Phytoremediation effect of Medicago sativa colonized by Piriformospora indica in the phenanthrene and cadmium co-contaminated soil

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Abstract

Background: Coexistence of polycylic aromatic hydrocarbons (PAHs) and heavy metals deleteriously threatens the quality of environmental health . F ew reports uncover the mechanism of inoculation plants with Piriformospora indica for remediating PAH- m etal co-contaminated soil by analyzing the chemical speciations of contaminants . This study investigated the influence of inoculation Medicago sativa with P. indica to remediate phenanthrene (kind of PAHs) , and cadmium (one of heavy metals) co-contaminated soil by analyzing the plant growth, physiological parameters and chemical speciation in rhizosphere and non-rhizosphere . Results: T he presence of P. indica significantly increased plants tolerance, Chlorophyll a , Chlorophyll b , maximum quantum efficiency of PSII photochemistry and electron transport rate values in phenanthrene an d /or cadmium contaminated soil. P. indica inoculation in M edicago sativa root increased f luorescein diacetate activities in phenanthrene, cadmium and both of that co-contaminated soil, especially in non-rhizosphere . The presence of phenanthrene hindered the inoculated plant from accumulating cadmium to some extent ; Whereas the presence of cadmium did not hinder the degradation of phenanthrene in both rhizosphere and non-rhizosphere after P. indica colonization. Although the poor bioavailability of cadmium in rhizosphere restricted the transportation into stem, P. indica colonization in plant efficiently increased cadmium accumulation in root in cadmium and phenanthrene co-contaminated soil. Conclusions: In conclusion, t he work provides the theoretical basis that Piriformospora indica combined with Medicago sativa contributed to the remediation of PAH-Metal co-contaminated soil.

Background

Coexistence of heavy metals and polycylic aromatic hydrocarbons (PAHs) in soil has caused worldwide environmental problems [1]. Heavy metals and PAHs are risky to human health because of their cytotoxicity, mutagenicity and teratogenicity [2]. Co-contamination of heavy metals and PAHs had higher toxicity in the environment, which increased the difficulty in remediating the polluted soil. For example, PAHs mineralization was inhibited by high content of heavy metals in co-contaminated soil [3]. Phytoremediation has been regarded as a cost-effective and environmentally-friendly technology and widely used for soil remediation [4]. Plant associated microorganisms significantly enhance the removal efficiency of PAHs and heavy metals from soil [5-6] The meaning of removal contains two aspects: one aspect is fixation and isolation of PAHs and heavy metals by plant root; the other aspect is degradation of PAHs by microbes in the rhizosphere [7]. The presence of microorganism could protect plants against damage from plant pathogens and promote plant growth; in turn, the presence of plants changes the microbial community structure and results in more conductive to remove the contamination in soil.

Arbuscular mycorrhizal fungi (AMF) have been introduced into phytoremediation of soil contaminated with diesel [8] or heavy metal [9] to enhance plant resistance and capacity to accumulate heavy metal. However, AMF are obligate endosymbionts which are unable to be cultivated *in vitro*. *P. indica* was first discovered in the rhizospheres of woody shrubs in the sandy desert soils of the Thar region in India [10]. The root endophytic basidiomycete *P. indica* belongs to the recently defined order Sebacinales [11].

Species of this order form a novel type of mutualistic mycorrhizal symbiosis and is able to colonize a broad spectrum of plant and conveys various beneficial effects to colonized host plants [12-15]. *P. indica* acts as a bioprotector against pathogens [16], alters plant secondary metabolites [17], increases nutrient uptake and promotes plant growth [18-19], confers drought tolerance to Arabidopsis and barley [20-21], and alleviates salt stress in barley and rice by increasing the activity of detoxifying enzymes and photosynthetic pigment content in colonized plants [22-23]; *P. indica* is different from AMF that can be cultivated *in vitro.* Considering the various beneficial effects caused by *P. indica,* the fungus is regarded to have significant agronomical and high ecological application value [24]. However, there are few reports about the utilization of this endophyte for the remediation of the soil contaminated by heavy metal and PAHs.

No matter the remediation of heavy metal or PAHs, bioavailability determined the remediation efficiency [25-26]. More organic acid production by root could efficiently increase the bioavailability of heavy metals and PAHs [27-28]. *P. indica* directly increases plant root biomass by producing indole-3 acetic (IAA) [29], which contribute to more organic acids produced by root. Parallel, biosurfactants produced by plant growth-promoting microorganism (PGPM) could increase the bioavailability of heavy metals and PAHs [27-28]. As far as the remediation of heavy metal, the presence of heavy metals stimulated PGPM to produce siderophores, which in turn increased the bioavailability of heavy metals [30-32]. In case of remediating PAHs, plants provide an effective platform to recruit more efficient microbes to digest or degrade the PAHs [1]. Therefore, PGPM combined with plant roots provide a major way for remediating the heavy metal and PAHs contaminated soil.

Some researchers have reported that using microorganism to help promote the remediation of single contamination caused by heavy metal or PAH [33], however, few studies focus on the influence of inoculation plants with *P. indica* on the chemical speciations of heavy metal and PAH in the metal and PAH co-contaminated soil. Notably, the selected *P. indica* was widely studied on its interaction with plant. However, the special views of this study were taken from other aspects: (1) to address the stress tolerance of *M. sativa* (*M. sativa*) against phenanthrene and cadmium after *P. indica* colonization; (2) to speculate the rhizospheric effect of *P. indica* inoculation by identifying microbial activity and enzyme activity in the rhizosphere and non-rhizosphere; (3) to evaluate the effect of *P. indica* inoculation on phytoremediation efficiency of phenanthrene-cadmium co-contaminated soil.

Results

P. indica inoculation increased the biomass of M. sativa in contaminated soil

The biomass of *M. sativa* was recorded to reflect the tolerance to pollutants in soil. *P. indica* inoculation significantly promoted plant biomass both on root and stem in contrast to non-inoculated plant (control) (Fig. 1A). Besides, the leaf area was increased compared to control (Fig. 1B). However, biomass (fresh weight) reduced was observed in Cd, Phe and Phe+Cd treatments without *P. indica* inoculation; whereas the addition of *P. indica* spores significantly enhanced the biomass in those respective treatments (Fig.

1C-D). One hundred plants for each different treatments were statistically recorded in Fig.2. Phe+Cd treatment led to the most serious biomass reduced. Contrastingly, plant biomass in these treatments, including Piri+Cd, Piri+Phe and Piri+Phe+Cd was higher than those respective treatments without *P. indica* inoculation. Notably, the growth inhibition on Cd treatment was more grievous than that of Phe treatment.

Chlorophyll (Chl) a, b contents and fluorescence parameters

The differences on Chl *a* and Chl *b* contents were significant ($p<0.01$) between *P. indica* inoculation or not when plant exposure on Cd, Phe contamination (Table 1). Without Cd and Phe contamination, *P. indica* inoculated plant increased Chl *a* and Chl *b* contents in *M. sativa* leaves. Either Cd or Phe contamination reduced Chl *a* and Chl *b* contents significantly. Phe effects on Chl *a* and Chl *b* contents was severe than Cd. However, *P. indica* inoculation significantly increased Chl *a* and Chl *b* contents under Cd, Phe, Cd+Phe treatments in compare to non-inoculated plants. Presence of *P. indica* significantly reduced F0 so that F0 values of inoculation plants were 8%, 12.9%, 14% lower than non-inoculated plants under Cd, Phe, Cd+Phe treatments, respectively. Additionally, all three fluorescence parameters including Fm (maximal fluorescence level in the dark-adapted state), Fv/Fm (maximum quantum efficiency of PSII photochemistry), and ETR (the relative PSII electron transport rate) were significantly decreased under Cd, Phe, Cd+Phe treatments. However, a noticeably increased on Fm, Fv/Fm, ETR was observed in *P. indica*-inoculated *M. sativa* in comparison to non-inoculated plants.

Auxin production of P. indica affected by phenanthrene and cadmium

The effects of IAA on the growth of plants showed promotion in low concentration and inhibition in high concentration. Therefore, we were interested in revealing whether IAA production in *P. indica* will be affected by phenanthrene and cadmium. Qualitative analysis by Salkowski-method showed that after adding the tryptophan, the solution containing spores of *P. indica* under each treatment changed to pink in three repetitions as compared to control (Fig.3A-B), which indicated that IAA was produced under Phe, Cd and both of Phe and Cd treatments. Additionally, IAA content produced by *P. indica* was quantified by HPLC. The result showed that either Phe or Cd treatments could affect IAA production (Fig. 3C). Especially, Phe and Cd co-treatments resulted in less IAA production (0.69 μM). This result explained why the biomass compensation of root treated by Phe and Cd was less than only Phe or Cd treatment, though all the treatments roots were colonized by *P. indica* (Fig. 1).

