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Growth, photosynthesis and antioxidant responses of endophyte infected and non-infected rice under lead stress conditions

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ABSTRACT

An endophytic fungus was tested in rice (*Oryza sativa* L.) exposed to four levels of lead (Pb) stress (0, 50, 100 and 200 μM) to assess effects on plant growth, photosynthesis and antioxidant enzyme activity. Under Pb stress conditions, endophyte-infected seedlings had greater shoot length but lower root length compared to non-infected controls, and endophyte-infected seedlings had greater dry weight in the 50 and 100 μM Pb treatments. Under Pb stress conditions, chlorophyll and carotenoid levels were significantly higher in the endophyte-infected seedlings. Net photosynthetic rate, transpiration rate and water use efficiency were significantly higher in endophyte-infected seedlings in the 50 and 100 μM Pb treatments. In addition, chlorophyll fluorescence parameters Fv/Fm and Fv/Fo were higher in the infected seedlings compared to the non-infected seedlings under Pb stress. Malondialdehyde accumulation was induced by Pb stress, and it was present in higher concentration in non-infected seedlings under higher concentrations of Pb (100 and 200 μM). Antioxidant activity was either higher or unchanged in the infected seedlings due to responses to the different Pb concentrations. These results suggest that the endophytic fungus improved rice growth under moderate Pb levels by enhancing photosynthesis and antioxidant activity relative to non-infected rice.

1. Introduction

Heavy metal pollution in the environment is a major ecological concern due to its impact on human health through the food chain [1]. According to the U.S. Environmental Protection Agency, lead (Pb) is the most common heavy metal contaminant in the environment [2]. Pb is not a necessary element for plant growth, and excess Pb causes a range of negative effects. Pb is known to suppress germination and growth, inhibit photosynthesis, affect membrane structure and permeability, and damage the structure and function of photosystem II [3,4]. In addition, Pb can induce the production of reactive oxygen species (ROS), including superoxide radicals (O^{-}_{2}), hydroxyl radicals (•OH) and hydrogen peroxide (H_2O_2), which react very rapidly with DNA, lipids and proteins to cause cellular damage [5]. To counter ROS, plants produce antioxidants and antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) [6].

An endophyte is a bacterium or fungus that lives within a plant, and some increase the host plant's tolerance to biotic and abiotic stress [7–9]. Research has shown that at least some fungal endophytes confer habitat-specific stress tolerance to host plants [10,11]. Kane [12] suggested a positive effect of endophyte infection on the growth of *Lolium perenne* under drought stress. Malinowski and Belesky [7,13] found that a fungal endophyte allowed a grass to withstand drought stress primarily by causing early stomatal closure and adjusting osmotic potentials. Zaurov et al. [14] reported that endophyte infection can enhance the aluminum tolerance of fine fescues (*Festuca* spp.). Fabien et al. [15] demonstrated that endophytic *Neotyphodium lolii* induced tolerance to Zn stress in *Lolium perenne*. Zhang et al. [16] reported that endophyte *Neotyphodium gansuense* improved the cadmium tolerance of infected *Achnatherum inebrians* compared to non-infected specimens. However, no reports have explored the interactions between endophytes and rice under lead stress.

Suaeda salsa, one of the most important halophytes in China, is native to saline soils and adapted to the high-salinity region in northern China [17]. Some of the unique adaptations of this plant, especially those related to stress tolerance, have been attributed to the presence of an endophyte. Teng et al. [18] reported that 1-aminocyclopropane-1-carboxylate (ACC) deaminase-containing endophytic bacteria isolated from *Suaeda salsa* have biological characteristics related to plant growth promotion and regulation of homeostasis under stress.

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We recently isolated an endophytic fungus from *Suaeda salsa*, designated EF0801. In this study, we tested whether the endophyte (E) affected rice (*Oryza sativa*) growth under Pb stress and normal conditions. We also measured photosynthesis, chlorophyll fluorescence and antioxidative enzymes in E+ and E− seedlings under Pb stress conditions to investigate if these physiological and biochemical indicators were impacted by the endophyte.

