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Running Title: PHYTOREMEDIATION OF CADMIUM

Mutualistic Fungus *Piriformospora indica* Modulates Phytoremediation Property of Host Plant via Concerted Action of Enzymatic and Non-enzymatic Biochemicals

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ABSTRACT

Soils and ecosystems contaminated with cadmium (Cd) threaten human health, and adversely affect morphological, physiological, and biochemical parameters of plants. Symbiotic association of endophytic fungi with their host plants is the best strategy to improve various plant characteristics and remediate soils polluted with heavy metals (HM). Being a well-known plant growth-promoting fungus, *Piriformospora indica* confers resistance against a number of abiotic stresses including heavy metals. This pot experiment explores the potential and ameliorative effects of *P. indica* on *Artemisia annua* L. plants treated with different concentrations (0, 40, 80 and 120 mg/kg) of Cd. *P. indica* significantly increased plant performance especially, by enhancing chlorophyll content, water potential and by decreasing electrolytic leakage as compared with un-inoculated plants despite of high Cd levels. Similarly, *P. indica* enhanced antioxidant enzymes activities, thereby, reduced the drastic effects of Cd in inoculated plants. Also, *P. indica* accumulated Cd in roots of colonized plants as revealed by atomic absorption spectroscopy and restricted Cd translocation to aerial parts. Furthermore, *P. indica* showed resistance (up to some level) *in vitro* condition to Cd stress, however, fungus growth was inhibited at very high Cd concentrations, proving it an excellent candidate for the use as a potential phytoremediator in fields affected with cadmium contamination. The transcriptional analysis showed that the signaling genes, artemisinin and flavonoids biosynthesis pathway genes were significantly up-regulated in *P. indica*-co-cultivated plants as compared with un-inoculated plants, suggesting a fine corroboration of primary and secondary metabolism, to modulate resistance capacity and to enhance the phytoremediation capability of *A. annua* against cadmium toxicity.

***Key Words*:** *Artemisia annua*; Cadmium; Endophytic fungi; *Piriformospora indica*; Transcripts
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INTRODUCTION

To fulfill the needs of the human population which is growing exponentially, urbanization, extensive mining, industrialization and intensive agriculture have accelerated, consequently threatening natural resources and causing environmental contamination on large scale (Wan *et al.*, 2012). As a severe threat to natural resources, heavy metal(loid)s (HM) are the main cause of environmental contaminations which are non-biodegradable, highly mobile, persistent in nature and having a number of life-threatening effects (Dong *et al.*, 2001; Huang *et al.*, 2015). Soil is the main medium on which plants grow and contamination of this medium with HM have adverse and sometimes lethal effects (direct/indirect) on human health (Dong *et al.*, 2001; Yousaf *et al.*, 2016). Soil contamination with HM is mainly due to rapid industrial development which is a burning issue around the globe (Ifthikar *et al.*, 2017). Additionally, HM once accumulate in the plant, especially in edible parts, beyond a certain level can cause a number of diseases such as bone health, cardiovascular, neurological systems, nervous, renal and several other disorders (Jolly *et al.*, 2013).

Like other HM, Cd is found ubiquitous in nature and can accumulate in the organisms, subsequently interrupt the metabolic processes (Iqbal *et al.*, 2016; Rehman *et al.*, 2017). Although the presence of Cd in the soil is in trace amount, however, due to a number of activities (both natural and man-made) including urbanization, industrialization, mine exploration and extensive application of pesticide in agricultural lands are increasing rapidly its concentration in the environment (Prapagdee *et al.*, 2013). On one hand, being a carcinogen and widespread pollutant (Haschek *et al.*, 2013) Cd affect the human health while on the other hand it affects the quality and safety of important staple crops such as maize (Liu *et al.*, 2018) and rice (Li *et al.*, 2016). In addition, once incorporated into the plant cell, Cd interfere with various physiological (photosynthesis, respiration, chlorophyll synthesis, enzyme activity and plant growth and nutrients uptake) and biochemical processes (Muradoglu *et al.*, 2015). Furthermore, Cd have a negative impact even at very low concentration on plant reproductive and vegetative organs (DalCorso *et al.*, 2010). Additionally, being a DNA destabilizer, Cd also retard roots and shoots' growth, cause nutrient imbalances, chlorosis and leaf withering and biomass reduction (Zhang *et al.*, 2010).

Plants have evolved a number of strategies to reduce the toxicity of HM and cope with other adverse environmental conditions (Luo *et al.*, 2017); these strategies include metal chelation, synthesis of metal binding proteins and compartmentalization (Nahar *et al.*, 2015; Xu *et al.*, 2017). *Artemisia annua* L., an important member of the family Asteraceae has been dragged into main stream research after the rediscovery of artemisinin, potent antimalarial compound produced by the plant (Pandey and Pandey-Rai, 2014) *A. annua* was used in current experiment because of its phytoremediation potential and even several metals have been shown as a stimulator for artemisinin biosynthesis (Rai *et al.*, 2011; Kumari *et al.*, 2017). In addition, many species of *Artemisia* such as *Artemisia herba, Artemisia vulgaris, Artemisia princeps, Artemisia aucheri* etc. have also been extensively reported with metals hyperaccumulation capacity (Ok and Kim, 2007; Rebele and Lehmann, 2011; Vahedi, 2013; Rebhi *et al.*, 2019).

Unfortunately, HM not only reduce the growth of non-hyperaccumulating plants but also that of hyperaccumulators, thereby restricting the potential (phytoextration) of such plants (Rajkumar *et al.*, 2010). Therefore, the development of other strategies for detoxification of soils contaminated with HM are necessary. Mycorrhizal association is considered to be the most effective strategy to reduce and or alleviate heavy metals phytotoxicity (Hashem *et al.*, 2016). Microbe-assisted phytoremediation is a possible and eco-friendly substitute improving the efficiency and HM tolerance level of plants (Rajkumar *et al.*, 2012). Being a relatively new approach, association of endophytic fungi with their host plants can enhance various plant characteristics including phytoremediation potential (Mei and Flinn, 2010). In addition, endophytes can enhance host plant growth by a number of mechanisms, for example, chemical and morphological changes in the plant tissues

triggered by different endophytes not only affect nutrients' composition but also play a key role in plant protection against various biotic and abiotic stresses (Singh *et al.*, 2011). Among these endophytes, *Piriformospora indica* is a well-known beneficial root endophytic fungus of the order Sebacinales which can confer resistance against both biotic and abiotic stresses. *Piriformospora indica* reside in the root cortex of host/inoculated plant in the form of pear-shaped chlamydospores (both intra-and intercellular), enhance plant growth by accelerating water and nutrients' uptake (Varma *et al.*, 2012). Furthermore, *P. indica* association with its host plant alleviates the detrimental conditions caused by desiccation, acidity and heavy metal toxicity (Yadav *et al.*, 2010). Also, *P. indica* has been extensively reported ameliorating heavy metal stress in various plants such as rice (Mohd *et al.*, 2017) *Cassia angustifolia* (Nanda and Agrawal, 2018), tobacco (Hui *et al.*, 2015), wheat and sunflower (Shahabivand *et al.*, 2012; Shahabivand *et al.*, 2017). Therefore, this study was carried out (i) to evaluate the effect of *P. indica* on physiological, biochemical and molecular responses of *Artemisia annua* L. grown under Cd stress; (ii) to determine the response/capability of *P. indica* to Cd stress (*in vitro*) (iii) to evaluate the ameliorative effect of *P. indica* on transcriptional regulation of artemisinin biosynthesis and flavonoid biosynthetic pathway genes under Cd stress and (iv) to evaluate the effect of *P. indica* on Cd uptake.

MATERIALS AND METHODS

In vitro analysis of P. indica for Cd stress

In order to analyze the growth/tolerance of *P. indica* under different concentrations of Cd stress, *P. indica* was grown on petri dishes having Hill and Kaefer agar medium supplemented with 0, 0.01, 0.05, 0.1, 0.2, 0.3, 0.6 and 0.9 mM Cd. Plates were incubated for 20 days in dark (30 ^{o}C) in incubator. Fungus growth/tolerance (on surface medium) was analyzed by measuring the radius of growing hyphae (from the center towards plate edges) at different intervals i.e. 5^{th}, 10^{th} ,15^{th} and 20^{th} day of fungal inoculation.

