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VESICULAR ARBUSCULAR MYCORRHIZA AND BRASSINOSTEROIDS AMELIORATESTHE EFFECTS OF STUNTED GROWTH AND OXIDATIVE STRESS INDUCED BY LEAD

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Abstract

The growth and metabolic activities in plants are prominently influenced by the biotic and abiotic stress. Different bio-fertilizers and phytohormone are used as bio-stimulant to mitigate these stresses to sustain plant physiological output. Trace metals such as lead (Pb) toxicity is a threat to vegetation and induce impairments in morphological and biochemical attributes. Vesicular arbuscular mycorrhizae (VAM) and Brassinosteroids (BRs) possess beneficial physiological responses and bioremediation capabilities to resist different stresses including heavy metals stress in plants. This study will assess the mitigating effects of VAM and BRs on metabolism and growth of *Luffa cylindrica* plants affected by the lead trace metal. Growth (shoot and root length, plant fresh and dry weight), biochemical analysis (protein, carbohydrate, phenol and photosynthetic pigments) and antioxidant levels (Malondialdehyde (MDA), Peroxidase (POD) and Superoxide dismutase (SOD) enzymes activities were examined to assess the efficacy of applied biostimulants. *L.cylindrica* plant treated with (Pb) showed a significant reduction in physiological and biochemical parameters compared to control, VAM and BRs treated plants. Plants grown under Pb trace metal showed highest MDA levels compared with other treatments. Plants exposed to the combined application of VAM and BRs bio-stimulant enhanced growth, biochemical and antioxidant defense compared to their individual treatments. The results of morphological and biochemical analysis revealed that the synergic treatment of VAM and Brassinosteroids enhanced the plant adaptations to resist metal toxicity caused by lead (Pb). In conclusion, the combined application of VAM and BRs could be applied as biofertilzer to enhance the plant growth and provide the formulations methods to remediate the Pb trace metal polluted soil to maintain environmental safety and ecotoxicology.

Key words: Antioxidants, Metal toxicity, Phytoremediation, Reactive oxygen species.

Introduction

Human-induced actions have considerably raised heavy metal toxicity in the environment and posed an unprecedented risk to the biosphere due to increasing industrialization and urbanization (Sharma *et al.*, 2021). Pollution due to trace metals in soils become an enduring problem because of its resistance to degradation and restricted mobility in soil particles (Zhong *et al.*, 2020; Munir *et al.*, 2023; Mujeeb *et al.*, 2023). The higher accumulation of trace metals reduce the plant productivity and ultimately cause food insecurities especially in the developing countries (Abideen *et al.*, 2022, 2023; Umer *et al.*, 2023). The effects of lead on human health and the environment have been widely acknowledged, making it one of the most dangerous heavy metal pollutants (Alengebawy *et al.*, 2021). Lead (Pb) influences the metabolic functions of different plant cells, which can result in decreased seed germination, nutrient transfer disruptions, decreased cell division, suppressed photosynthesis, eventually decrease shoot and root growth (Kohli *et al.*, 2020). There are several bio-stimulants and bio fertilizers that can help in reducing the trace metal toxicity and sustain the plant tissue metabolic status and bioremediation of soil toxicity (Hasnain *et al.*, 2023; Abideen *et al.*, 2020).

The roots of many terrestrial plants are colonized by Vesicular-arbuscular mycorrhiza (VAM) fungi. They have a major role in the plant growth, nutrients uptake, and the health of the soil. VAM fungus can increase the absorption of phosphorus and other nutrients by up to 10 times (Qi *et al.*, 2022). VAM may possibly play a significant role in bioremediation. The fungus can aid in the absorption of harmful metals and other contaminants as well as enhance soil structure and fertility. By lowering the demand for artificial fertilizers and pesticides, VAM can aid in the reduction of environmental pollution and the improvement of soil health (Chaturvedi & Malik, 2019). The potential of VAM fungus to boost plant development and production as well as their capacity to deal with heavy metal toxicity in various ecological situations has been established by (Koza *et al.*, 2022). It has been demonstrated that the VAM fungus improves plant tolerance to high levels of metal toxicity and reduces oxidative damage (Zou *et al.*, 2021).

Brassinosteroids are among the six classes of plant hormones that are needed for responses to biotic and abiotic challenges (Zhang *et al.*, 2023). The stimulation of particular macromolecules by BRs can change plant growth and development subjected to biotic and abiotic stress factors as salt, drought, heavy metals, and temperature (Dehghanian *et al.*, 2021). It also performs a pivotal function in nutrient deficiency by promoting osmolytes, macronutrient accumulation, photosynthesis, as well as antioxidant defense systems, and hormonal homeostasis (Dai *et al.*, 2023). BRs aid the reduction of heavy metals and radioactive elements via the regulation of ion uptake. The toxicity caused by excess heavy metals can be minimized with BRs (Madaan *et al.*, 2022). Following hypothesis were tested in this experiment to assess the efficacy of bio stimulant on the eco-physiological responses of *Luffa cylindrica* against the Pb trace metal stress.

1. Plant treated with VAM and BRs combination stimulates the plant growth and physiological attributes better than individual effects.
2. The bio stimulant VAM and BRs enhance plant antioxidant defense under Pb trace metals stress compared to control plants.

Material and Methods

Experimental protocol: The soil (sandy clay loam) taken from the nursery was dried, crushed, and sieved through a 2mm sieve. It was further autoclaved and sterilized for two hours at 121°C and 1 atmosphere or 15 pounds per square inch in a metallic autoclave. The experiment of this study was conducted in the greenhouse at the Department of Botany, University of Karachi, Pakistan, during the months of September to November 2022, with a photo periodic cycle of 11-12h Day light, 12-13h dark and 25-30°C day, 20-26°C night temperature along with 45%-75% relative humidity. Seeds of *Luffa cylindrica* L. were surface sterilized with mercuric chloride before being sown in earthen pots. In each of the six identical pots of treatments, a total of 10 seeds were sown. To keep the moisture level constant, the pots were routinely irrigated. This research was intended to examine the synergistic effects of VAM and BRs in terms of stunted growth patterns and oxidative stress on Pb-stressed *Luffa cylindrica* plants weekly for up to 12 weeks. Five separate sets of treatments were applied after the seedlings were allowed to establish for a month. One set of treatments consisted of non-contaminated control, a second set of treatments designated as Pb, and a third set of VAM solution treatments (164 ml in each pot, containing 1,640 VAM spores per plant given that 1 ml contained 10 VAM spores). The fourth set of treatments was BRs (24-epibrassinolide) (each pot was administered with 25 ml of this BRs solution), and VAM+BRs+Pb was maintained as fifth set of therapy.