2. Materials and methods

2.1. Microorganisms and plant material

An isolate of endophytic fungus (EF0801) was obtained from leaves of *Suaeda salsa*. The molecular identification of the fungus was based on internal transcribed spacer regions which showed that EF0801 is congeneric to *Sordariomycetes* sp. (99% similarity). EF0801 was maintained on potato dextrose agar (PDA) plates under refrigerated conditions. The initial pH value was 7.0–7.5, and the strain was inoculated at the 3 days instar stage for 5% into 75 ml PDA culture solution and grown in a 150 ml shaker flask for 12 days at 180 rpm and $24 \pm 1\,^{\circ}C$. The fermentation broth was used for seedling treatments.

Rice seeds were surface sterilized in 2.65% sodium hypochlorite, rinsed with distilled water and then transferred to Petri dishes for germination. After 2 days, germinated seeds (100 grains) were cultivated in a 500 ml beaker containing full Hoagland's nutrient solution. The Hoagland's nutrient solution consisted of 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 1 mM KH_2PO_4, 50 μM H_3BO_3, 1 mM $MgSO_4$, 4.5 μM $MnCl_2$, 3.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.1 mM $(NH_4)_6Mo_7O_{24}$ and 10 μM Fe-EDTA at a pH of 5.5. The seedlings were grown in a growth chamber (27 °C day/20 °C night; 16 h/8 h light/dark period; 600 $\mu mol\,m^{-2}\,s^{-1}$ photosynthetic photon flux density; and 80% relative humidity) for 2 days.

2.2. Treatments

On the second day in the growth chamber, Pb ($Pb(NO_3)_2$) and endophyte treatments were performed. Seedlings were divided into two groups. One group was inoculated with the fermentation broth by planting in full Hoagland's solution with 5% fermentation broth (E+, endophyte-infected seedlings), while the control group (not inoculated) was planted in full Hoagland's solution alone (E−, endophyte-uninfected seedlings). Each group (12 pots, 3 replicates × 4 treatments) was randomly assigned to four Pb treatments (i.e., concentrations of 0, 50, 100 and 200 μM Pb in Hoagland's solution). The degree of endophyte infection was determined using the method of Liu and Chen [19]. The endophyte colonized the plant roots, and colonization was >90% for E+, whereas the roots of the E− treatment were not colonized at all. Leaves were sampled to measure photosynthetic pigments, enzymes and malondialdehyde (MDA) 14 days after imposing the treatments.

2.3. Measurement of growth

The effects of endophyte infection and Pb stress on biomass production and growth parameters were determined over a 2-week period. The length and fresh weight of shoots and roots were recorded. Dry weight (DW) was obtained after drying at 80 °C until a constant weight was reached.

2.4. Measurement of photosynthetic parameters

The net photosynthetic rate (Pn), stomatal conductance (Gs) and transpiration rate (E) were determined after seedlings were treated for 14 days. In each replicate, three seedlings were selected randomly and the third leaf from the bottom was used to measure photosynthesis with a portable photosynthesis system (Li-6400, Li-Cor Lincoln, NE, USA). The average value of three plants was considered as one replicate. Water use efficiency (WUE) was defined as the ratio Pn/E.

2.5. Measurement of chlorophyll fluorescence

Chlorophyll fluorescence was measured with a portable fluorometer (Pocket PEA, Hansatech, England). The minimal fluorescence (Fo) with all PSII reaction centers (RCs) open was measured with modulated radiation low enough to not induce any significant variable fluorescence (Fv). The maximal fluorescence (Fm) with all RCs closed was measured with a 0.8 s pulse of saturating radiation of 3000 $\mu mol\,m^{-2}\,s^{-1}$ on dark-adapted leaves. The maximal photochemical efficiency (Fv/Fm) of PSII was expressed as the ratio $Fv/Fm = (Fm - Fo)/Fm$.

2.6. Quantification of photosynthetic pigments

Chlorophyll and carotenoids (Car) were extracted from 0.1 g leaf discs with 10 ml 80% acetone and quantified as described by Agrawal and Rathore [20].

2.7. MDA content

Estimation of MDA levels followed the method of Islam et al. [21]. 4.0 ml of 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) trichloroacetic acid (TCA) was added to a 1.0 ml aliquot of the supernatant. The mixture was incubated in boiling water for 30 min. The amount of MDA was measured by spectrophotometer at 532 nm and corrected for nonspecific turbidity at 600 nm.

