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ALLEVIATORY EFFECTS OF PGRS (PLANT GROWTH REGULATORS) PRIMING ON COBALT-INDUCED STRESS IN LENTIL VARIETY

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Abstract

Cobalt is heavy metal that possesses significant abiotic stress to plants adversely affecting growth and development by interfering with key physiological, biochemical and metabolic processes. Excess cobalt in soil can lead to reduced nutrients uptake, impaired photosynthesis and accumulation of reactive oxygen species ultimately compromising plant productivity. To investigate the impact of cobalt stress and evaluate strategies for its mitigation, a pot experiment was conducted at the Department of Botany, University of Balochistan, Quetta. The lentil variety Black Panjgur was grown under cobalt stress at a concentration of 30 μM , and seeds were primed with different plant growth regulators (PGRs), including salicylic acid (SA, 250 μM), moringa leaf extract (MLE, 10%), and thiourea (TU, 10 μM). The experiment was arranged in a completely randomized design (CRD) with three replicates. The result showed that cobalt stress inhibited seedling growth, reducing root and shoot length and decreasing chlorophyll and carotenoid contents. Metabolites such as soluble sugars, soluble phenolics and anthocyanins were also adversely affected indicating oxidative and metabolic stress. Application of PGRs alleviated these negative effects by enhancing morphophysiological attributes and boosting biochemical defense mechanisms. Among the treatments, MLE 10% exhibited the strongest positive impact, improving seedling growth, photosynthetic pigments, and stress-related biochemical compounds more effectively than SA or TU. These findings demonstrate that priming of PGRs particularly MLE can effectively mitigate cobalt induced toxicity in lentils. This strategy offers a practical approach to enhance plant tolerance against heavy metal stress and supports sustainable crop production in contaminated soils. The study provides valuable insights into the role of natural plant extracts and chemical regulators in improving plant resilience and productivity under metal-induced stress conditions.

Keywords: Cobalt toxicity, *Lens culinaris*, PGRs, Seed priming

INTRODUCTION

Cobalt (Co) is recognized one of important element for all living organism on earth and, whereas its significance for higher plants remains uncertain (1, 2). It is the core part of vitamin B12 (cobalamin), which is the source of protein in animal life (3). Cobalt exhibits a dual character, functioning both as a trace micronutrient and a heavy metal depending on its concentration and biological context (4). Nevertheless, previous research has demonstrated that elevated cobalt levels can have phytotoxic effects, impairing plant growth and physiology. Beneficial elements for plants i.e: aluminum (Al), cobalt (Co), sodium (Na), selenium (Se), and silicon (Si) positively effects on plant growth and stress resistance (5). It was reported that Co about 0.01–1 $\mu\text{g/g}$ dry matter, and but higher amount result in Co phytotoxicities (6). Plants grow in soil that contains less than 0.08 mg Co per kilogram (7). Co concentrations differ from 0.006 mg per liter to 0.43 mg per liter however the average soil Co levels move up to 40 ppm which is toxic for plants (8, 9).

The main sources of Co are industrial smokes, vehicle emission etc and other emissions (1). Excessive Co result in yellow-colored leaves, colorless veins in plants. Cobalt stress damages plants due to increase in reactive oxygen species (ROS), which are harmful chemicals (10, 11). These enzymes help prevent damage and maintain plant health (12, 13). Cobalt helps higher plants, but its necessity for growth remains



unclear. In primitive plants like *Euglena gracilis*, cobalt is crucial for photosynthesis and is found in chloroplasts. It is also essential for nitrogen-fixing bacteria.

Lentils (*Lens culinaris*), Legumes like lentils, garbanzos, and pea plant are affordable causes of protein and fiber. Public awareness of their antioxidant benefits supports their inclusion in diets to combat diseases linked to oxidative anxiety (14, 15). Lentils are particularly rich in zinc (44–54 mg/kg) and iron (73–90 mg/kg), making them ideal for biofortification (16, 17). But, micronutrient content varies by genotype (18). Micronutrient deficiencies affect crop yield and human/animal health globally, with zinc and iron deficiencies affecting large populations (19). These nutrients are vital for immune function, enzyme activity, and DNA/protein synthesis (20). Biofortification developments nourishing excellence and harvest productivity (18).