Extraction and preparation of treatments: VAM spores were extracted following Vilarino&Arines (1990) and identified Schenck & Perez (1990). *Glomus sinnosum*, *Acaulospora delicata*, *Gigaspora rosea*, and *Gigaspora albida* were the components of the VAM species. The 24-epibrassinolide (EBL) analog of brassinosteroids, which was obtained from Chem Cruz Biotechnology Inc., Dallax, was employed. As a source, lead chloride ($PbCl_2$) was utilized to create lead stress (1000 ppm).

Biochemical analysis

Total photosynthetic pigments: The entire photosynthetic contents were determined using the approach of Lichtenthaler *et al.*, (2005) comprising chlorophyll a, b, total chlorophyll, and carotenoids. The leaf homogenization was performed in 5 ml of 80% Acetone, followed by repeated centrifugation for 10 min at 4000rpm until the colourless supernatant was extracted. The absorbance was calculated at 646.8, 663.2 and 470nm.

Protein content analysis: Protein Estimation was done by using Coomassie brilliant blue dye and the technique of (Bradford, 1976). The leaf was extracted by deionized water. Then 5ml of the dye was added in 1ml extracted sample for 5 minutes incubation. The blue colour was later appeared after incubation and optical density was noted at 595nm.

Phenols estimation: The phenolic compound quantification was carried out by applying the methodology given by McDonald *et al.*, (2001) via the use of Folin-Ciocalteu reagent (FC-Reagent). The extraction of leaf was done using 80% acetone followed by centrifugation process at 4000 rpm for 5 minutes. Then this mixture was gone through under incubation period in dark for 30 minutes containing supernatant (1ml), FC-reagent (5ml) and Na_2CO_3 (4ml) and by using spectrophotometer the absorbance was recorded at 765nm.

Carbohydrate estimation: The carbohydrate content was estimated (Yemm & Willi's 1956) using Anthrone reagent. The leaf sample was extracted by using deionized water followed by twice centrifugation process at 2500 rpm for 5min.5ml Anthrone reagent was added in 1ml supernatant solution the mixture was placed in boiling water bath for 15min, cooled in ice cold water and optical density was recorded at 595nm.

Antioxidant analysis

To perform antioxidant biochemical analyses, a buffer with a pH of 7.8 labeled as potassium phosphate was used to create leaf homogenate followed by a 15-minute centrifugation process at 14000 rpm. After that, the supernatant was collected and used to calculate the redox analysis. Thiobarbituric acid (TBA) was used to measure the Malondialdehyde (MDA) level according to Jambunathan's (2010) procedure. 5% Trichloroacetic Acid (1.5ml) was used for leaf homogenization followed by centrifugation at 4ºC 12000 rpm for 5 minutes. (1ml) leaf extract +0.5% (1ml) TBA were transferred in test tubes then test tubes were placed for 30 min at 95ºC in boiling water bath. These tubes were then transferred in an ice bath to stop the reaction. The centrifugation of test mixture was 0.5% of TBA (1ml) and Trichloroacetic Acid (1ml) were warmed for 30 min at 90ºC. After cooling the mixture in an ice bath, the centrifugation of sample was performed for 5 min at 7500 rpm. The optical density was recorded at 532 and 600nm. The technique described by Beauchamp & Fridovich (1971) was used to calculate Super Oxide Dismutase (SOD). A solution mixture of 3ml containing 50mMNitroblue Tetrazolium (NBT), Ethylene DiamineTetraacetic Acid (EDTA) (1mM), Methionine (10 mM), Potassium Phosphate Buffer Ph 7.8 (50mM) + freshly prepared Riboflavin (20ml) and extract of sample were added in all test tubes except control. The test tubes of control and sample were covered with aluminium foil and exposed to fluorescent light for 15 minutes. To stop the reaction the fluorescent light was turned off then absorbance was taken at 560nm.

The estimate of Peroxidase (POD) enzyme activity followed the Polle *et al.*, (1994) technique. Freshly prepared 20mM H_20_2 (100 µl), 2.7mM (50µl) Guaiacol, (1750 µl) Potassium Phosphate Buffer and (100 µl) sample extract was taken in test tube. Mix this mixture thoroughly and absorbance recorded taken at 470nm.

Statistical Analysis

IBM SPSS (version 23) was used to assess the data's normality and equality of variances to make sure that the prerequisites for statistical analysis were met.

Results

Effect of VAM and BRs on morphological parameters: It was found that the morphological (fresh and dry weight, root and shoot length,) data was significant at (p<0.01). Over all other treatments, Pb-stressed plants showed a significant decrease in all morphological attributes (shoot length by 76%, root length by 31%, fresh weight by 82%, dry weight by 87%). The treatments of VAM and BRs alone showed mitigating effects to reduce Pb toxicity by an enhancement in all morphological attributes. VAM+Pb significantly increased shoot length by 76%, root length by 45%, fresh wt by 77% and dry wt by 85%. BRs+Pb treatment increase shoot length by 84%, root length by 46%, fresh wt by 80% and dry wt by 87%. Application of VAM+BRs to Pb-stressed plants was superior to all other treatments as it had exhibited an enhanced rise in morphological features of shoot length and root length by 87% and 49%, fresh and dry weight by 82% and 88% as shown in (Figs. 1-4).

Effect of VAM and BRs on biochemical parameters: The results of the experimental study for the *Luffa cylindrica* plant's biochemical (protein, carbohydrate, phenol, and total photosynthetic contents) data showed a significant effect at (p<0.01). When *L. cylindrical* Pb stressed plants were treated with VAM and BRs, there was a discernible rise; (VAM+Pb increase protein by 43%, carbohydrate by 38%, phenol by 41%, total photosynthetic pigments by 33%) (BRs+Pb increase protein by 23%, carbohydrate by 43%, phenol by 48%, total photosynthetic pigments by 35%) however, when VAM and BRs were applied in combination to the same plants, there was an even greater increase than with the other treatments (protein increased by 61%, carbohydrate by 55%, phenol by 57%, total photosynthetic pigments by 42%).Plants treated with Pb stress decreased protein by 22%, carbohydrate by 55%, phenol by 35%, total photosynthetic pigments by 29% as shown in (Figs. 5-8).

Effect of VAM and BRs on antioxidant analysis: In the current research, lipid peroxidation was assessed as a measure of oxidative stress. MDA, a lipid peroxidation marker, has been seen in comparison to controls and treatments. The results revealed the MDA levels in all of the treatments were substantially (p<0.01) higher than the controls. The combination group treatments (VAM+Pb and BRs+Pb), in contrast to the Pb-treated group demonstrated protective benefits against a considerably (p<0.01) reduced level of oxidative stress (Fig. 9).

In the current study, SOD and POX, two significant antioxidant enzymes were also calculated. The findings demonstrated that, in contrast to Pb-induced oxidative stress, the combined group treatments VAM+PB, BRs + Pb, and VAM+BRs+Pb significantly (p<0.01) exhibited higher antioxidant levels. (Figs. 10-11).