2.8. Enzyme extraction and activity assay

Fresh leaf samples were homogenized in extraction buffer (0.1 M phosphate buffer at pH 6.8) with a mortar and pestle on ice. The homogenate was then centrifuged at 12,000 × *g* for 15 min at 4 °C, and the supernatant was used as the crude extract for analysis of enzymes superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT). The amount of SOD, POD and CAT was estimated using the methods previously described by Beauchamp and Fridovich [22], Putter [23] and Aebi [24], respectively. The amount of protein was estimated using the method described by Bradford [25].

2.9. Statistical analyses

The differences in plant biomass and physiological parameters between infected and non-infected plants under different Pb concentrations were determined using two-way analysis of variance (ANOVA) (with Pb concentration as one factor and infection or not as the other factor) followed by LSD's multiple-range test for multiple comparisons. All analyses were made using the SPSS statistical software package (Ver.13.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Plant growth parameters and biomass production

The shoot and root lengths were significantly shorter at higher Pb concentrations in both infected and non-infected treatments (Fig. 1). Shoot length was observed to be significantly higher ($p < 0.05$) for E+ seedlings (relative to E− seedlings) growing under the control and high (100 and 200 μM) Pb concentrations (Fig. 1A). E+ seedlings had significantly shorter root length compared to E− seedlings (Fig. 1B).

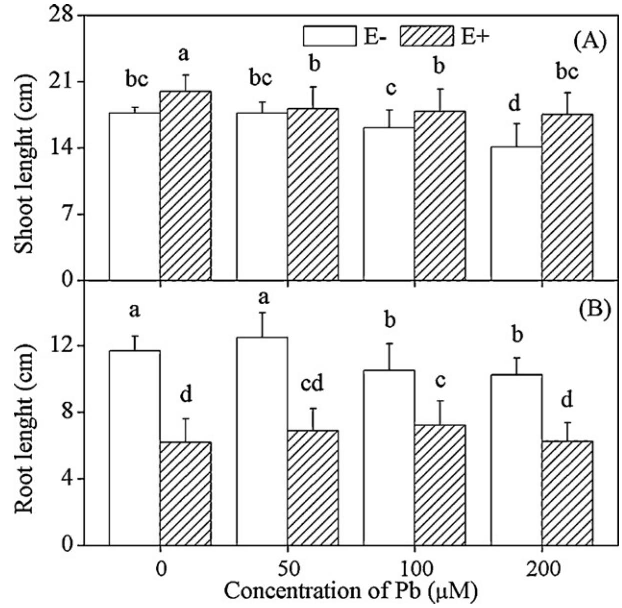

Fig. 1. (A) Shoot length and (B) root length of endophyte-infected (E+) and uninfected (E−) *Oryza sativa* after a 2-week treatment with 0, 50, 100 and 200 μM Pb in Hoagland's solution. Bars indicate standard deviations ($n = 3$). Columns with different letters indicate significant difference at $p < 0.05$ (LSD test).

Rice biomass significantly decreased with the increasing Pb concentrations both in infected and non-infected treatments (Fig. 2). There was significantly ($p < 0.05$) more above-ground biomass production in E+ compared to E− seedlings at 0, 50 and 200 μM Pb concentrations (Fig. 2A), with a relative increase of 16.9%, 15.3% and 8.0%, respectively (Fig. 2A). However, no significant ($p < 0.05$) difference in above-ground biomass production was observed between E+ and E− seedlings in the 100 μM Pb treatment. There was also significantly ($p < 0.05$) greater below-ground biomass production in E+ compared with E− seedlings in the 50 μM and 100 μM Pb treatments, an increase of 26.8% and 28.5%, respectively (Fig. 2B). However, in all cases, seedlings grown in the 200 μM Pb treatment had overall lower biomass production than seedlings in the 0 μM Pb treatment.

