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Research Paper

Impact of ascorbic acid exogenous application on growth, biochemical parameters, and membrane stability of (*Vicia faba* L.) under heavy metals stress

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ABSTRACT

The toxicity of Cd and Pb in *Vicia faba* species was investigated in this study with regard to growth characteristics and biochemical parameters in both the presence and absence of ascorbic acid treatment at varying dosages. Based on all the results reported, quantification of faba bean metabolites revealed that Cd and Pb led to a higher decrease in protein and soluble sugars compared to the control group. Whereas the increase in the level of proline is related to the effect of stress, since it is a stress marker in unfavorable conditions. Additionally, in batches treated with cadmium and lead, the treatment of two concentrations of ascorbic acid (0.5 mg/l and 1 mg/1) stimulated the growth of seedlings, resulting in an increase in average stem length, leaf growth and a highly significant root increase. These findings demonstrate ascorbic acid's potential as an affordable mitigation strategy for enhancing crop resilience in areas contaminated by metals.

Key words: Ascorbic acid, Cadmium, Growth parameters, Lead, *Vicia faba.*

INTRODUCTION

Heavy metals in different environments are the source of many types of pollution problems. The aggregation of metals in plants, through soil-plant exchange, and in people and creatures, through the food chain, is caused by contamination of both strong and sea-going situations. Oxidative stress is initiated when plants are exposed to high levels of heavy metals. (Al-Rashedy *et al.* 2023, Azeh *et al.* 2019, Luma *et al.* 2020, Madhu and Sadagopan 2020). The discharge of cadmium (Cd) into the environment is caused by human activities such as phosphate fertilizers, family transfer, urban systems and mechanical systems. This source can lead to improved soil and, consequently, calcium layer levels in crops, which can lead to increases in dietary exposure to the calcium layer. CD may be a particularly dangerous toxin due to its high hazard and high solubility in water (Mathew *et al.* 2022). At low concentrations, Cd is not harmful to plants, but at higher concentrations it is toxic. High concentrations of Cd are carcinogenic, mutagenic and teratogenic for many animal species (Jiang and Hou 2001). It specifically inhibits root growth and cell division in plants such as onion, spiderwort (*Tradescantia)*, and faba bean (*Vicia faba)* (Youssef *et al*. 2005). As far as lead is concerned, this substance is one of the heavy metals that are of no importance to the plant and its uptake from the environment is simple; it is found in the various parts of the plant. The transfer of lead into the plant depends on pH, particle size, and various physical and chemical factors (Farouk and Muhammad 2018). According to Marschner (2011), it is easily absorbed by plant roots. The environment is polluted by lead through the use of fertilizers, car exhaust, and various human and industrial activities (Saleem *et al*. 2021), In addition to lead smelting, paint, batteries and pipes contain lead (Karri *et al.* 2008). The influence of lead on plants can be seen in its influence on their morphological characteristics, physiological processes and the biochemistry of plant life (Pourrut *et al.* 2011). In order to protect themselves against stress, plants produce both antioxidant enzymes and non-enzymatic compounds thus, maintaining a high antioxidant capacity in the cells increases stress tolerance. The role of cellular antioxidant defense mechanisms against metalloids was described in some higher plants (Feng *et al.* 2009, Pan *et al.* 2011).

The essential role of ascorbic acid is to promote the detoxification of ROS. Available in small quantities, ROS play the role of messengers in the plant's response to stress. When the concentration of ROS increases, oxidative damage occurs, alteration of membrane fluidity and ion transport, reduction in the activity of certain enzymes, inhibition of protein synthesis, DNA degradation, and finally, cell death (Sharma *et al.* 2012). In the present study, the effects of two heavy metals, including cadmium and lead, were investigated on cultivated faba bean seeds for several morphological, physiological and biochemical variables.

