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Enhancing Anthocyanin Accumulation in *Zea mays* under Cadmium Stress through Azotobacter sp. and Azospirillum sp.

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ABSTRACT

Cadmium (Cd) contamination poses a major constraint to maize (*Zea mays* L.) cultivation, impairing growth and inducing oxidative stress. Anthocyanins, as potent antioxidants, play a key role in mitigating Cd toxicity, yet their regulation under biofertilizer application remains poorly understood. This study investigated the influence of *Azotobacter* sp. and *Azospirillum* sp. biofertilization on anthocyanin accumulation in maize seedlings exposed to Cd stress. Seeds were germinated under $CdSO_4$ concentrations (2, 5, and 10 mM) with or without bacterial inoculation, and anthocyanin content was quantified in leaves and roots after 21 days. Cd stress alone triggered a progressive increase in leaf anthocyanins, while root levels remained low and unchanged. In contrast, co-application of Cd and biofertilizers reduced excessive foliar anthocyanin accumulation and promoted a modest but stable increase in root pigments, suggesting a more balanced distribution of secondary metabolites. These findings indicate that *Azotobacter* and *Azospirillum* alleviate Cd-induced oxidative stress by enhancing nutrient status and antioxidant defenses, thereby reducing the need for compensatory anthocyanin overproduction. The results highlight the potential of microbial biofertilizers as eco-friendly strategies to stabilize pigment metabolism, improve stress resilience, and sustain maize productivity under heavy metal contamination.

تعزيز تراكم الأنثوسيانين في نبات الذرة (Zea mays) تحت ضغط الكادميوم باستخدام أنواع Azospirillum و Azotobacter

بسمة ا.البركي منى عبدالقادر عمر

يشكل تلوث الكادميوم (Cd) عائقًا رئيسيًا أمام زراعة الذرة (Zea mays L)، حيث يعيق نموها ويؤدي إلى الإجهاد الأكسدي. وتلعب الأنثوسيانينات، باعتبارها مضادات أكسدة قوية، دورًا رئيسيًا في التخفيف من سمية Cd، إلا أن آلية عملها عند استخدام الأسمدة الحيوية لا تزال غير مفهومة جيدًا. بحثت هذه الدراسة تأثير كل من بكتيريا Azotobacter sp. و Azospirillum sp على تراكم الأنثوسيانين في شتلات الذرة المعرضة لإجهاد Cd. تم إنبات البذور تحت تركيزات $CdSO_4$ (2 و 5 و 10 مليمولار) مع أو بدون تلقيح بكتيري، و تم قياس محتوى الأنثوسيانين في الأوراق والجذور بعد 21 يومًا. أدى إجهاد الكادميوم بمفرده إلى زيادة تدريجية في أنثوسيانين الأوراق، في حين ظلت مستويات الجذور منخفضة ودون تغيير. في المقابل، أدى الاستخدام المشترك للكادميوم والأسمدة الحيوية إلى تقليل التراكم المفرط للأنثوسيانين في الأوراق وتعزيز زيادة متواضعة ولكن مستقرة في صبغات الجذور، مما يشير إلى توزيع أكثر توازناً للمستقلبات الثانوية. تشير هذه النتائج إلى أن Azotobacter و Azospirillum يخففان من الإجهاد التأكسدي الناجم عن Cd من خلال تحسين حالة المغذيات والدفاعات المضادة للأكسدة، مما يقلل من الحاجة إلى الإفراط في إنتاج الأنثوسيانين التعويضي. تسلط النتائج الضوء على إمكانات الأسمدة الحيوية الميكروبية كاستراتيجيات صديقة للبيئة لتثبيت استقلاب الصبغات، وتحسين القدرة على تحمل الإجهاد، والحفاظ على إنتاجية الذرة في ظل تلوث المعادن الثقيلة.

INTRODUCTION

Anthropogenic cadmium (Cd) contamination is an emerging constraint for maize (*Zea mays* L.) production, causing yield losses and quality deterioration in many regions. Cd disrupts photosynthesis, nutrient balance and cellular redox homeostasis, leading to growth inhibition and oxidative damage in maize seedlings and mature plants (Kaleem *et al.,* 2022; Javed *et al.,* 2017; Ekmekçi *et al.,* 2008; Saleem *et al.,* 2022; Anjum *et al.,* 2015). Developing eco-friendly strategies that both mitigate Cd toxicity and improve nutritional/functional quality of maize grains is therefore a key goal for sustainable agriculture.