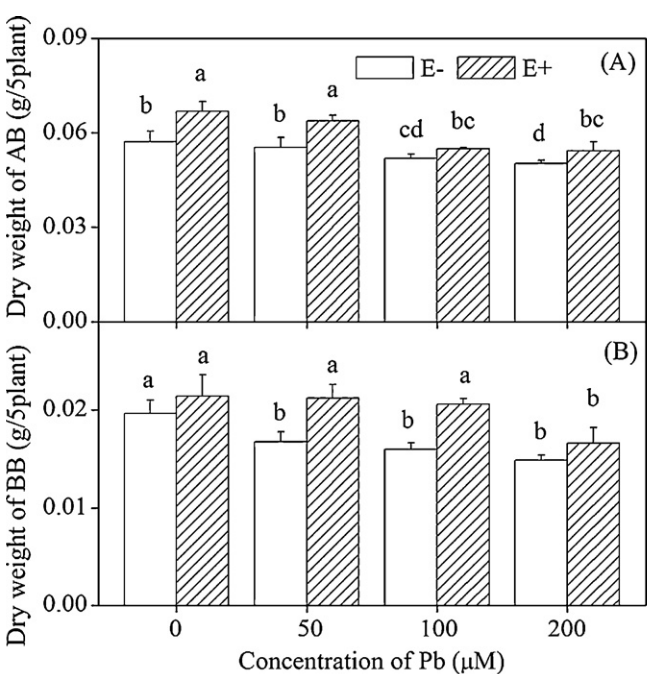

Fig. 2. Dry weight of (A) above-ground (AB) biomass and (B) below-ground (BB) biomass of endophyte-infected (E+) and uninfected (E−) *Oryza sativa* after a 2-week treatment with 0, 50, 100 and 200 μM Pb in Hoagland's solution. Bars indicate standard deviations ($n = 3$). Columns with different letters indicate significant difference at $p < 0.05$ (LSD test).

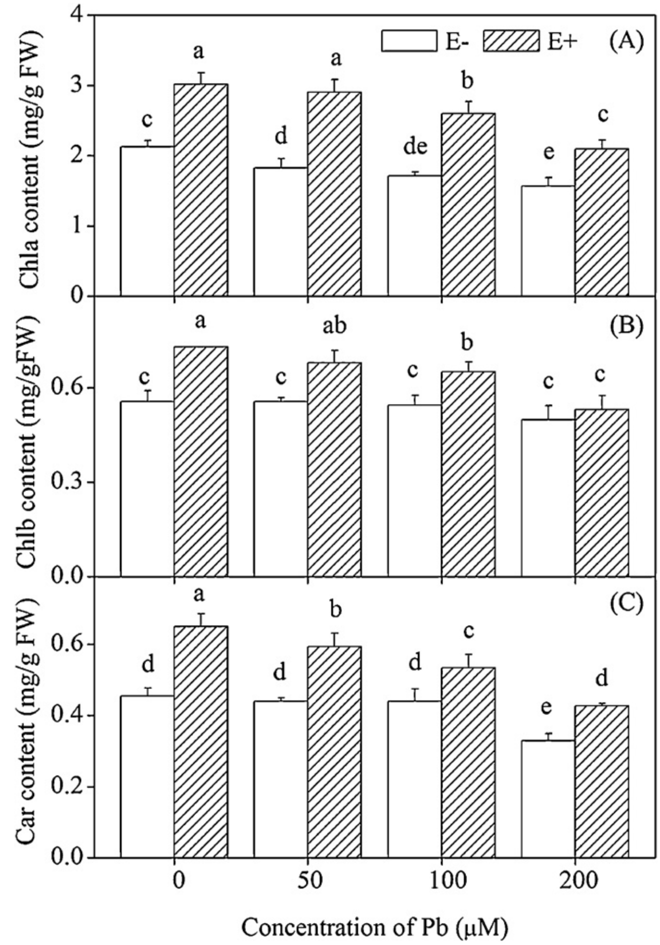

Fig. 3. (A) Chlorophyll *a* (Chla), (B) chlorophyll *b* (Chlb) and (C) carotenoid (Car) content of endophyte-infected (E+) and uninfected (E−) *Oryza sativa* after a 2-week treatment with 0, 50, 100 and 200 μM Pb in Hoagland's solution. Bars indicate standard deviations ($n = 3$). Columns with different letters indicate significant difference at $p < 0.05$ (LSD test).

3.2. Changes in the chlorophyll and carotenoid content of leaves

Levels of chlorophyll *a* and carotenoids were significantly decreased by increasing Pb concentration in both infected and non-infected treatments (Fig. 3). There was a significant ($p < 0.05$) chlorophyll *a* increase in E+ relative to E− seedlings under both Pb stress as well as no stress (Fig. 3A), although chlorophyll *a* decreased in all specimens with increasing concentrations of Pb. The chlorophyll *b* of E+ seedlings was significantly ($p < 0.05$) higher than that of E− seedlings under all Pb concentrations except the 200 μM treatment (Fig. 3B). Levels of carotenoids were significantly ($p < 0.05$) higher in E+ seedlings compared to E− seedlings with and without Pb stress (Fig. 3C). However, the presence of carotenoids diminished with increasing concentrations of Pb.