Cobalt is vital for nitrogen-fixing bacteria trendy legumes (21). In soils, Co accumulates from industrial sources and sewage use, affecting crops (22). High levels occur in serpentine and peaty soils, as well as near refineries and exhaust sources an important legume crop, are high in protein and contribute to soil nitrogen fixation. Under cobalt stress, lentils experience disrupted growth, photosynthesis, and enzyme functions (23-25). Riboflavin reported to mitigate this stress by supporting antioxidant enzyme systems (26-30). PGRs like salicylic acid, thiourea, and moringa leaf extract also help by modulating hormones and improving stress resilience (31, 34). The essentiality of Cobalt in plants remains debatable, except for its role in nitrogen fixation and rhizobial growth (35, 36). Legumes accumulate more Co than cereals and grasses, reflecting soil status (37). Excess Co causes variable plant responses: citrus shows growth reduction at 11 µg/g, while *Nyssa sylvatica* tolerates much higher levels (38). In beans, excess Co impairs carbohydrate transport (39) and iron deficiency from Co toxicity increases ROS production (40). Elevated Co reduces essential nutrient levels, photosynthesis, and chloroplast integrity (41, 42). It also affects Fe-based enzyme activity (43). Plants produce stress-response proteins under biotic and abiotic stress (44, 45).

Abiotic stresses can cause up to 42% yield loss globally (46). Metal toxicity is worsened by human activities and long-term metal persistence (47). Climate change intensifies stresses like drought, salinity, and temperature extremes, reducing crop productivity (48). These stresses affect plant morphology, physiology, and metabolism depending on severity and duration (49). Global warming has increased average temperatures by 0.9 °C since the 19th century, threatening agriculture (50, 51). Thus, Stress interferes with crop yield, quality, and molecular processes (53).

Salicylic acid (SA) contributes to plant defenses against pathogens and abiotic stress (54-56). SA improves antioxidant enzyme activities in stressed plants (57). Prior studies show SA enhances drought tolerance by maintaining photosynthesis and antioxidant defenses (58, 59). In India, lentils thrive in rainfed agro-ecologies like rice fallows and face moisture stress during reproductive stages (60, 61). Providing growth regulators at key stages enhances nutrient uptake, flowering, photosynthesis, and seed quality (62-65). Thiourea, a nitrogen-sulfur compound, helps manage oxidative stress and meets nitrogen demands (66). Cobalt toxicity induces oxidative stress and negatively affects growth and metabolism in lentils (*Lens culinaris* L.); however, limited information is available on the oxidative stress responses of lentil under excess cobalt conditions. While plant growth regulators (PGRs) are known to enhance stress tolerance in plants, their role in alleviating cobalt-induced oxidative stress in lentil has not been sufficiently explored. Additionally, the effectiveness of PGR seed priming as a pre-sowing strategy against cobalt stress remains poorly understood. Hence, there is a clear research gap in understanding how PGR priming mitigates cobalt-induced oxidative damage in lentils. Thus, this study focuses on evaluating the comparative efficacy of PGRs molecules in alleviating cobalt toxicity and highlighting the best priming treatment ameliorating cobalt toxicity in lentil variety.

MATERIALS AND METHODS

A pot experiment was conducted in the Department of Botany UoB, Quetta using 1kg pot. Lentil seeds (variety black Panjgur) was primed for 12 hours with different PGRs i.e MLE (10%), SA (250µM), TU (10mM), & water soaked. Primed seeds was sown in soil at 1inch depth, after 15 days of seed germination cobalt stress was applied in comparison to control. After 1 week of cobalt stress plant was harvested for

various morphological and physiological attributes. The design of the experiment was CRD with three 3 replicates. Seeds of Lentil (*Lens culinaris* M.) was supplied by the (Directorate of Pulses) ARI, Quetta.

DETERMINATION OF MORPHOLOGICAL ATTRIBUTES

Growth parameters of lentil were recorded by measuring the Root and Shoot length, fresh and dry weight of both root and shoot. Number of leaf per plants, root and shoot length was recorded by use of measuring tape in cm and Fresh and dry weight recorded by using electric balance and dry weight was recorded after shoot/root dry weight (g) was taken after oven drying the samples at 65-75°C.

PHYSIO-BIOCHEMICAL ATTRIBUTES

DETERMINATION OF PHOTOSYNTHETIC PIGMENTS

Leaf photosynthetic pigments was extracted by taking 0.1g of leaf sample and grinded 1 to 2mL in 80% acetone and making final volume up 10mL by 80% acetone. Absorbance was measured at 663, 645nm against 80% acetone blank.