MATERIALS AND METHODS

Plants and growing conditions

The study was carried out at the biology department of Mohamed Cherif Messaadia University in Souk-Ahras, Algeria. Faba bean (*Vicia faba)* seeds were germinated for 7 days in the dark at a temperature of 25 °C. Following germination, the plants were transferred to separate containers for hydroponics. Three liters of pots were used, each containing ten plants. The plants were grown in an air-conditioned room at a temperature of 24±2 °C and a relative humidity of 50%, with a lifespan of 16 hours and a light intensity of 150µmol m-2 s-1. The Hoagland nutrient solution consisted of hydroponic medium (Hoagland and Arnon 1959). All chemical products used in the present study were obtained from Sigma Aldrich company.

The medium was aerated continuously (100 ml min^{-1}) and replaced every 3 days. At the three-leaf stage, the 14-day-old (*Vicia faba*) seedlings were treated with two heavy metals, lead and cadmium, added separately to the nutrient solution in the form of Pb (NO3) 2 and CdCl2, respectively, at a constant concentration of 25 µmol/l and 150 µmol/l combined with different concentrations of ascorbic acid (0mg/1, 0.5mg/l, 1mg/l). The seedlings were harvested after 14 days of treatment and split into two parts: roots and stems.

Analytical methods

Growth parameters determination

We cleaned the plants, measured their fresh weight using a sensitive electronic balance and separated the roots and leaves. The lengths of the roots and leaves were measured using a scale.

Chlorophyll and carotenoid determination.

Pigment levels (chlorophyll A, chlorophyll B, carotenoids) were measured in the fresh weight of leaves using homogeneous sampling with 80% acetone. Absorbance was measured at 663 nm for chlorophyll A, 645 nm for chlorophyll B and 470 nm for carotenoids. Pigment content was calculated using the equations of Lichtenthaler and Wellburn (1983).

Determination of soluble sugars

Soluble sugar was extracted from the leaf after drying with 80% ethanol. Soluble sugars were determined using the sulphuric antron method described by Scott and Melvin (1956).

Proline and protein content

According to Bates *et al.* (1973), proline in the leaves was estimated. Absorbance was measured spectrophotometrically at 520 nm and toluene was used as a control. Bradford (1976) used the method to assess the protein content of leaves. Absorbance was measured at 595 nm using bovine serum albumin as the sample.

Determination of electrolyte leakage

According to Hassanein and colleagues (2012), the level of electroconductivity was measured as a function of electroconductivity (EC %). We cut the leaf samples into a homogeneous disc and placed them in 10 ml of distilled water at 40 °C for 30 minutes. We then recorded their conductivity (Cl) using a conductivity meter. After that, it was kept in a hot water bath (100 °C) for 15 minutes, and its conductivity was recorded (C2). The percentage electrolyte leakage was calculated using this formula: EC (%)= (C1/C2)*100, Cl and C2 are the electrolyte conductivities measured before and after the broth, respectively.

Determination of the Membrane Stability Index

The membrane stability index (MSI) was calculated by dissolving 200 mg of leaves in 10 ml of distilled water over two sessions. One was heated to 40 °C for 30 minutes in a water bath and the electrical conductivity (Cl) was measured. The second series was heated to 100 °C in a hot water bath for 10 minutes, then the conductivity (C2) was measured: both conductivities were measured using a conductivity meter. The MSI was calculated using the formula : MSI (%) = [1−(C2C1)]×100 described by Premchandra and colleagues (1990).

Statistical analysis

Results were given as the mean ± SD of three different trials. All samples were statistically analyzed using a single-way ANOVA and Tukey's multiple comparison method, using the computer program R 3.4.3.

RESULTS AND DISCUSSION

Morphological aspects

Figure 1A shows that bean seedlings treated with 150µmol/l Cd and 25µmol/l Pb show a delay in leaf development compared with the control. However, we observed that the addition of ascorbic acid at different concentrations (0.5mg/l and 1mg/l) to the nutrient solution induced an increase in the number of leaves. In the same way, both metals inhibited the growth of the aerial part (Figure 1B) and significantly reduced the growth of the root part (Figure 1C). Statistical analysis showed that there was no difference in variations in stem elongation ($p=0.998$, $p=1.000$), while a significant increase in leaf numbers ($p=0.0422$) and root elongation was highly significant for the stressed groups compared with the control ($p<0.001$).

