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Article

Bradyrhizobium sp. role in strengthening biochemical defense of trefoil under metal stress

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Abstract

This study explores the role of *Bradyrhizobium sp.* (*Lotus*) in enhancing the biochemical resilience of *Lotus ornithopodioïdes* during germination under metal stress conditions. Specifically, the effects of cadmium (Cd) and copper (Cu) on germination and various biochemical parameters were assessed. Seeds were inoculated with *Bradyrhizobium sp.* (*Lotus*) and subjected to different concentrations of trace metals. Key biochemical parameters, including proline, amino acids, total soluble sugars, #-amylase activity, and DNA content, were analyzed. The results demonstrated that inoculation with *Bradyrhizobium sp.* (*Lotus*) significantly mitigated the adverse effects of metal stress, leading to improved germination rates and enhanced biochemical responses. Notably, proline and amino acid levels were reduced, while #-amylase activity and DNA content increased in inoculated seeds compared to non-inoculated controls. These findings suggest that *Bradyrhizobium sp.* (*Lotus*) plays a vital role in promoting resilience in *Lotus ornithopodioïdes* under metal stress, highlighting its potential for use in bioremediation and sustainable agricultural practices.

Keywords:

biochemistry, cadmium, copper, germination, metal stress, PGPR, trefoil.

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Introduction

Heavy metal contamination of agricultural soils is a growing global concern, posing significant challenges to crop production and food security (Rizwan *et al* ., 2016). Toxic trace metals such as cadmium (Cd) and copper (Cu) can severely impair seed germination, plant growth and overall crop yield (Hossain *et al* ., 2012).

The rising presence of heavy and trace metals in agricultural systems calls for sustainable, eco-friendly strategies to reduce plant stress. One effective approach is the use of plant-microbiome interactions, particularly plant growth-promoting rhizobacteria (PGPR) (Etesami and Maheshwari, 2018).

Bradyrhizobium species are well-known PGPR that enhance growth, nutrient uptake, and stress tolerance in legumes, including mitigating metal stress (Ahemad and Kibret, 2014 ; Shameer and Prasad, 2018). However, the mechanisms by which *Bradyrhizobium sp.* (*Lotus*) modulates the biochemical responses of *Lotus ornithopodioïdes* L. during germination under metal stress remain poorly understood.

The research focuses on evaluating the impact of *Bradyrhizobium sp.* (*Lotus*) inoculation on key biochemical parameters, including proline, amino acids, total soluble sugars, #-amylase activity and DNA content, under varying concentrations of trace metals.

Material and methods

Bacterial strain

Bradyrhizobium sp. (*Lotus*) described by Degaichia *et al* . (2024) was used at a concentration of 10^8CFU ml^{-1}.

Inoculation and germination assay

The germination of the *Lotus ornithopodioïdes* L. seeds and the conduct of the test is carried out according to the work protocol of Degaichia *et al* . (2025) .

Inoculation and application of stress

Seed inoculation with *Bradyrhizobium sp.* (*Lotus*) was performed following Silini *et al* . (2016) . Seeds were germinated in Petri dishes (20 seeds per dish) on filter paper moistened with distilled water (control) or $CdCl_2$and $CuCl_2 2H_2O$ solutions (Degaichia *et al* ., 2025) (Table 1). Germination was conducted in the dark at 25 °C.

Table 1. Concentration (g L^{-1}) of copper and cadmium used (Degaichia *et al* ., 2025).

Cu(II)	Cd(II)	Cu(II):Cd(II)
1.5	2	1.5:2 …. I
2	3.5	2: 3.5…. II
3	6	3:6 …. III

After the third day of incubation, the analysis of the biochemical parameters was carried out (Degaichia *et al* ., 2025).

Dosage of Proline content

The proline content was determined by the method developed by Singh *et al* . (1973) . The absorbance was read at 520 nm. The results are reported on a proline standard curve.