Formula of developed by Arnon (1949) was used for estimation of chlorophyll and represented as:
 $\mu\text{g of chl/g FWT.}$

$$\text{Chl a} = \{(1.27 \times A_{663} - 2.69 \times A_{645})\} \times V/1000 \times W$$

$$\text{Chl b} = \{(22.9 \times A_{645}) - 4.68 \times A_{663}\} \times V/1000 \times W$$

The method of Kirk and Allen, (1965), was used for carotenoid estimation and described in units of its absorbance as Acar 480 FWT/g.

$$A_{\text{car}} / 480 / \text{wt of leaf} = A_{480} + 0.114 \cdot A_{663} - 0.638 \times A_{645} / 2500 \times 1000$$

DETERMINATION OF ANTHOCYANIN

0.1 g of root and shoot sample was taken to determine the anthocyanin contents. Sample was extracted in 1-2mL of acidified methanol that was placed in water bath at 50°C for 1 hour. The absorbance was noted 535nm by spectrophotometer. The acidified methonal was used as blank (Stark and Wray, 1989).

DETERMINATION OF SOLUBLE SUGARS

Method of Yoshida *et al.*, (1976) followed to determine the Soluble Sugar Shoot and root about 0.1 g was boiled for one hour in 5 mL of distilled water. 1mL from the extract was content and diluted by adding 9mL distilled water, and 0.5mL from this extract was taken and 2.5mLof anthrone reagent was added. sample was place water bath for about 20 min at 90°C. Reading was noted at 620nm using spectrophotometer. Distilled water was used as a blank.

DETERMINATION OF SOLUBLE PHENOLICS

Method developed by Julkunen-titto, (1985) was used to determine soluble phenolics, plant sample of 0.1g was taken and grinded into 1-2 mL of 80% acetone. In a test tube take 1 mL distilled water followed by the addition of 100 μ L of the sample and 0.5 mL Folin phenol reagent, then 2.5mL of 20% Na₂CO₃ (Sodium carbonate) was added. Reading at 745nm was noted using spectrophotometer with 80% acetone as blank.

NUTRIENTS ANALYSIS

The analysis of sulfur was carried out to record the differences among treatments. Plant sample was firstly oven dried and then was digested in nitric acid.

STATISTICAL ANALYSIS

Recorded data were analyzed by the help of "STATISTIX 8.1" while graphs and mean, standard deviation was calculated using MS. EXCEL.

RESULTS

MORPHOLOGICAL ATTRIBUTES



Data regarding the root length of *Lens culinaris* under control and cobalt stress conditions revealed statistically non-significant results among different treatments. The largest root length was observed in treatment of MLE 10% control (6.83 cm) however the smallest root length observed in the treatment of TU 10mM Cobalt (3.66 cm) (Fig. 1A). Data regarding the shoot length of *Lens culinaris* under control and cobalt stress conditions revealed statistically significant results among different treatments. The largest shoot length was observed in treatment of water primed control (22.17 cm) however the smallest shoot length observed in the treatment of MLE 10% Cobalt (10.33 cm) (Fig. 1B). Data regarding the number of branches of *Lens culinaris* under cobalt stress revealed statistically non-significant results among different treatments. Maximum NOB was observed in treatment of SA (250 μ M) control (2.66 cm) however the minimum NOB observed in the treatment of MLE 10% Cobalt stress (1.6667cm) (Fig. 1C). Data regarding the number of leaves of *Lens culinaris* under stress revealed statistically significant results among different treatments. Maximum NOL was observed in treatment of SA (250 μ M) control (8.33 cm) however the minimum NOL observed in the treatment of MLE 10% Cobalt stress (3.66cm) (Fig. 1D). Data regarding the shoot fresh weight of *Lens culinaris* under stress revealed statistically significant results among different treatments. Maximum SFW was observed in treatment of MLE10% control (0.21 cm) however the minimum SFW observed in the treatment of MLE 10% Cobalt stress (0.09cm) (Fig. 1E).