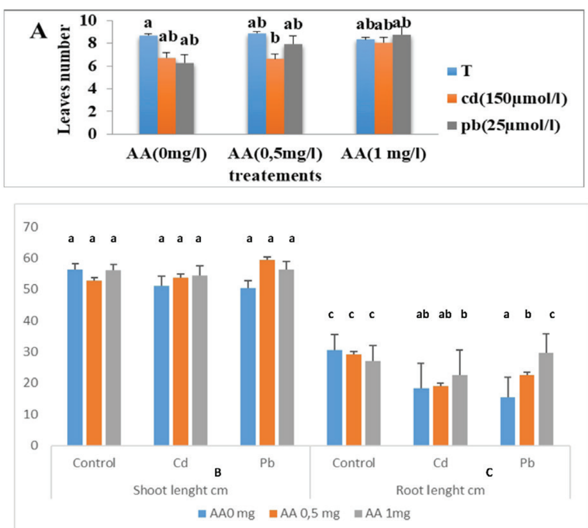

Fig. 1. Effects of ascorbic acid on leaf number (A), shelongation (B) and root elongation (C) in bean seedlings with and without metal stress (cadmium and lead).

Roots of seedlings treated with 150 µmol/l cadmium chloride and 25 µmol/l lead nitrate showed browning, in agreement with the observations of Mishra *et al.* (2006) in *Bacopa monnieri* exposed to Cd (100 nmol/l for 4 to 7 days) and Elloumi *et al.* (2007) in *Prunus dulcis* after 14 days of Cd treatment. Similar symptoms, including root browning, leaf yellowing, chlorosis, and necrosis, have also been reported under lead treatment (Seregin and Ivanov 2001, Sharma and Dubey 2005). Elongation and branching of fava bean roots were strongly inhibited (Aslam *et al.* 2013). A reduction in primary and secondary root growth was observed, similar to that described in *Pisum sativum* by Rodriguez-Serrano *et al.* (2006) after Cd treatment (50 µmol/l for 14 days), with a decrease in the number and length of lateral roots. These results confirm that the root system was more sensitive to metal stress than the leaves, as shown by the work of Abd Elgawad *et al.* (2020) and Souguir (2009).

Fresh biomass

The results showed that decrease in the fresh biomass of the stems (Figure 2A), while the fresh biomass of the roots was significantly reduced (Figure 2B), as was the fresh biomass of whole plants at a concentration of 150 µmol/l of Cd and 25 µmol/l of Pb (Figure 2C).

Statistical analysis indicated that there was a highly significant difference in variations in stem and root fresh biomass ($p<0.005$).

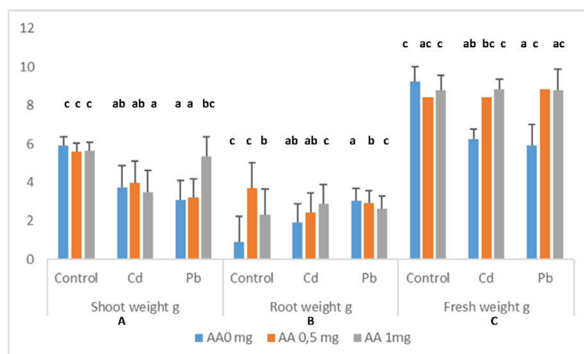

Fig. 2. Effects of ascorbic acid on fresh material of aerial parts (A), root (B) and whole plants (C) in bean seedlings under or without metal stress (Cadmium and Lead).

Dry biomass and moisture content

According to the results shown in (Figure 3), we observed that bean plants treated with 150µmol/l of Cd and 25µmol/l of Pb showed a greater decrease in the dry biomass of the above-ground part (Figure 3A), and of the root part (Figure 3B) compared with the control. The supplementation of ascorbic acid at different concentrations increased dry biomass in both stressed and unstressed parts of the bean.

