University of Mazandaran

Journal of Genetic Resources
J Genet Resour 2018;4 (1): 44-55
http://sc.journals.umz.ac.ir
doi: 10.22080/jgr.2018.14168.1102

Iranian Biology Society

The Cadmium Toxicity in *Helianthus annuus* can be Modulated by Endosymbiotic Fungus (*Piriformospora indica*)

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Received: 10 October 2017 Accepted: 16 November 2017

Abstract

Cadmium (Cd), as a widespread metal pollutant, readily accumulates in the food chain due to its easy absorption and high mobility. The endophytic fungi are cosmopolitan microorganisms that occur widely in association with plants in a heavy metal stress environment. A current pot experiment was conducted to evaluate the influence of *Piriformospora indica*, as a root endosymbiotic fungus, on the biomass and biochemical responses of sunflower cv. Zaria under excessive Cd concentrations (0, 40, 80 and 120 mg Cd/kg) in the soil, in a 2 × 4 factorial randomized block design in five replicates. In response to increasing Cd levels in soil, root colonization, growth parameters, and total chlorophyll and carotenoids contents were significantly reduced ($P < 0.05$), whereas root and leaf Cd accumulation, malondialdehyde (MDA) amount and the antioxidant enzymes activities of catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX) and superoxide dismutase (SOD) were increased ($P < 0.05$). Under different levels of Cd in soil, presence of *P. indica* (in inoculated sunflowers) had a significant increase ($P < 0.05$) on growth rate, photosynthesis pigments content, root Cd accumulation, and activities of CAT, POD, APX and SOD in compare to absence of *P. indica* (in non-inoculated ones). Also, *P. indica*-inoculated plants showed a reduced MDA concentration and leaf Cd accumulation than un-inoculated sunflowers. The results indicated that *P. indica*, as an appropriate fungal association, can improve tolerance of sunflower to Cd toxicity through increased levels of photosynthesis pigments and antioxidants, and reduced Cd accumulation and MDA content of the leaf. Therefore, this root endophyte can be modulated cadmium toxicity in *Helianthus annuus* under excessive cadmium in soil, and recommended as a complement crop-growing strategy in the fields under Cd-contaminated soils, for other studies.

Key words: Antioxidant enzyme assay; Cadmium pollution; *Piriformospora indica*; Stress mitigation; Sunflower

Introduction

The contamination of heavy metal in soil is one of the major environmental concerns due to high persistence and non-degradability nature of heavy metal and their harmful effect on human health through the food chain pollution (Valko *et al.,* 2007). Among the heavy metals, cadmium (Cd) is considered as a non-essential element that is toxic for all organisms including plants, animals, and human. It is continuously accumulated in soil through natural and anthropogenic activities such as weathering of metal-rich rocks, exceeding the use of phosphate fertilizers, and the use of sewage sludge and Cd-contaminated water for field watering (Zoffoli *et al.* 2013). Cd is taken up easily by roots and translocated to edible parts of plants due to its higher solubility and mobility (Groppa *et al.* 2012). Even at low amounts, Cd is toxic to most crops. Chlorosis, leaf rolling and stunting are the major visible symptoms of Cd toxicity (Benavides *et al.* 2005). Crops exposure to Cd stress causes many physiological disorders like the decline in respiration and photosynthesis, and large changes in the contents of reactive oxygen

species (ROS; such as superoxide anion radical, hydrogen peroxide, hydroxyl radical and singlet oxygen) and antioxidant enzyme activities (Maksymiec and Krupa 2006; Lin *et al.* 2007). Accumulation of ROS leads to peroxidation of membrane lipids and oxidative damage in plant cells.

Some soil beneficial fungi like endophytic fungi play an important impact in improving plant growth and yield and participate in stress tolerance of host plants and bioremediation of heavy metal polluted soils (Aishwarya *et al.,* 2014). In 1992, a root endophytic fungus was introduced with similarities to arbuscular mycorrhizal fungi (AMF) namely *Piriformospora indica* belongs to *Sebacinaceae* family and Hymenomycetes class that was easily cultivable, lacks host specificity and interacts with many different plant species (Varma *et al.,* 2001). This fungus stimulates growth and yield of a plant, promotes nutrient uptake, allows plants to survive under water, temperature and salt stress, and confers resistance to toxins, heavy metal ions and pathogenic organisms (Varma *et al.,* 2001).

Among crop plants, sunflower is one of the most important oleaginous crops in terms of cultivated area and oil production in some parts of the world (Cornu *et al.,* 2016). Sunflower, as a crop that produces a high biomass and extensive root system, is capable to grow on metal-contaminated soils (Awotoye *et al.,* 2009). The Cd problem is the main concern in sunflower since it accumulates more Cd than other cereals such as bread wheat and maize (Broadley *et al*., 2001; Grant *et al.,* 2008). Furthermore, sunflower is a suitable candidate for Cd extraction from Cd-contaminated soils, so that it produces the great biomass along with a high Cd accumulation (Faessler *et al.,* 2010; Yu *et al.,* 2011). The present work was conducted to investigate the potential role of endosymbiotic fungus *P. indica* in modulating growth, leaf pigments, lipid peroxidation, related antioxidant enzymes of Cd-induced oxidative stress and Cd accumulation in sunflower cv. Zaria plants under excessive Cd concentrations in soil.