Cadmium exposure decreases biomass, chlorophylls and anthocyanins in maize, while enhancing reactive oxygen species (ROS), membrane damage and lipid peroxidation (Kaleem *et al.,* 2022; Kaleem *et al.,* 2021; Anjum *et al.,* 2015). Nonetheless, increased anthocyanin accumulation can act as an antioxidant defense; in wheat, anthocyanins participate in protection against Cd by scavenging ROS and supporting stress tolerance (Shoeva & Khlestkina, 2018). This highlights anthocyanins as promising biochemical markers and targets in Cd-stress mitigation.

Free-living N-fixing *Azotobacter* spp. enhance crop nutrition, yield and stress tolerance through biological nitrogen fixation, phytohormone production, improved nutrient uptake, and heavy-metal binding by extracellular polymers and siderophores (Aasfar *et al.,* 2021; Sumbul *et al.,* 2020; Sagar *et al.,* 2022). Metal-tolerant *Azotobacter chroococcum* reduces oxidative damage and metal accumulation in maize under Cu and Pb, while improving growth and kernel traits (Rizvi & Khan, 2018). *Azospirillum* spp. similarly fix nitrogen and act as plant biostimulants, promoting root growth, nutrient acquisition, antioxidant activity, and tolerance to abiotic stresses, including heavy metals (Sun *et al.,* 2025; Fukami *et al.,* 2018; Raffi & Charyulu, 2020). The co-inoculation of Azotobacter and Azospirillum has been shown to enhance root development, water and mineral uptake, and biological nitrogen fixation. It has also been found to alleviate abiotic stresses, such as salinity, suggesting a strong synergistic potential as biofertilizers (Sumbul *et al.,* 2020; Prusty *et al.,* 2023).

In maize exposed to cadmium (Cd), a nitrogen biofertilizer containing *Azospirillum* and *Azotobacter* (Nitrobien) has been shown to improve biomass, photosynthetic pigments, nitrogen assimilation enzymes, amino acid levels and phytohormone levels, while reducing Cd-induced oxidative stress (Abou-Zeid, 2020). Similarly, cyanobacterial and bacterial biofertilizers improve maize growth and antioxidant capacity, while reducing Cd accumulation (Ismail *et al.,* 2020; Abou-Zeid, 2020). Since bacterial biostimulants can modulate antioxidant systems and secondary metabolism, including anthocyanins (Ismail *et al.,* 2020; Hussain *et al.,* 2024; Piao *et al.,* 2022; Sun *et al.,* 2025), *Azotobacter–Azospirillum* consortia could be an effective way to boost anthocyanin-based defense and quality traits in Cd-stressed maize. Cd stress severely impairs maize growth and induces oxidative damage, which is partly counteracted by anthocyanin accumulation and antioxidant defenses. Various factors, including calcium (Ca), silicon (Si), zinc oxide (ZnO) nanoparticles, humic acid and microbial biofertilisers, can alleviate Cd toxicity in maize. Combined use of these bacteria offers a biologically based approach to mitigating Cd toxicity, while potentially enhancing the anthocyanin content and functional quality of *Zea mays*.

MATERIALS AND METHODS

Source of seeds

The maize seeds (*Zea mays* L.) were supplied by the Agricultural Research Center in Giza, Egypt. A nitrogen biofertilizer comprising nitrogen (N)-fixing bacteria (*Azotobacter* sp. and *Azospirillum* sp.) was provided by the Biofertilizers Unit of the General Organization for Agriculture Equalization Fund, Agricultural Research Centre, Ministry of Agriculture, Giza, Egypt.

Seed preparation for sprouting

To determine the sub-lethal cadmium concentration that affects seed germination, a preliminary test was performed. Twenty grains were distributed in Petri dishes measuring 15 cm in diameter, which were lined with filter paper and soaked with 20 ml of CdSO4 solutions at concentrations of 0, 2, 5, 10, and 15 mM. The containers were covered with lids and kept under natural environmental conditions for a week (photoperiod: 16 hours of light and 8 hours of darkness, with the temperature being... The temperature was kept at 31/28 ± 2 °C in a 12-hour light/dark cycle, with a light intensity of PPFD 23 µmol m^2 s^{-1}. The evaluation of germination rate was determined by the appearance of the radicle. The findings showed that 15 mM CdSO4 was fatal, leading to the selection of 2, 5, and 10 mM for further experiments. Before use, grains underwent a standardisation process that included size, shape, and color. Before the commencement of the germination process, the seeds underwent a surface sterilization treatment. This required immersion in a 4% (v/v) sodium hypochlorite solution for two minutes. This was succeeded by multiple rinses with distilled water, intended to thoroughly remove any residual chlorine.

