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# Physiological and anatomical responses of *Helianthus annuus* and *Amaranthus tricolor* stressed by Cd under plant growth promoting rhizobacteria (PGPR) system

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## ABSTRACT

Using *Helianthus annuus* and *Amaranthus tricolor* grown in a hydroponic culture experiment, the aim of this study was to determine the ability of *Bacillus halotolerance* DSM 8802 (as Cadmium (Cd)-resistant bacteria isolate) to enhance Cd phytoextraction at Cd stress concentrations (30, 40, and 50 mg/L), and the interaction between them through physiology parameters such as enzyme activities, anthocyanin and proline contents, and structural anatomy of the plants used. According to our findings, at 50 mg/L only, the maximum activities of *A. tricolor*'s 84.45% and 250.19% catalase (CAT) and peroxidase (POX) were 614.73% and 155.44% higher in *H. annuus*, respectively. The inoculated *A. tricolor* also displayed the highest relative activity of polyphenol oxidase (PPO), at 50 mg/L. *B. halotoleranc* inoculum increases the tolerance index (TI) for anthocyanin concentration by 200.00 at 50 mg/L in *H. annuus*. After *B. halotolerance* inoculum, *H. annuus*'s proline TI was 106.03%, greater than *A. tricolor*'s 73.89% at 40 mg/L. This study showed that *B. halotolerance* DSM 8802 enhanced against Cd toxicity and succeeded in boosting the phytoremediation combination with plant growth promoting rhizobacteria (PGPB) system. *H. annuus* and *A. tricolor* are effective accumulator models in phytoremediation. Applying the bacterial strain improved certain stem features, particularly in *H. annuus* from *A. tricolor*, when compared to Cd concentration in the absence of bacterial inoculum.

**Keywords:** Cadmium, Phytoremediation, PGPR, Antioxidants, Anthocyanin, Proline.

## INTRODUCTION

Even at low concentrations, Cd can be harmful to plants, affecting them morphologically, physiologically, biochemically, and molecularly (Rizwan *et al*., 2017). This is particularly the case for plants that are under elevated oxidative stress, which is the overproduction of reactive oxygen species (ROS) (Moradi *et al*., 2019). In polluted soils, plants have a range of defense strategies and mechanisms to deal with the consequences of Cd stress. For instance, by developing morphological and physiological resistance systems, they can flourish in poisoned soils (Mei *et al*., 2018). Plants are frequently employed in Cd phytoremediation to absorb or translocate Cd into portions of the plant that can be harvested. In order to continue growing normally even in highly contaminated soils, plants have developed a wide range of adaptations, including detoxification systems. The trend in Cd content in plant parts is as follows: roots, stems, and leaves (Ahmadpour and Soleimani, 2015). Plant growth promoting rhizobacteria (PGPR), one type of biofertilizer, is crucial for increasing production. Plants that are subjected to a variety of abiotic stressors, including as drought, salt, cold, and heavy metal toxicity, inhibited by PGPR. By enhancing root and shoot growth, nutrient

absorption, chlorophyll content, vigor, and yield under abiotic stressors, PGPR helps plants develop osmotolerance. According to Subiramani *et al*. (2020), PGPR release acids, proteins, phytoantibiotics, and other chemical substances that help plants become resistant to hazardous heavy metal stress. In oilseed, legume, and cereal crops, transpiration was found to have a linear relationship with photosynthetic inhibition; this suggests that Cd buildup in leaves prevents stomata from opening (Zhang *et al*., 2019). Toxicity of Cd injures the photosynthetic apparatus, especially the light-harvesting complex and photosystems I and II (Hasan *et al*., 2009). Cd inhibits the photosynthetic rate; exposure to Cd caused chlorosis of leaf which is most common visual symptom of Cd toxicity (Hsu and Kao, 2007). By substituting the magnesium ion from the chlorophyll molecule (Kupper *et al*., 2007), which causes chlorophyll degradation (Otero *et al*., 2006), or by interacting with the sulfhydryll (-SH) group of the enzymes involved in the synthesis of chlorophyll, such as amino levulinic dehydratase, Cd reduces the amount of chlorophyll present in the chlorophyll (Touiserkani and Haddad, 2012).