Materials and methods

Preparation of materials

The seeds of sunflower (*Helianthus annuus* cv. Zaria) were obtained from the Dryland Agricultural Research Institute, Maragheh, Iran. Seeds were surface sterilized for 2 min in ethanol followed by 10 min in a 1% NaCl solution, then washed with distilled water several times. The sterilized seeds were germinated on wet filter paper at 4 °C for 48 h in order to synchronize germination. *P. indica* was cultured in Petri dishes on a Hill & Käfer medium (Hill and Käfer, 2001). The fungal samples were located in a temperature-controlled growth chamber at 29±1 °C in dark for 14 days.

For soil preparation, surface soil sample (0–25 cm depth) was collected from the uncontaminated farmland of Maragheh University Campus and then was physicochemically analyzed. Soil characteristics are shown in Table 1. After air-drying for 10 days, the soil was sieved through a 2-mm mesh, and then autoclaved-sterilized with steam at 100 °C for 1 h (3 times in 3 consecutive days) in order to eliminate indigenous AMF and other microorganisms. After this, four Cd concentrations (0, 40, 80 and 120 mg Cd/kg soil) were added to the soil and mixed thoroughly with soil samples. The soil samples then were incubated at 20 °C for 30 days in order to Cd distribution into various fractions of soil and metal stabilization in soil solid phase. Cd treatment was supplied as cadmium dichloride ($CdCl_2$). All chemicals and reagents used in this study were of reagent grade and purchased from Sigma-Aldrich Co. and Merck Ltd.

Table 1. Experimental soil properties.

Characteristics	Units	Value
Sand	%	68
Silt	%	20
Clay	%	12
EC	ds/m	1.3
pH	-	7.3
Organic matter	%	1.2
Total N	mg/kg	0.05
Available P	mg/kg	7
Available K	mg/kg	35
Total Cd	mg/kg	0.7

Experimental set-up

A 4×2 factorial randomized block design including two *P. indica* treatments (with or

without fungus inoculation) and 4 Cd levels (0, 40, 80 and 120 mg Cd/kg soil) was conducted in five replicates. Two uniform cultivated seedlings were transferred into each plastic pot (27 cm in diameter, 32 cm in height), which filled with 5 kg of sterilized sandy soil that contained four added Cd concentrations. Two fungal plugs of 10 mm in diameter was placed at a distance of 1 cm below the sunflower seedlings in the soil at sowing time. To provide similar conditions, non-*P. indica* treatments received an equivalent amount of autoclaved *P. indica* inoculum. Pots then were kept in a greenhouse at 28±2/18±2 °C day/night cycle, 60–70% relative humidity and a photoperiod of 14 h (14 h in the day and 10 h at night). The experimental pots were watered using deionized water once every three days to near field capacity. After 35 days of planting, sunflowers were harvested by cutting the shoots at the soil surface, and the roots were carefully separated from the soil. The shoots and roots were rinsed with distilled water, wiped with tissue paper and weighted (plant fresh weight: shoot fresh weight + root fresh weight). Then, plant height was determined (plant length: shoot length + root length), and finally dried at 75 °C for 48 h in order to determine the dry weights (plant dry weight: shoot dry weight + root dry weight) and Cd concentrations. Furthermore, some fresh samples of leaves were immediately stored in liquid nitrogen for the assay of chlorophyll, carotenoids and MDA contents, and antioxidant enzymes activities. The root subsamples were kept in 50% ethanol for assessment of root colonization.

Estimation of root colonization

To monitor the percent of colonization, the sampled roots were thoroughly washed in tap water, cut into 1 cm root pieces and were softened with a 10% (w/v) KOH solution at room temperature, and finally stained with 0.05% (v/v) Trypan blue in lactic acid according to Phillips and Hayman (1970) method. Under a light microscope, the chlamydospores distribution within the roots of *P. indica*-inoculated plants was estimated as an index for root colonization (Oelmüller *et al.,* 2009).

Estimation of Cd concentration

The method of Zhao *et al.* (1994) was used for determination of Cd amounts. The dried samples of root and leaf (0.1 g) were digested in an acid oxidative mixture of HNO_3 and $HClO_4$ (7:1 ratio, v/v). The contents of Cd in digested transparent solutions, after filtering using Whatman No. 42 filter paper, were measured by an atomic absorption spectrophotometer (Shimadzu, Japan).

Measurement of total chlorophyll (Chl) and carotenoids contents

Total Chl (Chl *a* + Chl *b*) and carotenoids contents in the youngest fully expanded leaves (0.1 g) was extracted by 80% acetone, centrifuged at 4000 rpm for 20 min, and then the optical density of the supernatant was read at 663, 645 and 480 nm wavelengths for Chl *a*, Chl *b* and carotenoids, respectively (Arnon, 1967).

Measurement of malondialdehyde (MDA)

The level of leaf MDA, a lipid peroxidation product, was analyzed by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Absorbance was recorded at 600 nm and 532 nm (The blank was 1% thiobarbituric acid in 20% trichloroacetic acid). The level of MDA was determined by an extinction coefficient of 155 mM^{-1} cm^{-1}.