Artemisia annua seedlings and growth conditions

After surface sterilization (for detail see (Khalid *et al.*, 2020)), *A. annua* seeds were sown in plastic trays filled with substrate of known composition (pH 7.32, EC (dS/m) 0.14, Avail. N (ppm) 111.6, Avail. P (ppm) 181.7, Avail. K (ppm) 181.7, CEC (cmol(+)/kg) 306.8, NH_4^+ (ppm) 7.86, NO_3^- (ppm) 2.67, Total C (%) 1.92, Total N (%),0.19, Total K (ppm) 2063) and sand (autoclaved) in a ratio 3:1 (w/w). After 21 days, uniform seedlings were shifted to plastic pots filled with the same substrate contaminated one week before with different concentrations (0, 40, 80 and 120 mg/kg) of Cd with/without *P. indica* (Shahabivand *et al.*, 2017). The treatments are: control (normal and un-inoculated); normal and inoculated; stressed and inoculated; and stressed and un-inoculated. Plants were harvested (30 days after shifting) and fresh samples, mostly leaves, were immediately used while also kept at -80 ^{o}C for further experiments. Three biological replicates were used for all samples.

Culture of P. indica and plant inoculation

Piriformospora indica was obtained from 'Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)'. *P. indica* was cultured at 28 ^{o}C for 10 days in Kafer agar medium (Hill and Kafer, 2001). The fungus was also cultured in modified Kafer liquid medium. Briefly, a 4-5 mm fungal plug from 10-days-old agar medium was inoculated in 200 ml conical flasks having 50 ml Kafer broth medium. The flasks were incubated in a shaking incubator for 15 days at 120 g at 28 ^{o}C. Artemisia seedlings were inoculated with the fungus from the liquid medium.

Root colonization assay

Root colonization assay was carried out following previously described method (Phillips and Hayman, 1970; Dickson and Smith, 1998) with slight modification using typhan blue kit (Sangon Biotech Shanghai Co., Ltd.).

Determination of cadmium concentration in plant parts

Dry samples (both leaves and roots, 0.2 g) were used for Cd determination. Roots and leaves of both inoculated and treated and un-inoculated treated plants were digested in $HCLO_4/HNO_3$ (1:4, v/v). The digested mixture was further extracted using 5 ml HNO_3 and adjusted to the final volume of 250 ml of ddH_2O. Inductively coupled plasma spectrometry (ICP, Thermo Fisher, ICAP7600, USA) technique was used for determination of Cd concentration in root and shoot.

Assessment of photosynthetic pigment and osmotic stress responses

Chlorophyll content was measured spectrophotometrically at 645 and 663 nm following a previously reported method (Hiscox and Israelstam, 1979). Electrolyte leakage was measured as described elsewhere (Lutts *et al.*, 1996). Fresh leaves (detached from the same position) were used for each treatment. Briefly, after washing with deionized water to remove (if any) electrolytes adhered with the surface, leaf samples were kept in closed vials filled with deionized water (15 ml). After incubation (25 ^oC for 24 h) period, the electrical conductivity of the solution (L_t) was determined. While the electrical conductivity (L_o) was measured by autoclaving the samples for 20 min at 120 ^oC.

Electrolyte leakage (%) = $(L_t / L_o) \times 100$

For leaf relative water content (LRWC) measurement, fresh leaves were weighed immediately to get fresh mass (FM). Leaf samples were placed in petri dishes filled with distilled water in order to determine turgid mass (TM) (Smart and Bingham, 1974). After turgid mass determination, oven dried samples (85 ^oC overnight) were used for dry mass (DM) measurement. Leaf relative water content was calculated using the values of DM, FM and TM (Khalid *et al.*, 2018).

LRWC (%) = FM – DM ÷ TM – DM

Proline, H_2O_2, MDA and antioxidant enzymes assays

Proline accumulation was determined spectrophotometrically at 520 nm using ninhydrin method (Bates *et al.*, 1973). Under acidic condition, toluene was used as a blank while for standardization process, purified proline was used and expressed as µmol per gram fresh weight. H_2O_2 accumulation was measured by following a previously described method (Junglee *et al.*, 2014) while malondialdehyde (MDA) content was measured using Thioarituric acid (TBA) protocol (Bao *et al.*, 2009). Different enzyme's activities were assayed by spectrophotometric analysis using kits (Nanjing Jiancheng Biotechnology Institute). After samples preparation, the activities of peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) were measured at 420, 405 and 550 nm, respectively. Likewise, the activities of ascorbate peroxidase (APX) and glutathione reductase (GR) were also measured at 340 and 290 nm, respectively.

Determination of phenolic compound, phenolic acid and flavonoid content

Total phenolics were determined using Folin-Ciocalteau reagent by adopting previously described method (Singleton *et al.*, 1999). In order to determine total phenolic acid, 1 ml sample was mixed with Arnov reagent, 1 ml 1 M NaOH, 1 ml 0.5 ml HCL and 5 ml water (Szaufer-Hajdrych, 2004). The amount of distilled water was reached to a final volume (10 ml). Absorbance was measured for phenolic acid and phenolic compound at 725 and 490 nm, respectively. Phenolic acid was expressed as caffeic acid µg/g fresh weight while

phenolic compound was expressed as gallic acid equivalent in mg/g of fresh weight. For total flavonoid determination, Lamaison and Carnet (Lamaison and Carnet, 1990) method was used. Flavonoid content was measured spectrophotometrically at 430 nm.

HPLC analysis of artemisinin and flavonoid content

Artemisinin was extracted from oven dried (50 ºC for 72 h) leaf samples. Leaf powder (0.1 g) was used by adjusting ultrasonic processor at 30 ºC for 30 min. After that, the sample was centrifuged at 12, 000 rpm for 10 min and the supernatants were filter through 0.25-µm filter. HPLC analysis was carried out using a Waters Alliance 2695 HPLC system (Milford, USA). An earlier described method was used for HPLC analysis of processed samples (Lu *et al.*, 2013). Flavonoids content from all samples (treated and untreated) were extracted and measured with high performance liquid chromatography (HPLC). An earlier described method was used for polyphenol extraction (Złotek *et al.*, 2014). Acidified methanol (0.1 M HCl, 15 ml 50% v/v) was used to macerate leaf samples for 20 min at 25 ºC and centrifuged at 9000 rpm for 30 min. Supernatant was collected (after repeated the procedure at least three times) and further evaporated under a vacuum till dryness at 40 ºC. The extract was prepared by adding methanol (100%) to the final volume of 10 ml and HPLC was carried out (Guo *et al.*, 2005). Phenolic compounds such as gallic acid, rutin trihydrate, hydroxycinnamic acids, quercetin, syringic acid, kaempferol, ferulic acid, chlorogenic acid and luteolin were used as standards.

Molecular analysis

Total RNA was extracted (at least 2 times) form treated and untreated leaf samples using RNA extraction kit (TIANGEN, RNAprep pure plant kit). RNA quality and quantity were checked via agarose gel electrophoresis and nanodrop spectrophotometer, respectively. Normal PCR was also carried out to amplify the studied genes and bands were checked through agarose gel electrophoresis. Complementary DNA (cDNA) libraries were constructed (prime script RT reagent kit, Takara) and q-PCR was carried out to check the expression level of the selected genes of different pathways. House-keeping gene (actin) was used as internal control while $2^{-\Delta\Delta CT}$ method was used to analyze q-PCR data (Table S1).

Statistical analysis

All the analytic determinations were carried out at least three times, and results are expressed as mean ± SD of triplicate samples. Data were statistically analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range (DMR) tests (SPSS Inc., Chicago, IL, USA). Differences were denoted statistically significant at $P<0.05$.