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Dosage of soluble amino acids

The samples were stored below -15 °C before analysis. The extraction was carried out according to the method described by Naidu *et al* . (1998) . The results were expressed in µmoles of equivalents leucine per gram of fresh matter (µM equiv leucine g^{-1}FM).

Total soluble sugar content

The extraction of sugars is carried out according to Babu *et al* . (2002) . The determination of total soluble sugars was carried out using the Dubois method (Dubois *et al* ., 1956). The results of the optical densities were reported on the standard curve of soluble sugars (expressed as glucose).

α- amylase content

Alpha-amylase was measured according to the method of Xiao *et al* . (2007) . The results were expressed in enzymatic units per milliliter (U ml^{-1}), calculated according to the following formula:

$$\alpha\text{-amylase content}\left(U\text{ml}^{-1}\right)=\frac{\left(A_{580T}-A_{580E}\right)}{A_{580\text{ET}}\times T\times V_{\text{ext}}}$$

Where: A_{580T}= absorbance of starch without addition of the enzymatic extract; A_{580E}= absorbance of starch after addition of the enzymatic extract; A_{580ET}= absorbance for 1 mg of starch derived from the standard curve; T= incubation time in minutes; Vext= volume of the added enzymatic extract in milliliters.

DNA content

DNA was measured according to the technique used by Burton (1956) . The optical density reading of the samples was carried out at 600 nm (OD_{600}). The DNA content is determined according to the formula:

$$\text{DNA}\left(\mu\text{mol}g^{-1}\text{DM}\right)=\frac{\left(\text{OD}_{600}-0.015\right)}{0.0059862}$$

Statistical analysis

Statistical analyses were performed using SPSS software (version 20.0.0). A Manova test was conducted at a 5% significance level to evaluate the effect of bacterial inoculation on biochemical parameters under metal stress conditions.

Results

In all tables, Conc denotes TM concentration; N ino and Ino indicate non-inoculated and inoculated seeds with *Bradyrhizobium sp.* (*Lotus*), respectively. Values represent means. Different uppercase letters within rows and lowercase letters within columns indicate significant differences according to student’s t-test and Tukey’s test, respectively (p# 0.05).

Proline content

In the control, 0.97 µg g^{-1}FM of Proline content is recorded, this value increases significantly with the increase in the Cd(II) concentration and reaches 1.41 µg g^{-1}FM at 6 g $L^{-1}$of Cd(II). In a copper medium, the Proline content is positively correlated with the concentration, where a maximum of 1.55 µg g^{-1}FM is recorded for the concentration 3 g L^{-1}. The mixture of the two elements also generates significant increases compared to the control (Table 2).

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Table 2. Effect of *Bradyrhizobium sp.* (*Lotus*) inoculation on proline content.

	Conc (g L^{-1})	Proline (µg g^{-1}FM)	
		N ino	Ino
Control	0	0.97aB	0.24aA
Cu(II)	1.5	1.06bB	0.76eA
	2	1.52fB	0.82fA
	3	1.55fB	0.74dA
Cd(II)	2	1.35dB	0.56bA
	3.5	1.28cB	0.61cA
	6	1.41eB	0.94iA
Cu(II) + Cd(II)	I	1.35dB	0.88gA
	II	1.52fB	0.9hA
	III	1.39eB	0.96jA

Bacterial inoculation significantly reduces the proline level when comparing it with noninoculated tests. Indeed, in the control, a Proline content equal to 0.24 µg g^{-1}FM is recorded, this value is significantly low compared to the inoculated test (0.97 µg g^{-1}FM).

Amino acid content

Metal stress significantly increased amino acid (AA) content in treated samples. Without inoculation, AA levels more than doubled (200.66%) at 2 g L^{-1}Cd(II) compared to the control. Under Cu(II) stress, AA content increased proportionally with concentration, reaching 0.135, 0.185 and 0.19 µM equiv leucine g^{-1}FM at 1.5, 2 and 3 g L^{-1}, respectively. In contrast, in the combined metal treatment, AA content increased as metal concentrations decreased (Table 3).