Extraction and assay of antioxidant enzyme activities

Frozen leaf tissue (0.5 g) was powdered by a pre-chilled mortar and pestle, and homogenized in 5 mL ice-cold extraction buffer (50 mM potassium phosphate, pH 7.0, 4% polyvinylpyrrolidone). The extract was centrifuged at 14.000 g for 30 min at 4 °C. The supernatant was used for assays of the activities of antioxidant enzymes. Total protein concentration in the leaf extracts was determined according to the Bradford method (Bradford, 1976) with bovine serum albumin as the standard. The superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by measuring SOD ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated at 560 nm,

according to Dhindsa and Matowe (1981). One SOD unit was considered as the amount of enzyme required to cause 50 % inhibition of the photochemical reaction of NBT. Catalase (CAT, EC 1.11.1.6) activity was assayed spectrophotometrically at 240 nm by Aebi (1984) method. The hydrogen peroxide decomposition rate was monitored for 1 min at 25 ºC. The activity of guaiacol peroxidase (POD, EC 1.11.1.7) was assayed according to Chance and Maehly (1955). The reaction of guaiacol oxidation was initiated by addition of H_2O_2 and the increase in absorbance was read at 470 nm. The activity of APX (EC 1.11.1.1) was assayed by Nakano and Asada method (1981). The reaction of ascorbic acid oxidation was started by addition of H_2O_2 and the decrease in absorbance was read at 290 nm. SOD, CAT, POD and APX activities were expressed as unit mg^{-1} protein.

Statistical analysis

Statistical analysis of all experimental data was carried out using Duncan's Multiple Range Test with two-way analysis of variance (ANOVA) by SAS 9.4 software version 2013. The variance was related to the main factors (*P. indica* and Cd levels) and to the interaction between them. Data were expressed as the means of replicates ± standard deviation (SD).

Results

Root colonization and plant biomass

Roots of non-inoculated plants did not establish colonization with *P. indica* fungus (Fig. 1). In the presence of *P. indica*, by increasing Cd concentration in the soil, root colonization (ranged from 39.7% to 60.8%) was significantly ($P < 0.05$) decreased and the lowest level of root colonization was observed under 120 mg Cd in the soil (Fig. 1). In both colonized and non-colonized sunflowers with *P. indica*, the growth parameters including plant length, plant fresh weight, plant dry weight and leaf number per plant were noticeably reduced ($P < 0.05$) *by* increasing Cd levels in the soil so that the highest concentration of Cd produced the lowest values of these growth parameters (Fig. 2A-D). The presence of endophytic fungus significantly increased ($P < 0.05$) the growth parameters at 0, 40, 80 and 120 mg Cd/kg soil compared to non-inoculated sunflowers at the same Cd levels. *P. indica*-colonized plants illustrated higher plant length by 1.7-1.9 times, plant fresh weight by 1.5-5.3 times, plant dry weight by 2.1-7.5 times and leaf number per plant by 1.3-2.5 times than those of non-colonized ones at different Cd levels in soil (Fig. 2A-D).

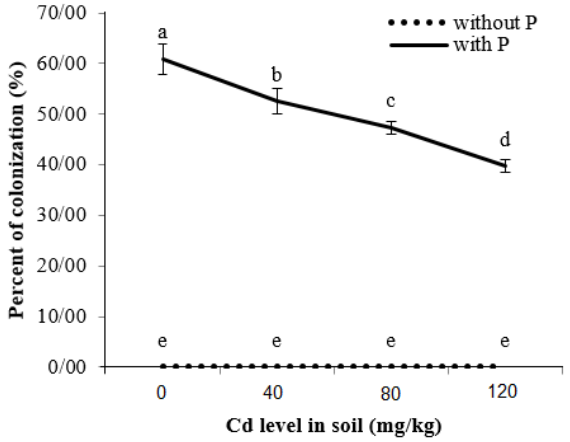

Fig. 1. The effect of different concentration of Cd in soil on root colonization of sunflower: P: *P. indica*. Values are mean ± SD; n = 5; the same letter indicates no significant difference among treatments using Duncan's Multiple Range Test at $P < 0.05$.

Pigments contents

In non-colonized and *P. indica*-colonized plants, in response to increasing external Cd in the soil, total Chl and carotenoids amounts were significantly decreased ($P < 0.05$) so that the largest Cd concentration in soil produced the lowest contents of total Chl and carotenoids in sunflower leaves (Fig. 3A and B). In compare to non-inoculated plants, fungus-inoculated sunflowers showed greater contents of total Chl by 1.14-, 1.33-, 1.12- and 1.19-fold under 0, 40, 80 and 120 mg Cd/kg soil, respectively (Fig. 3A). Also, inoculation of *P. indica* caused an increase ($P < 0.05$) on leaf carotenoids content by 1.22-, 1.22-, 1.29- and 1.19-fold at 0, 40, 80 and 120 mg Cd, respectively, in relation to non-inoculated ones (Fig. 3B).