RESULTS

In vitro studies of P. indica revealed high tolerance to heavy metal

The exposure of *P. indica* to different level of cadmium concentration showed that *P. indica* was capable of surviving in various level of Cd toxicity (0.01, 0.05,0.1, 0.2, 0.3 and 0.6mM concentrations). The maximum hyphal growth in terms of radius was shown by *P. indica* (3.93 ± 0.11cm) within15 days in uncontaminated control condition and in 0.01mM Cd stress condition (3.7 ± 3.9 cm). However, the hyphal growth gradually decreased with the increase of Cd concentration (Fig. 1, A -B). Cd concentrations 0.05, 0.01 and 0.2 restricted the fungal growth to 2.63 ± 0.27, 1.76 ± 0.20 and 1.3 ± 0.1 cm respectively after 20 days of hyphal growth measurement in petri plates. However, the hyphal growth dramatically decreased to 1.13 ± 0.15 and 1.03 ± 0.15 cm in 0.3 and 0.6 mM Cd stressed condition. While, no growth was recorded in medium having 0.9 mM Cd concentration (Fig. 1, A -B).

Fig 1 Effects of different concentrations of cadmium on the growth of *P. indica* (A-B) *in vitro*. *P. indicia* growth in terms of hyphal extension from the center towards plate edges under different Cd concentrations (0 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.6 mM and 0.9 mM) (A), Analysis of the radius of fungal hyphae after 5, 10, 15 and 20 days of inoculation (B). The data is the mean values of three biological replicates with ± standard error.

A. annua root colonization, P. indica/symbiotic development assay

Roots of *A. annua* grown in Cd fortified soil inoculated or un-inoculated with *P. indica* were stained with Typhan blue staining kit (Sangon Biotech Shanghai Co., Ltd.) and evaluated under microscope along with respective control plants. Successful roots colonization by beneficial fungus *P. indica* was prominently detectable in inoculated plants in the form of mycelia and mature piriform shaped chlamydospores while un-inoculated plants did not show any structure of mycelia in its primary and secondary roots. Thus, the presence of mature chlamydospores in the roots of inoculated plants evidenced the successful colonization by *P. indica* (Fig. 2).

Fig. 2 Colonization of *P. indica* in roots of *Artemisia annua* plants **A)** Control, **B**) Chlamydospores inside the root cells. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of Cd stress (40, 80 and 120 mg/kg fortified soil). Bars represent the means of replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test.

Cd accumulation in inoculated and un-inoculated A. annua roots

The influence of *P. indica* and Cd treatments on accumulation of Cd in leaf and root were prominent but there was no significant difference in the leaf and root Cd content in inoculated and un-inoculated plants under control condition. A significant increase of Cd content was observed in root and shoot of plants, with increasing Cd concentration in both *P. indica* inoculated and un-inoculated plants but the shoot Cd concentration was much lower in inoculated plants comparatively to un-inoculated plants (Fig. 3). Overall, the trend of Cd concentration in all Cd treated plants was root >shoot, in inoculated or un-inoculated experimental plants. The highest level of given Cd concentration (120 mg Cd/kg) induced the maximum accumulation of Cd in root (521.06 ± 5.55) and shoot (325.8 ± 13.67) in inoculated plants, also in root (376.5 ± 10.33) and shoot (445.6 ± 6.66) in un-inoculated plants (Fig. 3). With the excess of soil Cd concentration, *P. indica* co-cultivation significantly increased accumulation of Cd in root but decreased its distribution towards shoot. In *P. indica* inoculated plants, the increase of root Cd accumulation was 1.57, 1.23 and 1.28-fold higher at 40,80 and120 mg/kg fortified soil, respectively, comparatively to un-inoculated plants. While, *P. indica* co-cultivated plants, the shoot accumulated less Cd by 0.57, 0.72 and 0.73-fold under 40,80 and120 mg/kg fortified soil, respectively, in comparison with un-inoculated plants. Overall, results showed that *P. indica* altered the distribution of Cd from root to shoot at whatever Cd level applied by reducing its accumulation in shoot as compared with root (Fig. 3).

Fig 3 Cadmium concentration in *Artemisia annua* root (A) and shoot (B); *Artemisia annua* plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated control. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of Cd stress (40, 80 and 120 mg/kg fortified soil). Bars represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test.

Osmotic tolerance indices and photosynthetic performance

Data from table 1 shows the effect of *P. indica* and Cd exposure on electrolyte leakage, relative water and chlorophyll content in plants. In *P. indica* co-cultivated and un-inoculated plants, the electrolyte leakage was increased while relative water and chlorophyll content were prominently reduced with increasing the soil Cd concentration so that the highest Cd concentration (120 mg/kg) induced the higher electrolyte leakage and lowest

relative water and chlorophyll content in the leaves of *A. annua* plants (Table 1). There was no significant difference in the electrolyte leakage in plants with no Cd treatment while the relative water and chlorophyll content were enhanced by *P. indica* co-cultivation in control plants, comparatively. Furthermore, *P. indica* co-cultivation reduced the electrolyte leakage in all Cd treated plants as compared with un-inoculated plants by 0.65, 0.68 and 0.59 fold under 40,80 and120 mg/kg, respectively. *Piriformspora indica* co-cultivation elevated the relative water content under normal condition by 1.05 fold while under Cd stressed conditions (40,80 and120 mg/kg) relative water content was increased by 1.12, 1.15 and 1.04, respectively. Like that, *P. indica* co-cultivation increased the chlorophyll content by 1.48, 1.42, 1.25 and 1.08 folds with 0, 40,80 and 120 mg/kg treatments, respectively (Table 1).

Table 1. Effects of cadmium on electrolyte leakage, relative water content (%) and chlorophyll content in *Artemisia annua* plants. Plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated controls. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of Cd stress (40, 80 and 120 mg/kg fortified soil).

Treatments	Electrolyte leakage	Relative water content (%)	Total chl content
0 mg/kg Cd			
P.indica	6.6 ± 0.3e	63.81 ± 2.61a	370.21 ± 34.68
Un-inoculated	6.5 ± 0.09e	60.62 ± 1.68b	250.11 ± 10.31
40 mg/kg Cd			
P.indica	6.83 ± 0.83e	52.72 ± 4.98c	352.27 ± 20.21
Un-inoculated	10.4 ± 0.63c	46.81 ± 1.59d	246.54 ± 35.14
80 mg/kg Cd			
P.indica	8.4 ± 0.54d	49.4 ± 1.55d	280.74 ± 52.21
Un-inoculated	12.32 ± 0.7b	42.81 ± 2.41e	223.81 ± 25.21
120 mg/kg Cd			
P.indica	10.01 ± 0.92c	41.43 ± 1.12e	240.25 ± 30.47
Un-inoculated	16.69 ± 0.51a	39.72 ± 3.13e	220.78 ± 74.21

Within each column, means followed by the same letter do not differ significantly at $P < 0.05$ by Duncan's test.

The activity of Proline, MDA, H_2O_2 and antioxidant enzymes assays

In order to check the influence of *P. indica* co-cultivation on plant anti-oxidant enzymes system after exposure of plants to different levels of Cd stress conditions, activities of some antioxidative enzymes were also evaluated. Proline and MDA contents of the leaf were same as that of un-inoculated plants under normal condition. The proline content was increased with increasing Cd concentration in both inoculated and un-inoculated plants but its concentration was prominently higher in inoculated plants than un-inoculated ones: by 1.31, 1.07 and 1.12 folds under 40,80 and120 mg/kg treatments, respectively (Fig.4). On the other hand, the level of MDA content was reduced by *P. indica* co-cultivation to 1.25, 1.17 and 1.30 folds of that in the un-colonized plant leaves when the Cd concentration was 40,80 and120 mg/kg soil. This showed that, the level of membrane damage and extent of lipid peroxidation was less in *P. indica* co-cultivated *A. annua* plants. There was no significant difference in H_2O_2 concentration in all given treatments except the highest Cd concentration (120 mg/kg) in which *P. indica* induced significantly lower concentration of H_2O_2 by 1.20 fold than un-inoculated ones. Control and low concentrations of Cd of both inoculated and un-inoculated plants exhibited no prominent differences in APX content (Fig. 4). However, *P. indica* co-cultivation caused significantly higher APX content (1.06 fold) as compared with un-inoculated plants.