P. indica inoculation increased FDA activities in non- rhizosphere

Fluorescein diacetate assay (FDA) was an easy and convincing method to determine soil microorganisms activities. Fig. 4. showed that FDA activity was higher in *P. indica* inoculated plant than non-inoculated plant in Cd or (and) Phe treatments, including the control. The FDA activity in non-rhizosphere under *P. indica* inoculation was the highest, whereas Cd+Phe treatment in non-rhizosphere had the lowest FDA activity. An interesting phenomenon was observed that *P. indica* colonization in plant roots significantly increased microorganisms activity in non-rhizosphere than rhizosphere. The Cd and Phe contamination significantly reduced FDA activity both in rhizosphere and non- rhizosphere. By

comparing Cd and Piri+Cd treatments, no obvious differences of FDA activity were observed in rhizosphere, adding of *P. indica* remarkblely increased FDA activity in non- rhizosphere. Similar results were obtained between Phe and Piri+Phe treatments.

P. indica inoculation increased the polyphenol oxidase in rhizosphere

Polyphenol oxidase activity in the rhizosphere and non-rhizosphere was identified in Fig. 5. The activity of polyphenol oxidase in the rhizosphere was higher than that in non-rhizosphere. *P. indica* colonization significantly increased polyphenol oxidase activity either in rhizosphere or non-rhizosphere. The order of polyphenol oxidase activity form low to high among different treatments was: Phe+Cd < Phe < Cd < Piri+Phe < Piri+Phe+Cd < Piri+Cd < Con < Piri. According to these data, several aspects were revealed: 1) polyphenol oxidase activity in soil was easily affected by Phe than Cd contamination; 2) the addition of *P. indica* significantly increased polyphenol oxidase activity in the rhizosphere; 3) for Phe and Cd co-contamination soil, the polyphenol oxidase activity was affected grievously, whereas after *P. indica* colonization in root of *M. sativa*, the polyphenol oxidase was remarkably enhanced. '

P. indica inoculation reduced phenanthrene content in rhizosphere

Phenanthrene degradation rate was identified around the rhizosphere and non-rhizosphere (Fig. 6A). Generally, phenanthrene content in the rhizosphere was less than the non-rhizosphere. In the rhizosphere, among four different treatments, phenanthrene concentration in soil from high to low was Phe+Cd, Phe, Piri+Phe+Cd, Piri+Phe. The concentration gradient in non-rhizosphere was similar to those in rhizosphere. Those data imply that *P. indica* colonization could effectively reduce phenanthrene content in soil.

In order to prove this evidence, phenanthrene concentration in plant roots and stem was measured additionally. Phenanthrene concentrations in roots were 135 ± 6 mg kg^{-1}, 165 ± 4 mg kg^{-1}, 108 ± 10 mg kg^{-1}, 156 ± 7 mg kg^{-1} among the Phe, Piri+Phe, Phe+Cd and Piri+Phe+Cd treatments, respectively (Fig. 6B). Notably, phenanthrene concentrations in roots were higher than those in stem. In stem, the Phe+Cd treatment had the lowest phenanthrene content than the other treatment. There is no striking difference of phenanthrene content in the stem among Phe, Piri+Phe and Piri+Phe+Cd treatments.

P. indica inoculation reduced cadmium content in rhizosphere

The cadmium content in the rhizosphere and non-rhizosphere was identified as shown in Fig.7A. Similarly, cadmium content in the rhizosphere was less than non-rhizosphere. In the rhizosphere, among four different treatments, cadmium concentration from high to low was Phe+Cd, Piri+Phe+Cd, Cd, Piri+Cd. Adding of *P. indica* spores significantly reduced cadmium content in compare to the treatment without *P. indica* inoculation. For instance: either in the rhizosphere or non-rhizosphere, Piri+Cd treatment had lower cadmium content than Cd treatment; and Piri+Phe+Cd treatment had lower cadmium content than Phe+Cd treatment. Remarkably, Cd content in the rhizosphere of *P. indica* treatment was significantly reduced (less than 2 mg kg^{-1}) than the original Cd content in soil (10 mg kg^{-1}). The cadmium

concentration gradient in non-rhizosphere was similar to those in the rhizosphere. Those data imply that adding of *P.indica* colonization could effectively reduce the cadmium content in soil.

Additionally, cadmium concentration in plant roots and stem was explored in parallel (Fig.7B). Cadmium concentrations in roots were 30±4mg kg^{-1}, 34±6 mg kg^{-1}, 23±8 mg kg^{-1}, 29±5 mg kg^{-1} in the Cd, Piri+Cd, Phe+Cd and Piri+Phe+Cd treatments, respectively. Likewise, the cadmium concentrations in roots were notably higher than those in stem. In stem, Cd content was different from phenanthrene content: Piri+Cd treatment had lower Cd content than Cd treatment, and Cd content in Piri+Phe+Cd treatment was lower than Phe+Cd treatment. It suggested that adding of *P. indica* inhibit the transport of Cd into stem.

Discussion

Heavy metal and organic pollutants in soil do severe harm to plant growth and development. Cd treatment leads to the most grave affection to plant biomass accumulation. Shahabivand et al. (2017) reported that *P. indica* successfully colonized plants grown in 120 mg/kg of Cd contaminated soil, which suggest that this fungus was able to colonize roots under high Cd stress in soil. However, we found that the spores germination and hyphal growth of *P. indica* were seriously inhibited under 40 mg kg^{-1} of Cd (data not shown). Therefore, 10 mg kg^{-1} of Cd was selected as the test concentration.

Our data indicated that additional colonization of *P. indica* in Cd (10 mg kg^{-1}), Phe (40 mg kg^{-1}) and Phe+Cd treatments remits stress from heavy metal and PAHs. Though Cd concentration in roots of Piri+Cd treatment was higher than that in Cd treatment, the biomass was not significantly affected. The results implied that high concentration of Cd could be fixed or isolated in some degree by this fungus and plant co-living system and therefore reduced the harmful affection to plant. Cd accumulation in stem was reduced after phenanthrene contamination in soil, which indicated that the organic pollutants accumulated in roots might hinder Cd transport from root to above-ground parts. Piri+Phe treatment accumulated high concentration of phenanthrene in root, which decreased the biomass of roots and stem compared to Piri treatment. However, even high concentration of phenanthrene in root did not hinder the growth promotion effect of *P. indica* to host plant compared to Phe treatment. This result was different from the previous report that the plant-growth-promoting bacteria (PGPB) accumulation of pyrene decreased the biomass of stem [32]. The possible reason is that the endophytic fungus, which is different from bacteria, could accumulate and endure the phenanthrene, therefore reduce the toxicity to plant.

M. sativa plants subjected to Cd, Phe stress, showed reduced growth in terms of shoot and root lengths, and shoot and root fresh weight. Cd toxic effects have widely been reported in different plant species, and Cd is known to reduce or inhibit plant growth because of Cd harmful impact on the processes of photosynthesis, respiration and essential elements uptake [34-35]. Chaoui and Ferjani (2005) [36] reported that activity of indole-3-acetic acid (IAA) oxidase (as a growth-limiting enzyme) was increased under Cd toxicity, which results in the reduced plant growth due to diminution of endogenous content of plant growth-promoting hormone auxin. In our work, the elevation in photosynthesis efficiency of the host

via enhanced Chl *a* and *b* contents and elevated Fv/Fm and ETR values (Table 1) contributed to the *P. indica*-induced growth promotion and stress tolerance.