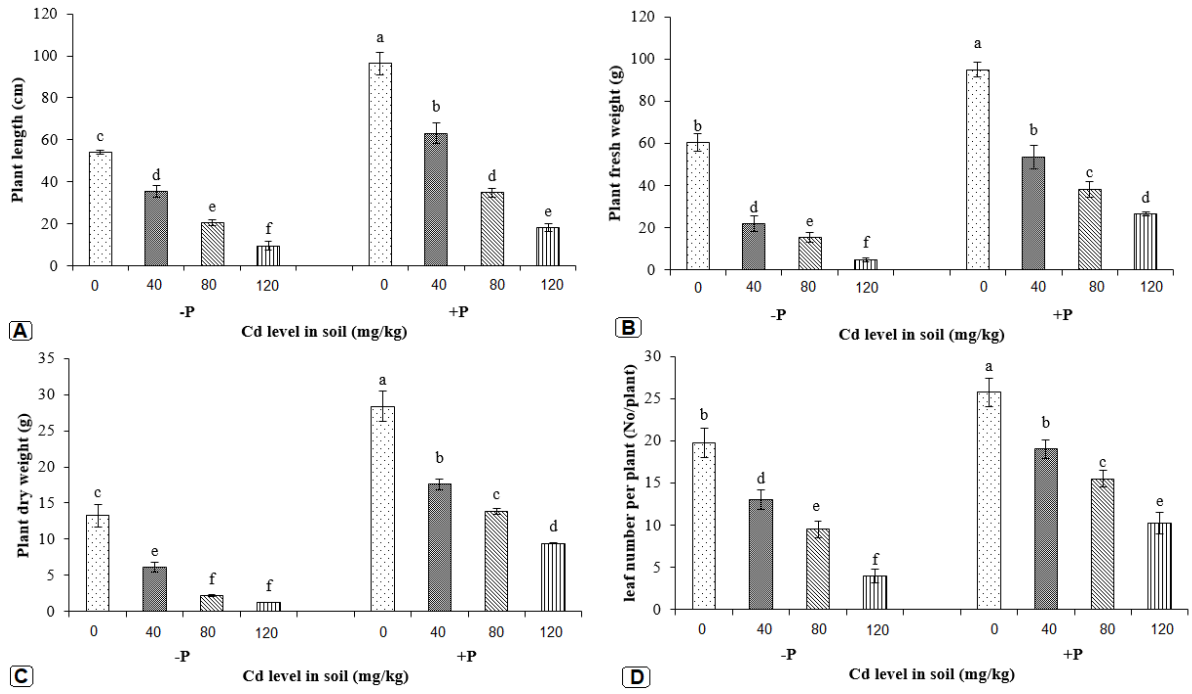

Fig. 2. The effect of *P. indica* on A) plant length; B) plant fresh weight; C) plant dry weight and D) leaf number per plant under different Cd concentrations in sunflower. P: *P. indica*. Values are mean ± SD; n = 5. The same letter within each column indicates no significant difference among treatments using Duncan's Multiple Range Test at $P < 0.05$.

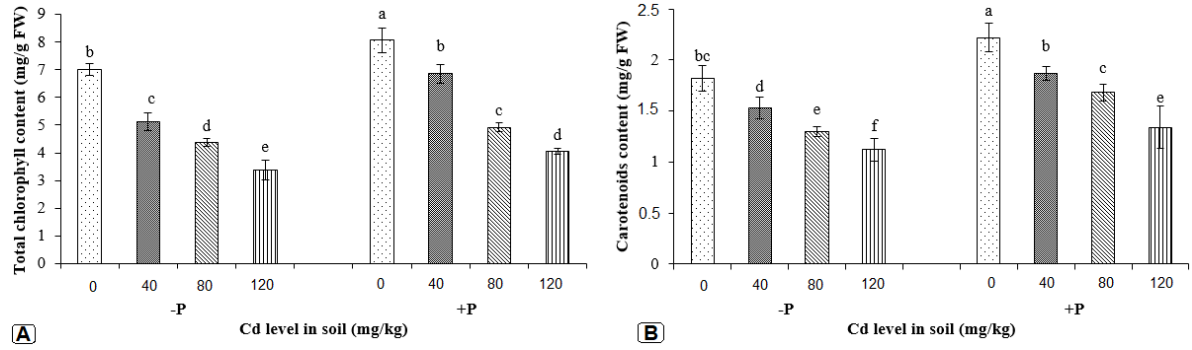

Fig. 3. The effect of *P. indica* on A) total chlorophyll and B) carotenoids contents in sunflower under different Cd concentrations in soil. P: *P. indica*. Values are mean ± SD; n = 5. The same letter within each column indicates no significant difference among treatments using Duncan's Multiple Range Test at $P < 0.05$.