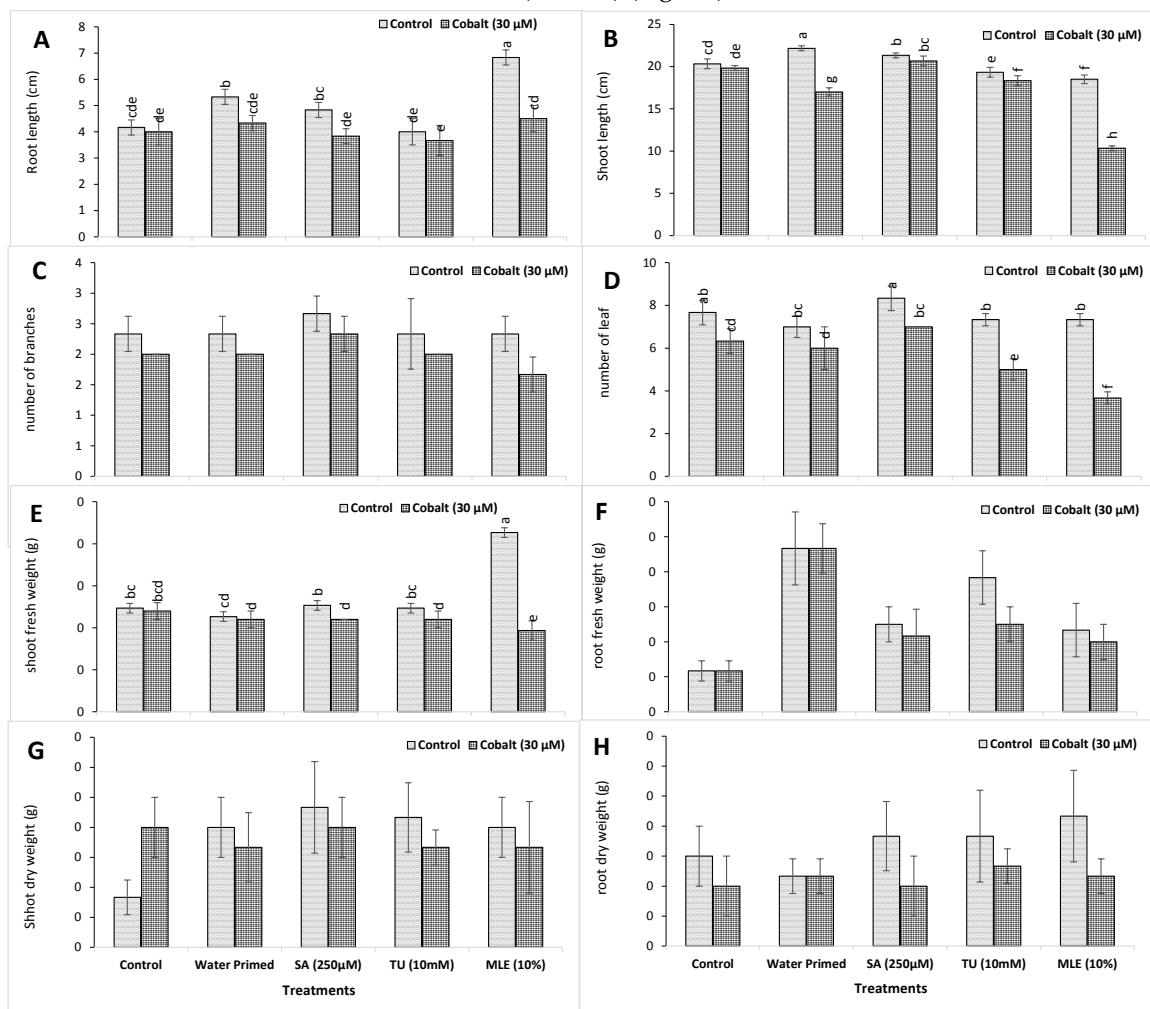


Fig. 1. Effect of PGRs priming on Growth parameter; (A) Shoot length; (B) Root length; (C) Number of Branches; (D) Number of leaves; (E) Shoot Fresh Weight; (F) Root Fresh Weight; (G) Shoot dry weight; (H) Root dry weight of *Lens culinaris* under Cobalt stress

Data regarding the shoot fresh weight of *Lens culinaris* under stress revealed statistically significant results among different treatments. Maximum RFW was observed in treatment of water primed control (0.09 cm) however the minimum RFW observed in the treatment of NFS Cobalt stress (0.02cm) (Fig. 1F). Data regarding the shoot dry weight of *Lens culinaris* under stress revealed statistically non-significant results among different treatments. Maximum SDW was observed in treatment of SA (250 μ M) – control (0.04 g) however the minimum SDW observed in the treatment of Control – Control (0.01g) (Fig. 1G). Data regarding

the root dry weight of *Lens culinaris* under stress revealed statistically non- significant results among different treatments. Maximum RDW was observed in treatment of MLE (10%) – Control (0.04 g) however the minimum RDW observed in the treatment of Control – cobalt (0.02g) (Fig. 1H).

