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Impact of phosphorus amendments on cadmium uptake by sunflower inoculated with *Rhizophagus irregularis* mycorrhizae in a contaminated soil

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ABSTRACT

Immobilization of heavy metals (HMs) by phosphorus compounds is an efficient and cheap technique in decreasing their phytoavailability in soil, depending highly on the type and rate of HMs. Greenhouse research was performed to evaluate the impact of various amendments on cadmium (Cd) absorbed by sunflower plants in HM-contaminated soil collected from a mining area. The experiment was performed as a randomized complete block design with two factors, namely mycorrhizal fungi (*Rhizophagus irregularis*) and amendments (di-ammonium phosphate, humic acid, bone meal, and humic acid +bone meal), in three replications. The results showed that applying all the amendments reduced the amount of soluble Cd and Cd^{2+} species in the soil. The highest decreases occurred with the di-ammonium phosphate treatment (51% for soluble Cd), probably through decreasing pH and increasing phosphorus solubility, as a consequence, forming insoluble Cd phosphates. Mycorrhizal inoculation significantly decreased ($p \leq 0.05$) the Cd in the soil solution and the plant shoot but increased it in the plant root, probably through Cd accumulation in the fungal hyphae as Cd phosphates. The use of phosphate compounds is strongly recommended in order to immobilize Cd in highly contaminated sites.

1. Introduction

In recent decades, water and soil resources polluted with HMs present an important challenge because of their serious environmental and human health threats [1,2]. Cadmium (Cd) is one of the most important environmental pollutants, and its toxicity poses ecological, evolutionary, nutritional, and environmental problems. HM in agricultural soils can be easily absorbed by plants and thus

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enter the food chain [3,4]. Cadmium, however, accumulates in the main organs of the human body, such as the kidneys and lungs, and leads to some serious diseases such as cancer [1]. The use of phosphorus-containing compounds is one of the most effective methods to immobilize Cd in soil [1,5,6]. In this method, the application of phosphorus (P) to contaminated soils decreases Cd bioavailability so that it remains unavailable for plant roots for a long time and decreases its entry into the food chain through groundwater and surface water [7]. In addition, the apatite, clay containing phosphate, phosphoric acid, bone meal, and phosphate fertilizers have been used to amend soil containing HMs [8,9]. Bone meal is an organic substance containing P that can be used to improve soil contaminated with HMs [10]. Rhizospheric microorganisms may increase plant biomass and tolerance to HMs through direct and indirect mechanisms. Arbuscular mycorrhizal fungi (AMF) apply some mechanisms to suppress HMs stress to plants through chelating and immobilizing HMs into the external mycelium, improving the mineral nutrition of host plants, especially P, and adjusting the rhizosphere pH [11]. In addition, the AMF influences metal uptake by plants from the soil and transfers it to the roots and shoots, which depends highly on plant and fungi species ecotypes [6]. For instance, *Yao et al.* [12] declared that the inoculation of AMF (*Rhizophagus irregularis*) to the roots of clover (*Trifolium ripense L.*) significantly reduced Cd accumulation and, consequently, its toxicity in the plant. You et al. [13] reported that inoculation of these fungi increased the Cd absorption and translocation in the *Phragmites australis* species. However, the use of P organic/inorganic fertilizers can negatively affect the colonization and mycorrhizal activity, thereby affecting the uptake and accumulation of cadmium in plants. There is little research about the combined effects of phosphorus-containing fertilizers and mycorrhizal activities on plant uptake of cadmium in contaminated soils. Yazici et al. [14] found that the application of di-ammonium phosphate caused more uptake of cadmium in wheat by reducing mycorrhizal activity. The objective of the present study was to evaluate the effect of the combined application of AMF and fertilizers with different rates of P (humic acid with negligible content of P and bone meal and di-ammonium phosphate with high content of P) on Cd uptake by sunflower plants in contaminated soil.