Discussions

Lead is a serious toxicant which is a major threat to agriculture. To minimize the consequences of its toxicity, different approaches have been made. The mycorrhizal fungi (VAM) and phytohormone (BRs) have phenomenol amelioration properties. The current study deals with the ameliorating effects of VAM and BRs in lead induced plants. As in previous study it was revealed that Lead impairs vegetative development and metabolism by disrupting the absorption of vital nutrients (Hafeez *et al.*, 2023). It reduces the capacity of plants to absorb carbon, which results in a reduction in biomass production as well as fresh and dry weight of plants Chauhan *et al.*, (2022). Obi-Iyeke & Ogbara (2022) deduced from their research these the impaired physiological and anatomical processes in lead plants resulted in reduction of shoot and root length.

Results of present study also demonstrated that by using BRs and VAM applications, Pb toxicity could be considerably reduced by escalating the growth pattern in comparison to lead-induced toxicity in plants. It was reported earlier that brassinosteroids enhance plant productivity, and perform protective role and assist in activation of singling cascade (Hussain *et al.*, 2020). These results are further justified by Ahammed *et al.*, (2020), that the exogenous administration of Brassinosteroids can effectively mitigate the harmful effects which promote plants tolerance to lead stress. These findings are consistent with the previously reports (Aslam *et al.*, 2021; Bakshi *et al.*, 2023) that BRs treatments tend to enhance a variety of physiological processes, including carbohydrate metabolism, antioxidant enzyme activities, photosynthetic capability, and the heavy metals detrimental effects on plants. So, it is extracted that BRs perform well and lessen the destructive effects of lead stress.

In the case of VAM, current study shows that, VAM can assist plants withstand environmental stress and encourage the regeneration and repair of heavy metal-contaminated soil. Prior researchers also observed that VAM speeded physiological and morphological changes, increasing metal ion absorption and lowering metal toxicity in the host plants (Chauhan *et al.*, 2022). VAM colonisation boosted plant biomass indicating that VAM was essential for plant nutrition and growth. VAM symbiosis in plants can better withstand with environmental challenges such as salt, drought, and heavy metal toxicity (Chen *et al.*, 2022). It is also noted in the earlier studies that, the leaf area, shoot, root, fresh and dry weight of licorice (*Glycyrrhiza glabra* L.) substantially enhance by mycorrhizal inoculation (Tabrizi *et al.*, 2021).

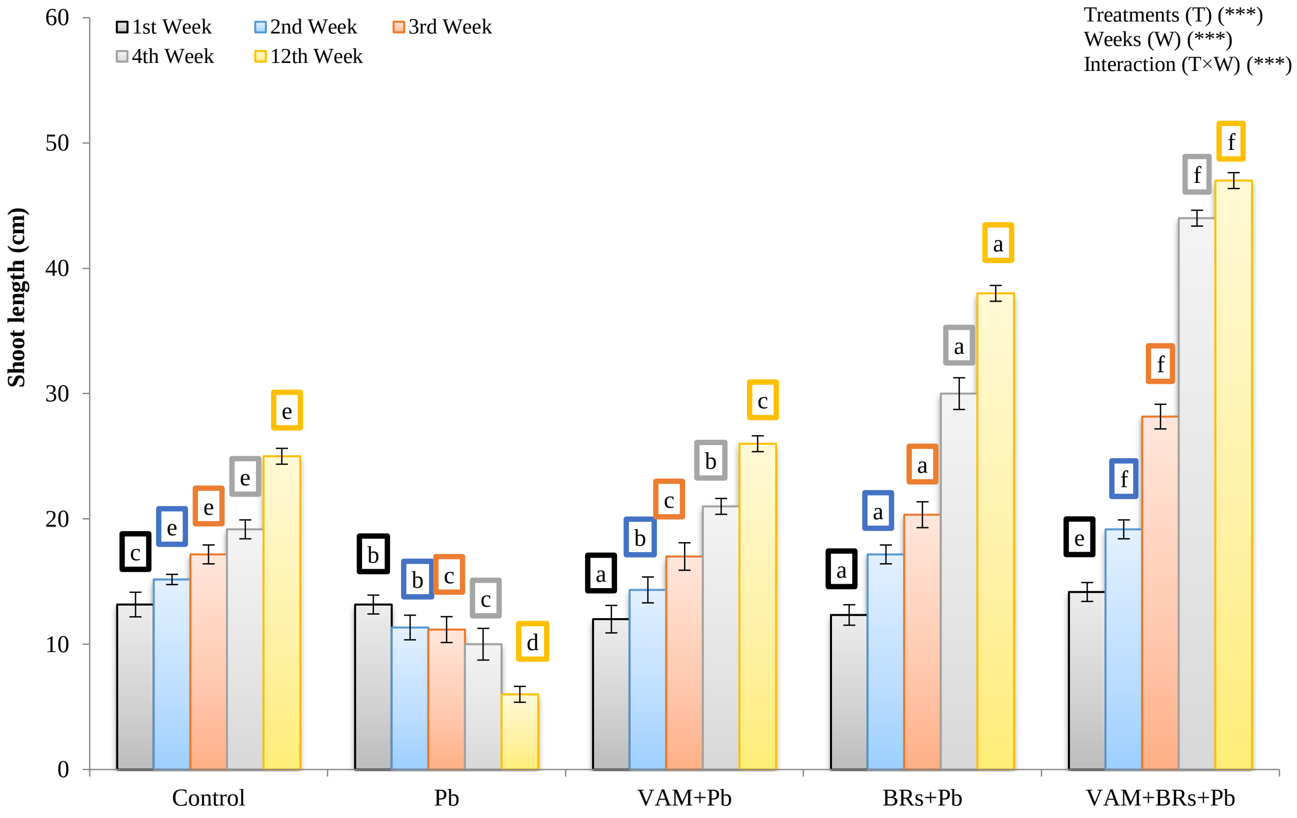

Fig. 1. Impact of Vesicular-arbuscularmycorrhizal (VAM) and Brassinosteriods (BRs) on shoot length (cm) in Lead (Pb) induced *L. cylindrica*. Data are represented as mean ± S.D. The bars show significant differences among treatments labeled with different letters at *p*<0.05.