Like that, the level of SOD, POD, GR and CAT were increased steadily with increasing Cd concentration but their activities were prominently higher in inoculated plants than un-inoculated plants (Fig. 5). In contrast to SOD, the POD, GR and CAT activities were not affected by the low-level concentration of Cd (40 mg/kg)

assayed. Figure 4 portrays that presence of *P. indica* significantly enhanced the level of SOD by 1.40, 1.21 and 1.09 folds when Cd concentration was 40, 80 and120 mg/kg, respectively. On the other hand, a significant enhancing effect was observed for POD by 1.12 and 1.24 folds under 80 and120 mg/kg Cd stress in soil, respectively (Fig. 5). The same increment was detected for GR by 1.06 and 1.08 folds in inoculated plants under 80 and120 mg/kg soil Cd level comparatively with un-inoculated stressed plants, respectively. Likewise, *P. indica* co-cultivation imparted a significant increase in CAT activity level by 1.23 and 1.19 folds under 80 and120 mg/kg Cd soil as compared with un-inoculated plants, respectively (Fig. 5). Thus, from the above data it can be suggested that *P. indica* co-cultivation with *A. annua* can ease the damage caused by the heavy metal.

Fig. 4 Proline (A), MDA (B), H_2O_2 (C), APX concentrations (D); *Artemisia annua* plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated control. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of cd stress (40, 80 and 120 mg/kg fortified soil). Bars represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test.

Fig. 5 Antioxidant enzymes concentrations (A-D); *Artemisia annua* plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated control. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of cd stress (40, 80 and 120 mg/kg fortified soil). Bars represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test.

Flavonoids content

In the present study, the secondary metabolites in *A. annua* (especially the antioxidants including total phenolic compounds, phenolic acids and flavonoids content) were also determined via spectrophotometry and HPLC under given treatments. Spectrophotometric based analysis showed that total phenolic compounds, phenolic acids and flavonoids content were significantly augmented in Cd treated plants in both *P. indica* inoculated and un-inoculated plants but the quantity of these antioxidants were lower in un-inoculated plants than inoculated plants (Fig. 6). HPLC analysis indicated that *P. indica* co-cultivation caused the elicitation of some major flavonoids including ferulic acid, luteolin, syringic acid, chlorogenic acid, quercetin, kaempferol, as compared with control and Cd stressed (40, 80 and 120 mg/kg) plants. Moreover, the level of luteolin, quercetin and kaempferol elicitation were observed at a higher degree in *P. indica* co-cultivat ed plants than that of un-inoculated ones in either natural control conditions or after exposure to different level of Cd (Table 2).

Fig. 6 Phenolic compounds (A-D); *Artemisia annua* plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated control. Plants were cultivated in the absence of cd for the entire experiment (0 mg/kg) or were subjected to three levels of Cd stress (40, 80 and 120 mg/kg fortified soil). Bars represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test.

Table 2. Effects of cadmium on phenolic compounds in *Artemisia annua* plants. Plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated control. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of Cd stress (40, 80 and 120 mg/kg fortified soil).

Treatments	Ferulic acid	Luteolin	Syringic acid	Rutin trihydrate	Chlorogenic acid	Quercetin	Kaempferol	Hydroxycinnamic acids	Gallic acid
0 mg/kg									
P. indica	2.71 ± 0.12e	0.34 ± 0.02de	3.39 ± 0.134d	7.29 ± 4.77bc	9.38 ± 0.58e	2.87 ± 0.20de	3.38 ± 0.33b	2.3 ± 1.38b	3.34 ± 0.37ab
Un-inoculated	1.36 ± 0.041f	0.17 ± 0.04e	2.44 ± 0.011e	1.77 ± 0.65e	5.64 ± 3.01e	1.17 ± 0.11e	1.27 ± 0.16cd	8.43 ± 5.9a	12.43 ± 0.16ab
40 mg/kg									
P. indica	8.43 ± 0.21a	0.51 ± 0.21d	5.40 ± 0.076b	9.26 ± 0.14b	141.5 ± 19.8c	9.29 ± 2.28a	4.30 ± 0.10a	ND	2.59 ± 0.04b
Un-inoculated	5.38 ± 0.45c	0.18 ± 0.02e	2.50 ± 0.232de	4.42 ± 0.50de	110.5 ± 3.61d	4.66 ± 0.41cd	1.79 ± 0.60c	0.31 ± 0.01b	1.32 ± 0.13ab
80 mg/kg									
P. indica	8.53 ± 0.52a	1.64 ± 0.61c	6.71 ± 0.92a	15.88 ± 2.03a	168.4 ± 4.93b	7.36 ± 0.66b	3.27 ± 0.16b	0.50 ± 0.03b	2.37 ± 0.11ab
Un-inoculated	6.47 ± 0.41b	0.37 ± 0.06de	4.41 ± 0.82c	5.58 ± 2.45cd	143.3 ± 14.7c	4.73 ± 0.03cd	1.18 ± 0.10cd	0.23 ± 0.09b	16.47 ± 0.038ab
120 mg/kg									
P. indica	5.72 ± 0.85c	6.38 ± 0.91a	2.59 ± 0.31e	9.19 ± 0.92bc	232.6 ± 2.43a	5.56 ± 0.61c	1.11 ± 0.01cd	0.26 ± 0.014b	11.50 ± 7.66a
Un-inoculated	4.41 ± 0.51d	4.43 ± 0.81b	1.59 ± 0.25f	9.72 ± 0.02b	227.3 ± 6.84a	3.54 ± 0.51d	1.76 ± 0.56c	0.28 ± 0.02b	2.32 ± 0.05ab

Within each column, means followed by the same letter do not differ significantly at $P < 0.05$ by Duncan's test. Here, ND stand for not detected

Artemisinin content

Data from fig. 7 shows that the influence and interaction of beneficial fungus *P. indica* on artemisinin content was significant, while the exposure of given Cd concentrations did not have a marked effect on the artemisinin content in un-inoculated plants. *P. indica* co-cultivation with *A. annua* increased the synthesis of artemisinin content by 1.18, 1.06, 1.27 and 1.30 folds under the Cd concentration of 0, 40, 80 and 120 mg/kg soil as compared with un-inoculated Cd stressed plants, respectively (Fig. 7).

Fig. 7 Artemisinin content; *Artemisia annua* plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated control. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of cd stress (40, 80 and 120 mg/kg fortified soil). Bars represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test.

Encoded secondary metabolites genes expression analysis

In order to understand the molecular mechanism of response to given treatments the transcriptomic analysis of relevant pathways was carried out including the key genes from flavonoid biosynthetic pathway (*DFR, CHS, CHI, F3H*), signaling genes (*MAPK, WRKY, LOX* and *MYC*), isoprenoid and terpenes pathway (*FDS, HMGR, CPS, GAS* and *BFS*) and artemisinin synthesis pathway (*ADS, CPR, DBR2, CYP71AV1* and *RED*) was carried out. Analysis from RT-PCR showed that *P. indica* augmented the expression profile of most (if not all) of the genes under whatever Cd concentration applied as compared with the un-inoculated Cd stressed plants. However, there was no prominent difference in the expression profile of signaling gene *WRKY* and artemisinin biosynthesis pathway gene *CYP71AV1* under given treatments (Fig. 8). Overall, this analysis revealed that *P. indica* co-cultivation with *A. annua* prominently augmented the expression profile of signaling, flavonoid and artemisinin biosynthesis pathway genes under given treatments comparatively.

Fig. 8 Expression analysis of *MYC, LOX, WRKY* and *MAPK* (Signaling genes), *BFS, GAS, CPS, FDS* and *HMGR* (Isoprenoid and Terpenes pathway genes), *RED, CYP71AV1, DBR2, ADS* and *CPR* (Atemisinin biosynthesis related genes) *CHS, CHI, F3H* and *DFR* (Flavonoid biosynthesis related genes); *Artemisia annua* plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated control. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of cd stress (40, 80 and 120 mg/kg fortified soil). Clustering and Heat Map analysis of genes mentioned above. The expression patterns of genes are showed on color scale provided at the left top of Heat Map.