Table 3. Effect of *Bradyrhizobium sp.* (*Lotus*) inoculation on amino acids content.

	Conc (g L^{-1})	Amino acids (µM equiv leucine g^{-1}FM)	
		N ino	Ino
Control	0	0.08aB	0.01aA
Cu(II)	1.5	0.14cB	0.05gA
	2	0.2fB	0.03fA
	3	0.19eB	0.07hA
Cd(II)	2	0.2fB	0.0243eA
	3.5	0.11bA	0.1iA
	6	0.17dB	0.12jA
Cu(II) + Cd(II)	I	0.22gB	0.018bA
	II	0.16dB	0.024cA
	III	0.08aB	0.024cA

Inoculation of seeds by *Bradyrhizobium sp.* (*Lotus*) reduces A.A contents significantly. In fact, we note a significant drop from 8.37 to 18.66% in AA content. for all concentrations compared to non-inoculated tests. We also note that the AA contents increase with the increase in the concentration of the medium in Cd(II) and associated TM.

Total soluble sugar content

In non-inoculated seeds, total soluble sugar content increased significantly in all treatments with individual metals and their mixtures compared to the control (3.74 µg g^{-1}FM). Sugar levels rose

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with increasing metal concentrations. In contrast, inoculation with *Bradyrhizobium sp.* (*Lotus*) significantly reduced total soluble sugar content compared to non-inoculated seeds (Table 4).

Table 4. Effect of *Bradyrhizobium sp.* (*Lotus*) inoculation on soluble sugars content.

	Conc (g L^{-1})	Soluble sugars (µg g^{-1}FM)	
		N ino	Ino
Control	0	3.77aA	4.08eA
Cu(II)	1.5	6.73hB	5.21gA
	2	8.16iB	3.6bA
	3	5.21dB	2.4aA
Cd(II)	2	5.69eB	3.97dA
	3.5	4.83cA	4.8fA
	6	4.16bB	4.08eA
Cu(II) + Cd(II)	I	6.7hB	5.32iA
	II	6.29gB	5.28hA
	III	6.22fB	3.71cA

α-amylase content

In the absence of bacterial inoculation, very low amylase activity is noted of the control (5.74 x 10^{-1}U ml^{-1}). The #-amylase content increases slightly with the increase in the concentration of Cd(II) and Cu(II) where the increase in the concentration of the mixture of the two elements causes a drop in #-amylase and reaches 2.55 x 10^{-5}U ml^{-1}(Table 5).

Table 5. Effect of *Bradyrhizobium sp.* (*Lotus*) inoculation on α-amylase content.

	Conc (g L^{-1})	#-amylase (10^{-5}U ml^{-1})	
		N ino	Ino
Control	0	5.74dA	61.1jB
Cu(II)	1.5	2.42aA	49.2hB
	2	2.44aA	11.4cB
	3	0.12gA	40.8gB
Cd(II)	2	6.72eA	51.3iB
	3.5	7.94fA	25.5fB
	6	0.15hA	18.4dB
Cu(II) + Cd(II)	I	12.2gA	24.9eB
	II	5.49cA	7.57bB
	III	2.55bB	0.97aA

Inoculation of trefoil seeds with *Bradyrhizobium sp.* (*Lotus*) significantly increased #-amylase activity compared to non-inoculated controls, which showed 61.1×10^{-1}U ml^{-1}. Amylase activity decreased progressively with increasing Cu(II) concentration, with the lowest activity observed in the Cu(II)+Cd(II) mixture treatments (0.97×10^{-1}U ml^{-1}at concentration III).

DNA content

Without bacterial pre-inoculation of the trefoil seeds, low DNA contents were noted in the treated tests compared to the control (26.35 µmol g^{-1}DM) except for concentration II of the medium containing the mixture Cu(II)+Cd(II) where the DNA content increases significantly by 58.56% (41.76 µmol g^{-1}DM) (Table 6).

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Table 6. Effect of *Bradyrhizobium sp.* (*Lotus*) inoculation on DNA content.