2. Materials and methods

Our study was performed in a research greenhouse on sunflower plants (*Helianthus annus L.*) as a randomized complete block design with two factors and three replications. A Xeric Haplocalcids [15] HM-contaminated soil (sandy loam [16] with a pH of 8.22, organic matter of 2.2% [17], and a total Cd [17], Olsen-P [18], and total P [17] of 17, 14, 173 mg/kg, respectively) was collected from an area around a zinc mine in the Dandy region, Zanjan province, Iran. Some pots (2 kg of soil in each pot) were inoculated without AMF (M_0) or with *Rhizophagus irregularis* fungus (M_1) as the first factor; the others were treated without amendment (C) and with di-ammonium phosphate (D), humic acid (H), bone meal (B), and humic acid + bone meal (HB) as the second factor. Humic acid was applied at the rate of 0.01 g/kg; bone meal (produced from a Mashhad livestock industrial slaughterhouse) and the $(NH_4)_2HPO_4$ amendment were applied at the same equivalent rates of P (0.125 g P/kg). Some properties of the organic substances used in this study are shown in Table 1.

Table 1. Chemical properties of the bone meal and humic acid used.

Type	pH	EC (1:2.5)	K	P	N	C	Humic acid	Fulvic acid
		(dS/m)				(%)		
Bone meal	5.9	3.9	0.01	12	0.05	21.4	-	-
Humic acid	6.0	0.24	0.02	0.02	0.01	54.6	75.2	5.1

The pH and EC were determined in 1:2.5 humic acid or bone meal: water suspension; the total K, P, N, and C were measured by flame photometry, spectrophotometry, oxidation with potassium dichromate with Kjeldahl, and CNS elemental analyzer, respectively. The humic and fulvic acids

were isolated on the basis of differences in their solubility, and C was determined in two fractions by the CNS elemental analyzer [17]. The species of AMF (*Rhizophagus irregularis*) was purchased from the Shahrood Turan Biotechnology Company. The mycorrhizal inoculum was trapped culture of subterranean clover containing 50 spores per gram of soil. The mycorrhizal inoculum was mixed with this soil at a depth of 5 cm, and then sunflower seeds were sown in 2-kg pots. The pot moisture was kept at 60% water holding capacity by weighing the pots periodically and was irrigated with tap water when needed. All pots received 25 mg N/kg as urea one week after thinning the seedlings. After a two- month growing period, different parts of the plant in each treatment were removed and oven-dried at 72 °C for 48 hours; then, it was ground to a fine powder to determine the concentration of P and Cd. Mycorrhizal colonization in the roots of the sunflower treated with *Rhizophagus irregularis* inoculations was determined using the gridline intersect method [19] to confirm mycorrhizal associations. Soil subsamples were taken from the rhizosphere of each pot for analysis, collected from soil adhered to the root surface so that it was not removed by loose shaking [20]. For determining Cd and P rates in the soil solution, 20 mL of magnesium nitrate ($MgCl_2$ 0.01 M, extracts soluble and, to some extent, exchangeable Cd) was added to a 2 g soil sample [17,21,22]. The mixture was shaken for 24 h, and, eventually, the pH, concentrations of Cd, anions of Cl^-, $H_2PO_4^-$, and SO_4^{2-} were determined in the extract. Visual MINTEQ, a chemical equilibrium speciation software, was used to estimate the activity of the Cd species in the soil solution [22]. The total P content was spectrophotometrically determined using phosphomolybdate blue [17]. The amount of plant Cd and soil soluble Cd was determined using atomic absorption spectrometry (Perkin Elmer, AAnalyst 200). The data were analyzed using SPSS software, and significances were on the basis of probability level ≤ 0.05 for the Least Significant Difference (LSD).

3. Results and discussion

Mycorrhizal colonization (Figure 1) was observed in the roots of sunflower treated with *Rhizophagus irregularis* inoculations (M_1) and, to some extent, in the roots of non-inoculated plants (M_0) that belonged to a population of indigenous mycorrhizae in the soil researched.

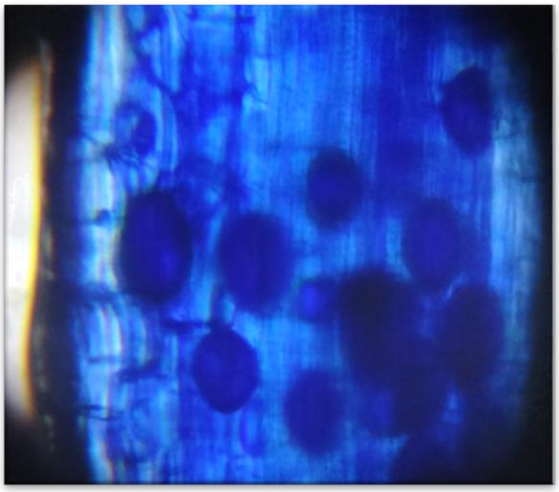

Fig. 1. Vesicular arbuscular mycorrhizal fungi (*Rhizophagus irregularis*) within the root tissues of sunflower under optical microscope.