3.3. Changes in photosynthetic characteristics

Net photosynthetic rate (Pn) and transpiration (E) of leaves decreased with increasing Pb concentration (Fig. 4A and C). No significant ($p > 0.05$) differences in Pn and E were observed between

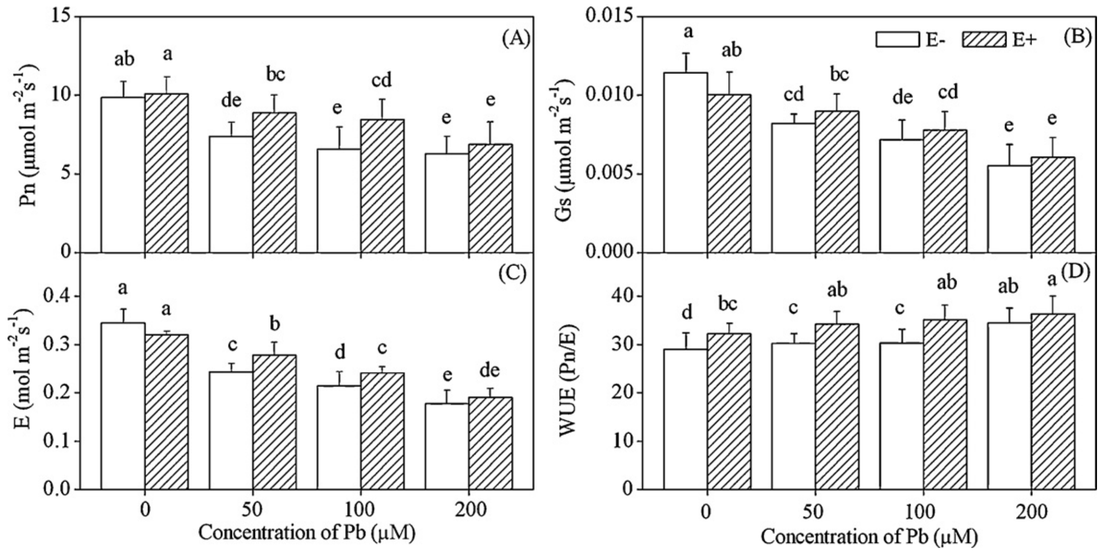

Fig. 4. (A) Net photosynthetic rate (Pn), (B) stomatal conductance (Gs), (C) transpiration rate (E) and (D) water use efficiency (WUE) of endophyte-infected (E+) and uninfected (E−) *Oryza sativa* after a 2-week treatment with 0, 50, 100 and 200 μM Pb in Hoagland's solution. Bars indicate standard deviations ($n = 3$). Columns with different letters indicate significant difference at $p < 0.05$ (LSD test).

E+ and E− seedlings treated with 0 and 200 μM Pb concentrations; however, there were significant ($p < 0.05$) increases in E+ seedlings compared with E− seedlings treated with 50 and 100 μM Pb concentrations. The endophyte had no significant effect on stomatal conduction (Gs) both with and without Pb stress (Fig. 4B); however, Gs decreased in all specimens with increasing concentrations of Pb. The water use efficiency (WUE) increased in all specimens with increasing concentrations of Pb, and WUE of E+ seedlings was significantly ($p < 0.05$) higher than E− seedlings treated with concentrations of 0, 50 and 100 μM Pb (Fig. 4D).

3.4. Changes in chlorophyll fluorescence

There were no significant changes in the Fv/Fm ratios in E+ seedlings under Pb stress (Fig. 5A); however, the Fv/Fm ratios in E− seedlings significantly decreased as the concentration of Pb increased. The Fv/Fm ratios of E+ seedlings were significantly ($p < 0.05$) higher than those in E− seedlings under Pb stress. A similar trend was also observed for Fv/Fo values (Fig. 5B).

3.5. Changes in the level of MDA and the activity of antioxidant enzymes

A positive correlation between Pb concentration and MDA levels was observed (Fig. 6). There was significantly ($p < 0.05$) higher MDA in E− seedlings vs. E+ seedlings in the 100 and 200 μM Pb treatments, with increases of 18.2% and 25.0%, respectively.