	Conc (g L^{-1})	DNA (µmol g^{-1}DM)	
		N ino	Ino
Control	0	26.41iB	20.88aA
Cu(II)	1.5	2.51bA	130.3hB
	2	8.52eA	58.3eB
	3	25.56hA	47.94cB
Cd(II)	2	2.34aA	151.35jB
	3.5	7.35cA	48.28dB
	6	7.52dA	126.46gB
Cu(II) + Cd(II)	I	16.04gA	112.43fB
	II	41.76jA	25.73bB
	III	12.70fA	133.47iB

In the absence of *Bradyrhizobium sp.* (*Lotus*), DNA content was notably low, with slight increases observed at higher TM concentrations. Inoculation with *Bradyrhizobium sp.* (*Lotus*) significantly enhanced DNA content across all metal treatments compared to the control (29.23 µmol g^{-1}DM). However, DNA levels in trefoil seeds decreased significantly with increasing concentrations of individual metals and their mixtures.

Discussion

Proline constitutes less than 5% of the total free amino acids, it is recognized as one of the most prevalent stress metabolites (Matysik *et al* ., 2002 ; Sharma and Dietz, 2006 ; Lei *et al* ., 2007). It plays multiple roles, including osmoregulation, chelation and detoxification of heavy metals, enzyme protection, cytosolic pH regulation, stabilization of protein synthesis machinery and scavenging of reactive oxygen species (ROS) (Sharmila *et al* ., 2002).

In our study, proline content increased under cadmium (Cd) exposure, consistent with reports that Cd induces proline synthesis in various plants, including radish cotyledons (Biteur, 2012). Similar proline accumulation has been observed in response to other heavy metals, such as Cd in peanuts (Anuradha and Rao, 2007) and Cu in rice and carrots (Chen *et al* ., 2004 ; Szafrariska *et al* ., 2011).

Our results indicate that Cd and Cu exposure significantly inhibited #-amylase activity, although treatment with a specific Cu/Cd mixture concentration stimulated the enzyme. These findings align with Mihoub *et al* . (2005) , who reported a marked decrease in amylolytic activity in the presence of these metals. The observed inhibition may result from direct interactions of Cd and Cu with #-amylase or its regulatory proteins, impairing carbohydrate mobilization during germination.

Additionally, DNA content in *Lotus ornithopodioïdes* L. cotyledons decreased under metal stress, indicating increased sensitivity to heavy metals. This observation aligns with previous studies reporting reduced DNA content in plants such as mustard and beans exposed to heavy metals under saline conditions (Chatterjee *et al* ., 1985 ; Bouzid, 2009). Lower DNA levels suggest impaired cellular development and protein synthesis, which are critical for plant growth and stress tolerance (Bouzid, 2009).

Inoculation with *Bradyrhizobium sp.* (*Lotus*) led to a significant reduction in total amino acids, proline and soluble sugars in the presence of copper or cadmium ions. This contrasts with previous findings by Silini *et al* . (2016) , which indicated an increase in osmoprotectants in wheat seeds after inoculation with Azotobacter under abiotic stress.

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Conclusion

The presence of cadmium (Cd) and copper (Cu) adversely affected germination and various biochemical parameters; however, the inoculated seeds exhibited improved performance compared to non-inoculated controls. Specifically, the inoculation led to a reduction in proline and amino acid levels, while promoting higher #-amylase activity and DNA content, indicating a more robust metabolic response to metal stress.

These findings suggest that *Bradyrhizobium sp.* (*Lotus*) plays a critical role in mitigating the negative effects of heavy metals on plant germination and growth. The mechanisms by which this bacterium enhances resilience may involve the modulation of osmoprotectants and enzymatic activities, which are essential for maintaining cellular homeostasis under stress conditions. Overall, this research highlights the potential of using *Bradyrhizobium sp.* (*Lotus*) as a biotechnological tool in sustainable agriculture to improve the resilience of crops in metal-contaminated environments.

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