Statistical analysis revealed that there was no significant difference in stem dry biomass variation ($p>0.005$), whereas there was a significant difference in the roots of samples treated with lead ($p=0.0232$) compared to the control and in combination with ascorbic acid at 1mg/l (p= 0.0467) compared to the stressed group.

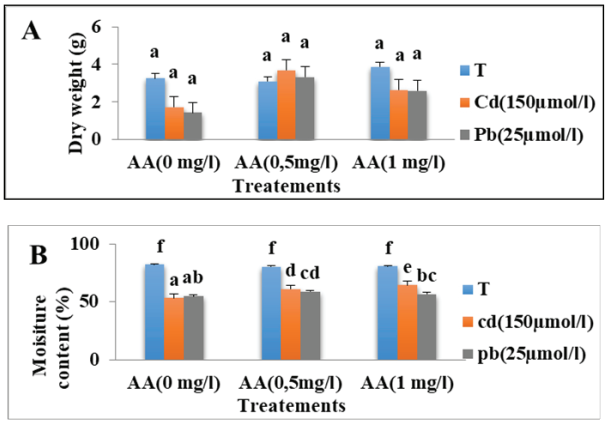

Fig. 3. Effects of ascorbic acid on dry matter and on the moisture content of the aerial part (A) root part (B) in bean seedlings under or without metal stress (Cadmium and Lead).

The study indicates that the reduction in fresh and dry biomass in (*Vicia faba* L.) is earlier and more pronounced in the roots than in the aerial part. However, Youssef *et al.* (2005) found a more significant decrease in dry biomass in leaves compared to roots of Cd-exposed rapeseed, indicating that these effects differ according to plant species and duration of exposure.The results corroborate those of Sereguine and Ivanov (1998), who observed a 50% inhibition of stalk growth in maize subjected to lead nitrate. Other research corroborates these findings (Ait Hamadouche *et al.* 2012, Anuradha and Rao 2007, Biteur *et al.* 2011, Wang *et al*. 2012, Garg *et al.* 2004, Gopal and Rizvi 2008), reporting a decrease in biometric parameters in (*Vicia faba)* under metal stress. Cd and Pb stress results in obstructed growth of faba bean seedlings, manifested by reduced biomass, plant height and number of leaves (Saadaoui *et al.* 2022). Relative leaf water content, an indicator of tissue turgor and stress tolerance, is significantly reduced by metal stress. This decrease is similar to that observed with ascorbic acid treatments, whether used alone or in combination (Keyvan 2010, Mahmud *et al.* 2020).

Photoreceptor pigments

Our results revealed a significant decrease in chlorophyll a content after treatment with 25µmol/l Pb (Figure 4A) relative to the stressed group, whereas chlorophyll b (Figure 4B) and carotene (Figure 4C) were not affected. After statistical analysis, the effect of Pb on chlorophyll a was significant in all (*Vicia faba* L.) seedlings (p=0.0405), although no significant difference was noted between samples of chlorophyll b (p=1.000, p=0.967) and carotene (p= 1.000).

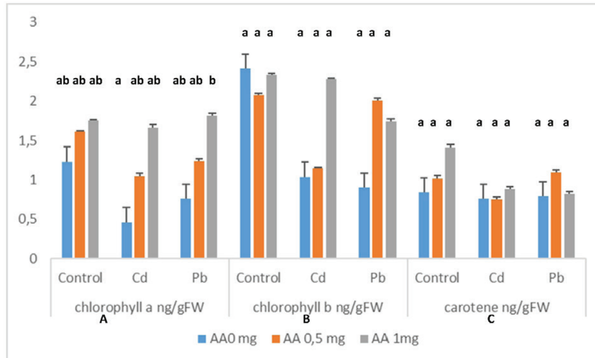

Fig. 4. Effects of ascorbic acid on chlorophyll a (A), chlorophyll b (B) and carotene (C) content in bean seedlings with and without metal stress (cadmium and lead) with the stressed group.