Cd amounts in root and leaf

According to our previous work (Shahabivand *et al.,* 2017), data from Fig. 4A and 4B showed that Cd augmentation in soil significantly elevated ($P < 0.05$) root and leaf Cd in both the absence and presence of the endophytic fungus. The largest concentration of Cd in soil (120 mg Cd/kg soil) caused maximum accumulation of Cd in root (813.9 mg Cd/kg DW) and leaf (130.87 mg Cd/kg DW) of non-inoculated plants, also in root (931.3 mg Cd/kg DW) and leaf (105.6 mg Cd/kg DW) of inoculated ones with *P. indica* (Fig. 4A and B). The presence of root endophyte remarkably increased ($P < 0.05$) Cd accumulation in root but decreased Cd content in leaf with the excess of Cd in the soil (Fig. 4A and B). In *P. indica*-inoculated sunflowers, the

increases for root Cd amounts were by as much as 47.8, 10.9, 15.2 and 14.4% at 0, 40, 80 and 120 mg Cd/kg soil, respectively, related to non-inoculated plants. On the other hand, in inoculated plants, the reductions in leaf Cd amounts were 34.9, 34.3, 25 and 19.4% under 0, 40, 80 and 120 mg Cd/kg soil, respectively, in relation to un-inoculated ones (Fig. 4A and B).

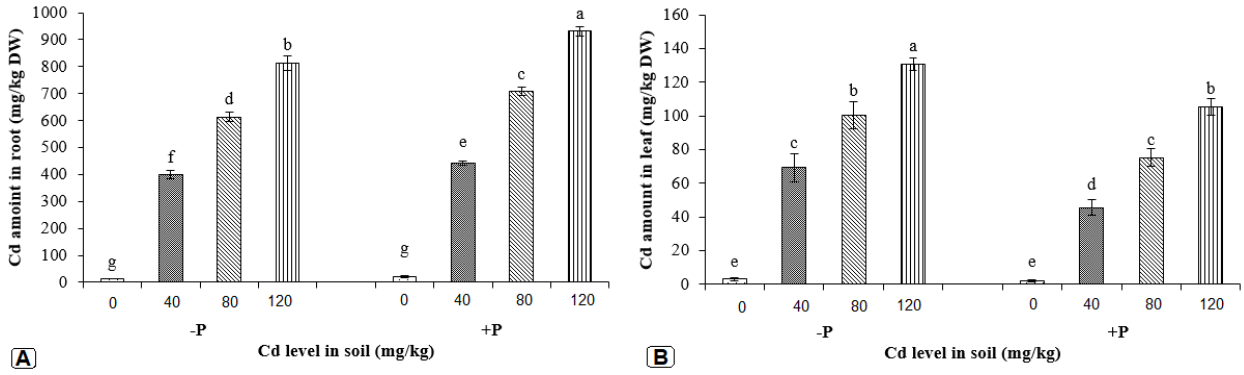

Fig. 4. The effect of *P. indica* on A) root Cd and B) leaf Cd accumulation in sunflower under different Cd concentrations in soil. P: *P. indica*. Values are mean ± SD; n = 5. The same letter within each column indicates no significant difference among treatments using Duncan's Multiple Range Test at $P < 0.05$.

MDA concentration and antioxidant enzymes activities

In response to increasing Cd levels in soil, MDA concentration in sunflower cv. Zaria leaf (in both non-colonized and colonized ones) was significantly elevated ($P < 0.05$), in contrast, in response to fungal inoculation, MDA amount was notably decreased ($P < 0.05$) by 21.5, 23.3, 20.2 and 23% under 0, 40, 80 and 120 mg Cd, respectively, compared to non-colonized plants (Fig. 5).

The addition of Cd to soil and *P. indica* association resulted in alteration in enzymatic antioxidants levels in sunflower cv. Zaria. In both non-inoculated and fungus-inoculated sunflowers, by increasing soil Cd levels, an increase in CAT and POD activities were observed, but this increase on CAT activity from 0 to 40 mg Cd in non-inoculated and from 40 to 80 mg Cd in fungus-inoculated plants, and on POD activity from 0 to 40 and from 80 to 120 mg Cd in non-inoculated ones was significant ($P < 0.05$; Fig. 6A and B). In general, fungal plants exhibited higher CAT and POD activities in leaves than their non-fungal homologous. However, presence of *P. indica* caused a significant elevation ($P < 0.05$) on POD activity by 39% at 0 mg Cd in soil, but had no significant enhancement on CAT and POD activities under other Cd levels in soil (Fig. 6A and B).

In non-inoculated and inoculated sunflowers with fungus, by increasing Cd levels in soil, APX activity was increased (especially in non-colonized ones) except at 40 mg Cd/kg soil in inoculated ones, but this increasing tendency was significant ($P < 0.05$) only from 0 to 40 mg Cd in non-colonized plants (Fig. 6C).

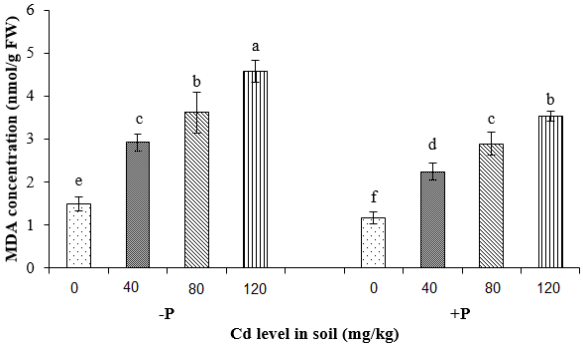

Fig. 5. The effect of *P. indica* on leaf MDA concentration in sunflower under different Cd concentrations in soil. P: *P. indica*. Values are mean ± SD; n = 5. The same letter within each column indicates no significant difference among treatments using Duncan's Multiple Range Test at $P < 0.05$.