DISCUSSION

Piriformospora indica was exposed to various levels of Cd stress toxicity**.** *In vitro* assay revealed the survival potential of *P. indica* under various concentrations of Cd (0.01, 0.05 and 0.1 concentrations), however, the hyphal growth gradually decreased with the increase of Cd concentration (Fig. 1, A-B). Growth pattern of *P. indica* varied under different Cd levels i.e. full growth (radical growth of fungal hyphae from the center of petri plates towards edges) was attained in 15 days under low levels of Cd while growth under high levels was achieved in 20 days. Notably, other fungi of the genera *Penicillium*, *Aspergillus*, *Mucor* and *Trichoderma* have been reported with survival ability under heavy metal stress condition (Hussain *et al.*, 2018; Oladipo *et al.*, 2018). Cadmium tolerance by *P. indica* could be due to the presence of different functional groups (particularly in the cell walls), which may bind with metal ions (Ferrol *et al.*, 2016). Moreover, various studies have been shown a decrease in the growth rate of phosphate solubilizing fungi (soil born micromycetes) with increasing the concentration of heavy metal stress (Zúñiga-Silva *et al.*, 2016). In the current study, *P. indica*

showed very normal growth in the Cd concentration of 0.01, 0.05 and 0.1mM as compared with control. In accordance with our study, *P.indica* have also been shown the tolerance capability and maintenance of its normal mycelial growth under arsenic toxicity up to 1mM concentration, while reduction of its growth at higher concentrations (Mohd *et al.*, 2017).

In this study, *A. annua* plants were treated with different concentrations of cadmium in the presence or absence of beneficial root endophytic fungus *P. indica*. Assessment of *A. annua* roots showed the successful colonization by beneficial fungus *P. indica*, prominently detectable in inoculated plants in the form of mycelia and mature piriform shaped chlamydospores while un-inoculated plants did not show any structure of mycelia in its primary and secondary roots (Fig. 2). In accordance with our study, the symbiotic colonization by *P. indica* under adverse environmental conditions has been documented in many other plants such as *Arabidopsis thaliana, Oryza sativa, Hordeum vulgare, Zea mays* and several other monocots and dicots (Gill *et al.*, 2016). Furthermore, *P. indica* accumulated more Cd in the roots of inoculated plants as compared with un-inoculated ones while Cd concentrations in the leaf of *P. indica* co-cultivated plants were lower than un-inoculated plants, indicating that *P. indica* possessing metal sequestration and or chelation systems or suitable degradation pathways (Fig. 3). Using these systems, *P. indica* can chelate or adsorb Cd to chitin in the fungal cell wall. A number of fungal species, especially of genus *Aspergillus* and *Trichoderma,* have been investigated for their contribution to plant heavy metal acquisition or distribution (Firmin *et al.*, 2015). The tolerance of *A. annua* plants to Cd stress is directly associated with the successful colonization by *P. indica* that accumulated more Cd in the root system and also prevents the translocation of Cd to the leaves. Thus, *P. indica* sequestered and alleviated Cd stress in inoculated *A. annua* plants. These results are also in agreement with previous studies where *P. indica* showed the same results co-cultivated with *Nicotiana tabacum* (Hui *et al.*, 2015), sunflower (Shahabivand *et al.*, 2017) and wheat plants (Shahabivand *et al.*, 2012). Our results showed that Cd accumulated in the roots as *P. indica* adsorbed and chelate Cd ions inside the fungal cell walls and inhibited Cd translocation to above ground parts. Likely, other studies have been suggested that fungal hyphae components, particularly in arbuscular mycorrhizal fungi (AMF), which may provide additional mechanisms for detoxification of heavy metals (Göhre and Paszkowski, 2006). Endophytes possess specific strategies, for example, metal sequestration, degradation pathways and chelation systems which can increase host plant tolerance towards heavy metals.

Cadmium stress adversely affect the chlorophyll content of un-inoculated stressed plants while increment in chlorophyll *a* and *b* and plant growth promotion was significantly induced by *P. indica* in our study (Table 1). Similarly, genes involved in chlorophyll biosynthesis were upregulated in rice plants co-cultivated with *P. indica* (Jogawat *et al.*, 2016). Therefore, it is suggested that the increase in chlorophyll content in the present study might be due to elevated expression of chlorophyll biosynthesis related genes. Similarly, chlorophyll content has been reduced by Cd in a number of plant species (Mangal *et al.*, 2013; Liu *et al.*, 2014) while diminution in chlorophyll (particularly under metal stress conditions) has been related to oxidative stress, chlorophylase activity leading to chlorophyll degradation, disorganization of chloroplasts and accelerated senescence and prevention in chlorophyll biosynthesis. Cd stress and other abiotic stresses, induces the production of reactive oxygen species (ROS) which are toxic for plants, causing severe damage to carbohydrates, lipids and proteins. To cope with such situations, plants activate both enzymatic (SOD, CAT, POD etc.) and non-enzymatic (proline, phenolic compounds and glutathione) antioxidants (Gill and Tuteja, 2010). Likewise, in our study these antioxidants were increased (consistent with previous studies) under Cd stress condition. While, *P. indica* further improved antioxidant enzymes system in leaves which in turn scavenge ROS (Fig. 1 and 2) (Vadassery *et al.*, 2009). There are overwhelming evidences that *P. indica* can augment the

antioxidant enzymes system such as monodehydroascorbate reductase, dehydroascorbate reductase and other enzymatic and non-enzymatic ROS-scavenging systems (Vadassery *et al.*, 2009; White Jr and Torres, 2010). Particularly, the antioxidant enzymes activities have been targeted by *P. indica* in finger millet and Chinese cabbage leaves, resulting in stress alleviation and growth promotion (Sun *et al.*, 2010; Tyagi *et al.*, 2017). A biomarker of oxidative stress, MDA was accumulated in Cd-stressed *A. annua* plants, indicating that plants were exposed to stress. Increase in MDA content is a major consequence of increasing ROS level that leads to lipid peroxidation of cell membrane (Sun *et al.*, 2010). Malondialdehyde content was lower in *P. indica* co-cultivated plants, indicating that the fungus may counteract stress response. In addition, proline content was produced in large amount under different levels of Cd, indicating plant resistance to Cd exposure. However, proline content was remarkably increased by *P. indica* in colonized plants as compared with un-colonized stressed plants. Proline not only serve as ROS scavenger but also acts as chelating substance under heavy metal stress conditions while enhancement in proline content under Cd stress has been shown by different plants species (Aghababaei and Raiesi, 2015). Also, high accumulation of proline is thought to be one of the best strategies adapted by plants to survive under stress condition. Heavy metal's toxicity is lethal for plants, therefore, proline accumulation under stress condition removes H_2O_2 content, protect enzymatic component and also maintain GSSG/GSH ratio (Anjum *et al.*, 2014). In addition, abiotic stresses are the main causative agents of oxidative stresses and ROS generation in plants which may lead to cell death (Gill and Tuteja, 2010; Rasool *et al.*, 2013). In current study, oxidative stress was reduced by *P. indica*, thereby improving antioxidant enzymes and proline content in colonized plants despite the presence of high Cd levels. Our results are in accordance with the previous study (Nanda and Agrawal, 2018), where *P. indica* sequestered heavy metal (copper) and reduced oxidative stress. Moreover, H_2O_2 content was significantly increased in Cd-stressed and un-inoculated *A. annua* plants while H_2O_2 accumulation was significantly decreased in *P. indica* co-cultivated plants. H_2O_2 content might be decreased due to increased activities of antioxidant enzymes. Decrease in H_2O_2 content has also been reported in Arabidopsis plants co-cultivated with *P. indica* (Camehl *et al.*, 2011).

Most importantly, artemisinin and flavonoid content (secondary metabolites) were significantly increased in *P. indica* co-cultivated *A. annua* plants. Such an induction of secondary metabolites associated with beneficial fungi has been extensively documented in other plants that in turn make the host plant resistant to biotic and abiotic stresses (Khalid *et al.*, 2019). Like that, genes of artemisinin and flavonoids biosynthetic pathways, terpenes, isoprenoids and signal molecules were up-regulated in *P. indica* co-cultivated plants as compared with un-inoculated stressed plants. It might be due to the simultaneous regulation of both flavonoid and artemisinin pathways via the concerted action of transcription factors and structural genes that can regulate both pathways at the same time (Hassani *et al.*, 2020). Moreover, the augmentative impact of *P. indica* on the expression level of defense related genes has been the subject of numerous studies for instance, the expression level of Cd-related genes (*Oas1*, *Gsh2* and *TaPcs1*) was increased in *N. tabacum* plants colonized with *P. indica* co-cultivation (Hui *et al.*, 2015).