The SOD activity first increased and then decreased with increasing Pb concentration both in infected and non-infected treatments (Fig. 7A). SOD activity was significantly ($p < 0.05$) different between the E+ and E− seedlings at all Pb concentrations except 200 μM, with greater activity in E+ than E− seedlings.

The CAT activity of E− seedlings declined significantly ($p < 0.05$) compared to the control at 50 μM, but it remained unchanged at higher levels (Fig. 7B). CAT activity in E+ seedlings was induced after exposure to the 50 μM Pb treatment; however, at 200 μM Pb, a significant ($p < 0.05$) decline in the CAT activity was noted and values were close to the control.

The POD activity levels were significantly lower as Pb concentration increased in both infected and non-infected treatments (Fig. 7C). POD activity in the E+ and E− seedlings was not significantly different in the 0, 50 and 100 μM Pb treatments, but there were significant ($p < 0.05$) differences in POD activity in the 200 μM Pb treatment, with greater activity in the E+ seedlings.

4. Discussion

This study of the interaction between an endophytic fungus, lead toxicity and *O. sativa* demonstrates that the presence of an endophyte can ameliorate the effects of lead toxicity.

Exposure of the seedlings to increasing lead concentrations resulted in reductions in shoot and root length (Fig. 1), which were both associated with the observed reductions in dry biomass

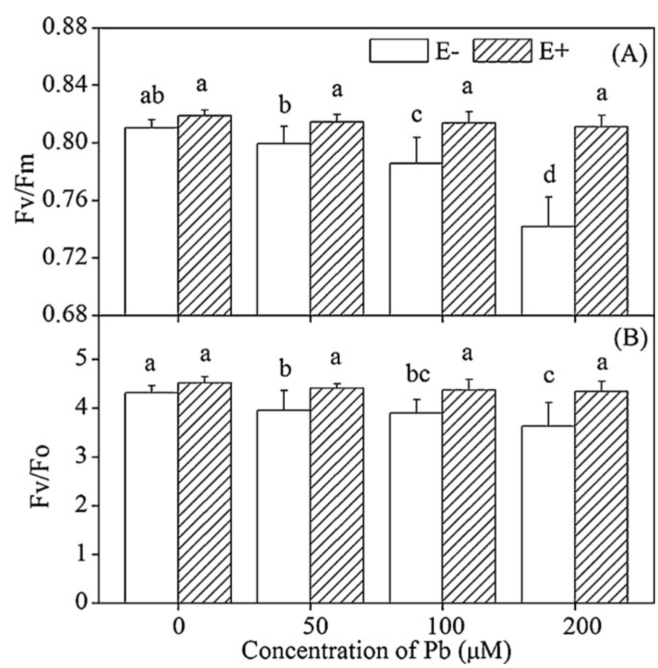

Fig. 5. (A) Fv/Fm and (B) Fv/Fo of endophyte-infected (E+) and uninfected (E−) *Oryza sativa* after a 2-week treatment with 0, 50, 100 and 200 μM Pb in Hoagland's solution. Bars indicate standard deviations ($n = 3$). Columns with different letters indicate significant difference at $p < 0.05$ (LSD test).

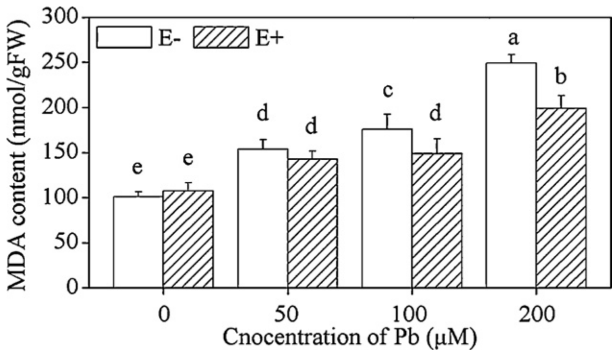

Fig. 6. MDA content of endophyte uninfected (E−) and infected (E+) *Oryza sativa* treated with 0, 50, 100 and 200 μM Pb in Hoagland's solution. Bars indicate standard deviations ($n = 3$). Columns with different letters indicate significant difference at $p < 0.05$ (LSD test).

production. The results show that endophyte infection is beneficial both without lead stress and with moderate lead stress, increasing the dry weight of above-ground and below-ground organs (Fig. 2). Researchers have reported the beneficial effects of endophytes on plant growth in the presence of cadmium [16], aluminum [14], zinc [15], lead [27], salt [26] and drought stress [28].