On the other hand, the presence of *P. indica* caused a significant elevation ($P < 0.05$) in APX activities by as much as 66% and 21.1% under 0 and 40 mg Cd/kg soil, respectively than those un-inoculated plants under the same Cd levels in soil (Fig. 6C). APX activity was approximately similar in *P. indica*-colonized and non-colonized plants under 80 and 120 mg Cd in soil. In the case of SOD activity and in non-inoculated plants, the increase in the soil Cd from 0 to 40 mg Cd did not significantly influence on SOD activity, but increasing soil Cd from 40 to 80 significantly elevated ($P < 0.05$) SOD activity and then from 80 to 120 mg Cd, this value was constant (Fig. 6D). Also, in fungus-inoculated plants, Cd augmentation in the soil had no significant influence on SOD activity and these treatments had approximately similar values (Fig. 6D).

SOD activity of sunflower leaves was significantly influenced by fungal inoculation so that the presence of *P. indica* enhanced SOD activity by as much as 44.9%, 44.2%, 23.5% and 20.3% at 0, 40, 80 and 120 mg Cd/kg soil, respectively than non-inoculated ones under the same soil Cd concentrations (Fig. 6D).

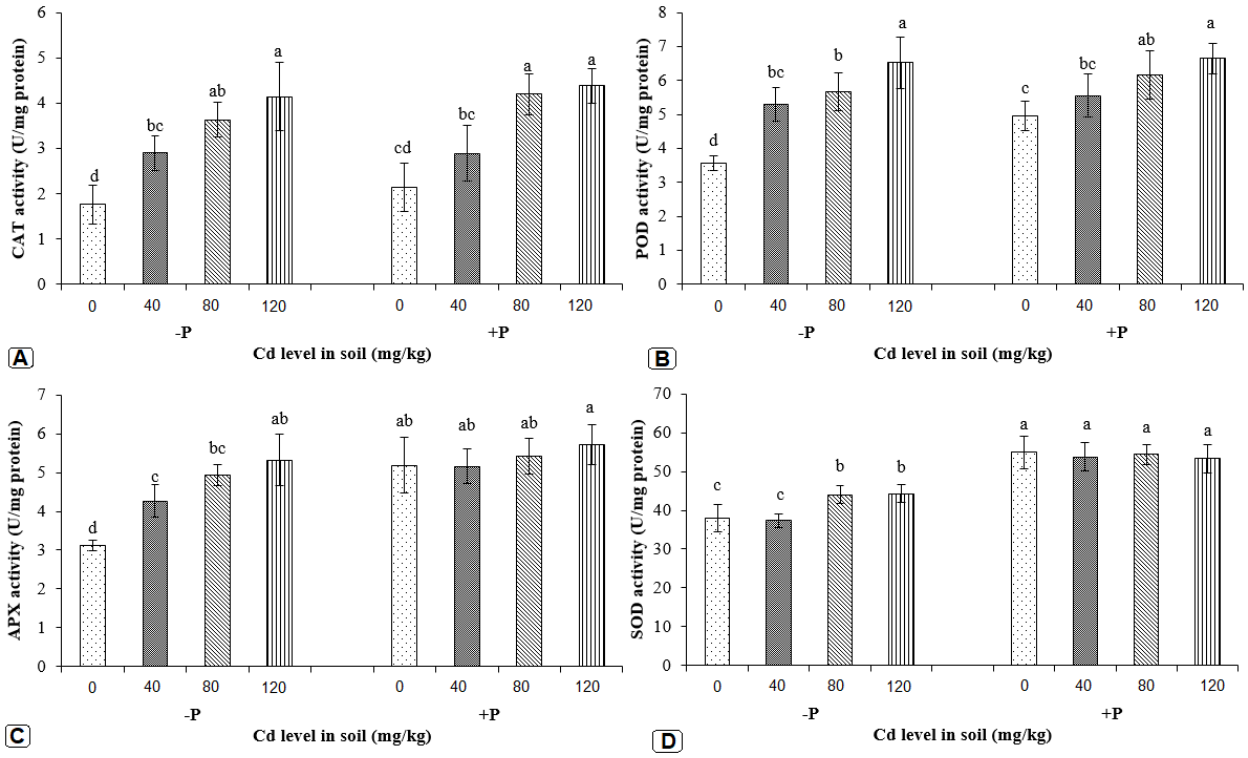

Fig. 6. The effect of *P. indica* on enzyme activities of A) CAT; B) POD; C) APX and D) SOD in sunflower leaves under different Cd concentrations in soil. P: *P. indica*. Values are mean ± SD; n = 5. The same letter within each column indicates no significant difference among treatments using Duncan's Multiple Range Test at $P < 0.05$.