Soil microorganism activity could effectively reflect soil fertility. Soil microorganism activity was determined to speculate the phytoremediation efficiency. We noticed that the FDA activity in non-rhizosphere was higher than rhizosphere in *P. indica* colonization plant. We analyzed the possible reason is that, because of the presence of *P. indica* in plant root, much more heavy metal was accumulated in rhizosphere than non-rhizosphere, which resulted in harmful effect to the microorganism and therefore reduced the FDA activity in rhizosphere. Anyhow, the FDA activity in *P. indica* treatment was higher than non-*P. indica* treatment either in rhizosphere or non-rhizosphere. Phe treatment had lower FDA activity than Cd treatment which was in accordance with the previous report that petroleum hydrocarbons inhibit the microorganism activity in soil [37]. Though similar FDA activities in Cd and Piri+Cd or Phe and Piri+Phe were obtained in the rhizosphere, the mechanism might be different. The reduction of FDA activities in Cd or Phe treatments was probably due to heavy metal or PAHs stress, whereas the reduction of FDA activities in Piri+Cd or Piri+Phe treatments probably depends on the phytopathogens growth inhibition. Enzymes, as part of soil composition, the degree of its active sensitively reflects the direction and strength of a biochemical reaction in soil [38]. The changes of enzyme activity effectively reflect the degradation ability of organic pollutants by microorganism and plant roots in soil. In fact, in our experiment, we detected several enzymes activities including urease, invertase and so on, and the results indicated those enzymes activity were better in the rhizosphere of *P. indica* colonization root (data not shown). Polyphenol oxidase is an important oxidoreductase enzyme in soil which participates in the process of decomposition and transformation of aromatic compounds. Enhanced polyphenol oxidase in this study implied that *P. indica* had a strong repairing ability for the contaminated soil.

Co-contamination of heavy metals and PAHs result in deleterious influence on microorganism activity in soil compared to single heavy metals or PAHs contamination [39], which explained that Phe+Cd treatment had the lowest FDA activity in all treatment. The Piri+Cd and Piri+Phe+Cd treatments had higher FDA activities than Cd and Phe+Cd tratments respectively, which implied that the endophyte and plant co-living system contribute to a well-established micro ecosystem for microorganism in soil.

As for Phe, Piri+Phe, Cd+Phe and Piri+Phe+Cd four different treatments, Phe accumulation in plants were 135 ± 6 mg kg^{-1}, 165 ± 4 mg kg^{-1}, 108 ± 10 mg kg^{-1}, 156 ± 7 mg kg^{-1} respectively. The high concentration of Phe accumulation in plant means the less Phe residual in soil. Besides plant, microorganism in soil played key roles in the removal of the PAHs. For Phe+Cd treatment, Phe concentration left in the rhizosphere or non-rhizosphere was higher than Phe treatment, which was reasonable due to higher microorganism activity in Phe treatment. Though no significant difference of FDA activities was obtained between Phe and Phe +Piri treatment, *P. indica* colonization obviously reduced Phe concentration in the rhizosphere and non-rhizosphere. Similar data was collected from Phe+Cd and Phe +Cd +Piri treatments. Totally, Phe concentration in the rhizosphere was less compared to non-rhizosphere, which explained that the rhizospheric effect was responsible for the less accumulation of PAHs in the rhizosphere soil than non-rhizosphere [40].

The order of Cd concentrations in root from high to low was as follows：Piri+Cd, Cd, Piri+Phe+Cd, Phe+Cd. The data indicated that *P. indica* colonization increased Cd accumulation in roots. However, by comparing Piri+Cd and Piri+Phe+Cd treatments, it was notably that the addition of Phe reduced *P. indica* accumulation Cd in a certain degree. Bioavailability of heavy metal determines the phytoremediation efficiency [41]. Microorganism in soil like fungi and bacteria is capable of producing biosurfactants, for instance, rhamnolipid which could increase the mobility of heavy metals, to enhance its bioavailability [42]. Therefore, it was reasonable to understand that *P. indica* colonization significantly reduced Cd concentration in the rhizosphere than non-rhizosphere. In the other hand, plant roots exudate also contribute to the acidification of heavy metals, which increase the efficiency of phytoremediation. *P. indica* colonization could significantly promote root growth and development, the roots in turn secret exudates to increase bioavailability of heavy metals and enhance phytoremediation efficiency in the rhizosphere, which a virtuous cycle was formed.

Conclusions

In conclusion, *P. indica* colonization posed positive effects on the processes of photosynthesis and increased the tolerance of *M. sativa* in phenanthrene and cadmium co-contaminated soil, especially in single cadmium contaminated soil. Phenanthrene and cadmium co-contaminated soil affected soil microorganism grievously than single phenanthrene or cadmium contamination. *P. indica* inoculation in *M. sativa* root increased the FDA activity in phenanthrene, cadmium and both of that co-contaminated soil, especially in the non-rhizosphere. The addition of *P. indica* stimulated root of *M. sativa* to accumulate higher concentration of phenanthrene and cadmium, and thereby enhanced phytoremediation efficiency. One vividly conclusion picture（Fig. 8.）was drawn about phytoremediation effect of *M. sativa* colonized by *P. indica* in phenanthrene and cadmium co-contaminated soil. This study suggested the application of *P. indica* combined with *M. sativa* contributed to the remediation of PAH-metal co-contaminated soil for sustainable agriculture.

Methods

Chemicals

Phenanthrene with a purity of 98% was purchased from Sigma (USA). The rest chemicals were purchased from Dingguo (Tian jin, China).

Soil used in the study

The experiment soil, never contaminated by PAHs or heavy metals was collected from the topsoil (0-20 cm) of Hebei University of Technology, China. The soil was a kind of phaeozems (alfisol) in Hebei Province [43].The test soil was sieved by 2 mm sieve after dried by air. Soil samples were measured using standard methods [44] before phytoremediation. The composition of the sample soil was physico-

chemically characterized: 55.6 ± 2.1% silt, 30.8 ± 1.8% sand and 13.6 ± 1.5% clay, 0.06% total N, 8 mg/kg available P, 40 mg/kg available K, 1.1% organic matter.The soil pH was 7.36 ± 0.06.

Preparation of phenanthrene contaminated soil

Phenanthrene was dissolved in acetone and added into a small part soil, one day later, after acetone volatilized, the small part soil was added into the whole sample soil and incorporated thoroughly, the final concentration of phenanthrene in soil was measured as 40 ± 3 mg kg^{-1}.

Preparation of cadmium contaminated soil

The aqueous solution of cadmium nitrate was added to the prepared soil, the final concentration of cadmium in soil was measured as 10 ± 2 mg kg^{-1}.

Preparation of phenanthrene and cadmium co-contaminated soil

The acetone stock solution containing phenanthrene was firstly added to the test soil. After acetone evaporated, cadmium nitrate aqueous solution was added to the previous soil polluted by phenanthrene. The final concentration of cadmium and phenanthrene in soil were measured as 10 ± 2 mg kg^{-1} and 40 ± 3 mg kg^{-1} respectively. The well prepared soil and a control soil without any pollution were shifted to boxes and aged in the dark for 15 days.

Fungal culture

In work, the isolate of *P. indica* DSM11827 (German collection of microorganisms and cell cultures in Braunschweig, Germany) was applied. *P. indica* was supplied by Karl-Heinz-Kogel (Institute of Plant Pathology and Applied Zoology, Giessen, Germany). *P. indica* was maintained at 23°Con CM medium [45]. For solid medium, 14 g L^{-1} agar was added; For liquid cultures, 100 mL medium was inoculated in a 300-mL Erlenmeyer flask. To test whether Cd and Phe will affect IAA production contents, 5 mg kg^{-1} and 20 mg kg^{-1} of cadmium and phenanthrene were added into the liquid cultures, respectively. CM medium was inoculated with 20 mycelium plugs from the margin of a growing colony of *P. indica* on CM solid medium; liquid cultures were incubated at 23°C at 150 rpm on a rotary shaker.

Quantification of IAA in fungal growth media by HPLC

IAA production ability of *P. indica* under Phe, Cd and Phe+ Cd treatment was performed using Salkowski-method according to the literature [46]. Quantification of IAA in fungal growth media was performed by HPLC. Fungal culture filtrates were harvested, acidified and extracted twice with ethyl acetate as described in [29]. The parameters used for HPLC are as follows: 50% methanol: 45% water: 5% acetonitrile (v/v) was used as the mobile phase. The 0.2 mL min^{-1} of flow rate was applied. The injection volume was 10 µl. Column temperature was kept at 40°C.The content of IAA was quantified by Agilent HPLC which equipped with an HC^{R} C18 (5 µm, 4.6 x 250 mm, Agilent, USA) reversed phase

chromatographic column. IAA concentrations were always determined in parallel in medium in which no fungus had been cultured but which had been incubated under the same conditions.