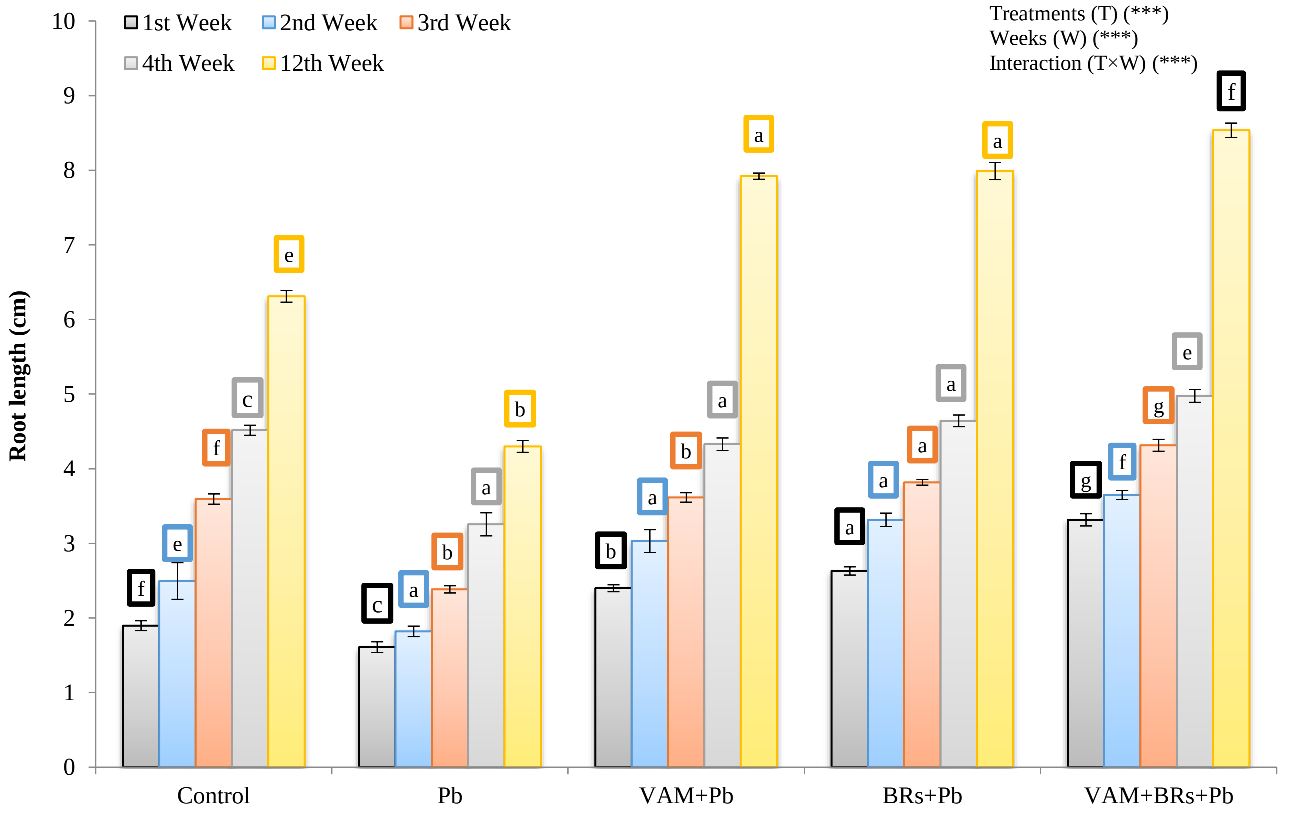

Fig. 2. Impact of Vesicular-arbuscularmycorrhizal (VAM) and Brassinosteriods (BRs) on Root length (cm) in Lead (Pb) induced *L. cylindrica*. Data are represented as mean ± S.D. The bars show significant differences among treatments labeled with different letters at *p*<0.05.

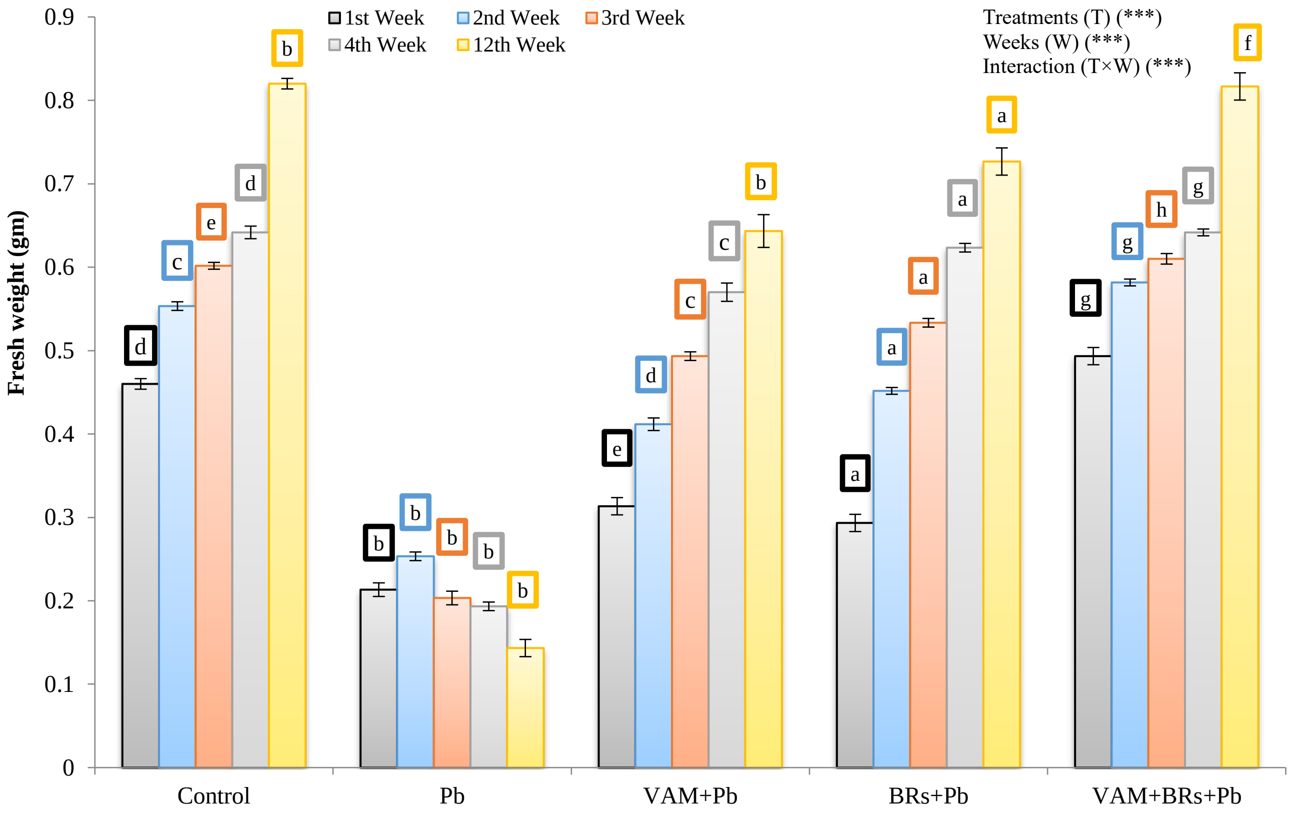

Fig. 3. Impact of Vesicular-arbuscularmycorrhizal (VAM) and Brassinosteriods (BRs) on Fresh wt.(gm) in Lead (Pb) induced *L. cylindrica*. Data are represented as mean ± S.D. The bars show significant differences among treatments labeled with different letters at $p<0.05$.