Discussion

It has been well known that association of different plants with beneficial soil microorganisms such as mycorrhizal and endophyte fungi modifies plant response to heavy metal stress, resulting in enhanced tolerance in heavy metal polluted soils (Göhre and Paszkowski, 2006; Shahabivand *et al.,* 2012). In this study, both symbiotic partners including endophytic fungus and sunflower cv. Zaria plants exhibited relative tolerance to excessive soil Cd up to 120 mg/kg soil, indicating that both two symbionts had partly an inherent tolerance or a low sensitivity to Cd toxicity. *P. indica* successfully interacted with sunflower roots, resulting in efficient colonization. Even though with increasing soil Cd levels, a reduction in root colonization was

observed, but *P. indica* formed was still functional.

Shahabivand *et al.* (2012) and Shahabivand *et al.,* 2017 found a reduction in *P. indica* colonization with increased soil Cd contamination in wheat and sunflower plants. It can be said that Cd toxicity has influenced chlamydospore germination and hyphal growth of *P. indica*, resulting in a decline on colonization of root by this endophyte.

The results from the present study demonstrate that Cd exposure resulted in reduced growth indicators including plant length, plant fresh weight, plant dry weight and leaf number per plant. This decline in growth parameters was probably occurred due to a remarkable increase in Cd amounts in root and leaf (Fig. 4), enhanced level of MDA (Fig. 5) and decreased light-harvesting pigments (Chl and carotenoids) contents (Fig. 3) under various Cd levels in the soil. Furthermore, according to Chaoui and Ferjani (2005), and Bashri and Prasad (2015) findings, Cd stress reduced growth parameters due to depletion of auxin which happened due to increased activity of auxin degrading enzyme. Sunflower plants inoculated with *P. indica* clearly showed an overall higher biomass and other growth parameters than those non-inoculated ones under different soil Cd concentrations. Similar results were achieved by the researchers in heavy metal-stressed crops such as tobacco and wheat (Shahabivand *et al.,* 2012; Hui *et al.,* 2015). These results support the plant growth-promoting impact of this root endophyte and its role in mitigation of adverse effect of Cd ions under Cd toxicity in soil. It has been showed that *P. indica* can be modulated the phytohormones involved in plant growth such as auxins and cytokinins. Also, this fungus enhanced uptake of macro- and micronutrients in the host (Sirrenberg *et al.,* 2007; Hartley and Gange, 2009; Padash *et al.,* 2016).

Heavy metals have a harmful effect on the physiology of plants, which can result in a reduction on the content of photosynthetic pigments, and damage to the process of photosynthesis (Borišev *et al.,* 2012). Under Cd stress, chlorophyllase enzyme activity goes up, resulting in the reduced biosynthesis of photosynthetic pigments as well as their degradation (Singh and Prasad, 2014). Besides, Cd is known to downregulate the expression of genes involved in the synthesis of enzymes of chlorophyll biosynthesis (Qian *et al.,* 2009). In this work, Cd treatments at different used doses reduced the Chl and carotenoids contents in both the presence and absence of *P. indica*, but we observed that the Cd caused more serious damage to the non-colonized sunflowers and *P. indica* can mitigate Cd negative effect on pigments content. An increase in photosynthetic pigments in colonized plants with this endophyte under heavy metal stress was also observed by Hui *et al.* (2015) in tobacco and Padash *et al.* (2016) in lettuce. With respect to *P. indica* impact on the enhancement of nutrient elements (Padash *et al.,* 2016), it is likely that this endophytic fungus has increased magnesium (Mg) uptake, as an important part of the chlorophyll molecule. These results suggest the positive influence of this plant-fungus beneficial interaction on the photosynthetic apparatus of sunflower cv. Zaria plants under Cd toxicity.

However, AMF is known to have a significant impact on the uptake and accumulation of heavy metals in host plants, but the information on the effects of endophytic fungi (such as *P. indica*) inoculation on this field is presently limited. Data from our study showed that Cd amount in the roots of sunflower was higher than soil Cd concentration, demonstrating that Cd uptake mechanism for roots, where might occur via available members of the ZIP transporter (Lin and Aarts, 2012), is an active process in sunflower cv. Zaria. Also, root accumulated remarkably more Cd than leaf under different Cd concentrations in soil. Rascio *et al.* (2008) showed that a little amount of the total Cd accumulated in roots translocated to shoot. These results demonstrated that sunflowers cv. Zaria tends to avoid toxicity in the physiologically most active parts (i.e. leaves to support the vital process of photosynthesis) by decreasing Cd translocation to the above-ground parts. In comparing to non-colonized sunflowers, colonized plants with *P. indica* had a higher accumulation of Cd in the root, but a lower Cd amounts in leaves. This finding is consistent with previous researchers' observations in wheat, tobacco and sunflower where they showed an increased Cd accumulation in root and reduced Cd accumulation in leaf under *P.*

indica inoculation (Shahabivand *et al.,* 2012; Hui *et al.,* 2015; Shahabivand *et al.,* 2017). The fungal cell surfaces contain polymers such as chitin and chitosan that are an abundant source of functional groupsan like carboxyl, hydroxyl, amino and phosphate that act as binding sites for the adsorption of certain heavy metals. These results indicated that chelation and sequestration of Cd ions inside the fungus or adsorption of Cd to the polymers via functional groups in the fungal cell wall resulted in agglomeration of Cd in root and reducing the mobility of heavy metal from root to leaf of sunflowers.