CONCLUSIONS

Our findings suggest that *P. indica* is responsible for mitigation of negative effects of ROS engendered by Cd stress as well as modulation of Cd sequestration/distribution from root to shoot. Further, *P. indica* induce the expression of signaling genes (*MAPK*, *WRKY*, *LOX* and *MYC*) and the concerted action of these transcriptome factors have a regulatory positive impact on the flavonoid and artemisinin biosynthesis pathways, simultaneously (Hassani *et al.*, 2020). Thus, *P. indica* co-cultivation augments the tolerance capability of *A. annua* and improves the secondary metabolism

along with enhanced phytoremediation property against Cd toxicity. However, studies are required to find the molecular mechanism(s) of *P. indica*-host plants' interaction and biosorption/tolerance profiles with other heavy metal(loid)s. In addition, field trials for large and commercial application of *P. indica* are highly recommended.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article can be found in the online version.

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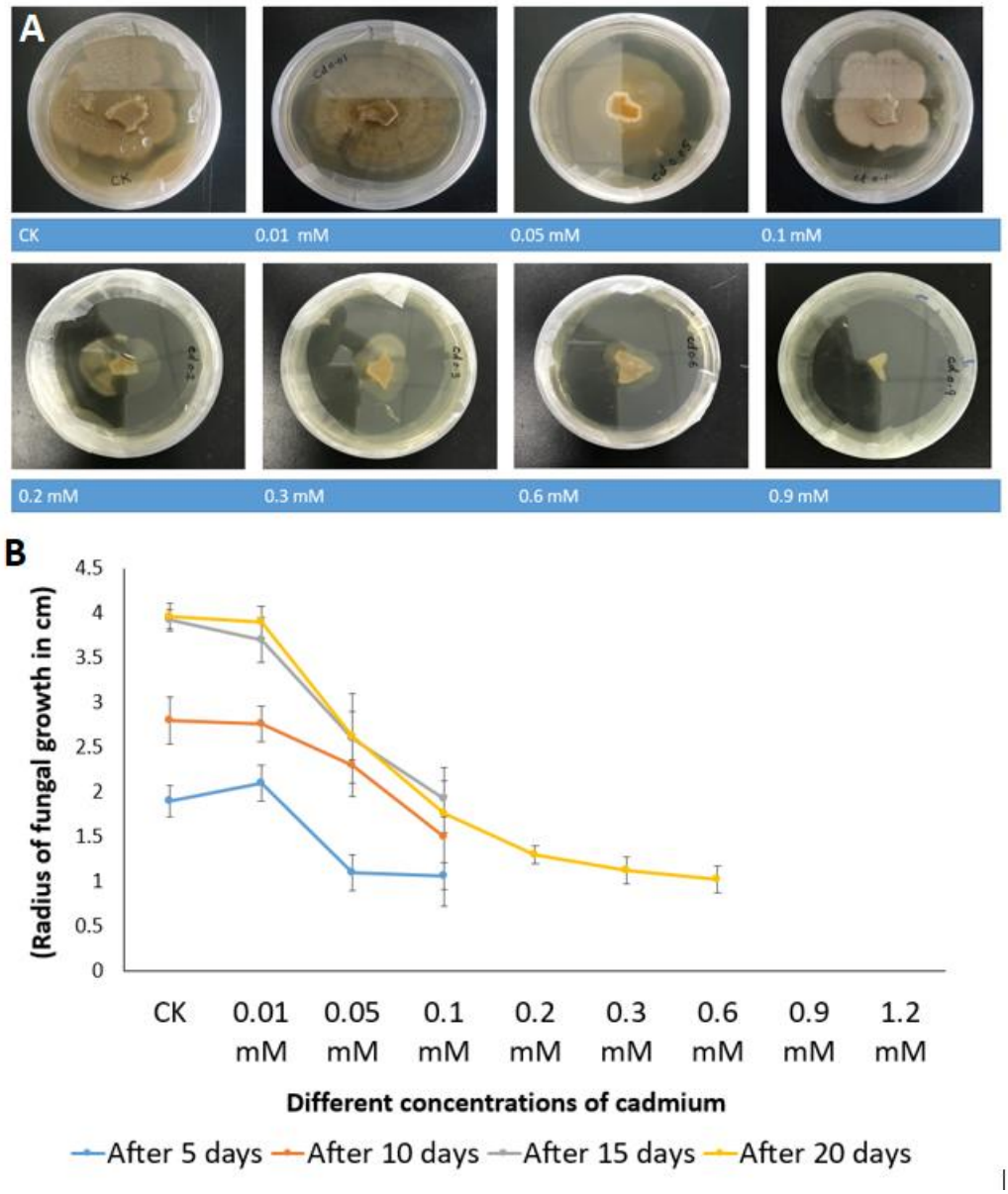

Fig 1 Effects of different concentrations of cadmium on the growth of *P. indica* (A-B) *in vitro*. *P. indicia* growth in terms of hyphal extension from the center towards plate edges under different Cd concentrations (0 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.6 mM and 0.9 mM) (A), Analysis of the radius of fungal hyphae after 5, 10, 15 and 20 days of inoculation (B). The data is the mean values of three biological replicates with ± standard error.

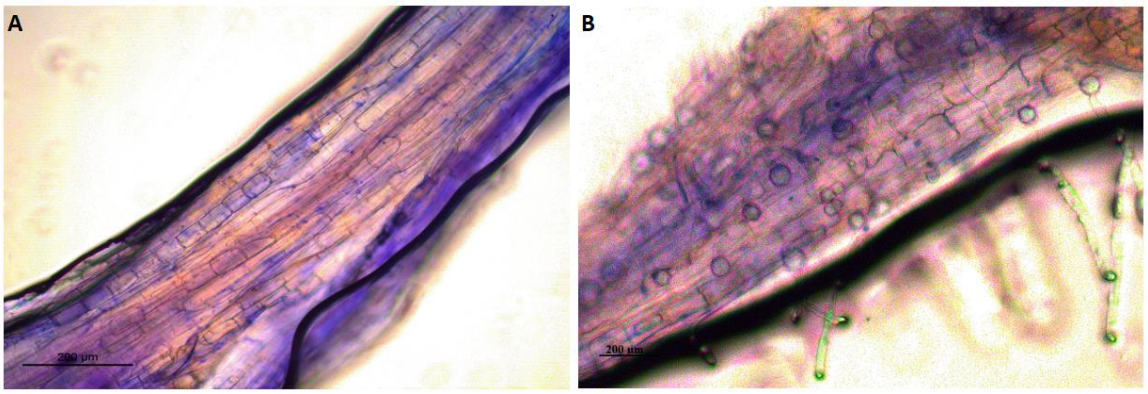

Fig. 2 Colonization of *P. indica* in roots of *Artemisia annua* plants **A)** Control, **B**) Chlamydospores inside the root cells. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of Cd stress (40, 80 and 120 mg/kg fortified soil). Bars represent the means of replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test.