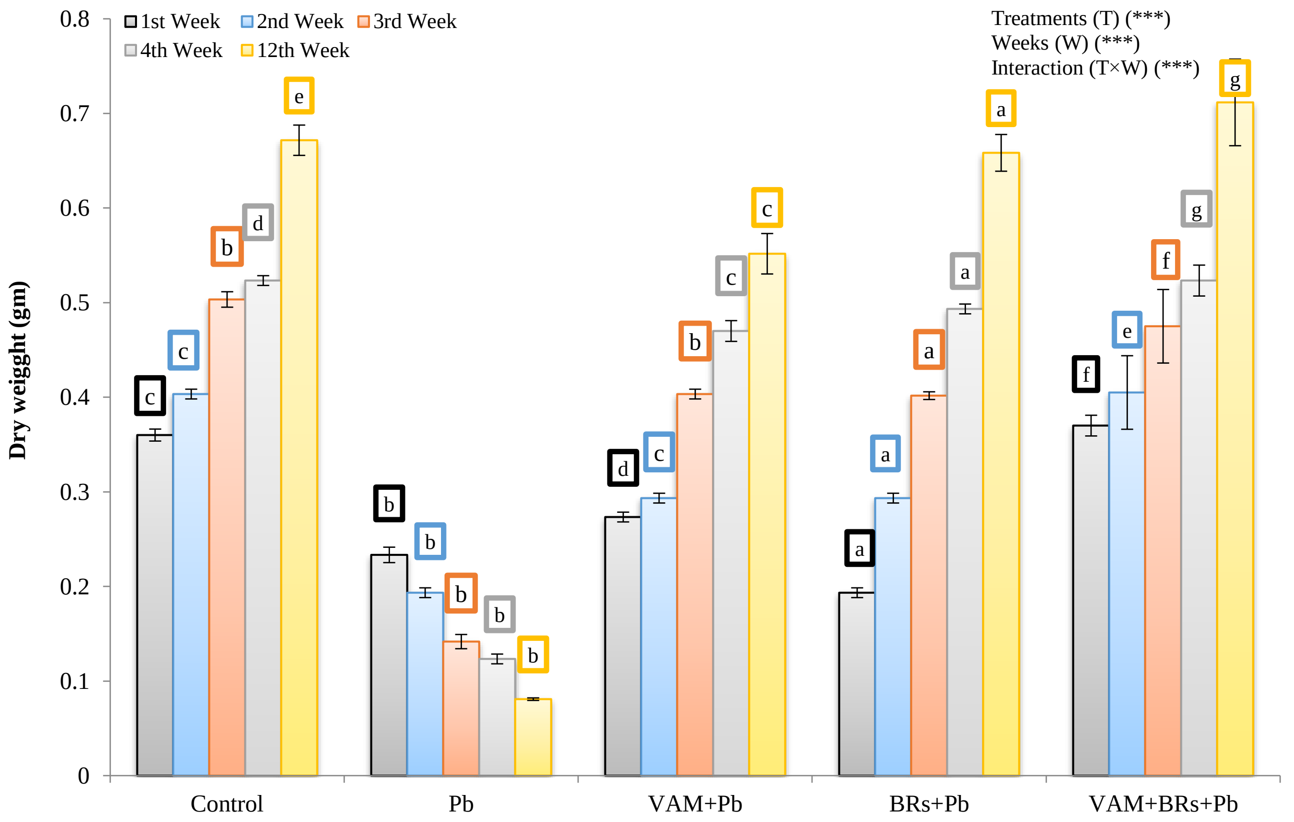

Fig. 4. Impact of Vesicular-arbuscularmycorrhizal (VAM) and Brassinosteriods (BRs) on Dry wt. (gm) in Lead (Pb) induced *L. cylindrica*. Data are represented as mean±S.D. The bars show significant differences among treatments labeled with different letters at $p<0.05$.

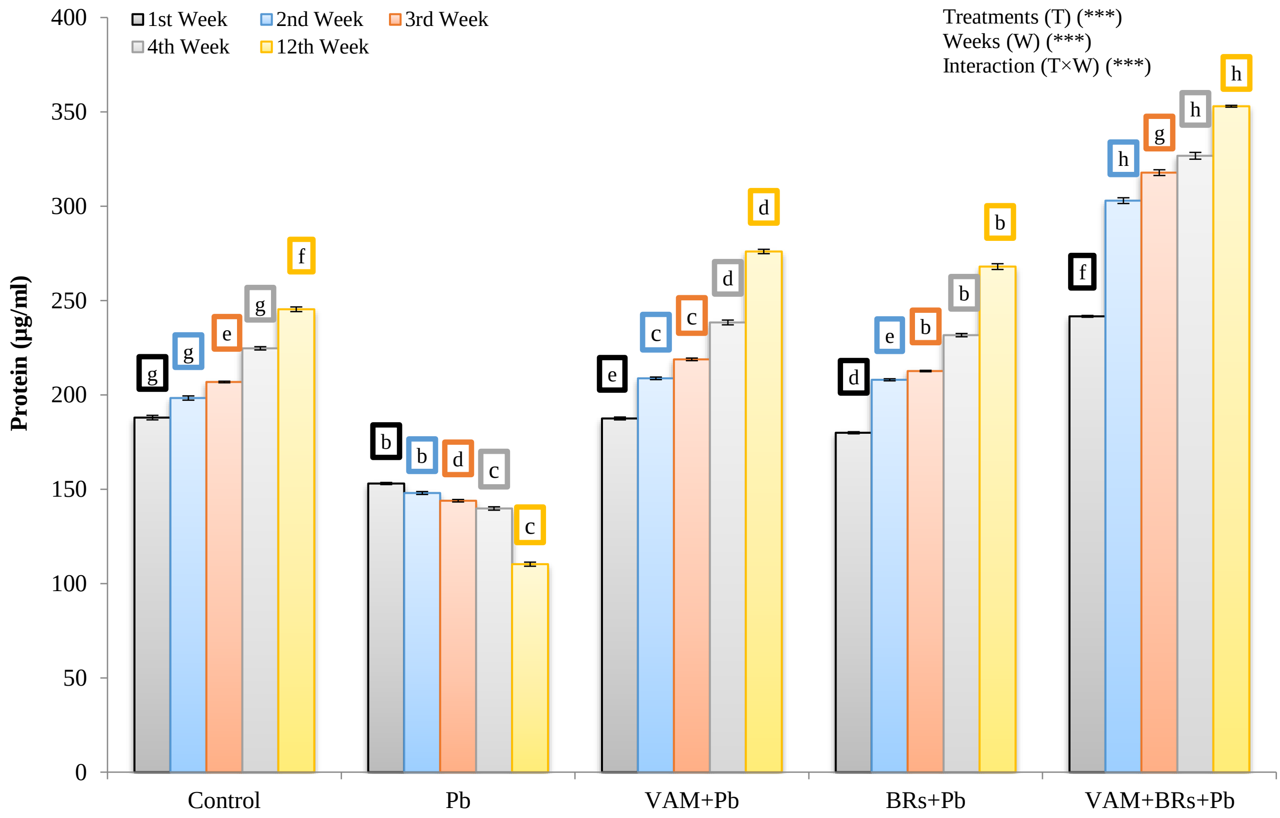

Fig. 5. Impact of Vesicular-arbuscularmycorrhizal (VAM) and Brassinosteriods (BRs) on Protein content (µg/ml) in Lead (Pb) induced *L.cylindrica*. Data are represented as mean±S.D. The bars show significant differences among treatments labeled with different lettersat$p<0.05$.