The ROS production degrades polyunsaturated lipids in cellular membranes (peroxidation of lipids that causes the loss of membrane integrity), as a result forming MDA where is used as a biomarker to the evaluation of oxidative stress level and its magnitude (Del Rio *et al.,* 2005). In this work, MDA accumulation in Cd-treated sunflowers clearly demonstrated that these plants were exposed to heavy metal stress. Elevated MDA levels as a result of Cd exposure was also observed in two kenaf varieties (Li *et al.,* 2013), sunflower plants (Saidi *et al.,* 2014) and tobacco (Hui *et al.,* 2015). These results demonstrated that antioxidant enzymes and other non-enzymatic antioxidants for combating excessive generated ROS are not a sufficient defense system under higher Cd levels in the soil. Data from Fig. 5 illustrated that MDA concentrations in leaves were notably lower in fungus-inoculated sunflowers in compare to control plants, therefore *P. indica* could partially counteract Cd stress response. The presence of *P. indica* inhibited MDA accumulation in leaves of Chinese cabbage under drought stress (Sun *et al.,* 2010). Also, Hui *et al.* (2015) reported that leaf MDA amount in both *P. indica*-inoculated and un-inoculated tobacco plants was increased with the increase in the concentration of Cd, but the quantity in inoculated plants was lower than in un-inoculated ones. Based on these results, we can say that this fungus could inhibit or retard the formation of MDA by preventing excess ROS generation, probably directly or indirectly via the regulation of antioxidants.

The cells have evolved a variety of defense systems based on both water-soluble and lipid soluble antioxidants, and on antioxidant enzymes (Mohadjerani *et al.,* 2016). In order to the support of this reason, i.e. whether *P. indica* can alter antioxidant enzymes activities in sunflower cv. Zaria under Cd toxicity, we focused on four classes of antioxidant enzymes including CAT, POD, APX, and SOD. SOD, as a metalloenzyme in various cellular organelles, detoxifies superoxide radicals and produces H_2O_2 and O_2 in the first line of defense because superoxide radicals can act as a precursor to other ROS. CAT is located in the peroxisomes, cytosol, and mitochondria, and decomposes H_2O_2 to H_2O and O_2. POD detoxifies H_2O_2, organic hydroperoxides or lipid peroxides to produce alcohols. APX catalyzes the H_2O_2-dependent oxidation of ascorbate as an electron donor. In the present work, by increasing Cd concentration in the soil the activities of antioxidant enzymes were enhanced. Gill and Tuteja (2010) resulted that under various stress conditions, plant stress tolerance may be improved by an enhancement in the in-vivo levels of antioxidant enzymes, resulting in scavenged ROS and avoiding the oxidative stress. John *et al.* (2009) in mustard, Hui *et al.* (2015) in tobacco and Khan *et al.* (2017) in *Solanum nigrum* also showed a gradual elevation in the activity of antioxidant enzymes as Cd concentration increased. With respect to this fact that most of the antioxidant enzymes are regulated post-translationally under oxidative stress (Sun *et al.,* 2010), we evaluated the activity level of antioxidant enzymes instead of the expression of the gene of antioxidants in response to *P. indica* inoculation under Cd toxicity. Also in our previous study in wheat, we suggested that *P. indica* can function more via enhancing enzyme activity than inducing transcription of antioxidant genes under cadmium stress (Shahabivand *et al.,* 2016). Data summarized in Fig. 6A-D demonstrated that presence of *P. indica* conferred protection, and reduced the harmful influence of oxidative stress caused by Cd, as evidenced by enhanced activity of four important enzymatic antioxidants i.e. CAT, POD, APX, and SOD. *P. indica*-induced increases in the activity of antioxidant enzymes have been identified in wheat (Shahabivand *et al.,* 2016) and in tobacco (Hui *et al.,* 2015) under Cd exposure. It is known that activation in antioxidant enzyme systems is the main target of *P. indica* in leaves (Baltruschat *et al.,* 2008). The further enhancement in the activities of

enzymatic antioxidants suggests the impact of *P. indica* in mediating quick scavenging of ROS induced by Cd, thus strengthening the plant's defense system. Nevertheless, more information is needed to determine the contribution of *P. indica* to the antioxidative system of Cd toxicity.

In conclusion, our findings demonstrated that Cd exposure at higher levels could significantly affect the growth rate as the plant length and biomass, photosynthesis pigments, lipid peroxidation and antioxidant enzymes activity in important crop sunflower. Inoculation with *P. indica* improved plant tolerance to Cd stress by lowering Cd accumulation and MDA amount in leaf, and enhancing its pigment content and antioxidant enzymes activity, consequently alleviating the Cd-induced oxidative stress. As regards, *P. indica*, unlike AMF, can easily reproduce on a large scale in the absence of a host, there seems to be needed to demonstrate the effectiveness of the extensive application of this root endophyte fungus on mitigating excessive Cd effects by sunflower under the field conditions.

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