3.1. Soil pH

Humic acid (H) and di-ammonium phosphate (D) treatments significantly decreased the soil pH value ($p \leq 0.05$) relative to the control (C) (Figure 2A). The use of P compounds, especially P fertilizers consisting of ammonium can decrease the pH value in alkaline soils. Because the ammonium is easily oxidized to nitrate, H^+ is released [9]. Other researchers also found significant decreases in the soil's pH by using different ammonium phosphates and calcium phosphate [5,23]. However, there was no significant difference ($p \leq 0.05$) in the soil pH values between the M_0 and M_1 treatments (data not shown).

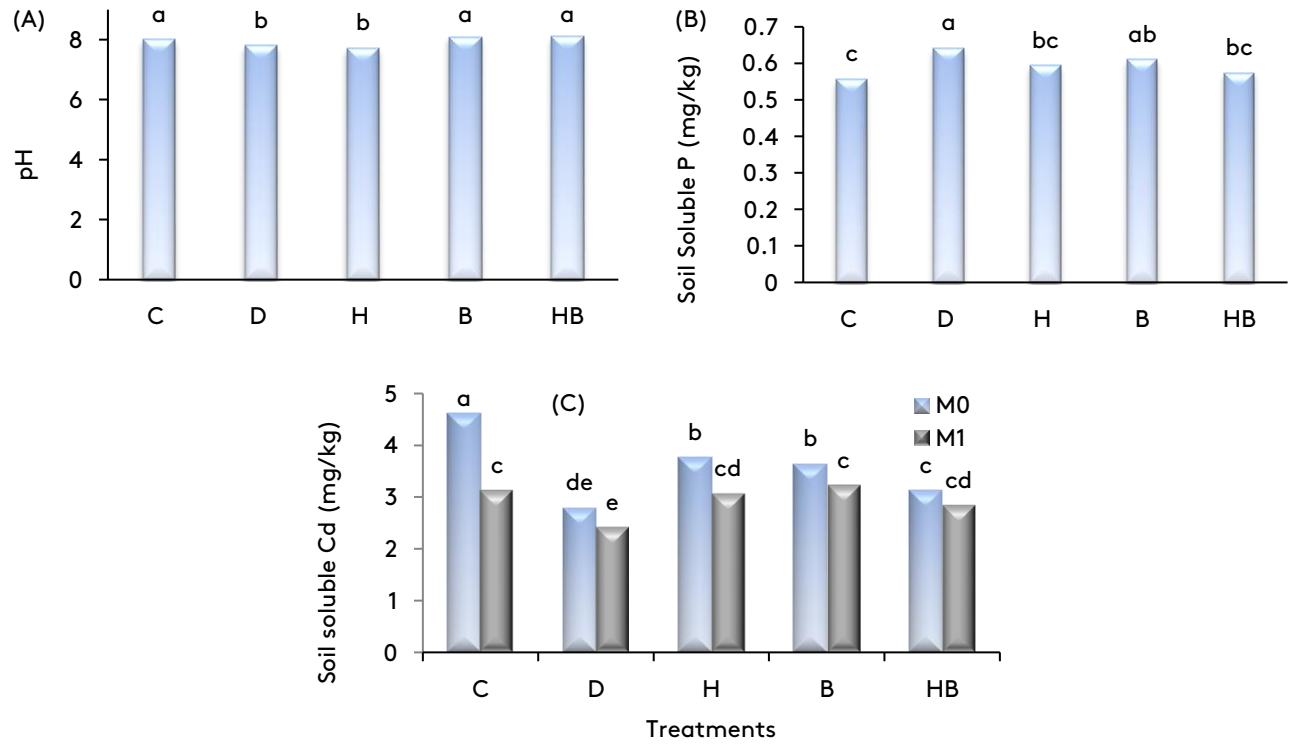

Fig. 2. Effects of different amendments and mycorrhizal inoculation on the soil pH (A), soil soluble P (B) and Cd (C). Different letters on the columns refer to significant differences ($P \leq 0.05$) based on LSD test. The pots were treated without amendment (C) and with di-ammonium phosphate fertilizer (D), humic acid (H), bone meal (B), and humic acid + bone meal (HB). M_0: The pots without inoculation, M_1: The pots inoculated by *Rhizophagus irregularis* fungus.