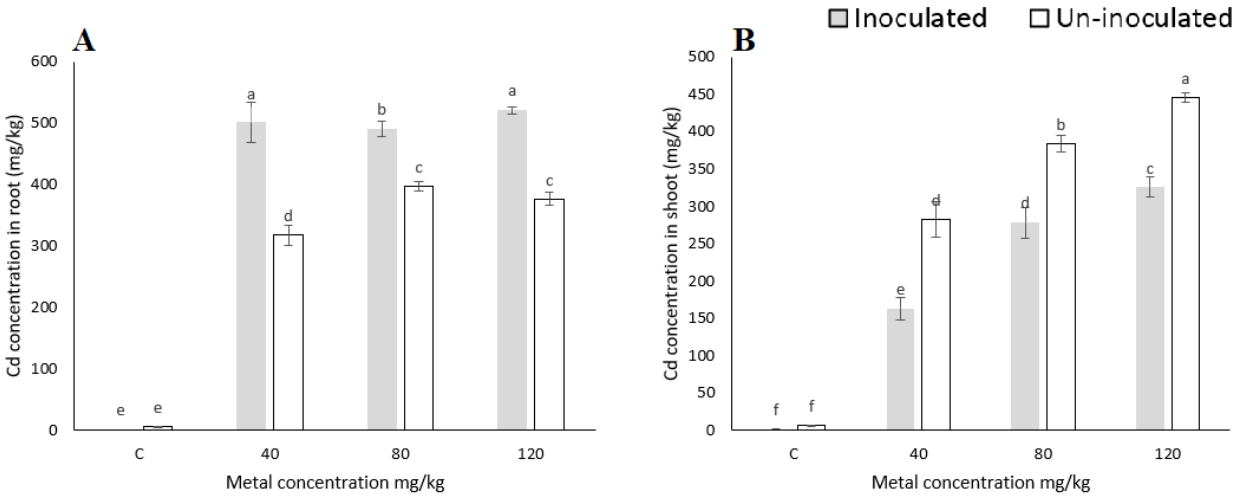

Fig. 3 Cadmium concentration in *Artemisia annua* root (A) and shoot (B); *Artemisia annua* plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated control. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of Cd stress (40, 80 and 120 mg/kg fortified soil). Bars represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test.

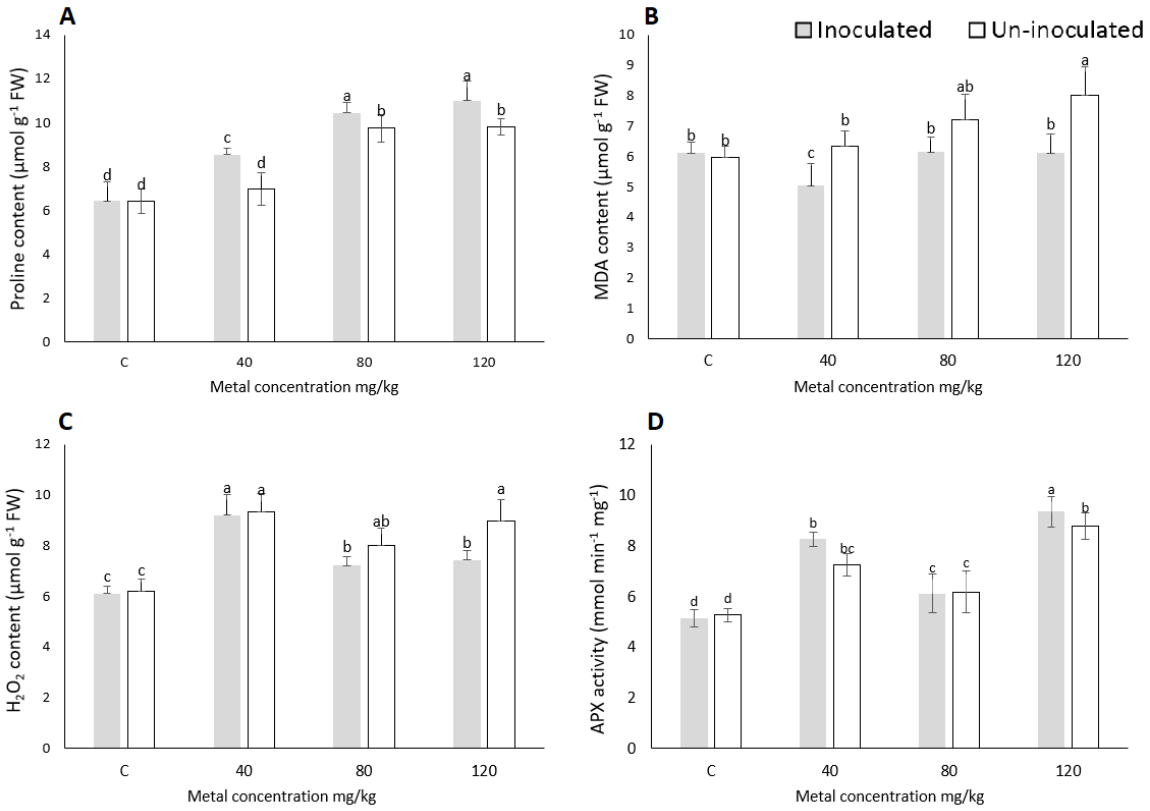

Fig. 4 Proline (A), MDA (B), H_2O_2 (C), APX concentrations (D); *Artemisia annua* plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated control. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of cd stress (40, 80 and 120 mg/kg fortified soil). Bars represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test.

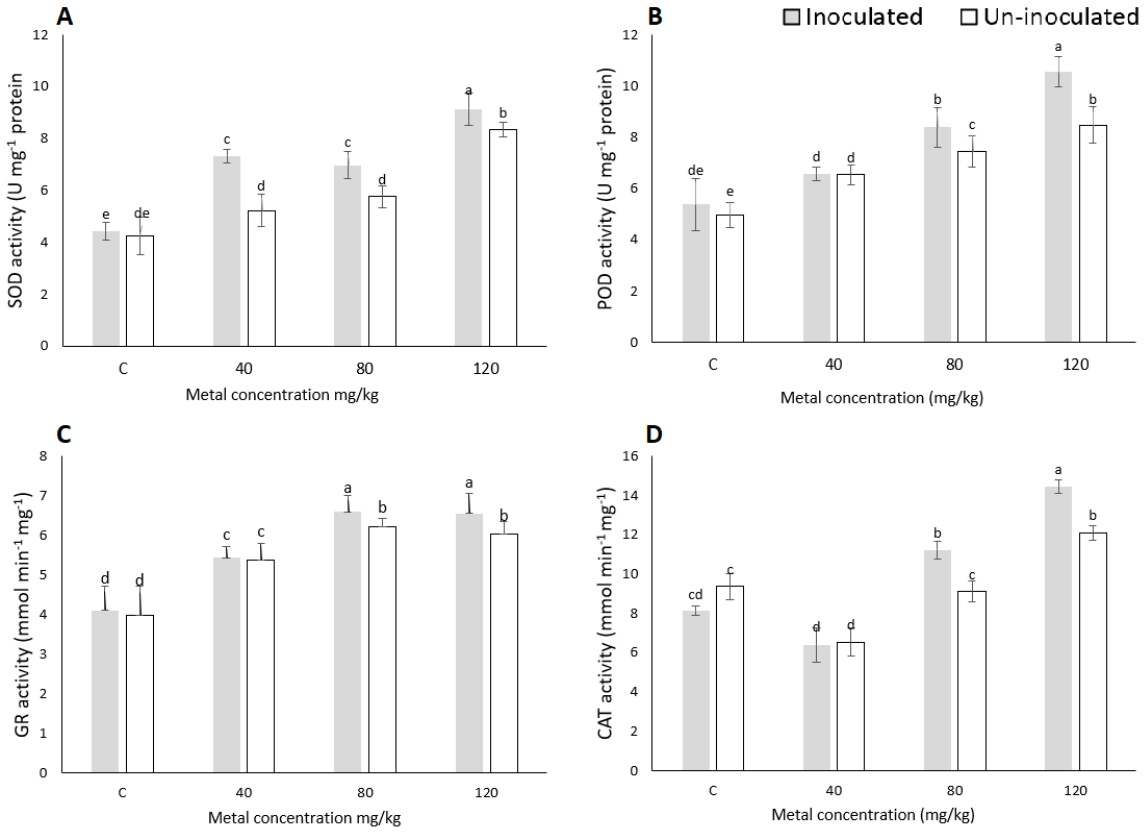

Fig. 5 Antioxidant enzymes concentrations (A-D); *Artemisia annua* plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated control. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of cd stress (40, 80 and 120 mg/kg fortified soil). Bars represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test.