Numerous studies have shown that lead (Pb) and cadmium (Cd) reduce chlorophyll pigment content (chlorophylls and carotenoids), mainly due to oxidative stress that inhibits the enzyme aminolevulinic acid dehydratase (ALAD), essential for chlorophyll biosynthesis (*Tan et al.* 2022). After treatment, these significant variations in pigment content were observed between the control and the exposed plants. A slight drop in chlorophyll A and B levels was observed, which could be attributed to a stimulating effect of low-dose heavy metals on metabolism (Kasim *et al.* 2017, Lui *et al.* 2023). This type of effect has also been reported by (HS 2012) in rice and sunflower grown on soils containing traces of lead. Conversely, Burzyinski (1984) noted a net reduction in pigments in bean seedlings grown on medium containing $PbCl_2$ at two concentrations (10^{-6} M and 10^{-4} M). The increase in chlorophyll content after treatment can be explained by the antioxidant properties of this molecule, which is capable of trapping and directly eliminating reactive oxygen species in the cytoplasm (Beyer 1994). It also plays a role in the preservation or repair of oxidised carotenoids or tocopherols (Shao *et al.* 2009).

Under stress, there is instability in the pigment complex and an increase in chlorophyllase activity, which contributes to a drop in chlorophyll levels (Mohamed and Akladious 2014). In addition, carotenoids decrease in response to lead poisoning, in proportion to the metal concentration, probably due to oxidative stress that disrupts the balance between oxidant and antioxidant molecules, thus affecting this protective pigment.

According to Talaat (2019), foliar application of ascorbic acid promotes the uptake of lead nitrate because of its positive effects on plant growth. Similar results have been observed in radish (*Raphanus sativus* L.) and oilseed rape (*Brassica napus*) grown on contaminated soils (Abid *et al.* 2016, Marchiol *et al.* 2004), as well as in nickel-treated wheat (Gajewska and Sktodowska 2007) and in maize and barley (*Hordeum vulgare* L.) exposed to cadmium.

Biochemical parameters

Proteins

Figure 5 shows the impact of ascorbic acid on protein levels in the aerial and root parts of (*Vicia faba.* L) stress and non-stressed plants. Samples treated with both concentrations of ascorbic acid and stressed by cadmium and lead revealed significant increases in protein synthesis in the roots and stems. The difference in protein content was significant in the roots (p=0.0321) and stems (p=0.0235) compared.

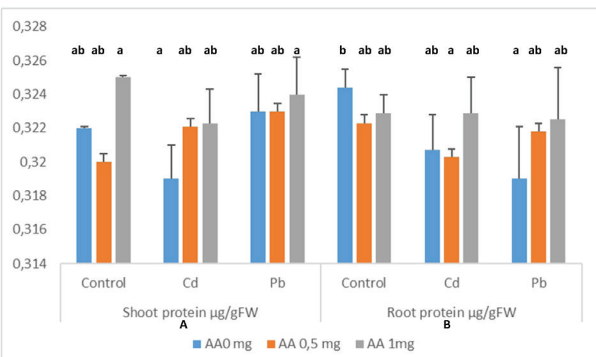

Fig. 5. Effects of ascorbic acid on the protein content of the aerial part (A) and root part (B) in faba bean seedlings under metal stress (cadmium and lead).

The decrease in protein content is due to the accumulation of heavy metals, which leads to the formation of free radicals that denature, oxidise, or degrade these proteins to form carbonyl derivatives. A decrease in protein content in wheat exposed to heavy metals may be due to the effect of ROS. The reduction in protein content under metal stress could be due to the disruption of nitrogen metabolism or inhibition of nitrate uptake, as reported by El-Zeiny *et al.* (2007), Singh *et al.* (2015). Lead and cadmium present in the cytoplasm interact with proteins. They can decrease protein pools (Mishra *et al.* 2006, Jacobson *et al.* 2017).