3.2. Soil soluble P

The applications of di-ammonium phosphate (D) and bone meal (B) led to significant increases ($p \leq 0.05$) in the soil soluble P compared to C (Figure 2B). It should be pointed out that the concentration of P in soil solution is very low, and it is strongly dependent on the pH. In general, the solubility of P in calcareous soils increases with decreasing pH until about 6.5, and then it decreases due to the formation of some insoluble P compounds [24]. It seems that di-ammonium phosphate could increase the soil solution P, probably due to having high P, as well as reducing the soil pH (Figure 2A). Bone meal also consisted of high P with an acidic pH (Table 1), thereby enhancing P in the soil solution.

3.3. Soil soluble Cd

The application of all organic and inorganic amendments and mycorrhizal inoculation significantly reduced ($p \leq 0.05$) the concentration of soil soluble Cd, in which the lowest concentration was found in the D+M_1 treatment (Figure 2C). In spite of lowering the pH in D+M_1 treatment, the soil soluble Cd was reduced about 38% relative to the C+M_0 treatment. In an incubation experiment, Abbaspour and Golchin [9] also found that applying di-ammonium phosphate to contaminated calcareous soil reduced the soil pH and Cd availability. The reduced Cd availability was mainly attributed to the formation of insoluble Cd phosphates. The results of another study showed that di-ammonium phosphate applied to contaminated soil transformed the soluble compounds of Cd into insoluble Cd phosphates [25]. *Dheri et al.* [26] reported that the use of P decreased soluble Cd concentrations in soil because of stable deposition of Cd phosphate ($Cd_3(PO_4)_2$). Xiao et al. [27] also showed that the addition of P fertilizer to contaminated soil could reduce Cd accumulation in the shoot of the *Trifolium repens* plant and EDTA-available Cd. The B+M_0 and H+M_0 treatments decreased the soil soluble Cd by 20.56% and 18.94%, respectively, when compared to C+M_0 (Figure 2C). The decreased solubility of Cd in the soil by applying organic amendments such as biochar may be due to the tendency of Cd for bonding with ligands released

during the decomposition process [28]. This finding was in agreement with several researchers [27,29,30]. The mycorrhizal inoculation to sunflower roots (C+M_1) decreased the soil soluble Cd by 31.02%, compared to the C+M_0 (Figure 2C). Some studies have confirmed that rhizospheric microorganisms, including AMF, detoxify heavy metals. They achieve this by producing chelators and metallothioneins, altering soil pH, or dissolving P compounds, thereby stabilizing heavy metals in the soil rhizosphere [31-33]. The activity of Cd^{2+} species was estimated by the Visual MINTEQ software on the basis of EC, pH, the amount of Cd, and some anions measured in the soil solution (Figure 3). The Cd^{2+} activity in the C and, to some extent, in B and HB treatments was probably supersaturated with octavite ($CdCO_3$) and controlled by soil-Cd or Cd phosphate in equilibrium with tri-calcium phosphate (TCP, $Ca_3(PO_3)_2$) and calcite, assuming that CO_2 gas pressure equals 0.003 atmosphere. In the D and H treatments, Cd^{2+} activity was probably controlled by Cd phosphate in equilibrium with di-calcium phosphate dihydrate (DCPD, $CaHPO_4.2H_2O$) and was undersaturated with activate and soil-Cd. It should be pointed out that P solubility in calcareous soils is probably controlled by calcium phosphates, whose solubility decreases proportionally to the increased pH value [34]. It is evident that DCPD is more soluble than TCP [34]. Therefore, the addition of di-ammonium phosphate (D) and humic acid (H) increased P solubility probably by decreasing the soil pH, thereby decreasing Cd solubility in the soil.