M. sativa treatment and P. indica inoculation

P. indica growing on CM medium plates for 3-4 weeks, was ready for preparation of spore suspensions. To collect spores from CM agar plates, sterilized water containing 0,05% Tween-20 was added. Through gently scratching the surface of plates with a spatula, the spores were released and the suspension solution was filtered through miracloth (Calbiochem, Bad Soden, Germany) in order to remove mycelium. After that, spores were collected by centrifuging suspension solution at 3500 rpm for 7 minutes. Then, spores were washed at least 3 times with sterilized Tween-H2O. By using a hemacytometer in combination with a microscope, spore densities were determined. The spore concentration was adjusted to 500,000 spores/ml with sterilized Tween-H2O. For inoculation, three ml spore suspension was pipetted on top of plant roots in one squared petri-dish. The seeds of *M. sativa* were surface sterilized in 70% alcohol for 1 min and then 3% NaClO for 15 min. After sterilized, seeds were washed by sterile deionized water repeatedly and planted into the MS medium [47] for germination. Seven days when the roots were presence, *P. indica* spores were added into the root surface. Then the seedlings contained *P. indica* spores were transferred into different types of soil in pot (5 kg). Three replicates were applied for each treatment.

The plants were rejuvenated for one week in shade. After then, pots were transferred to green house with natural light and watered daily to keep the soil moisture (approximate 300 mL water/ pot). Three months later, plants were harvested and soil from the rhizosphere and non- rhizosphere were collected respectively from the roots of *M. sativa .*

Measurement of chlorophyll contents and chlorophyll fluorescence

Chlorophyll content 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 and 645 nm wavelengths for Chl *a* and Chl *b*, respectively [48]. The following parameters of chlorophyll fluorescence were measured by analyzing the first fully grown leaves of *M. sativa* using a portable fluorometer (Hansatech, Instruments LTD, UK): F0 (minimal fluorescence level in the dark-adapted state), Fm (maximal fluorescence

level in the dark-adapted state), Fv/Fm (maximum quantum efficiency of PSII photochemistry) and ETR (the relative PSII electron transport rate). *M. sativa* plants were dark-adapted for 30 min to measure the influence of factors on photosystem II (PSII) efficiency.

Measure of soil microorganism activity

Soil microorganism activity was measured referenced to the literature [31]. Firstly, the soil was freeze-dried by Freeze drying machine (Alpha 1-2 L D plus, Germany). The 5 g freeze-dried soil was dissolved in 15 mL phosphate buffer solution (NaCl–8.5 g, Na_2HPO_4–2.2 g, NaH_2PO_4–0.1 g, pH 7.6) at room

temperature. The turbid liquid was shaked at 180 rpm for 30 min and then 0.5 mL of fluorescein diacetate (FDA) (2 g L^{-1}, in acetone) solution was added into the mixture. The absorbance value was recorded at OD_{490nm}.

Analysis of phenanthrene in the rhizosphere and non- rhizosphere

Similar three-step sequential extraction method was used to detect the concentrations of phenanthrene in different chemical speciations in the soil [40]. The ultrasonic extraction and high performance liquid chromatograph (HPLC) ultraviolet detector detection method was used. Ten gram freeze-dried soil samples was dissolved in 50 mL of acetone- hexane (1:1, v/v) mixed extraction solvent. The progress of extraction was performed for 1 h in the ultrasonic cleaners. Extracted liquid was filled with filter funnel containing 10 g anhydrous Na_2SO_4. The extracted liquid was concentrated to 5 mL by rotary evaporation instrument in 60℃water bath. Then 5 mL concentrated extracted liquid was transferred to silica gel-Alumina column chromatography and washed with methylene chloride-hexane (1:1, v/v) mixture elution. The condensed elution nearly drying was diluted to a final volume of 1mL and used for HPLC determination. The parameters used for HPLC are as follows: methanol and water (87:13, v/v) was used as the mobile phase. The 1 mL min^{-1} of flow rate and 254 nm of detection wave length was applied. The content of phenanthrene was quantified by Agilent HPLC which equipped with an HC^R C18 (5 μm, 4.6 x 250 mm, Agilent, USA) reversed phase chromatographic column. The recoveries for soil was 97 ± 3%.

Analysis of phenanthrene in plants

The phenanthrene in plants was extracted by acetone and dichloromethane (v/v, 1:1). After centrifugation and rotary evaporation, the concentrated phenanthrene was exchanged to 1 mL hexane to be analyzed. The content of phenanthrene was quantified by HPLC which equipped with a HC^R C18 (5 μm, 4.6 x 250 mm, Agilent, USA) reversed phase chromatographic column. The oven temperature was firstly maintained at 100℃for 2 min, then increased to 300℃ at a rate 10℃ min^{-1}, and finally kept at 300℃for 10 min. Phenanthrene standard was added to the uncontaminated plants and soil to measure the phenanthrene recovery. Procedural blank together with spiked blank and duplicate samples were included with every batch of ten samples in the analysis. The recoveries for plants was 99 ± 5%.

Analysis of cadmium in soil and plant

Cadmium was determined according to the references [40, 49]. Soil about the rhizosphere and non-rhizosphere of *M. sativa* was extracted by mixing 0.5 g soil with 10 mL HCl solution and then heating for 3 h (45℃). After cool down, Cadmium was extracted by mixing the soil with HNO_3 and $HClO_4$ (v/v, 4:1) for digestion (220℃, 1 h) and then adding HF and $HClO_4$ (v/v, 5:1) for further digestion (220℃, 2 h). The same method was used for cadmium extraction from plants. At the end of extraction step, the supernatant was harvested by centrifuging at 6000 rpm, 20 min. Then the supernatant was filtered through 0.45 μm microfiltration membrane and quantified by Inductively Coupled Plasma Optical Emission Spectrometry (ICPOES).

Analysis of enzyme activity in the rhizosphere and non- rhizosphere

Enzyme activity of polyphenol oxidase was determined according to the protocol book of soil enzymes and analytical methods [50]. Ten gram freeze–dried soil was dissolved in 10 mL pyrogallol (1%) and shaked with 150 r. min^{-1} for 1 min. Then the sample was kept in dark at 30℃ for 2 h. Four milliliter citric acid - phosphate buffer (disodium hydrogen phosphate-35.61g/ L, citric acid-21.01g/ L, pH4.5) was added into the sample. Finally, 35 mL ether was added and shaked for 2 min. The absorbance value at 430 nm was recorded after 30 min extraction.

Statistical analysis

In this study, all data are expressed as means ±SE and represent at least three independent biological experiments. The significant differences were analyzed by using a one-way analysis of variance (ANOVA), which referred to Duncan's multiple range test.

Abbreviations

AMF: Arbuscular mycorrhizal fungi; Cd:cadmium; Chl *a/* Chl *b: Chlorophyll (Chl) a, b*; ETR: the relative PSII electron transport rate; F0: minimal fluorescence level in the dark-adapted state; FDA: Fluorescein diacetate assay; Fm: maximal fluorescence level in the dark-adapted state; Fv/Fm: maximum quantum efficiency of PSII photochemistry; IAA: indole-3 acetic; *M. sativa*: *M. sativa*; PAHs: polycyclic aromatic hydrocarbons; *P. indica: P. indica*; PGPM: plant growth promoting microorganism; Phe: phenanthrene.

Declarations

Authors' contributions

L. L. designed and guided the whole experiment, also performed the work of analyzing the data and writing the manuscript. P. Z. was responsible for the *M. sativa* and *P. indica* cultivation. X W. was responsible for the phenanthrene (Phe) and cadmium (Cd) co-contaminated soil preparation and Fluorescein diacetate (FDA) activities analysis. Z. Z. performed the Cd and Phe concentration analysis in soil and other relative work. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. The effects of *P. indica*, cd and phe treatment on Chl a, Chl b, F0, Fm, Fv/Fm and ETR in *M. Sativa.*

− P: non-inoculation (control), + P: *P. indica.* Values are mean ± SE, n = 3. The same letter within each column indicates no significant difference among treatments using Duncan's Multiple Range Test.