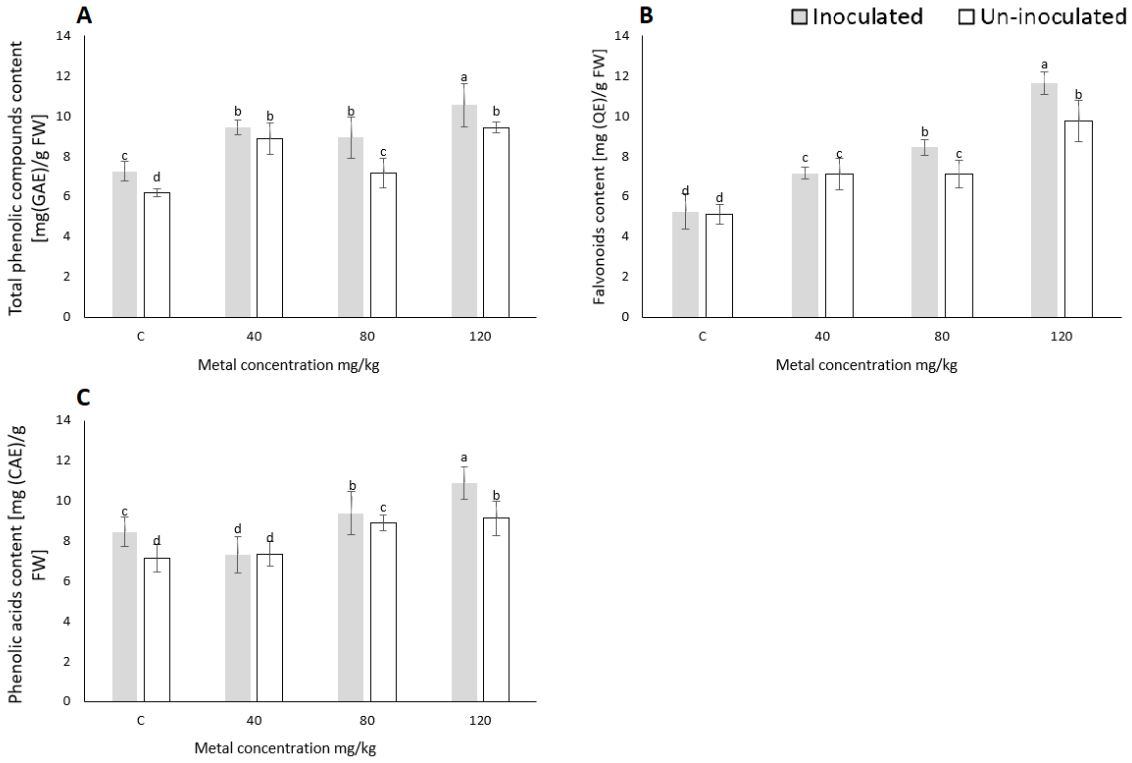

Fig. 6 Phenolic compounds (A-D); *Artemisia annua* plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated control. Plants were cultivated in the absence of cd for the entire experiment (0 mg/kg) or were subjected to three levels of Cd stress (40, 80 and 120 mg/kg fortified soil). Bars represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test.

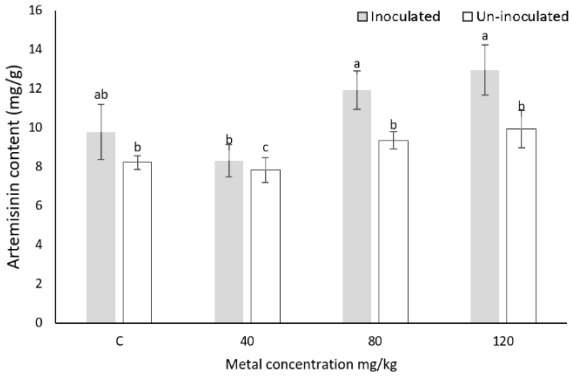

Fig. 7 Artemisinin content; *Artemisia annua* plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated control. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of cd stress (40, 80 and 120 mg/kg fortified soil). Bars represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test.

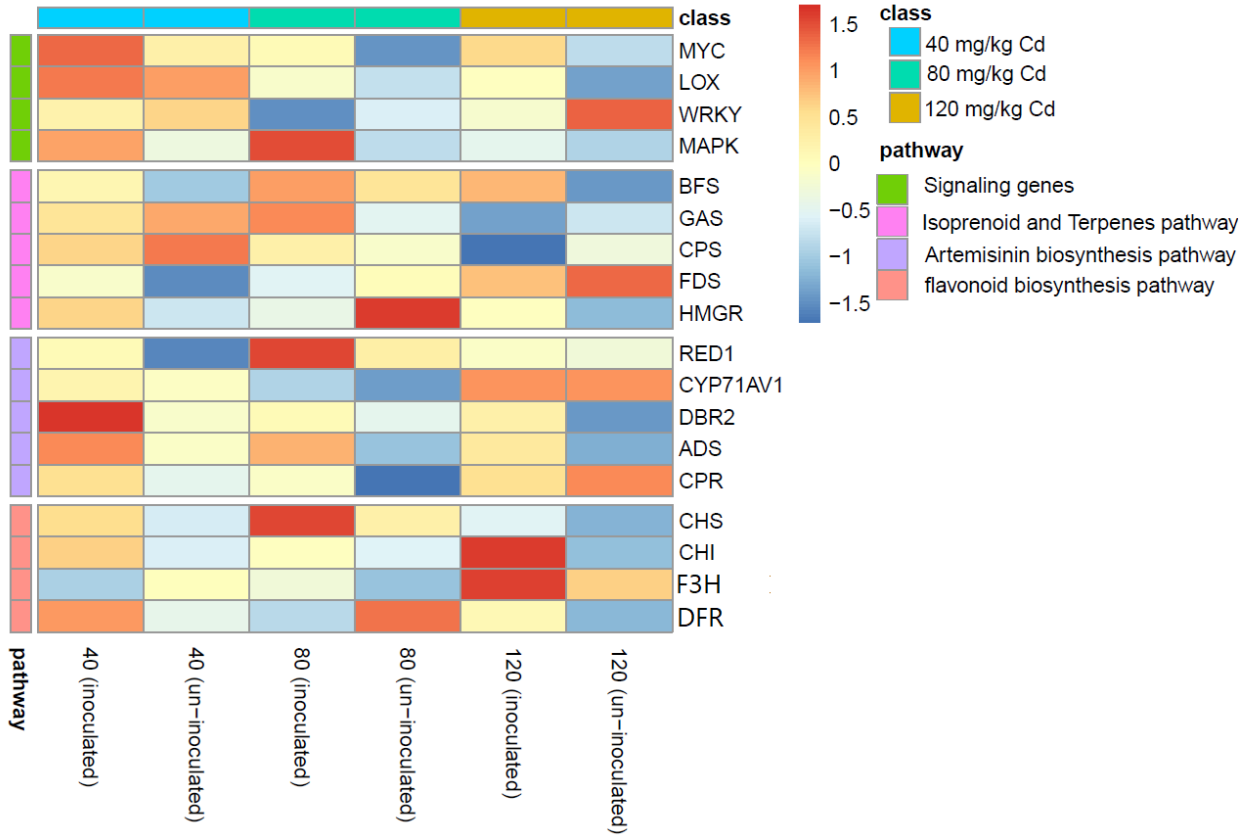

Fig. 8 Expression analysis of *MYC*, *LOX*, *WRKY* and *MAPK* (Signaling genes), *BFS*, *GAS*, *CPS*, *FDS* and *HMGR* (Isoprenoid and Terpenes pathway genes), *RED*, *CYP71AV1*, *DBR2*, *ADS* and *CPR* (Atemisinin biosynthesis related genes) *CHS*, *CHI*, *F3H* and *DFR* (Flavonoid biosynthesis related genes); *Artemisia annua* plants were inoculated with the fungus *Piriformospora indica* or remained as un-inoculated control. Plants were cultivated in the absence of Cd for the entire experiment (0 mg/kg) or were subjected to three levels of cd stress (40, 80 and 120 mg/kg fortified soil). Clustering and Heat Map analysis of genes mentioned above. The expression patterns of genes are showed on color scale provided at the left top of Heat Map.