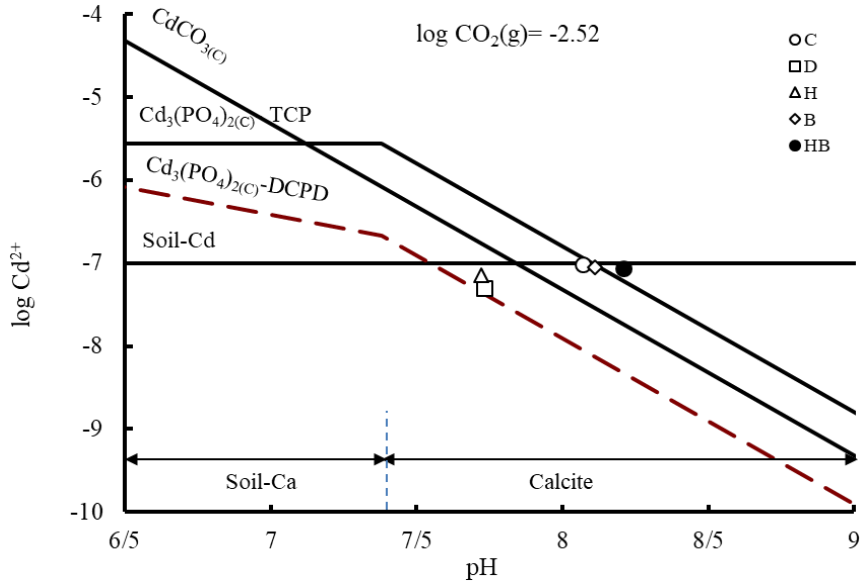

Fig. 3. Soil Cd^{2+} activity in the treatments as a function of pH in equilibrium with various Cd minerals. TCP: $Ca_3(PO_4)_2$, DCPD: $Ca(HPO_4).2H_2O$. The treatments are described in Figure 2.

3.4. Dry weights and P and Cd rates in the plant tissues

The highest dry weights of the shoot and root were observed in the D+M_1 treatment, indicating the effective application of di-ammonium phosphate associated with mycorrhizal inoculation on the plant growth (Figures 4A and 4B). It is clear that the appropriate addition of P fertilizers to soil improves plant growth. Several researchers have reported that plants inoculated with mycorrhizae have better growth and development through more efficient uptake of essential nutrients, especially P [24,33-37]. As well as promoting plant growth through better uptake of nutrients, species of AMF protect their host plants from harmful influences induced by heavy metals in contaminated soils [27]. It seems that AMFs are more tolerant than plants to high heavy metal concentrations in soil [31].