Table 2. The effects of *P. indica*, cd and phe treatment on Chl a, Chl b, F0, Fm, Fv/Fm and ETR in *M. Sativa.*

Fungal treatment	Phe and Cd treatment	Chl a (mg/g FW)	Chl b (mg/g FW)	F0	Fm	Fv/Fm	ETR
-P	0	4.57±0.01^{g}	2.63±0.02^{a}	0.76±0.03^{a}	2.78±0.03	0.74±0.07^{b}	92.5±2.4^{c}
	Cd	3.58±0.10^{a}	2.10±0.05^{a}	0.85±0.04^{a}	2.66±0.04^{a}	0.58±0.07^{c}	88.5±1.12^{b}
	Phe	3.21±0.02^{b}	1.93±0.04^{a}	0.93±0.01^{b}	2.41±0.02^{b}	0.45±0.06^{d}	71.6±2.07^{de}
	Cd+Phe	3.10±0.03^{b}	1.76±0.02^{b}	1.07±0.02^{c}	2.17±0.03^{c}	0.31±0.05^{e}	67.5±3.02^{a}
+P	0	5.01±0.02^{c}	2.85±0.04^{c}	0.63±0.05^{d}	2.86±0.04^{d}	0.86±0.02^{f}	98.3±3.17^{b}
	Cd	4.26±0.04^{d}	2.55±0.06^{d}	0.78±0.06^{f}	2.72±0.06^{e}	0.76±0.02^{a}	104±2.45^{c}
	Phe	4.02±0.02^{e}	2.41±0.05^{e}	0.81±0.03^{e}	2.62±0.05^{f}	0.61±0.03^{b}	101.1±3.14^{d}
	Cd+Phe	3.78±0.04^{f}	2.17±0.03^{f}	0.92±0.02^{g}	2.31±0.03^{g}	0.52±0.04c	93.8±2.53^{e}

Fungal treatment	Phe and Cd treatment	Chl a (mg/g FW)	Chl b (mg/g FW)	F0	Fm	Fv/Fm	ETR
-P	0	4.57±0.01^{g}	2.63±0.02^{a}	0.76±0.03^{a}	2.78±0.03	0.74±0.07^{b}	92.5±2.4^{c}
	Cd	3.58±0.10^{a}	2.10±0.05^{a}	0.85±0.04^{a}	2.66±0.04^{a}	0.58±0.07^{c}	88.5±1.12^{b}
	Phe	3.21±0.02^{b}	1.93±0.04^{a}	0.93±0.01^{b}	2.41±0.02^{b}	0.45±0.06^{d}	71.6±2.07^{de}
	Cd+Phe	3.10±0.03^{b}	1.76±0.02^{b}	1.07±0.02^{c}	2.17±0.03^{c}	0.31±0.05^{e}	67.5±3.02^{a}
+P	0	5.01±0.02^{c}	2.85±0.04^{c}	0.63±0.05^{d}	2.86±0.04^{d}	0.86±0.02^{f}	98.3±3.17^{b}
	Cd	4.26±0.04^{d}	2.55±0.06^{d}	0.78±0.06^{f}	2.72±0.06^{e}	0.76±0.02^{a}	104±2.45^{c}
	Phe	4.02±0.02^{e}	2.41±0.05^{e}	0.81±0.03^{e}	2.62±0.05^{f}	0.61±0.03^{b}	101.1±3.14^{d}
	Cd+Phe	3.78±0.04^{f}	2.17±0.03^{f}	0.92±0.02^{g}	2.31±0.03^{g}	0.52±0.04c	93.8±2.53^{e}

− P: non-inoculation (control), + P: *P. indica.* Values are mean ± SE, n = 3. The same letter within each column indicates no significant difference among treatments using Duncan's Multiple Range Test.

Figures

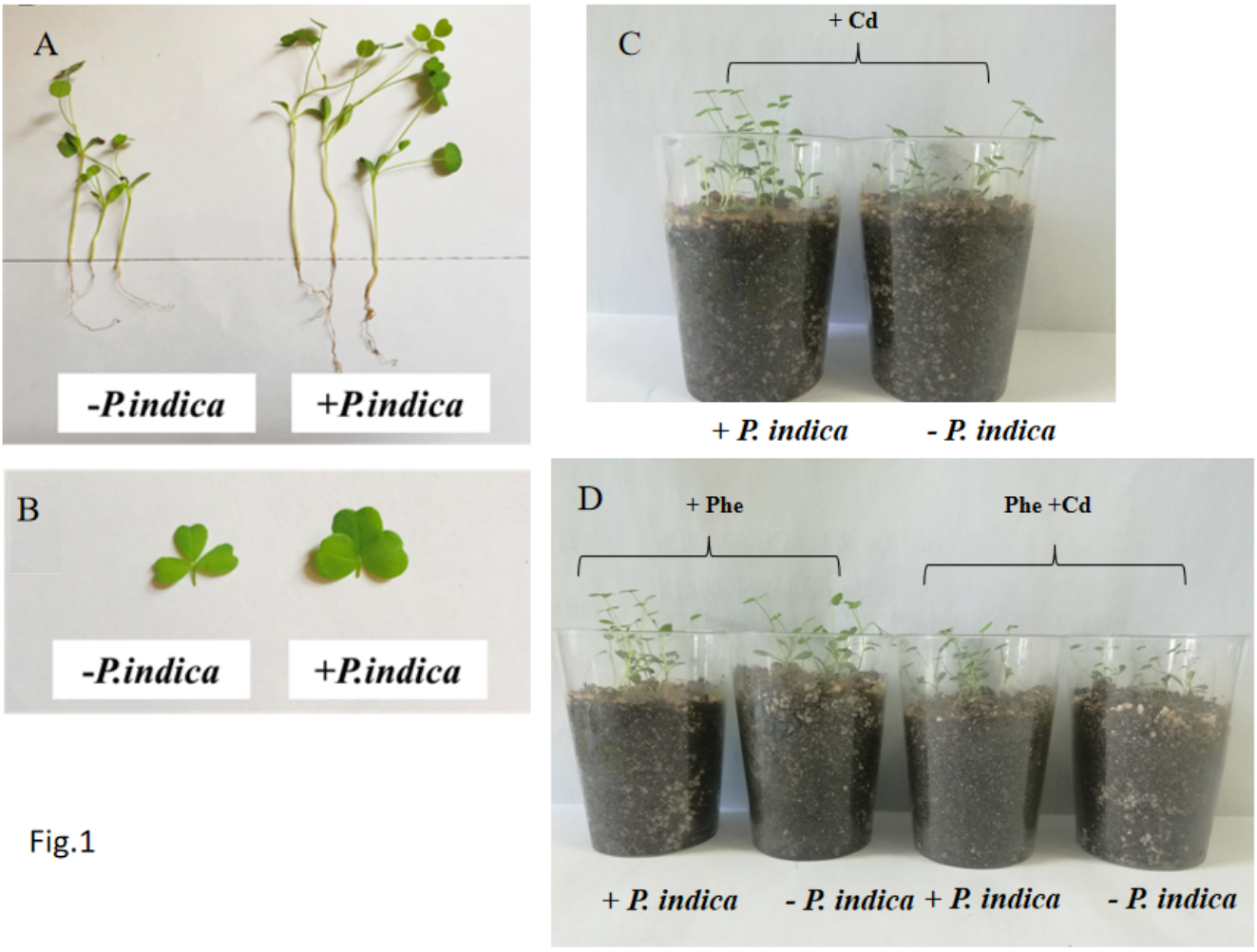

Fig.1

Figure 1

Biological effect on root and stem in Phe, Cd, and Phe-Cd co-contaminated soil with or without P. indica inoculation. A: Control with or without P. indica inoculation. B: Leaves area were compared between P. indica inoculation and non- P. indica inoculation without any contamination. C: Above-ground parts were compared between P. indica inoculation and non- P. indica inoculation in Cd contaminated soil. D: Above-ground parts were compared between P. indica inoculation and non- P. indica inoculation in Phe, and Phe-Cd contaminated soil.