Levels of chlorophyll *a* and carotenoids (Fig. 3) decreased with increasing lead concentration. Gou [27] found accumulation of chlorophylls in endophyte-infected *Achnatherum inebrians* under salt stress. Zhang et al. [16] also found higher concentrations of chlorophyll in E+ *A. inebrians* under Cd stress. Zhang and Nan [29] observed higher accumulation of chlorophyll in E+ compared to E− *Elymus dahuricus* under low water conditions. In this study, similar results were observed. Carotenoids protect the lipid phase of the thylakoid membranes and are quenchers of the excited triplet state of chlorophyll and singlet oxygen. A reduction in the carotenoid levels in leaves was observed with exposure to Pb [30]. In the present study, the higher carotenoid content of E+ seedlings might protect them against Pb stress.

Photosynthesis is one of the most Pb-sensitive plant processes. Photosynthesis is adversely affected by Pb, which could be due to metal-induced reductions in the levels of photosynthetic pigments [31], inhibition in the electron transport system [32], changes in the fine structure of chloroplasts, and stomatal closure [21]. In the present study, the Pb-induced reduction in photosynthesis in E− seedlings was probably caused by stomatal closure; since Pb-treated seedlings were accompanied by a lower stomatal conductance and transpiration rate, especially at higher levels of Pb (Fig. 4). According to Ahmad et al. [33] and Islam et al. [21], there is a strong relationship between Pb application and a decrease in whole plant photosynthesis, which is believed to result from stomatal closure. Swarthout et al. [28] found higher water use efficiency in E+ seedlings relative to E− seedlings under severe drought stress. Our study confirms that the presence of the endophyte allowed seedlings to maintain water use efficiency, whereas E− seedlings showed a significant decrease in water use efficiency. In E+ seedlings, a slow reduction in the net photosynthetic rate of Pb-treated plants could contribute to the higher level of photosynthetic pigments and higher stomatal conductance and water use efficiency.

Fv/Fm is an indicator of the efficiency of the photosynthetic apparatus, while Fv/Fo indicates the size and number of active photosynthetic centers in the chloroplast and, therefore, the photosynthetic strength of the plant [34]. An Fv/Fm ratio of 0.8 or higher and Fv/Fo ratios of 4.0 or higher indicate that the plant is healthy and not suffering from photosynthetic stress [35]. In the present study, Fv/Fm and Fv/Fo ratios were higher than 0.8 and 4.0 in E− seedlings under no stress and in E+ seedlings in all treatments. However, Fv/Fm and Fv/Fo ratios were lower than 0.8 and 4.0 in E− seedlings under Pb stress. The results indicated that the endophyte may enhance the plant's tolerance of Pb without significantly affecting its growth and development.

Heavy metals exert toxic effects through the production of reactive oxygen species (ROS), which have a variety of harmful effects in plant cells, including lipid peroxidation. Significant increases in leaf MDA content, a measure of oxidative stress inducible peroxidation of membrane lipids, were observed. Gupta et al. [36] found that MDA concentration in *Zea mays* seedlings increased linearly with increasing Pb levels. Thus, increased MDA indicates the presence of oxidative stress, and perhaps this is one mechanism by which Pb toxicity is manifested in the plant tissues. The level of MDA was lower in E+ seedlings compared to E− seedlings. Similar results were reported by Zhang et al. [16] with *A. inebrians* under Cd stress. To cope with ROS or alleviate their damaging effect, plants have evolved enzymatic and nonenzymatic antioxidant mechanisms. Improvement of stress tolerance is often related to an increase in the activity of antioxidant enzymes [37], and plants may experience oxidative damage due to the inability of the antioxidative enzymes to tolerate severe stress [38]. In the present study, a significant decrease in POD and CAT activities under Pb stress were observed. However, activities of antioxidant enzymes (SOD, POD and CAT) were higher in E+ seedlings compared to E− seedlings. Zhang et al. [16] also found that endophytic infection enhanced anti-oxidative mechanisms in *A. inebrians* under high Cd. Kohlera et al. [39] reported that inoculation with selected plant growth-promoting bacteria could increase levels of antioxidant enzymes in response to severe salinity. Plants containing high activities