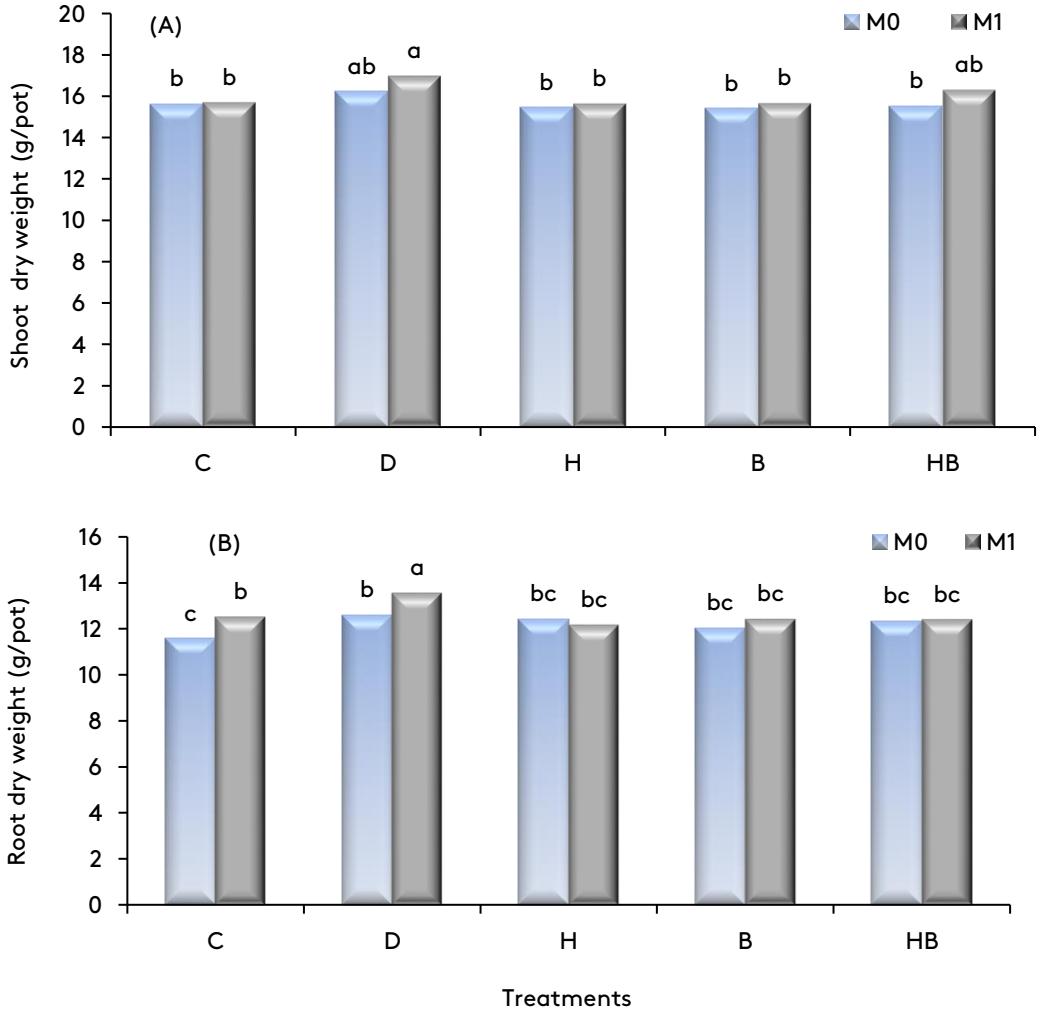

Fig. 4. Effects of different amendments and mycorrhizal inoculation on the shoot (A) and root (B) dry weights of sunflower. Different letters on the columns refer to significant differences ($P \leq 0.05$) based on LSD test. The treatments are described in Figure 2.

Compared to C+M_0, the shoot P rate increased significantly ($p \leq 0.05$) only in B+M_0 and HB+M_1 (Figure 5A). Although the soluble P concentration was the highest in the D treatment (Figure 2B), the shoot P rate did not significantly ($p \leq 0.05$) change relative to C+M_0. It may be attributed to the biomass dilution effect [38] caused due to the higher shoot dry weight in the D treatment (Figure 4A). The root P rate increased significantly ($p \leq 0.05$) in all treatments, except in D+M_0, H+M_0, and HB+M_0 treatments relative to C+M_0 (Figure 5B), indicating the better growth of the roots inoculated by mycorrhizae.

