retrieval from the stress and as a stabilizer for plasma membrane and some macromolecules, and free radical scavenger (Saha et al. 2015) thereby, protecting the plants in stress conditions. In this study, Cd treatment significantly improved proline content in leaves of wheat plants. It could be suggested that free proline might play an important protective role against Cd stress and wheat cv. Sardari39 had the stronger self-protection capacity. Although metal-induced proline accumulation in plant tissues has been detected (Andrade et al. 2009; Fariduddin et al. 2009), reports on the effect of *P. indica* symbiosis in proline content are scarce under metal stress conditions. Proline contents in leaves of *P. indica*-inoculated plants showed a more pronounced increase in response to Cd in soil when compared to non-inoculated homologues indicating the possible role of proline in Cd toxicity response in *P. indica*-colonized plants which showed higher mass accumulation than non-*P. indica* plants.

Increasing MDA that mainly formed by the degradation of polyunsaturated lipids (Del Rio et al. 2005), in Cd-treated wheats clearly demonstrated that the plants were exposed to oxidative stress. The rate of MDA was significantly lower in *P. indica*-inoculated plants than control treatment and thus root endophytic

fungus could alleviate the stress via prevent or retard the degradation of these lipids by preventing excess ROS formation. SOD catalyses the dismutation of  $O_2^-$  to  $H_2O_2$ , CAT dismutates  $H_2O_2$  to oxygen and water, and APX reduces  $H_2O_2$  to water by ascorbate as specific electron donor (Gara et al. 2003). A significant reduction in CAT and APX activities of wheat plants suggest that detoxification of  $H_2O_2$  by these enzymatic antioxidant is not sufficient under Cd stress. The study revealed a significant increase at SOD activity which imply its induction to quench higher levels of  $O_2^-$  generated due to Cd stress. The results in agree with Garnier et al. (2006) in tobacco plant. They found the plant cells under Cd stress results in rapid  $O_2^-$  generation that lead to oxidative damage. The presence of *P. indica* significantly increased CAT, APX, SOD and GR activities in wheat plants. The data from our study are consistent with previous reports that activation of the antioxidant enzymes is a major target of this fungus in leaves (Vadassery et al. 2009). According to Waller et al. (2005) report, induction of GR activity was observed during the *P. indica*–barley plant interaction, and it was suggested that the higher GR activity maintains an enhanced level of reduced glutathione which is involved in maintaining antioxidant capacity. The current investigation suggests that *P. indica* could contribute to the detoxification of  $H_2O_2$  and  $O_2^-$  by increasing the activities of CAT, APX and SOD under Cd stress.

## Conclusions

The present work provides insights into the impact of *P. indica* in physiological and biochemical response to Cd toxicity. *P. indica*-inoculation had a positive effect on growth and physiology of wheat plants, decreasing the concentrations of leaf MDA and Cd contents of shoot, and increasing antioxidant enzymes, and pigment and proline contents in wheat leaves. The beneficial effects of the root endophytic fungus, observed in this study suggest that the promotion of this symbiotic association could aid plants to cope with high Cd conditions in soils.

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