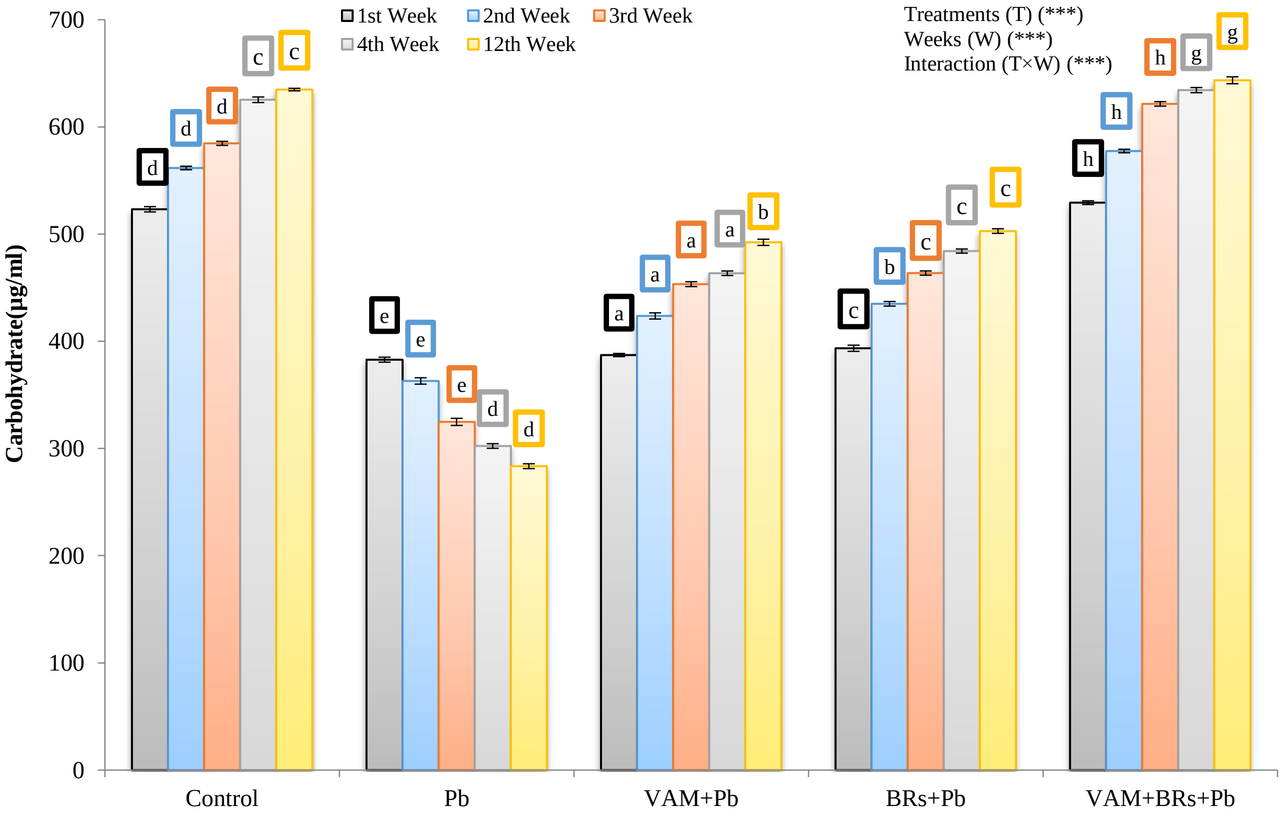

Fig. 6. Impact of Vesicular-arbuscularmycorrhizal (VAM) and Brassinosteriods (BRs) on Carbohydrate content (µg/ml) in Lead (Pb) induced *L. cylindrica*. Data are represented as mean±S.D. The bars show significant differences among treatments labeled with different lettersat$p<0.05$.