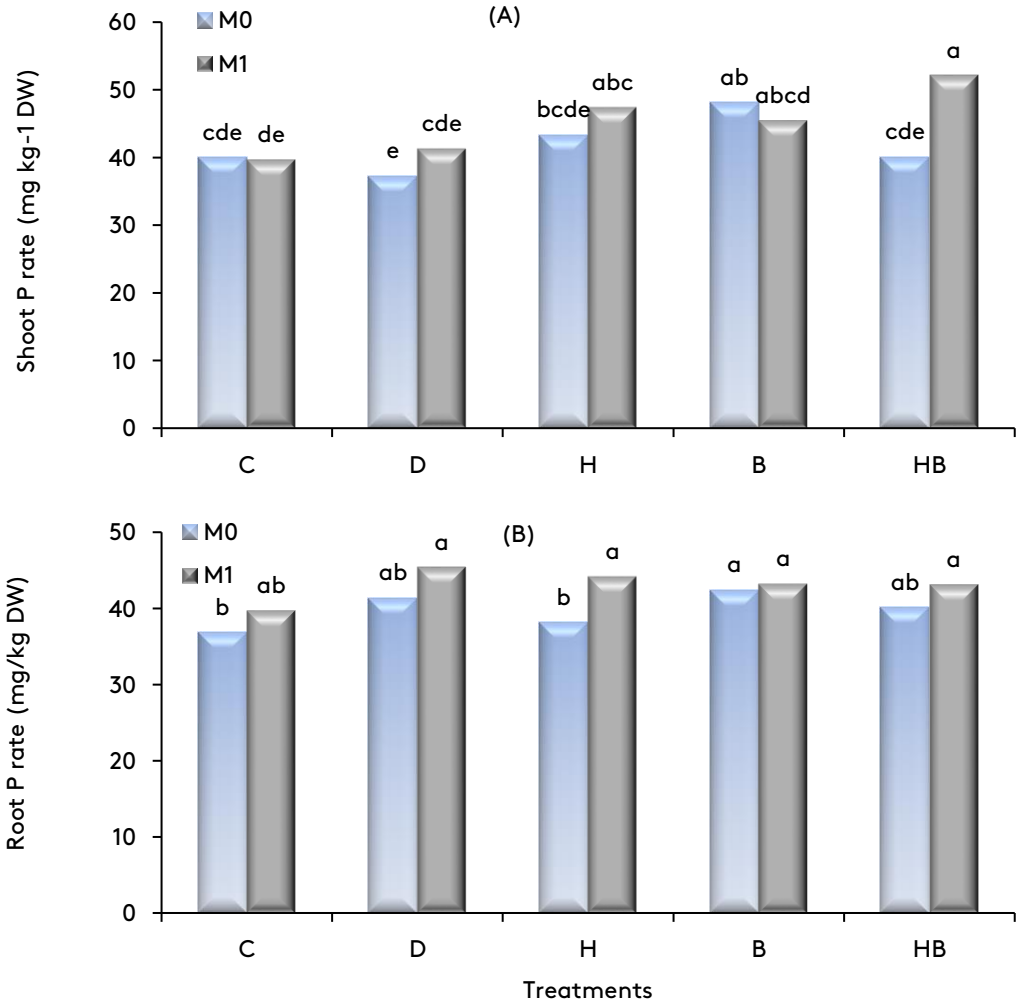

Fig. 5. Effects of different amendments and mycorrhizal inoculation on P rates in the shoot (A) and root (B) of sunflower. Different letters on the columns refer to significant differences ($P \leq 0.05$) based on the LSD test. The treatments are described in Figure 2.

All treatments, especially D, reduced the Cd accumulation in the sunflower shoot (Figure 6A), which was in agreement with *Brown et al.* [39]. They showed that the toxicity of some HMs such as Cd was reduced by P addition as a result of the precipitation reaction of P with Cd in the rhizosphere. The mycorrhizal inoculation (M_1) was more effective in the reduction of the shoot Cd than the non-inoculated treatments (M_0). Janoušková *et al.* [40] also showed that AMF inoculated into contaminated soils decreased Cd accumulation in tobacco stems. There is a general agreement that AMF can increase plant resistance against the HMs. Some researchers have also reported that mycorrhizal colonization can reduce the transfer of Cd to the stems and shoots of plants [11,12]. For instance, better uptake of nitrogen and phosphorus by the roots of bread wheat and less transfer of Cd from the roots to the shoots in symbiotic condition with AMF were also reported by Baghaie et al. [41]. However, the effectiveness of AMF on the plant accumulation of Cd depends highly on the soil's chemical and physical properties, Cd concentration in the soil and plant species, and genotypes [33,41,42]. Cadmium accumulated in the roots decreased by the application of all treatments, especially in non-inoculated treatments (Figure 6B). It seems that the roots inoculated by AMF prevented Cd transmission into the shoots. Andrade *et al.* [43] indicated that mycorrhizal plants absorbed HMs, with more accumulation in the roots but less transfer into the shoots when compared to the non-mycorrhizal plants. However, some possible mechanisms related to plants' tolerance to Cd by AMF have been explained by some researchers,

e.g., the retarded transfer of Cd from roots to aerial parts, the immobilization of Cd in soils, Cd uptake or complexation by some organic anions produced by hyphae, and the chelation or precipitation of Cd by polyphosphates presented inside hyphae, depending on plant species and environmental conditions [12,44]. The latter mechanism is considered to be the most probable process in this study because both the Cd (Figure 6B) and P rates (Figure 5B) in the plant root increased by the mycorrhizal inoculation.