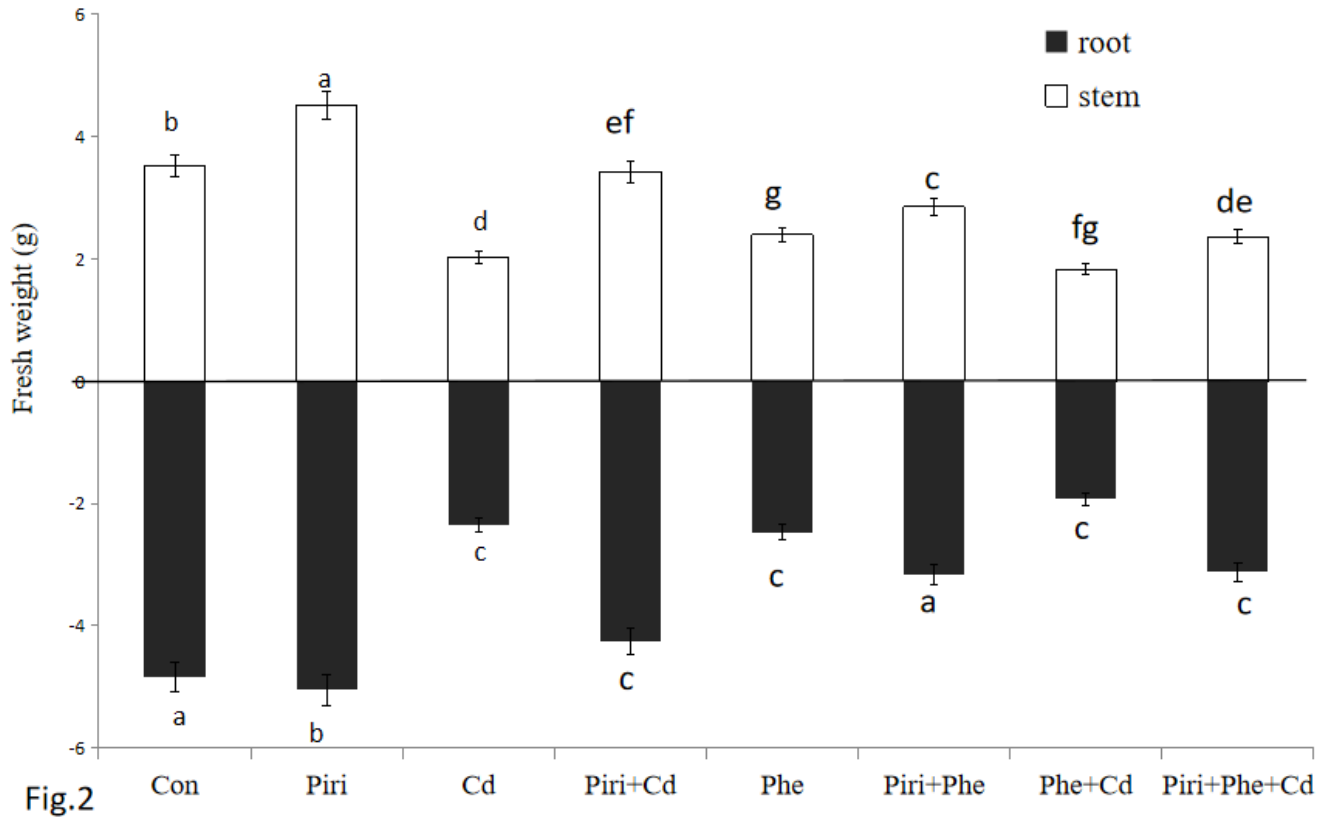

Fig.2

Figure 2

The fresh weight of stems and roots of M. sativa was statistically analyzed in control, Phe/Cd and Phe-Cd contaminated soil with or without P. indica colonization. Control with (Piri) or without (Con) P. indica inoculation; Phe contaminated soil with (Piri+Phe) or without (phe) P. indica inoculation; Cd contaminated soil with (Piri+Cd) or without (Cd) P. indica inoculation; Phe-Cd co-contaminated soil with (Piri+Cd+Phe) or without (Cd+Phe) P. indica inoculation. M. sativa was planted in all treatments. Each value of fresh weight is the mean of three replicates. Error bars show standard error. The fresh weight in different treatments were significantly reduced or increased as compared to the control according to Duncan's Multiple Range Test ($P < 0.05$).

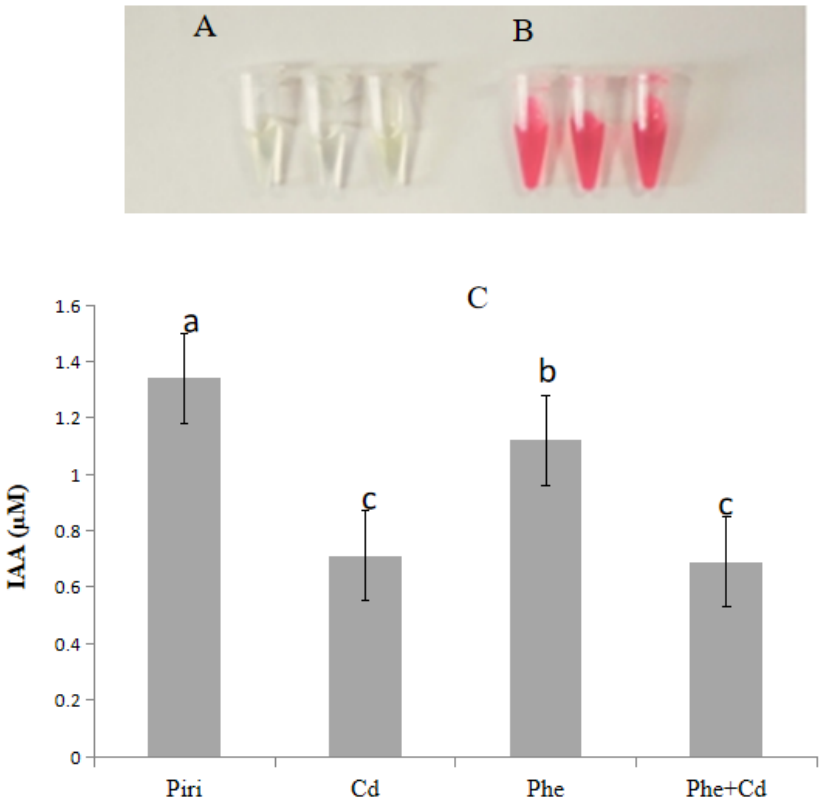

Fig.3

Figure 3

IAA production by P.indica during growth in CM medium containing Cd or Phe contamination. Qualitative analysis by Salkowski-method was performed to identify IAA production under phenanthrene, cadmium and both of phenanthrene and cadmium treatment. A: After adding the tryptophan, the solution not containing spores of P. indica under each treatment does not changed colour in each three repetitions. B: After adding the tryptophan, the solution containing spores of P. indica under each treatment changed to pink colour in each three repetitions. C: IAA was determined in culture supernatants by HPLC-MS after 6 weeks containing Cd, Phe or Cd and Phe contamination medium respectively. IAA concentrations in CM medium were 1.34+0.03 μM (n=3), but in Cd containing medium were 0.71+0.05 μM (n=3); in Phe containing medium were 1.12+0.07 μM (n=3), in Cd and Phe containing medium were 0.69+0.04 μM (n=3), respectively. Error bars show standard error. The IAA concentration was significantly reduced in the Cd, Phe and Cd+Phe treatments as compared to the control (P. indica) according to Duncan's Multiple Range Test ($P < 0.05$)

