



Decreasing Plant Abiotic Stress in the Soil Treated with Sewage Sludge Using *Piriformospora indica* Fungus and Multi Walled Carbon Nanotubes

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Abstract

Today, finding suitable methods to decrease plant abiotic stress such as heavy metals or salinity is very necessary in arid and semi-arid regions. Thus, this research was conducted as a factorial experiment in the layout of a completely randomized block design to evaluate the role of *Piriformospora indica* fungus and multi-walled carbon nanotubes (MWCNTs) on decreasing plant abiotic stress in the soil treated with lead (Pb)-polluted sewage sludge. The treatment consisted of applying sewage sludge at the rates of 0, 15, and 30 t/ha, 3 levels of cadmium spiked in to the applied sewage sludge (0, 800 and 1600 mg Pb/kg), as well as 0%, 0.5%, and 1.5% (W/W) MWCNTs in the presence and absence of *P. indica*. The plant used in this experiment was barely. After 90 days of the experiment, the plant was harvested and its Pb concentration was measured using atomic absorption spectroscopy. Applying 0.5 and 1% (W/W) MWCNTs in the soil treated with 15 t/ha sewage sludge significantly decreased plant Pb concentration by 8.1% and 12.3%, respectively. In addition, the presence of *P. indica* had significant effects on decreasing plant Pb concentration since the lowest plant Pb concentration was observed in soil amended with 30 t/ha sewage sludge (800 mg Pb/kg) by receiving 1.5% (W/W) MWCNTs in the presence of *P. indica*. The results of this study showed that applying MWCNTs has significant effects on decreasing soil heavy metals or soil salinity that is a positive point in environmental studies.

Keywords: Multi-walled carbon nanotubes, Sewage sludge, Pb, Microbial respiration

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1. Introduction

Organic matter is the soil organic fraction that is formed from the decomposition of plants, animals, and microorganisms. In addition, it is considered to be a major component of the earth ecosystem that plays an important role in improving the physical, chemical, and biological properties of the soil (1) and is considered as a source of flux and energy for the soil micro-organism population (2). Soil organic matter is also an important food source for plant growth. Due to the lack of organic matter in arid and semi-arid soils, the use of any organic compound, especially sewage sludge is essential for improving the soil quality and structure, as well as soil permeability, water holding capacity, and aggregate stability. In addition, this type of compound contains high amounts of macronutrient elements such as nitrogen, phosphorus, and potassium or micro-nutrients including iron, zinc, copper (Cu), and manganese (Mn) that are essential for the human food chain (3). The application of sewage sludge to soil has been widespread and many researchers

have confirmed the positive influence of sewage sludge on soil and crop production, including increasing the nutrient and organic matter content of the soil, as well as soil structure and porosity, soil cation exchange capacity, and enzymatic activities (4), which is regarded as a positive point in environmental studies. However, the negative effect of applying sewage sludge cannot be ignored as well. The application of sewage sludge can result in excessive concentrations of heavy metals such as lead (Pb) and cadmium (Cd) if its quality is not controlled (5), which threatens human health via entering high amounts of heavy metals in the environment (6).

Soil salinity and contamination with heavy metals are 2 main problems of many arid and semi-arid regions of the world that may be related to applying organic amendments such as sewage sludge (7). In industrial countries where large amounts of sewage sludge are produced by urban populations, the use of biosolids in the field is a common practice (8,9). However, increasing its application in the soil may increase soil heavy metals

such as nickel or the soil salinity (10,11). In addition, the contamination of soils with heavy metals has become a serious environmental issue in the present times. The important point is that soil salinity can increase soil heavy metal that is considered as a negative point in environmental studies. Zhao et al reported that salinity can increase Cd, Cu, Mn, and Pb availability in the soil (12). Although Pb is not an essential nutrient element, it is easily absorbed through plant roots which is dangerous to the food chain. The accumulation of Pb in plant tissues can also be toxic and cause growth retardation. Further, the presence of Pb in the food chain can lead to a disease in humans, animals, and microorganisms (13).

Furthermore, heavy metals are highly toxic environmental pollutant which causes major effects on human health even at lower concentrations. Although some heavy metals are used for various biological functions in human beings, they become toxic when they exceed certain threshold limits. For instance, Pb is one of the toxic heavy metals that enters water bodies and is a major pollutant through various industrial and agricultural sectors. Moreover, heavy metals such as Cd and Pb are toxic trace pollutants for many organisms including the plants via the production of reactive oxygen species (ROS) in the plant that are very toxic to the plant cells. Antioxidant activities such as catalase, superoxide dismutase, ascorbate peroxidase, and glutathione reductase, as well as glutathione, ascorbate, and proline in the plants protect their cells against the damaging influence of ROS (14). Thus, finding a suitable method for decreasing the abiotic stress is necessary.