Soluble sugars

Results obtained (Figure 6) reveal that in bean plants, accumulation of total soluble sugars (TSS) varied according to the concentration of Cd 150 µmol/l, lead 25 µmol/l and ascorbic acid (0.5 mg/l, 1 mg/1). There was a significant difference between the groups (p<0.05). Cadmium chloride and lead nitrate added to the nutrient medium decreased the carbohydrate content compared with the control, while ascorbic acid treatment alone increased the TSS content compared with the control (p<0.01).

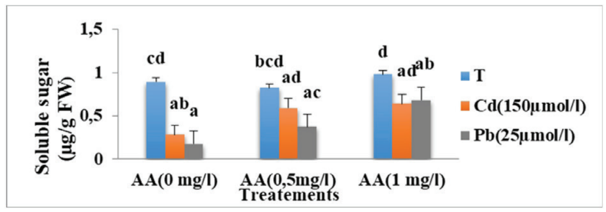

Fig. 6. Effects of ascorbic acid on soluble sugar content in bean seedlings under metal stress (cadmium and lead).

The application of ascorbic acid at 0.5 mg/l and 1 mg/l showed opposite trends to the effects of stress, mainly at 1 mg/1, leading to significant increases in this parameter not only compared to stressed plants, but also to untreated plants. (Tekle and Alemu 2016). Soluble sugars play an essential role in plant structure, metabolism and function. They are also involved in numerous mechanisms of response to biotic stresses. (Afza *et al.* 2021), and may play a role in protecting chlorophylls and carotenoids against the aggressiveness of metallic stress (Zhang *et al.* 2024, Ali *et al.* 2019), attributing this accumulation to the plant's possible adaptation to adjust and maintain a favorable osmotic potential.

Proline

The results reported in (Figure 7) reveal a significant difference in proline content between the groups for the aerial part (p=0.0114), and a highly significant difference for the aerial part of *Vicia faba* L. (p=0.0035). The root part (Figure 7B) showed a higher accumulation of this acid than the aerial part (Figure 7A).

According to the study, exposure to 150 µmol/l of cadmium chloride and 25 µmol/l of lead nitrate causes a significant build up of proline in the bean's root and aerial parts. This result is consistent with the observations of Sharma and Dietz (2006), who reported that heavy metals stimulate the synthesis of various metabolites, in particular amino acids such as proline. This accumulation is thought to be

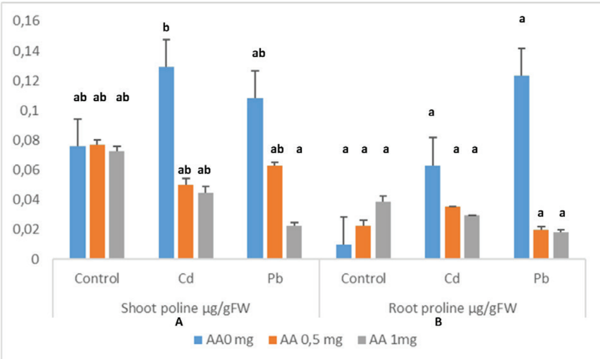

Fig. 7. Effect of ascorbic acid on proline content in leaves (A) and roots (B) of bean seedlings subjected or not to metal stress (cadmium and lead).

linked to the degradation of stress-sensitive proteins and the synthesis of new amino acids (Pena *et al.* 2006). Increases in proline in response to cadmium have been reported in various species, such as radish (Chen *et al*. 2003) and soya (Balestrasse *et al.* 2005). According to Sharma and Dietz (2006), other metals such as zinc, lead and copper can also induce this accumulation. Proline plays an important role in maintaining osmotic balance, preserving membrane integrity and stabilising cytoplasmic structures under stress. It also helps to reduce lipid peroxidation. Our results are in agreement with those observed in (*Canavalia ensiformis)* under the effect of copper (after 11 weeks) and in (*Coffea arabica*) (after 30 weeks). In this sense, proline is often considered a biomarker of stress (Hosseinifard *et al.* 2022).

Electrolyte leakage

Results (Figure 8) clearly indicated that metal stress (Cd and Pb) reduced electrolyte leakage from leaf membranes in Faba bean plants compared with the control. Statistical analysis showed that there was a highly significant difference between the nine groups ($p<0.001$).