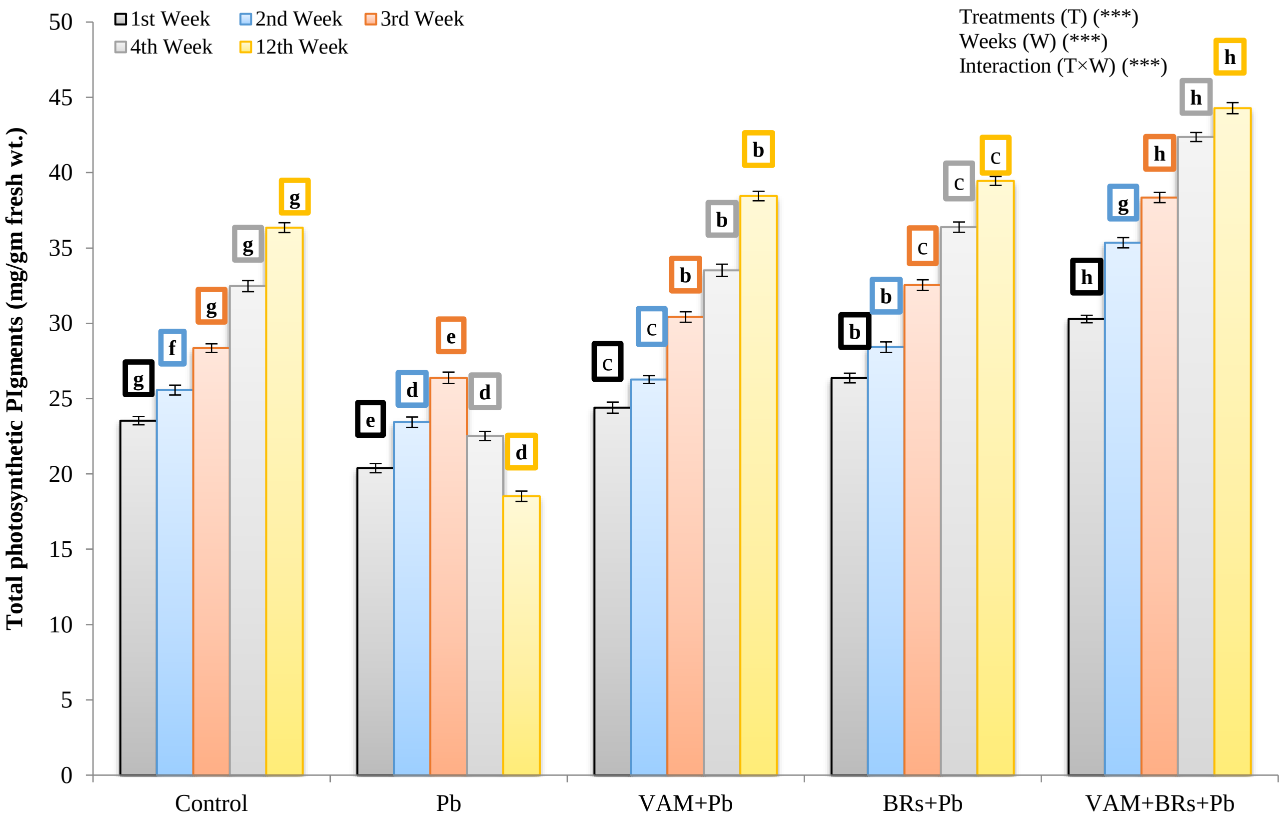

Fig. 7. Impact of Vesicular-arbuscularmycorrhizal (VAM) and Brassinosteriods (BRs) on Total Photosynthetic Pigments (mg/gm fresh wt.) in Lead (Pb) induced *L. cylindrica*. Data are represented as mean±S.D. The bars show significant differences among treatments labeled with different lettersat$p<0.05$.

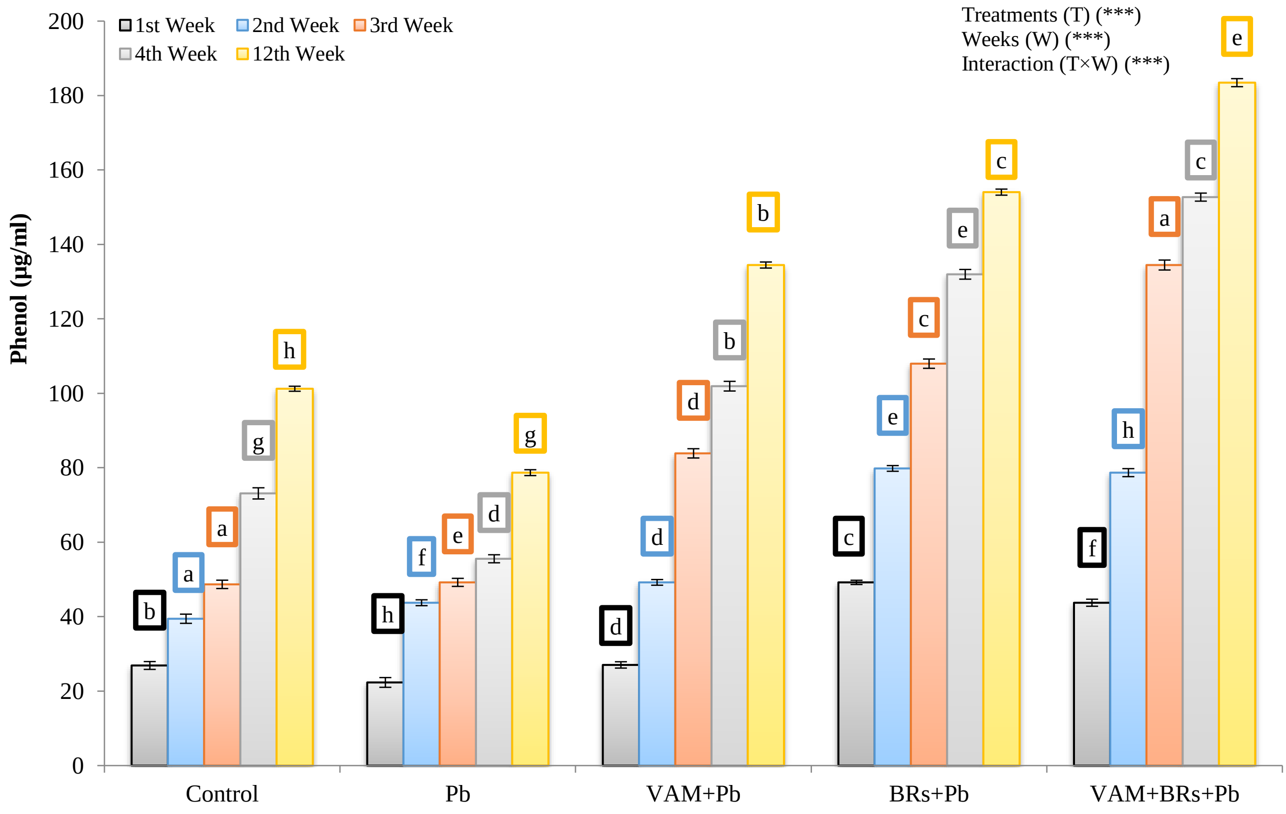

Fig. 8. Impact of Vesicular-arbuscularmycorrhizal (VAM) and Brassinosteriods (BRs) on Phenolic content (µg/ml) in Lead (Pb) induced *L.cylindrica*. Data are represented as mean ± S.D. The bars show significant differences among treatments labeled with different lettersat$p<0.05$.

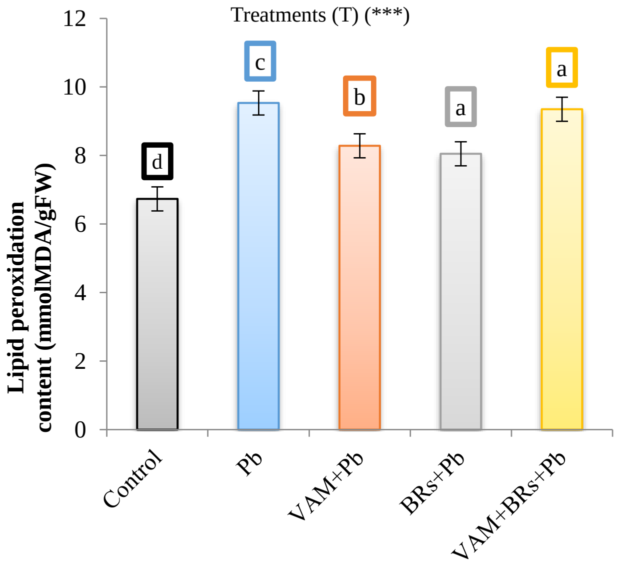

Fig. 9. Impact of Vesicular-arbuscularmycorrhizal (VAM) and Brassinosteriods (BRs) on Lipid peroxidation content (mmolMDA/gFw) in Lead (Pb) induced *L. cylindrica*. Data are represented as mean±S.D. The bars show significant differences among treatments labeled with different lettersat$p$<0.05.