Piriformospora indica is a root endophytic fungus that colonizes the roots of a wide variety of plant species and promotes their growth and tolerance against abiotic and biotic stresses (15). The activation of the antioxidant enzyme systems is the main target of *P. indica* in the leaves of barley and Arabidopsis (16). Sun et al indicated that *P. indica* induced drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes and reducing the malondialdehyde content (17). Additionally, the symbiotic microbial association can enhance the ROS-antioxidant defense system, and ultimately, improve the plant fitness under the stress (18). The beneficial effects for the plant, including increased shoot dry matter, stem diameter, and leaf area can be a result of improved nutrient supply by the root endophytic fungus.

On the other hand, nano-materials (NMs) are defined as the materials with at least one dimension of 100 nm or less. Due to their unique size and extraordinary properties, NMs have potential applications for reducing heavy metal availability. Similarly, carbon nanotubes (CNTs) are cylindrical molecules that are composed of carbon atoms. In addition, these nanotubes may vary in length, diameter, chirality (the symmetry of the rolled graphite sheet), and the number of layers. According to their structure, CNTs maybe classified into 2 main groups including single-

walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). Recent developments have increased the potential applications of CNTs in the field of environmental remediation. Further, the interactions between CNTs with contaminants influence the bioavailability of contaminants to organisms. Dehghani et al investigated the role of SWCNTs and MWCNTs in decreasing the heavy metals from the polluted aqueous source and concluded that these NMs would be suitable adsorbents for heavy metal ions in wastewater (19).

Although some studies have investigated the role of *P. indica* fungi in reducing the abiotic stress caused by salinity or heavy metals, only some of them have reported about the function of the fungus in the presence of the organic amendment soil including sewage sludge. On the other hand, there is limited information about the role of MWCNTs in the removal of heavy metals from the aqueous solution. However, no study has assessed the effect of these NMs on reducing the heavy metal concentration in the presence of the plant. Thus, this research aimed to evaluate the interaction effect of MWCNTs, *P. indica*, and sewage sludge on decreasing plant abiotic stress in the soil treated with sewage sludge.

2. Materials and Methods

A factorial experiment in the layout of randomized completely block design was set up to investigate the effect of applying sewage sludge, Cd concentration, and MWCNTs on decreasing the abiotic stress. The treatments consisted of applying sewage sludge at the rates of 0 (S_0), 15 (S_{15}), and 30 (S_{30}) t/ha, the 3 levels of Cd spiked in to the applied sewage sludge (0 (Pb_0), 800 (Pb_{800}), and 1600 (Pb_{1600}) mg Pb/kg), as well as 0 (M_0), 0.5 ($M_{0.5}$), and 1.5 ($M_{1.5}$) % (W/W) MWCNTs in the presence and absence of *P. indica*. To investigate the effect of sewage sludge on the changes in soil sorption properties, a sandy loam, non-saline, non-calcareous soil with low organic carbon content was collected from soil surface layer (0-15 cm) around Pakal village in Markazi province. The sewage sludge was collected from the Isfahan north wastewater (Shahinshahr). The selected physic-chemical properties of the soil and sewage sludge in this experiment are provided in Table 1.

On the other hand, the sewage sludge was polluted with Pb at the rates of 0 (Pb_0), 800 (Pb_{800}), and 1600 (Pb_{1600}) mg Pb/kg sewage sludge and incubated for 2 weeks. Then, the sewage sludge was added to the soil and incubated for 2 weeks. During the incubation, the sewage sludge amended soil was wet and dried to reach the equilibrium. In addition, the MWCNTs were added to the soil at the rates of 0, 0.5, and 1.5% (W/W).