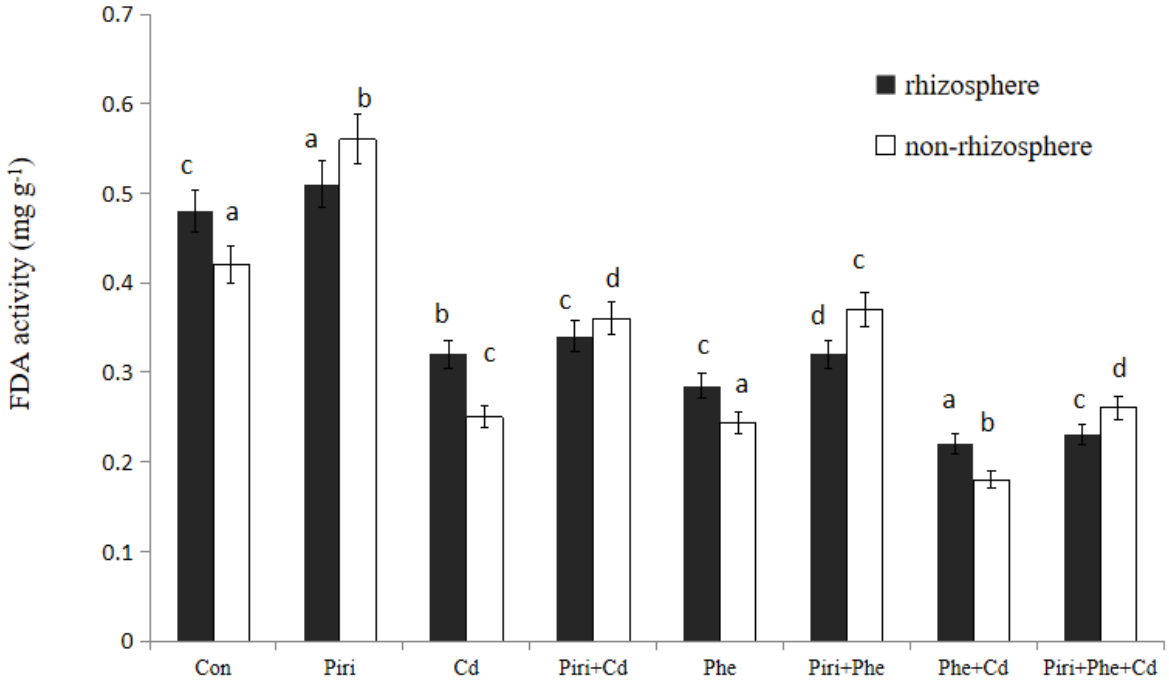

Fig.4

Figure 4

FDA activities were identified in control, Phe/Cd and Phe-Cd contaminated soil with or without P. indica colonization. Control with (Piri) or without (Con) P. indica inoculation; Phe contaminated soil with (Piri+phe) or without (phe) P. indica inoculation; Cd contaminated soil with (Piri+Cd) or without (Cd) P. indica inoculation; Phe-Cd co-contaminated soil with (Piri+Cd+Phe) or without (Cd+Phe) P. indica inoculation. M.sativa was planted in all the treatments. Each value of FDA activities is the mean of three replicates. Error bars show standard error. For the control, Cd, Phe and Cd+Phe treatments, the FDA activity in non-rhizosphere was significantly reduced as compared to the rhizosphere; For the Piri, Piri+Cd, Piri+Phe and Piri+Cd+Phe treatments, the FDA activity in non-rhizosphere was significantly increased as compared to the rhizosphere; for Control and Piri, Cd and Piri+Cd, Phe and Piri+Phe, Phe+Cd and Piri+Phe+Cd, the adding of P. indica spores significantly increases the FDA activity both in rhizosphere and non-rhizosphere as compared to the treatment without P. indica inoculation. . Different letters (a-d) in the columns indicate signifificant difference in FDA activities between treatments according to Duncan's Multiple Range Test ($P < 0.05$).

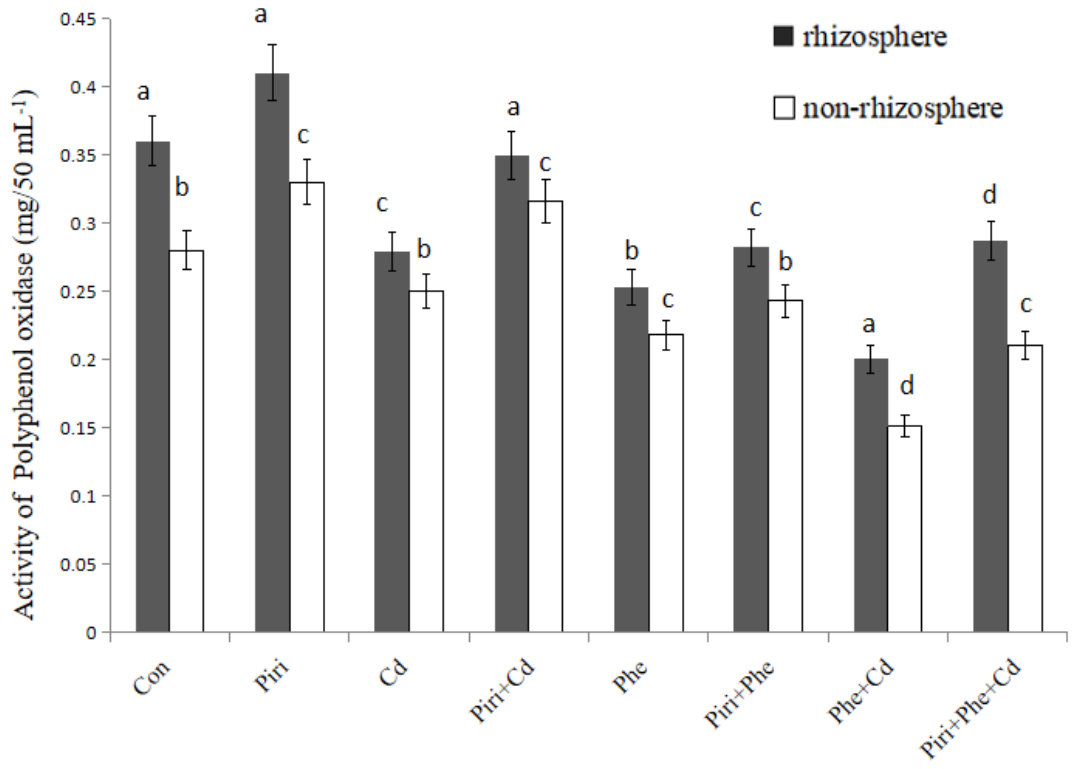

Fig.5

Figure 5

Polyphenol oxidase was identified in different treatments in the rhizosphere and non-rhizosphere. Control with (Piri) or without (Control) P. indica inoculation; Phe contaminated soil with (Piri+Phe) or without (Phe) P. indica inoculation; Cd contaminated soil with (Piri+Cd) or without (Cd) P. indica inoculation; Phe-Cd co-contaminated soil with (Piri+Cd+Phe) or without (Cd+Phe) P. indica inoculation. M. sativa was planted in all treatments. Each value of fresh weight is the mean of three replicates. Error bars show standard error. For Control and Piri, Cd and Piri+Cd, Phe and Piri+Phe, Phe+Cd and Piri+Phe+Cd, the adding of P. indica spores significantly increases the Polyphenol oxidase activity both in rhizosphere and non-rhizosphere as compared to the treatment without P. indica inoculation. Different letters (a-d) in the columns indicate signifificant difference in polyphenol oxidase between treatments according to Duncan's Multiple Range Test ($P < 0.05$).