PHOTOSYNTHETIC PIGMENTS

Data regarding the chlorophyll A of *Lens culinaris* under stress revealed statistically significant results among different treatments. Maximum CHL.A was observed in treatment of MLE10% cobalt (2.62 cm) however the minimum CHL.A observed in the treatment of SA (250 μ M) – Control (1.62cm) (Fig. 2A). Data regarding the chlorophyll B of *Lens culinaris* under stress revealed statistically significant results among different treatments. Maximum CHL.B was observed in treatment of control-control (2.18 cm) however the minimum CHL.B observed in the treatment of TU (10 mM) – Cobalt (1.05cm) (Fig. 2B). Data regarding the carotenoids of *Lens culinaris* under stress revealed statistically significant results among different treatments. Maximum carotenoid was observed in treatment of MLE (10%) – Cobalt (1.69 cm) however the minimum carotenoid observed in the treatment of Control – Cobalt (1.08cm) (Fig. 2C).

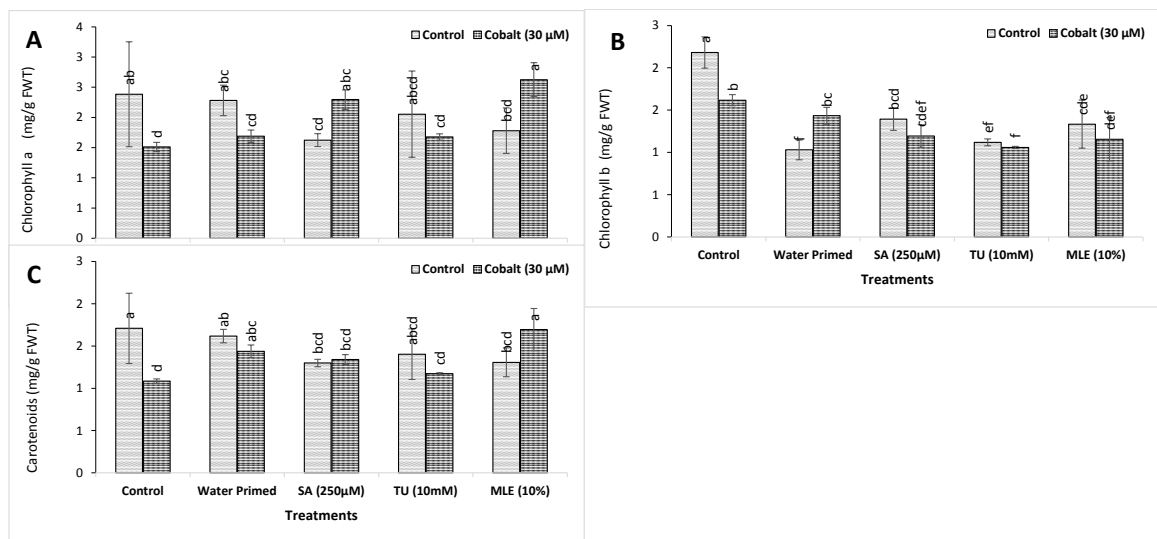


Fig. 2. Effect of PGRs priming on Photosynthetic Pigments; (A) Chlorophyll a; (B) Chlorophyll b; (C) Carotenoids of *Lens culinaris* under Cobalt stress

METABOLITES

Data regarding the shoot soluble phenolics (SSP) of *Lens culinaris* under stress revealed statistically significant results among different treatments. Maximum SSP was observed in treatment of MLE (10%) – control (0.33 cm) however the minimum SSP observed in the treatment of SA (250 μ M) – control (0.25cm) (Fig. 3A). Data regarding the root soluble phenolics (RSP) of *Lens culinaris* under stress revealed statistically significant results among different treatments. Maximum RSP was observed in treatment of MLE (10%) – control (0.33 cm) however the minimum RSP observed in the treatment of SA (250 μ M) – control (0.25cm) (Fig. 3B). Data regarding the root soluble sugar (RSS) of *Lens culinaris* under stress revealed statistically significant results among different treatments. Maximum RSS was observed in treatment of MLE (10%) – control (22.23 cm) however the minimum RSS observed in the treatment of Control – Control (11.64cm) (Fig. 3C). Data regarding the shoot soluble sugar (SSS) of *Lens culinaris* under stress revealed statistically non-significant results among different treatments. Maximum SSS was observed in treatment of SA (250 μ M) – Cobalt (19.9 cm) however the minimum SSS observed in the treatment of Control – Control (11.08cm) (Fig. 3D). Data regarding the anthocyanin (ANTH) shoot of *Lens culinaris* under stress revealed statistically non-significant results among different treatments. Maximum ANTH shoot was observed in treatment of MLE (10%) – Control (0.879 g) however the minimum ANTH shoot observed in the treatment of TU (10 mM) – Control (0.417g) (Fig. 3E). Data regarding the anthocyanin root of *Lens culinaris* under stress revealed statistically non- significant results among different treatments. Maximum ANTH root was observed in