The fungal strain of *P. indica* was obtained from the soil biology of water and soil research institute. According to the methods of Zamani et al, inoculum for the experiments was prepared in the soil biology laboratory of Isfahan University of Technology (20). Barely seeds (Afzal

Table 1. Selected Physicochemical Properties of the Soil and Sewage Sludge

Characteristic	Unit	Amount	
		Soil	Sewage Sludge
Soil Texture	---	Loamy Sand	---
pH	---	7.4	6.7
EC	dS/m	1.2	11.3
Available Pb	mg kg ⁻¹	ND	3*
Available Cd	mg kg ⁻¹	ND	0.3
Available As	mg kg ⁻¹	ND	2
Available Ni	mg kg ⁻¹	ND	1.6
Organic carbon	%	0.8	18.2
CaCO ₃	%	7	10

Note. *Before enrichment.

Cv.) were sterilized for 5 minutes in 70% ethanol solution, followed by sterilizing for eight minutes in NaClO solution (0.75% Cl) and then washed with the deionized water. The seeds were placed on an agar surface medium in closed Petri-dishes (diameter: 120 mm) and incubated for 2 days at 25°C for germination. Next, 2 uniform sets of seedlings with the radicles of about 1 cm length were selected for the experiment, one of which was inoculated with *P. indica* by immersion for 3 hours in inoculums (adjusted nearly to 2×10^6) under gentle shaking. The non-inoculated seedlings were dipped in sterilized distilled water containing Tween 0.02%. Then, the 5 kg pots were filled with the treated soils. The inoculated or non-inoculated seedling (10 seeds) was planted at a depth of 1 cm in the uncontaminated top soil layer in the center of each pot and irrigated to reach near field capacity. The plant requirement water was determined according to the moisture curved (21). At the end of the experiment (90 days), barely plants were harvested and the soil and plant above the ground of each pot were transferred to the laboratory. According to Baghaie et al (22), the soil and plant Pb concentrations were also measured using atomic absorption spectroscopy (Perkin Elmer, model: 3030).

Soil microbial respiration was determined by measuring the soils that released CO₂ during 48 hours of incubation (23). Accordingly, the soil samples of each treatment in

3 replications were incubated (3 days) at 25°C in 250-mL glass containers that were closed with rubber stoppers. The CO₂ produced by the soil microbial respiration was collected into a 10 mL NaOH 0.1 N solution. Finally, the remaining NaOH was titrated with HCl and 3 glasses containing NaOH without any soil were also considered as the control treatment (23).

2.1. Statistical Analysis

Statistical analyses were calculated according to the ANOVA procedure using SAS software, version 9.1. The mean differences were considered based on the least significant difference (LSD test). The 95 percentage ($P=0.05$) probability value was considered for determining the significant difference.

3. Results and Discussion

Applying MWCNTs had no significant effect on the soil pH (Fig. 1a) while it increased soil sorption properties such as cation exchange capacity (CEC) so that applying 0, 5, and 1.5% (W/W) MWCNTs significantly increased the CEC by 12 and 16%, respectively (Fig. 1b).

Farghali et al investigated the role of MWCNTs in removing heavy metals from the soil solution and concluded that applying these amendments can increase sorption properties and thus decrease the availability of heavy metals, which is similar to our results (24). However, their study was carried out merely in aqueous solutions. The positive point of using MWCNTs in the present study is the increasing effect of soil sorption properties and the non-significant effect of the use of these compounds on the soil pH that can affect heavy metal availability. Several studies reported an increase in soil Pb availability by decreasing pH (25-27). Based on the results of our study, increasing the soil CEC had a positive effect on decreasing soil Pb availability that is a positive point in environmental studies.

On the other hand, applying sewage sludge had a significant effect on decreasing the soil pH (Fig. 2a),

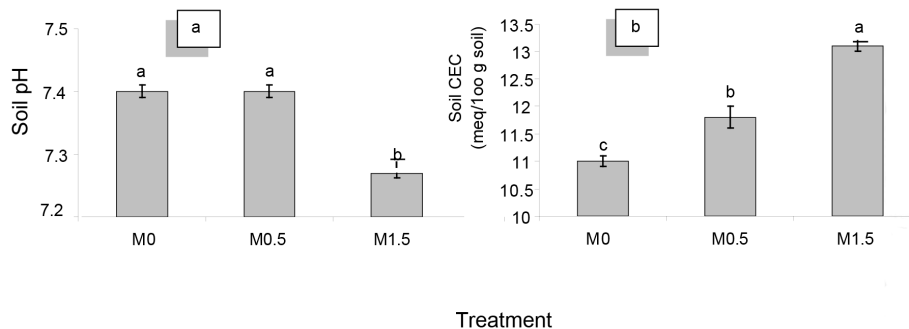


Fig. 1. Effect of Treatments on Soil pH (a) and Soil CEC (b).