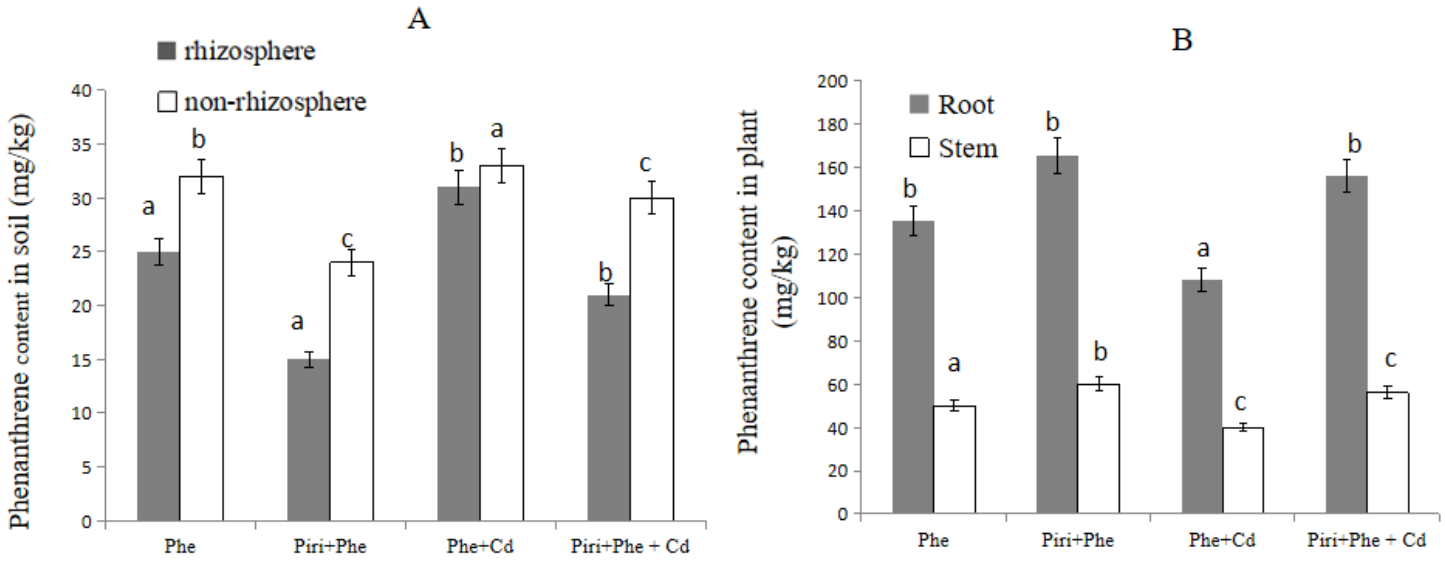

Fig.6

Figure 6

Phenanthrene content was identified in soil and plant. A: Phenanthrene content was identified different treatments in the rhizosphere and non-rhizosphere. B: Phenanthrene content was identified in different treatments in root and stem. Phe contaminated soil with (Piri+phe) or without (phe) P. indica inoculation; Phe-Cd co-contaminated soil with (Piri+Cd+Phe) or without (Cd+Phe) P. indica inoculation. M.sativa was planted in all treatments. Each value of phenanthrene content is the mean of three replicates. Error bars show standard error. For Phe and Piri+Phe, Cd+Phe and Piri+Cd+Phe, the adding of P. indica spores significantly reduced/increased Phenanthrene content in rhizosphere and non-rhizosphere/plant (root and stem) as compared to the treatment without P. indica inoculation. Different letters (a-c) in the columns indicate signifificant difference in phenanthrene content between treatments according to Duncan's Multiple Range Test ($P < 0.05$).

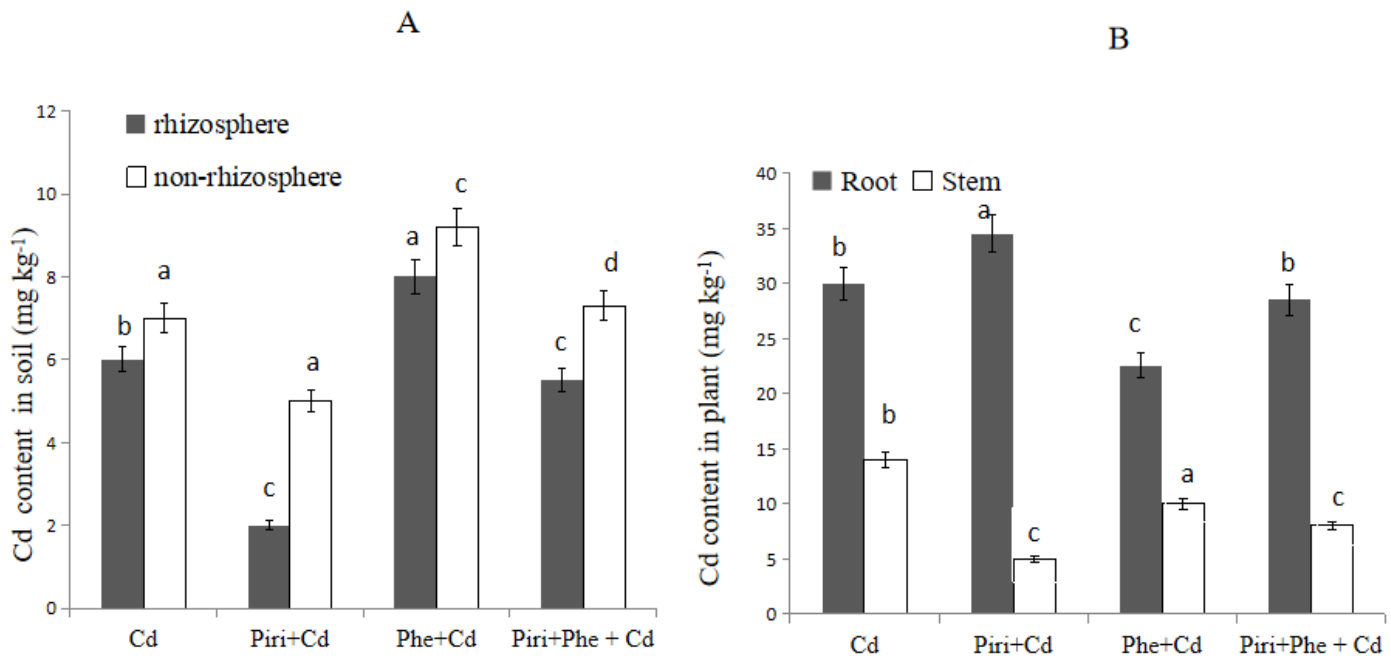

Fig.7

Figure 7

Cadmium content was identified in soil and plant. A: Cadmium content was identified in different treatments in the rhizosphere and non-rhizosphere. B: Cadmium content was identified in different treatments in root and stem. Cd contaminated soil with (Piri+Cd) or without (Cd) P. indica inoculation; Phe-Cd co-contaminated soil with (Piri+Cd+Phe) or without (Cd+Phe) P. indica inoculation. M. sativa was planted in all treatments. Each value of cadmium content is the mean of three replicates. Error bars show standard error. For Cd and Piri+Cd, Cd+Phe and Piri+Cd+Phe, the adding of P. indica spores significantly reduced/increased cadmium content in rhizosphere and non-rhizosphere/plant (root and stem) as compared to the treatment without P. indica inoculation. Different letters (a-c) in the columns indicate signifificant difference in cadmium content between treatments according to Duncan's Multiple Range Test ($P < 0.05$).

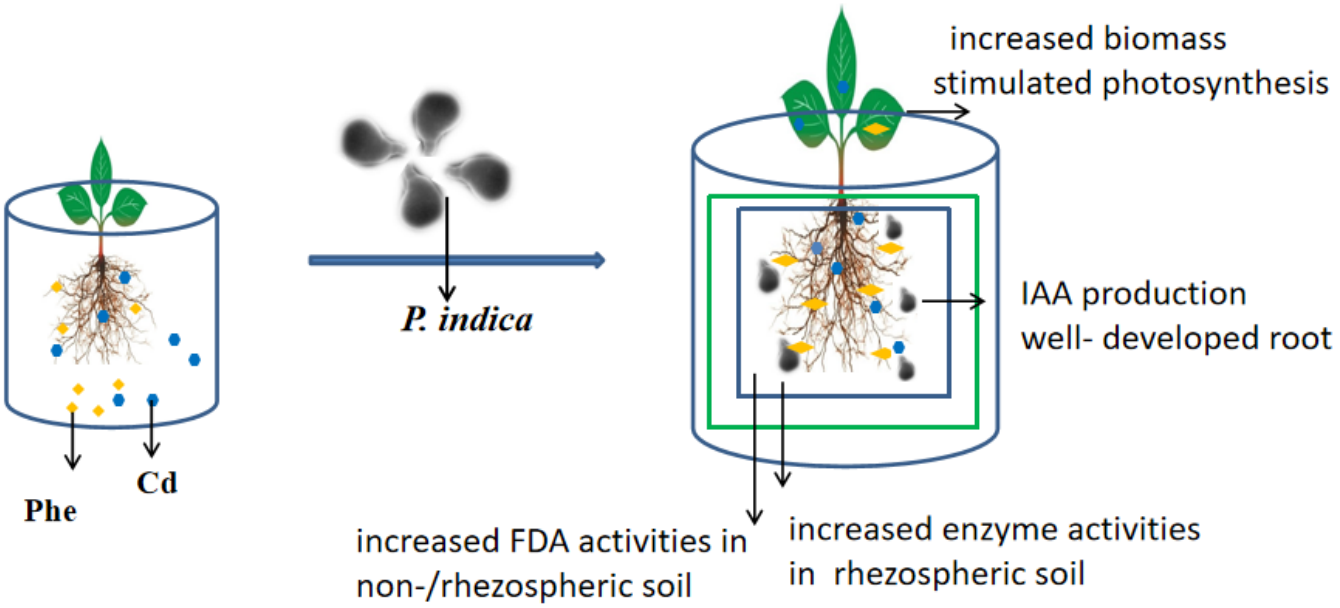

Fig.8

Figure 8

The phytoremediation effect of M. sativa colonized by P. indica in phenanthrene and cadmium co-contaminated soil. Four benefits aspects: including increased biomass, well- developed root, increased FDA activities in the rhizosphere and non-rhizosphere and increased enzyme activities in rhizosphere were obtained after P. indica colonization in M. sativa root under phenanthrene and cadmium co-contaminated soil.