treatment of MLE (10%) – cobalt (1.062 g) however the minimum ANTH root observed in the treatment of Control – Control (0.529g) (Fig. 3F).

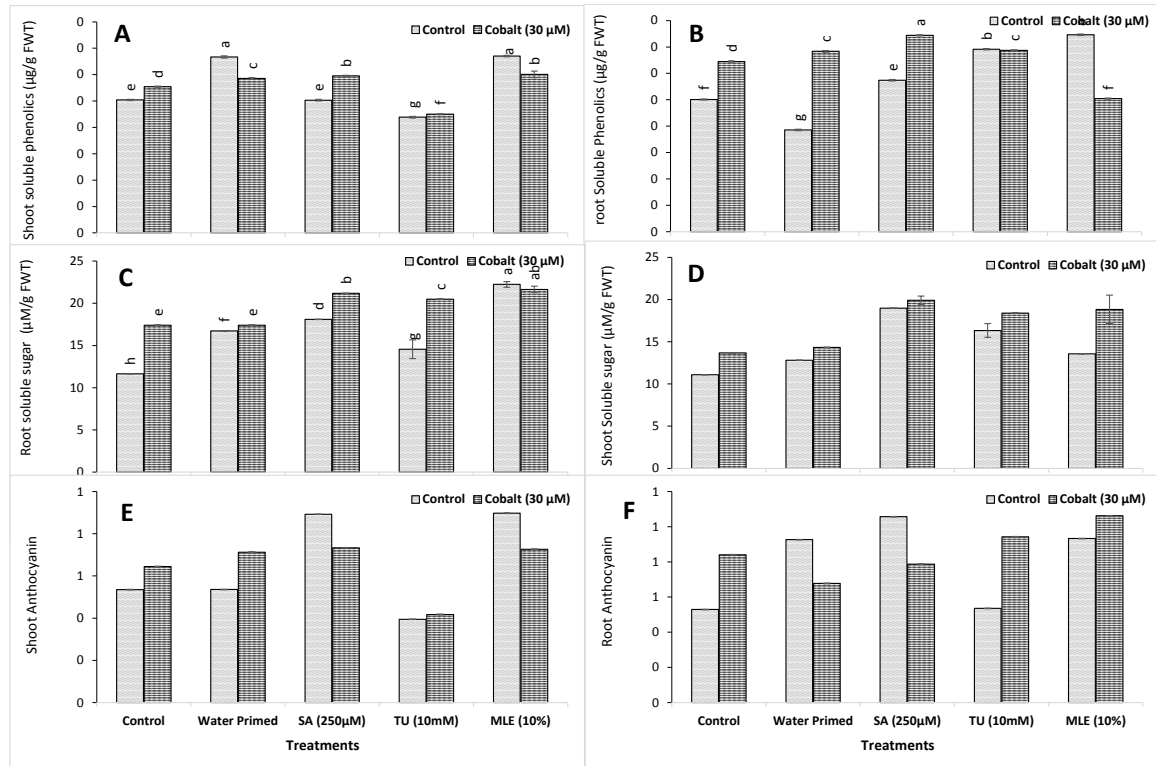


Fig. 3. Effect of PGRs priming on Metabolites; (A) Shoot soluble phenolics; (B) Root Soluble Phenolics; (C) Shoot Soluble Sugar; (D) Root Soluble Sugar; (E) Shoot Anthocyanin; (F) Root Anthocyanin under cobalt stress

NUTRIENTS

Data regarding the sulphate shoot conc of *Lens culinaris* under stress revealed statistically non-significant results among different treatments. Maximum sulphate shoot conc was observed in treatment of Control – Control (2.380 g) however the minimum sulphate shoot conc observed in the treatment of Water Primed – Control (1.841g) (Fig. 4A). Data regarding the sulphate root conc of *Lens culinaris* under stress revealed statistically non-significant results among different treatments. Maximum sulphate root conc was observed in treatment of SA (250 µM) – Cobalt (2.239 g) however the minimum sulphate root conc observed in the treatment of MLE (10%) – Control (1.573g) (Fig. 4B).