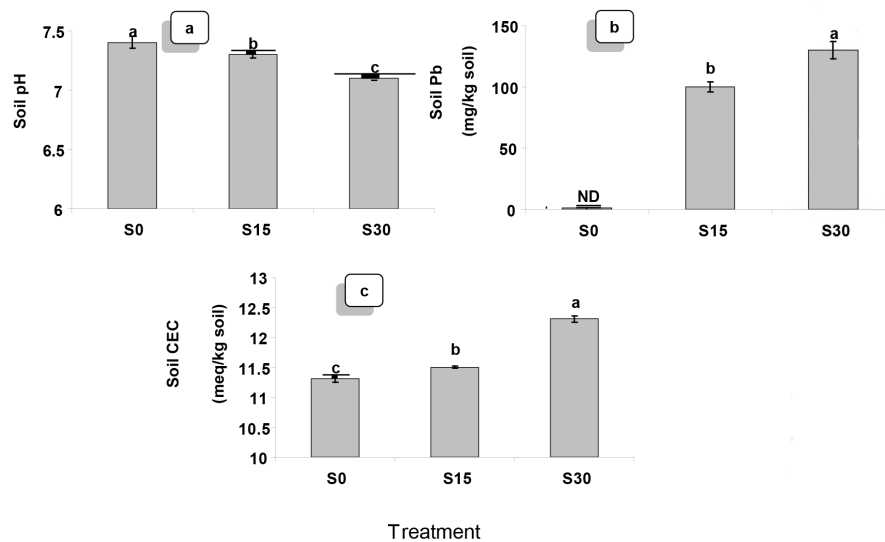


Fig. 2. Effect of Sewage Sludge on Soil pH (a), Soil Pb Availability (b), and Soil CEC (C)

leading to an increase in soil Pb availability (Fig. 2b) that is a negative point in environmental studies. Karami et al (28) evaluated the heavy metal uptake by the wheat from a sewage sludge-amended calcareous soil and concluded that applying this organic amendment cause a significant decrease in the soil pH and thus increased soil heavy metal availability, which is similar to our results. However, the role of sewage sludge in increasing soil sorption properties such as CEC (Fig. 2c) is undeniable.

Applying 30 t/ha Pb-enriched sewage (10 and 15 mg Cd/kg sewage sludge) significantly decreased the soil pH by 0.3 units and the pH decrease significantly increased soil Pb availability. However, the CEC significantly increased by 1.2 units due to applying 30 t/ha sewage sludge. Today, sewage sludge is used as a common practice method of fertilization to increase the soil organic matter in arid and semi-arid soils of the central of Iran. Nevertheless, its negative aspect regarding increasing the heavy metal concentration of the soil is extremely more than its positive effects on increasing the soil organic matter. Sohrabi et al studied the effects of sewage sludge on heavy metal concentrations and some morphological characteristics of lettuce and found that sewage sludge should not be used for growing edible food crops. However, it should be a difference based on the local conditions, vegetation type, and soil characteristics (29).

Regardless of the negative effect of heavy metals on sewage sludge, applying that amendment significantly increased the soil nitrogen. In other words, applying 15 and 30 t/ha organic amendment significantly increased soil N by 0.2 and 0.4 %, respectively. Fathololomi et al investigated the effect of municipal sewage sludge on the concentration of macronutrients in the soil and plant and

some agronomic traits of the wheat (30) and concluded that sewage sludge can be recommended as a suitable application rate for improving the chemical quality of the soil and soil nutrient concentration. However, the sewage sludge electrical conductivity (EC) and its role in plant growth are irrefutable. Similarly, Latare et al examined the effect of direct and residual application of sewage sludge on yield, heavy metal content, and soil fertility under rice-wheat system and observed a significant buildup of P, S, Zn, Fe, and Mn by using the sewage sludge (31) although the negative effect of heavy metals on sewage sludge is unquestionable.

The interaction effect of sewage sludge, Pb concentration, the presence of *P. indica*, and the use of MWCNTs on plant Pb availability was significant (Table 2). Further, the greatest plant Pb concentration belonged to the soil that was obtained in 1600 mg Pb/kg soil inorganic Pb salt without receiving MWCNTs in the absence of *P. indica* (Table 2). Likewise, Baghaie et al evaluated the role of the organic and inorganic fractions of sewage sludge and cow manure in plant availability and concluded that metals added to the organic amendment are less phyto-available compared to metal salts added to the soils without organic amendment (22), which is similar to our results.