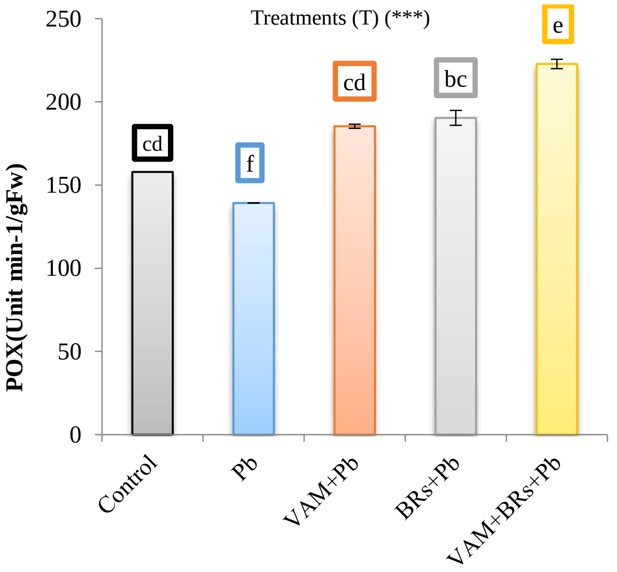

Fig. 10. Impact of Vesicular-arbuscularmycorrhizal (VAM) and Brassinosteriods (BRs) on POX (Unit min-1/gFw) in Lead (Pb) induced *L. cylindrica*. Data are represented as mean±S.D. The bars show significant differences among treatments labeled with different lettersat$p$<0.05.

The current findings show the drastic reduction in carbohydrate, protein, total photosynthetic pigments and phenols in lead induced plants whereas, ameliorating effects are shown in lead induced plants when subjected to VAM and BRs treatments. According to previous study of (Ikkonen & Kaznina 2022) Pb toxicity has a negative impact on protein and carbohydrate content in plants. A reduction in the amount of carbohydrates is brought about by a disturbance in the function of photosynthetic pigments. Further, Ashraf *et al*., (2022) reported decrease in photosynthetic pigments due to lead. VAM can enhance plant defense systems, reduce stress, and increase phenolic content in plants highlighting the critical function of VAM in boosting phenol synthesis under stress (Pratyusha, 2022). Mycorrhizal inoculated plants have higher photosynthetic pigment and protein concentrations (De Mandal *et al*., 2022). Previous analysis revealed that BRs application increased the number of phenolic compounds in grapefruit Babalik *et al*., (2020). Sharma *et al*., (2020) reported the enhanced biochemical aspects in Pb-induced plants such as total photosynthetic pigments, protein, carbohydrate, and nucleic acid content by the application of BRs.

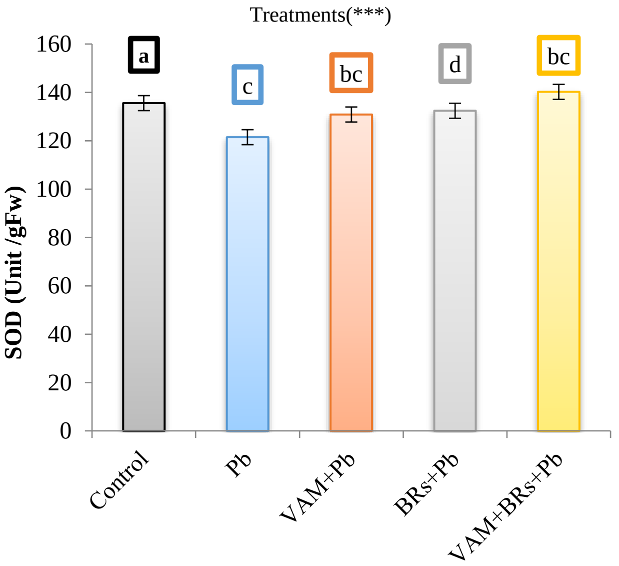

Fig. 11. Impact of Vesicular-arbuscularmycorrhizal (VAM) and Brassinosteriods (BRs) on onSOD (Unit /gFw) in Lead (Pb) induced *L. cylindrica*. Data are represented as mean ± S.D. The bars show significant differences among treatments labeled with different letters at p<0.05.

In the case of MDA the present study reveals that, MDA is a reactive aldehyde that develops from lipid peroxidation when there is oxidative stress. High MDA may be a sign of oxidative stress and increased lipid peroxidation. The analysis of current study revealed that the Pb-induced plants attained increased MDA levels compared to all other treatments whereas VAM and BRs-treated lead-toxic plants showed reduction in MDA levels. The results of this study are consistent with those from Bakhtiari *et al*., (2023) that exposure to lead increases the levels of MDA in Sage plants, indicating oxidative damage. In plants that are exposed to heavy metals, the exogenous application of brassinosteroids reduces the levels of MDA, indicating a reduction in oxidative damage and lipid peroxidation (Bali *et al*., 2021). Albqmi *et al*., 2023 analyzed the oxidative effect of VAM in arsenic stress. They reported that in arsenic induced plants MDA levels increased whereas lower MDA levels were found in arsenic induced plants subjected with VAM proved amelioration effect of VAM towards heavy metal stress. The present study shows that there is a retardation of Pb toxic plaints in anti-oxidative enzyme efficacy of Superoxide dismutase (SOD) and Peroxidase (POX). The results of the current study are supported by the previous researches that, Lead negatively impacts upon antioxidant defense by disrupting the metabolic activity and oxidative stress. SOD and POX antioxidant enzyme activity is decreased in Pb-induced plants (Guedes *et al*., 2021). Moreover, this study reveals that VAM and BRs can counter various stresses in plants through the enhancement of antioxidant enzymes. The results of this study illustrated that VAM and BRs treatments in Pb-stressed plants alleviated the Pb toxicity by the antioxidant enzymes regulation. The ability of VAM and BRs to enhance anti-oxidative enzyme capacity to sequester reactive oxygen species must be controlled to combat oxidative stress. These findings are in accordance with the results of Sheteiwy *et al.*, (2021) that VAM inoculation increased SOD and POX antioxidant enzyme capacity. According to Rodrigues *et al*., (2020) BRs promoted SOD and POX enzyme activity.

Conclusion

Lead pollution in plants could be reduced by using VAM and BRs synergistically resulting in the encasement of plant growth. In a vast variety of crops, VAM fungi have the ability to positively influence plant development and soil strength. The utilization of phytohormones as an additive for the treatment of toxicity of heavy metals have highlighted in many studies. Additionally, owing to the buildup of heavy metals, VAM and BRs have great biological and preventive properties against oxidative stress. Under stressful circumstances, they can prevent lipid breakdown caused by excessive ROS generation. They help to protect photosynthesis, and plant growth by raising the levels of anti-oxidative systems that in turn can increase a plant's tolerance. Therefore, VAM and BRs have the potential to improve agricultural production and sustainability when used as organic fertilizers.

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