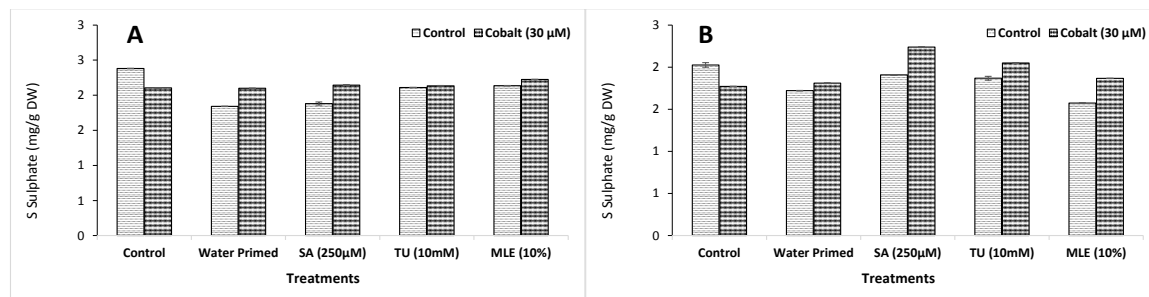


Fig. 4. Effect of PGRs priming on Sulphate; (A) Shoot sulphate; (B) Root Sulphate under cobalt stress

DISCUSSION

Cobalt (Co) though not categorized as an essential nutrient for plants has shown several positive effects on legume when present in trace amount. The current study demonstrated that cobalt (Co) stress negatively impacted the growth of *Lens culinaris*, as evident from reductions in root and shoot length, number of branches and leaves, shoot fresh weight, and root fresh weight (Reskin, 2023). Excess cobalt (Co) in the growth by generating reactive oxygen species (ROS), leading to lipid peroxidation protein and nucleic acid damage and disruption of cellular membrane (67). Cobalt toxicity further inhibits chlorophyll biosynthesis and damage chloroplast ultrastructure, thereby reducing photosynthetic efficiency and gas

exchange. However, seed priming with plant growth regulators (PGRs) such as salicylic acid (SA), thiourea (TU), and moringa leaf extract (MLE) showed ameliorative effect particularly with SA (250 μM) which improved shoot-related parameters under both control and stress condition. These findings are in line with (Zanganeh *et al.*, 2019) (68) on *Zea mays* under lead (Pb) stress which reported that Pb significantly reduced shoot and root length as well as root fresh weight while SA pretreatment alleviated these negative effect by enhancing shoot growth and biomass. Although the improvements in root parameters were less pronounced or statistically non-significant SA still conferred some protective effect. The similarity between the two studies (our and Zanganeh *et al.*, 2019) despite the difference in plant species and heavy metal type highlights SA's potential role in mitigating heavy metal stress through improved physiological responses and growth promotion. Therefore, SA-mediated seed priming may be considered an effective approach for enhancing plant tolerance to various heavy metal toxicities, including both Co and Pb.

The present study demonstrated that cobalt (Co) stress adversely affected both morphological and physiological traits of *Lens culinaris* including reduction in shoot and root lengths, number of leaves and branches, shoot and root fresh weight and photosynthetic pigments specially chlorophyll a, chlorophyll b and carotenoids declined under cobalt stress, though treatments with plant growth regulators (PGRs) such as salicylic acid (SA), thiourea (TU) and moringa leaf extract (MLE) alleviated these effects. Notably MLE (10%) under CO stress yielded the highest levels of chlorophyll a and carotenoids indicating enhanced protection of the photosynthetic machinery. These observations are consistent with (Zanganeh *et al.*, 2019) (68) on *Zea mays* under lead (Pb) stress, where Pb significantly reduced total chlorophyll content, but SA seed priming restored chlorophyll levels under stress conditions interestingly in both studies. SA did not significantly increase chlorophyll levels under control (non-stressed) conditions suggesting its specific role in activating defense responses only under heavy metal stress. Overall, the results indicate the PGRs, particularly SA and MLE, enhance pigments stability and photosynthetic performances in legumes like lentil and cereals like maize under heavy metal toxicity, highlighting their potential in mitigating abiotic stress-induced physiological decline.