The lowest plant Pb concentration was observed in the soil amended with 30 t/ha sewage sludge (800 mg Pb/kg) by receiving 1.5% (W/W) MWCNTs in the presence of *P. indica*. On the other hand, plant Pb concentration in the control soil or without adding Pb salt was undetectable by atomic absorption spectroscopy. The important point is that applying MWCNTs can decrease soil Pb concentration due to increasing the soil CEC (Fig. 1b) while decreasing Pb plant uptake. In other words, its

Table 2. The Effect of Sewage Sludge, Pb Concentration, the Presence of *P. indica*, and the Use of MWCNTs on Plant Pb Availability

MWCNTs	Pb Concentration	+ <i>P. indica</i>			- <i>P. indica</i>		
		Sewage Sludge			Sewage Sludge		
		0	15	30	0	15	30
0		ND	ND	ND	ND	ND	ND**
0.5	0	ND	ND	ND	ND	ND	ND
1.5		ND	ND	ND	ND	ND	ND
0		44.1i	42.2k	39.9m	47.3	45.9h	43.4j
0.5	800	41.8l	40.0m	36.4o	44.2i	42.1k	40.3m
1.5		39.3m	37.2n	33.9p	42.7k	39.9m	36.8o
0	1600	54.3b	52.2c	49.7f	57.1a	54.7b	51.4d
0.5		52.1c	49.4f	47.3g	54.3b	51.2d	49.4f
1.5		50.7e	47.2g	44.6i	52.7c	49.3f	47.1g

Note. *Date with the similar letter are not significantly different ($P=0.05$, LSD test); **ND: Not detectable by atomic absorption spectroscopy.

application can diminish the negative effect of using Pb polluted sewage sludge on increasing soil heavy metals that may be related to the role of increasing the soil sorption properties. Generally, MWCNTs have a high adsorption capacity due to their high surface area and can immobilize heavy metals in the soil, which can help remediate the contaminated soils. On the other hand, the application of these compounds can have a persistence role in reducing the availability of heavy metals in the soil due to their non-degradability (32). According to the results of this study, applying 0.5 and 1% (W/W) MWCNTs in a soil treated with 15 t/ha sewage sludge significantly decreased plant Pb concentration by 8.1 and 12.3% that is a positive point in environmental studies.

In addition to increasing the soil heavy metals due to applying sewage sludge, using this organic amendment had a significant effect on soil salinity so that The greatest soil EC (Table 3) has belonged to the soil treated with 30 t/ha Pb polluted sewage sludge (800 mg Pb/kg). The important point is the increase of the soil EC by increasing the plant Pb availability (Table 2) which is a negative point in environmental studies. In other words, the application of 30 t/ha Pb polluted sewage sludge (1600 mg Pb/kg) significantly increased the soil EC and plant Pb availability by 14 and 11.5%, respectively, and this can reduce the plant biomass (No data are available in this regard in this study).

The greatest soil microbial respiration was observed in a soil treated with 30 t/ha non-Pb polluted sewage sludge under *P. indica* inoculated plant (Table 4) while the lowest soil microbial respiration belonged to the Pb polluted soil (1600 mg Pb/kg soil) with any organic amendment in the absence of *P. indica*. Based on the results, increasing Pb concentration in sewage sludge amended soil had a negative effect on soil microbial respiration so that applying sewage sludge polluted with a ratio of 15 to 5 mg Pb/kg sewage sludge significantly decreased the soil microbial respiration by 14%.