Treatments

Seeds that had been sterilized were sown in plastic pots (12 cm wide, 17 cm tall, with drainage holes) filled with a standardized blend of acid-washed quartz sand and clay in a 3:1 proportion. Every pot contained twenty seeds and was kept in the environmental conditions outlined earlier. Soil moisture was maintained at 80% of its water-holding capacity during the entire experiment. The pots were organized into two treatment categories, with each category containing three pots. The initial group received irrigation with $CdSO_4$ solutions at concentrations of 2, 5, and 10 mM, while the second group had grains inoculated with *Azotobacter* sp. and *Azospirillum* sp. biofertilizers and was irrigated with a one-tenth strength modified Hoagland solution enriched with 2, 5, and 10 mM $CdSO_4$. Irrigation using the corresponding treatment solutions occurred every two days. Following 21 days, the plants were collected, thoroughly washed to eliminate sticking soil particles, meticulously separated into roots and leaves, and promptly preserved for future growth evaluations and chemical analysis.

Measurement of Anthocyanin

To determine the anthocyanin content, 0.1 g of powdered plant material was extracted using 5 ml of methanol with 1% HCl and was left to sit for 24 hours. The substance was subsequently homogenized, and the extraction volume was set to 10 ml. After centrifugation at 12,000 g for 10 minutes, the absorbance of the supernatant was measured at 530 and 657 nm. The concentration of anthocyanins in the leaves and roots (3 replicate for each in all pots) was then determined using the formula: (A_{530} – 0.33 × A_{657}), as outlined by Mancinelli *et al.,* (1975).

Analysis of Statistics

One-way ANOVA statistical analysis from SPSS (version 25 software) was utilized to determine the significance of variance at $P<0.05$ regarding anthocyanin concentration in the leaves and roots of *Zea mays* across the two treatments.

RESULTS AND DISCUSSION

The results indicate that cadmium stress alone markedly increased anthocyanin accumulation in maize leaves, with concentrations rising progressively from control to 10 mM Cd, while root levels remained comparatively low and statistically unchanged. In contrast, the combined treatment with cadmium and nitrogen fixing biofertilizers (*Azotobacter* sp. and *Azospirillum* sp.) moderated this response: leaf anthocyanin levels were consistently lower than under cadmium alone, and root concentrations showed slight but stable increases. This pattern suggests that biofertilizer application alleviated cadmium-induced oxidative stress by reducing excessive anthocyanin buildup in leaves, while promoting a more balanced distribution of pigments between leaves and roots. Overall, the data support the role of Azotobacter and Azospirillum as biofertilizers in mitigating cadmium toxicity and stabilizing secondary metabolite production in Zea mays.

Table 1: Anthocyanin concentration (µmol/g) in the two treatments.

	Treatment by Cadmium		Treatment by Cadmium & Biofertilizer	
	Leaves	Roots	Leaves	Roots
Control	0.256± 0.20**ab**	0.012±0.00**a**	0.099±0.01 **a**	0.113±0.01**a**
2mM	0.828±0.05**bc**	0.039±0.01**a**	0.388±0.05**ab**	0.021±0.01**a**
5mM	1.216±0.64**c**	0.036±0.02**a**	0.428±0.14**ab**	0.032±0.00**a**
10mM	2.407±0.32**d**	0.064±0.03**a**	0.464±0.03**ab**	0.043±0.00**a**

The observed increase in anthocyanin accumulation in maize leaves under cadmium (Cd) stress aligns well with the established role of anthocyanins as key secondary metabolites involved in plant defense against abiotic stresses, including heavy metal toxicity. Cd exposure can induce oxidative stress in plants by generating reactive oxygen species (ROS), which leads to lipid peroxidation, protein oxidation, and disruption of cellular homeostasis. In response, plants often upregulate antioxidant compounds such as anthocyanins, which have potent ROS-scavenging properties, to mitigate oxidative damage and maintain cellular integrity. The progressive rise in leaf anthocyanin content with increasing Cd concentration, as found in your results, suggests an active defense mechanism triggered by Cd-induced oxidative stress specifically in the photosynthetically active aerial tissues. This pattern is consistent with prior findings that Cd toxicity leads to enhanced oxidative stress and related antioxidant responses in maize leaves (Anjum *et al.,* 2015; Malčovská *et al.,* 2014).