In the present study, cobalt (Co) stress significantly influenced the biochemical profile of *Lens culinaris*, including shoot and root soluble phenolic (SSS, RSS). Co stress generally reduced these biochemical parameters, while seed priming with plant growth regulators (PGRs) such as moringa leaf extract (MLE), salicylic acid (SA) and thiourea (TU) alleviated the negative impacts. MLE (10%) treatment under both control and stress conditions consistently produced higher SSP, RSP and RSS levels, suggesting enhanced antioxidative potential and osmotic protection. SA (250 μM) also showed considerable improvement in SSS, especially under cobalt stress, indicating its role in osmotic balance and sugar metabolism. These results are in agreement with (Lwalaba *et al.*, 2019) (69) on barley under cobalt stress where Co increased proline levels while reducing antioxidants such as glutathione (GSH), protein thiols (PT) and ascorbic acid (AsA). Furthermore Panday *et al.*, 2015 (65) TU pretreatment in that study helped restore antioxidant levels and improve proline accumulation, suggesting mitigation of oxidative stress in Brassica, although the specific biochemical markers differ across studies—phenolics and sugars in *Lens culinaris* versus proline and antioxidant in Brassica—the underlying protective role of TU remains consistent along with SA. Additionally, both PGRs appear to regulate stress responses by modulating osmolyte content and antioxidant systems, enhancing resilience under heavy metal toxicity. However, MLE's strong performance in the current study may reflect its natural antioxidant and growth-promoting properties, making it a promising bio-stimulant under cobalt stress. These physiological disturbances collectively result in reduced biomass, impaired nutrient uptake and weakened stress tolerance. Plant growth regulators (PGRs) have been shown to alleviate heavy-metal-induced stress by enhancing antioxidant defense system, stabilizing photosynthetic pigments and maintaining osmotic balance (70). In particular brassinosteroids (BRs) have been reported to mitigate Co-induced toxicity in maize by improving enzymatic antioxidant activity and protecting photosynthetic parameters. Similarly salicylic acid (SA) plays a pivotal role in regulating plant defense mechanisms under abiotic stresses including heavy metal exposure by enhancing ROS scavenging and protecting chlorophyll content (68).

In the present study shoot and root sulphate concentration of Lentil under control and cobalt stress condition showed statistically non- significant difference among treatment yet clear trends were observed. Cobalt stress generally reduced sulphate accumulation in both shoot and roots; however, seed priming with plant growth regulators (PGRs) such as SA and enhanced sulphate content compared to unprimed stressed plants. These findings portray that sulphate assimilation can enhance plant tolerance to abiotic stress by supporting normal metabolic function maintaining antioxidant activity which is vital for stress tolerance. In present study however, a statistically non- significant result was observed in S level but improvements with PGR priming suggest that enhanced root-to-shoot S transport and metabolic integration may have occurred noting sulphate's role in modulating oxidative stress responses and interacting with other nutrients to mitigate toxicity.

CONCLUSION

The study demonstrated that cobalt stress negatively impacts various growth and physiological parameters of *Lens culinaris* including root length, shoot length number of branches, number of leaves fresh and dry biomass, chlorophyll content, carotenoids, anthocyanin levels and sulphate concentration. While the extent of reduction varied among traits cobalt exposure consistently caused growth inhibition compared to control condition. However, seed priming with plant growth regulators (PGRs) such as MLE (10%), SA (250µM), TU (10mM), and water priming significantly mitigated these adverse effects. In many causes primed seeds under cobalt stress outperformed the unprimed control under stress highlighting the potential of PGRs- based seed priming as an effective strategy to enhanced cobalt stress tolerance in lens culinaris. Overall, seed priming improved both morphological and biochemical attributes, suggesting its applicability for sustainable crop production in cobalt-contaminated soils.

Conflict of interest:

There is no conflict of interest in this study.

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Authors' contribution:

FK Performed the experiments and wrote initial draft; KS Supervised and analyzed the data, reviewed and edited the manuscript; AS Co-supervised, lab analysis and editing of manuscript TB Edited and revised the manuscript; MM Data analysis and revised the manuscript; TA Lab analysis and data handling; NA Lab analysis and graph generation; MS Lab analysis and graph generation; AA Revised and edited manuscript; TI Provided technical outputs and correspondence.

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