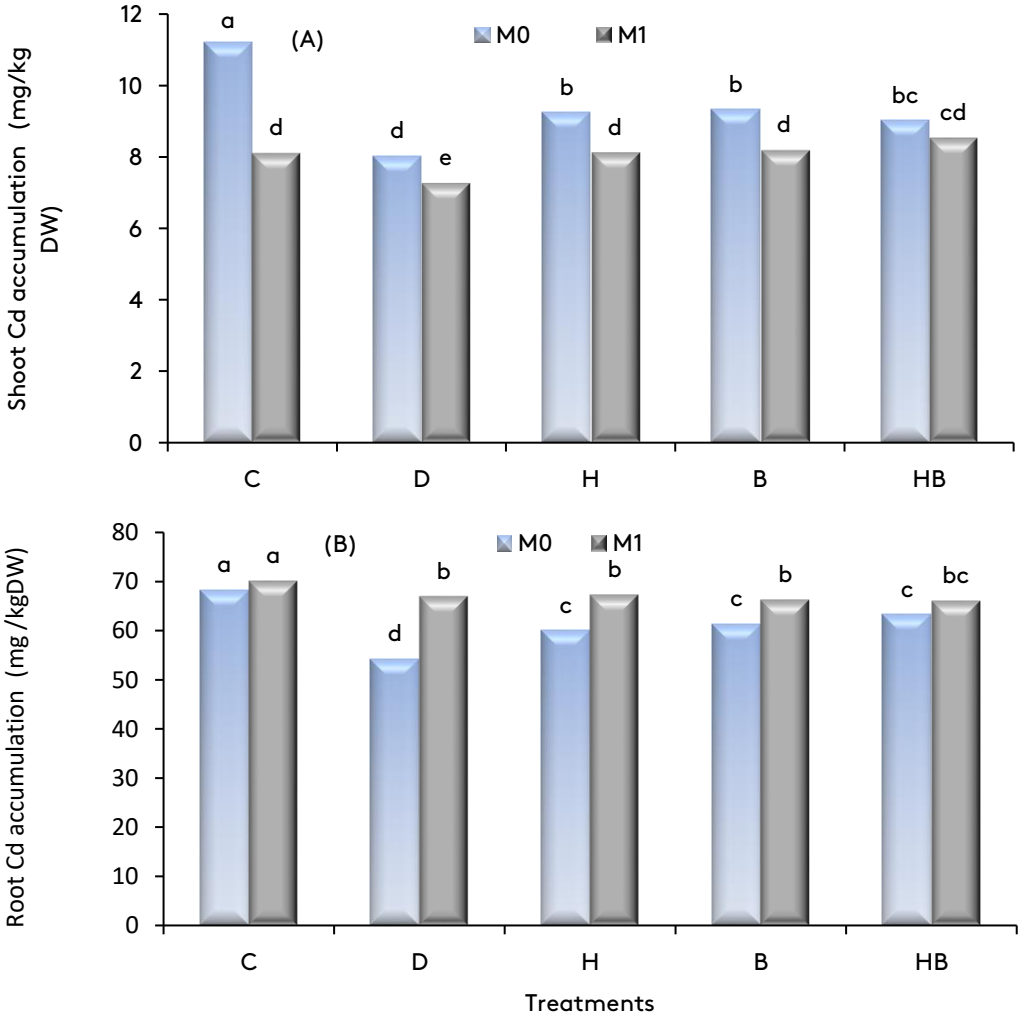

Fig. 6. Effects of different amendments and mycorrhizal inoculation on Cd accumulation in the shoot (A) and root (B) of sunflower. Different letters on the columns refer to significant differences ($P \leq 0.05$) based on the LSD test. The treatments are described in Figure 2.

4. Conclusions

The root Cd levels were 5-9-fold higher than those of the shoot Cd in almost all treatments, in which the lowest (55.3 mg/kg) and the highest (69.5 mg/kg) were observed in D+M_0 and C, respectively. This result denoted the fact that applying di-ammonium phosphate decreased the Cd amount in the roots, probably due to the formation of sparingly soluble minerals such as Cd phosphates. The inoculation of the plant roots by *Rhizophagus irregularis* mycorrhizae caused a significant increase in the roots' Cd levels despite a reduced Cd concentration in the soil solution. It seems that the AMF can develop mechanisms to detoxify Cd in the root and/ or protect the plant from the excessive translocation of Cd from the root to the shoot. The most probable mechanism that occurred in the plant root was the reaction of aqueous Cd with phosphates absorbed by the fungal hyphae and, ultimately, the formation of insoluble Cd phosphates like what happened in the soil. However, it should be pointed out that this

occurrence may not be found inside the roots of non-inoculated plants.

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