Notably, the relatively low and unchanged anthocyanin levels observed in roots, even under increasing Cd exposure, may reflect tissue-specific differences in stress perception or metabolic capacity (Fang *et al.,* 2025). Roots are the initial site of Cd uptake and accumulation and are known to accumulate Cd more intensely than shoots; however, they may rely on other detoxification mechanisms such as increased glutathione and phytochelatin synthesis to chelate and sequester Cd ions, as indicated in previous studies (Cobbett, 2000; Wahid *et al.,* 2009; Gupta *et al,* 2013). The absence of significant anthocyanin accumulation in roots might also be due to constraints in biosynthetic pathways or the allocation of metabolic resources toward maintaining root viability under stress.

The mitigating effect of nitrogen-fixing biofertilizers *Azotobacter* sp. and *Azospirillum* sp. on anthocyanin accumulation under combined Cd and biofertilizer treatments provides important insights into the mechanisms of biofertilizer-mediated stress alleviation in maize. *Azotobacter* and *Azospirillum* are well-documented plant growth-promoting rhizobacteria (PGPR) that enhance nutrient availability (notably nitrogen), improve plant physiological activities, and elevate antioxidant enzyme activities under abiotic stresses, thereby enhancing plant resilience (Al-Turki *et al.*, 2023). The observed reduction of leaf anthocyanin levels in the combined treatment suggests that biofertilizer inoculation may reduce the intensity of Cd-induced oxidative stress in leaves, possibly by enhancing the overall antioxidant defense system and nutrient status, which attenuates the need for elevated anthocyanin synthesis as a compensatory response.

Moreover, the slight but stable increase in root anthocyanin concentrations upon biofertilizer co-application hints at a more balanced distribution or modulation of secondary metabolites between above- and below-ground tissues. This dynamic allocation could reflect improved root health and metabolic activity facilitated by *Azotobacter* and *Azospirillum*, which can fix atmospheric nitrogen and produce phytohormones, thereby promoting root growth and function even under heavy metal stress (Aasfar *et al.*, 2021). The enhanced root biochemical status may provide a buffering capacity against Cd toxicity, reducing excessive oxidative damage in leaves and resulting in lower foliar anthocyanin accumulation.

These findings resonate with reports that microbial inoculants can stimulate antioxidant mechanisms, including enzymatic antioxidants like superoxide dismutase, catalase, and peroxidases, which collectively detoxify ROS more effectively than reliance solely on non-enzymatic antioxidants such as anthocyanins (Anjum *et al.*, 2015). By complementing plant metabolism, biofertilizers hence contribute to maintaining redox homeostasis without necessitating over-accumulation of anthocyanins, which can be metabolically costly.

Additionally, the stabilization of secondary metabolite production observed indicates that biofertilizer application helps maintain metabolic equilibrium under Cd stress, preventing potentially deleterious overproduction of anthocyanins that could otherwise interfere with normal growth and photosynthetic processes. This highlights the potential of Azotobacter and Azospirillum as biofertilizers in sustainable agriculture to alleviate heavy metal stress, not only by enhancing nutrient uptake and growth but also by modulating stress-induced secondary metabolite pathways.

Collectively, these results emphasize the multifaceted role of biofertilizers in mitigating Cd toxicity. They appear to shift the plant's defense strategy from an overreliance on anthocyanin accumulation to a more integrated antioxidant network, supporting both shoot and root health. Such modulation aligns with previous observations where amendments such as silicon, chitosan, humic acid, similarly improved antioxidant enzyme activities and reduced ROS accumulation, leading to stabilization of phenolic and pigment compounds in maize under Cd stress (Malčovská *et al.*, 2014; Rao & Zheng, 2025; Shehzadi *et al.*, 2025; Song *et al.*, 2025).

CONCLUSION

The data support a model whereby cadmium-induced oxidative stress triggers increased anthocyanin synthesis in maize leaves as a protective response. However, when maize is inoculated with biofertilizers such as *Azotobacter* and *Azospirillum*, the resultant improvement in antioxidant defenses and nutrient status alleviates oxidative stress, reducing the necessity for excessive anthocyanin accumulation. This leads to a more stable and balanced pigment distribution between leaves and roots, which likely contributes to improved plant tolerance and growth under cadmium toxicity. These insights reinforce the potential application of these biofertilizers in managing heavy metal stress in maize cultivation and underscore the complex interplay between microbial inoculants and plant secondary metabolism under abiotic stress conditions.

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