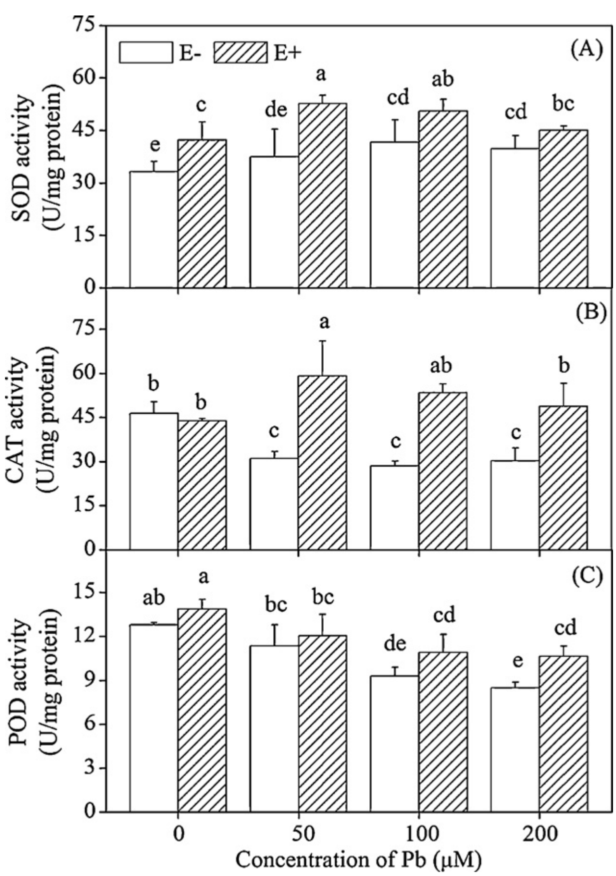

Fig. 7. Activity of (A) SOD, (B) POD, and (C) CAT of endophyte uninfected (E−) and infected (E+) *Oryza sativa* treated with 0, 50, 100 and 200 μM Pb in Hoagland's solution. Bars indicate standard deviations ($n = 3$). Columns with different letters indicate significant difference at $p < 0.05$ (LSD test).

of antioxidant enzymes have considerable resistance to oxidative damage [40,41]. Thus, the results indicate that endophyte infection was beneficial to the antioxidative mechanisms in rice exposed to high concentrations of Pb. A more efficient antioxidative system likely resulted in lower oxidative stress and reduced membrane damage. Analysis of the physiological mechanism showed that the improved tolerance of rice to Pb was due to an overall promotion of photosynthesis and the protective role of antioxidant enzymes.

It is now well recognized that inoculation with endophytes influences heavy metal uptake, translocation and accumulation in host plants. Mycorrhizal inoculation (*Glomus* sp. and *G. mosseae*) has been shown to significantly reduce the uptake and accumulation of heavy metal in host plant roots [42]. Galli et al. [43] proposed that the retention of heavy metals by fungal mycelia may involve adsorption to cell walls, thereby minimizing metal translocation to the shoots. Furthermore, analysis of molecular mechanisms has shown that the improved tolerance of plants to heavy metal is due to an overall upregulation of stress-related genes and the protective role of polyamines [44]. Moreover, the differential transcription expression patterns are induced by mycorrhizal fungi occurred in the leaves and roots of mycorrhizal (*Gl. intraradices*) and non-mycorrhizal tomato [45].

5. Conclusions

The positive effect of endophyte infection on Pb stressed rice has many possible explanations. (i) Inoculation with the endophyte increased the levels of photosynthetic pigments and water use efficiency, which promoted photosynthesis and exerted a beneficial effect on plant growth. (ii) Inoculation with the endophyte alleviated the oxidative damage induced by Pb. E+ seedlings had lower levels of MDA than E− seedlings. (iii) Inoculation with the endophyte exerted a protective effect by increasing antioxidant enzyme activity. Taken together, this evidence supports the conclusion that endophytes may indirectly attenuate Pb toxicity through a development of a general antistress response, which probably includes the regulation of photosynthesis and the antioxidant system. This suggests that inoculation with endophyte could serve as a useful tool to improve plant growth under Pb stress conditions.

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