Qiu et al investigated the changes in soil microbial

biomass C, adenosine triphosphate (ATP), and microbial ATP concentrations due to increasing soil Pb levels in Chinese paddy soils growing rice and concluded that increasing soil Pb concentration has significantly negative effects on soil microbial respiration (33). However, they did not consider the role of other soil physicochemical properties such as soil salinity in soil microbial respiration. Base on the results of this study, using 30 t/ha Pb-polluted sewage sludge significantly decreased the soil microbial respiration by 14% while the soil Pb concentration and EC increased by 8% and 4.5%, respectively. Raiesi et al evaluated the interactive effect of salinity and cadmium toxicity on soil microbial properties and enzyme activities and found that the multiple stresses induced by salinity and Pb pollution may synergistically affect soil microbial processes and attributes (34). Further, increasing metal and salinity levels alone or in combination can lower the soil microbial respiration and enzyme activity but with a greater reduction when combining these stressors (35,36). Furthermore, salinity can enhance the solubility of heavy metals and their bioavailability in the soils as a result of the formation of heavy metal complexes with organic amendments (37). Therefore, using a suitable method is necessary for decreasing the abiotic stress such as heavy metal or salinity. For instance, the soil microbial respiration was significantly greater in the soil by receiving 15 t/ha sewage sludge polluted with a ratio of 5 to 15 mg Pb/kg that can be related to the interactive role of Pb toxicity and salinity in soil microbial respiration (Table 4). The effect of sewage sludge-induced soil salinity significantly increased the soil Pb availability while it decreased the soil microbial respiration. Several studies also reported the effect of the soil salinity of increasing the soil Pb availability (38-40). Furthermore, the plant root exudate can affect a soil microbial activity that is subject to further investigation in future studies.

Table 3. The Effect of Sewage Sludge, Pb Concentration, the Presence of *P. indica*, and the Use of MWCNTs on Soil EC

MWCNTs	Pb Concentration	<i>+P. indica</i>			<i>-P. indica</i>		
		Sewage Sludge			Sewage Sludge		
		0	15	30	0	15	30
0		1.32l	3.38f	3.54d	1.34l	3.35f	3.57d'
0.5	0	1.24m	3.21g	3.44e	1.25m	3.24g	3.45e
1.5		1.17n	3.19h	3.29g	1.19n	3.15h	3.27g
0		1.49k	3.51d	3.71b	1.44k	3.53d	3.75b
0.5	800	1.33l	3.41e	3.55d	1.35l	3.42e	3.51d
1.5		1.27m	3.33f	3.43e	1.29m	3.30f	3.41e
0	1600	1.69i	3.71b	3.86a	1.65i	3.73b	3.88a
0.5		1.53j	3.55d	3.61c	1.54j	3.52d	3.63c
1.5		1.35l	3.42e	3.53d	1.33l	3.45e	3.56d

Note. 'Date with the similar letter are not significantly different ($P=0.05$, LSD test); MWCNTs: Multi-walled carbon nanotubes; EC: Electrical conductivity.

Table 4. The Effect of Sewage Sludge, Pb Concentration, the Presence of *P. indica*, and the Use of MWCNTs on Soil Microbial Respiration

MWCNTs	Pb Concentration	<i>+P. indica</i>			<i>-P. indica</i>		
		Sewage Sludge			Sewage Sludge		
		0	15	30	0	15	30
0		10.41v	13.55g	14.93a	10.47v	13.53g	14.95a'
0.5	0	10.44v	13.50g	14.91a	10.43v	13.52g	14.90a
1.5		10.43v	13.52g	14.90a	10.45v	13.50g	14.92a
0		9.32y	13.12j	14.41d	9.12 a'	12.35q	14.12f
0.5	800	9.48x	13.33i	14.59c	9.28z	12.53o	14.35e
1.5		9.59w	13.44h	14.76b	9.41x	12.69n	14.49d
0		8.11d'	12.13	12.77m	7.64f'	11.33u	12.45p
0.5	1600	8.55c'	12.55o	12.91k	7.83e'	11.61t	12.73m
1.5		8.87b'	12.88l	13.31i	8.19d'	11.92s	13.12j

Note. 'Date with the similar letter are not significantly different ($P=0.05$, LSD test).

Pb: Lead; MWCNTs: Multi-walled carbon nanotubes.

4. Conclusion

Based on the results of this study, applying MWCNTs can decrease the negative effect of using Pb-polluted sewage sludge. However, the positive role of applying sewage sludge in increasing soil nutrition availability is undeniable. The interesting point of this research is that although using sewage sludge could increase the soil cation exchange capacity, and consequently decrease plant Pb availability, the decomposition of that amendment could redistribute the soil Pb availability, which is a negative point in environmental studies. Among this, applying MWCNTs can help decrease soil and plant Pb availability. Furthermore, the presence of *P. indica* can decrease the abiotic stress such as heavy metals or salinity by preventing the Pb plant uptake or changes in uptake cation ratio that needs further investigation in future studies. Moreover, it is necessary to consider the role of other heavy metals of organic amendments in plant availability. However, it should be noted that organic amendments positively contribute to increasing soil and plant nutrition availability.

Conflicts of Interest Disclosure

The authors declare that they have no conflicts of interest.

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