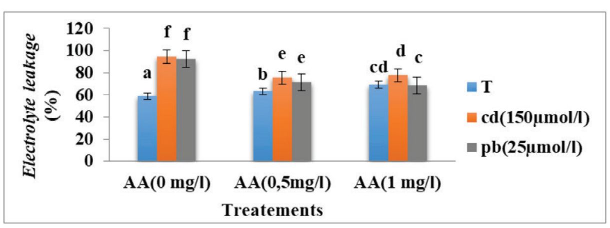

Fig. 8. Effects of ascorbic acid on leaf electrolyte leakage in bean seedlings with and without metal stress (cadmium and lead)

Membrane stability index (MSI)

Although there was no significant difference between the groups for the membrane stability index of the leaves ($p=0.770$, $p=0.783$), Figure 9 shows a slight difference.

The results indicate that a decreasing effect on stability was recorded in the group stressed with 150 µmol/1 $CdCl2$ and 25µmol Pb $(NO3)2$ without treatment.

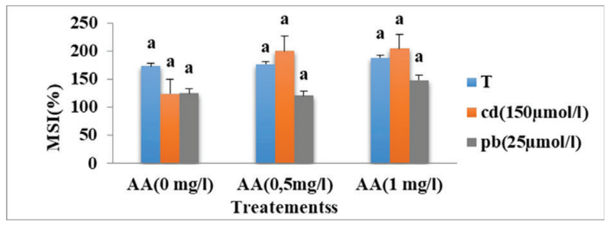

Fig. 9. Effects of ascorbic acid on the leaf membrane stability index in bean seedlings subjected or not to metal stress (cadmium and lead).

Proline accumulation, as a compatible solution, fulfils several vital roles in response to metal stress: it helps with osmotical regulation, metal detoxification, enzymatic protection, cytosolic pH regulation, stabilisation of the protein synthesis machinery and the removal of oxygen-reactive species like hydroxyl radicals or oxygen singlet (Hanif *et al.* 2021). The results indicate that the rate of electrolyte leakage was higher in stressed plants, as a result of altered membrane integrity. This is reflected in a decrease in the membrane stability index (MSI) under the effect of cadmium chloride and lead nitrate. Previous studies have observed similar results under salt stress in (*Lycopersicon cheesmanii)* and (*L. peruvianum)* (Uddin *et al.* 2023). A reduction in lipid peroxidation and improved membrane stability have also been observed in maize (*Zea mays*) and sugarcane (Rabêlo *et al.* 2019). The accumulation of ROS (reactive oxygen species) observed in this study could therefore explain the loss of cellular integrity observed in bean roots and leaves at the end of treatment. This phenomenon is also induced by other metals, such as mercury, capable of disrupting cellular integrity in (*Medicago sativa)* (Ortega-Villasante *et al.* 2007, Zhou *et al.* 2007). Metal ions exert their toxicity by promoting the creation of reactive oxygen radicals. These species damage vital molecules such as nucleic acids, proteins, or lipids, leading to enzymatic dysfunction or lipid peroxidation. Metal stress thus has a marked effect on biochemical, physiological and chemical parameters. Metal stress thus has a marked effect on the biochemical, physiological and cytogenetic parameters of plants (Briat and Lebrun 1999).

CONCLUSION

The present study shows that broad bean seedlings exhibit noticeable morphological and physiological alterations in response to lead and cadmium exposure. Root length proved particularly sensitive because of its direct function in metal uptake, making it a good biomarker of phytotoxicity. Exposure to heavy metals decreased biomass output, root elongation, and leaf expansion. In addition, proline, a definite indicator of stress, rose while Cd and Pb decreased protein and chlorophyll levels, interfered with water balance and carotenoids, and damaged membrane integrity. Crucially, by functioning as an efficient antioxidant, ascorbic acid application lessened these adverse effects and maintained development and physiological equilibrium. These findings highlight how effective antioxidant therapies can be in lowering heavy metal stress and maintaining plant productivity in impacted areas.

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