Plants' capacity react to biotic and abiotic environmental stresses is largely dependent on flavonoids. In particular, plants are protected from reactive oxygen species (ROS) produced in response to various abiotic stresses by the powerful antioxidant qualities of anthocyanins (Lee *et al*., 2016). Furthermore, studies have demonstrated that anthocyanin content rises in response to heavy metal exposure (Chen *et al*., 2015). It has been documented that anthocyanin-enriched plants improve antioxidant activity and stress tolerance in a variety of plant species (Ai *et al*., 2018). Plants exposed to metals increased their anthocyanin content overall in their stems and leaves (Krupa *et al*., 1996), which strengthened the plants' defenses against heavy metal pollution (Gould, 2004).

Research has indicated that an excess of Cd leads to an overproduction of ROS in cells, including OH, $O_2$, and $H_2O_2$ (Asad *et al*., 2019). Ascorbate peroxidase (APX), CAT, POD, and PPO activities are all increased when free radicals are produced (Pandey and Dubey, 2019). According to Yilmaz and Parlak (2011), elevated CAT activity contributed observed high tolerance to Cd stress. Under Cd stress, its activity decreased in soybean, *Phragmites australis*, *Capsicum annuum*, and Arabidopsis roots and rose in rice, mustard, wheat, chickpea, and black bean (*Vigna unguiculata* subsp. cylindrica) roots (Gill and Tuteja, 2010). It has been shown that Cd causes a decrease in POD activity in mustard (*Brassica juncea*) (Markovska *et al*., 2009). In radish (*Raphanus sativus L*.), Cd stress boosts POD activity (El-Beltagi *et al*., 2010). The activities of POD and CAT varied according to the plant species and concentration of Cd. Numerous plants treated with Cd, including peas (Sandalio *et a*l., 2001) and wheat (Milone *et al*., 2003), have been shown to have increased superoxidase (SOD) activity. *Phaseolus vulgaris*, *Helianthus annuus*, and *Pisum sativum* have all been linked to Cd toxicity due to a decline in the enzymatic activity of CAT and SOD (El-nabarawy et a*l.*, 2017).

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According to Kishor and Sreenivasulu (2014), plants have the ability to synthesize proline both in the presence and absence of abiotic stressors such exposure to metals. Proline content has been shown to rise in response to Cd exposure in a variety of plant species, including cucumber (Sun *et al*., 2007), cucumber (Semida *et al*., 2018), hackberry, cucumber (Semida *et al*., 2018), bean (Rady *et al*., 2019) and (*Celtis australis*) (Hatamian *et al*., 2020). The latter authors

showed that applying exogenous proline increases the content of proline, decreases oxidative stress markers, and significantly lowers the amount of Cd in olive roots and leaves. However, they also showed an increase in gas exchange parameters, photosynthetic pigment content, and macronutrients (Ca, Mg, and K). In addition, proline plays a protective role by acting as a source of C and N, forming a nontoxic Cd-proline complex, and stimulating the antioxidant system (Zouari *et al*., 2016). Al-Saadi *et al.* (2013) discovered alterations in the aquatic plant *Potamogeton sp*.'s cortical parenchyma and air space stems, and they came to the conclusion that these changes were caused by metal bind-ing to the cell wall and the intercellular space, It illustrates how marsh plants' ability to withstand metals interacts with their ability to retain oxygen in the stem's structure.

This suggests that *Bacillus altitudinis* SrN9 may have a role in phytoremediation of Cd without affecting plant productivity. Similarly, following inoculation with the bacterial strain *Rhodococcus erythropolis* NSX2, increased Cd absorption was noted in the roots of the Sedum plumbizincicolaa plant (Liu *et al*., 2015). Numerous researches have demonstrated how PGRB aids in the phytoremediation of hazardous metals, such as Cd. Plant roots can receive vital nutrients (such as N, P, and K) through bioinoculation with PGPR, which can also speed up germination and boost biomass content (Manoj *et al*., 2021). Additionally, they aid in the synthesis of hormones like auxin, which is produced when PGPR uses tryptophan derived from roots in the rhizospheric region to make indole-3-acetic acid. gibberellins, 1. Aminocyclopropane plant ethylene precursor ACC is broken down by 1-carboxylate (ACC) deaminase into ammonia and ketobutyrate. (Honma and Shimomura, 1978). Indole-3-acetic acid (IAA) is important in plant–microbe relations, especially between plants and rhizobacteria which stimulate plant growth through extensive root systems and protect the plant against abiotic stress (Liu *et al*., 2013). To decrease the negative effects of Cd, *Arthrobacter sp*. SrN1 and *Bacillus altitudinis* SrN9 were given to rapeseed. The inoculation of *Arthrobacter sp*. SrN1 and *Bacillus altitudinis SrN9* boosted Cd absorption and translocation in addition to boosting rapeseed's tolerance to Cd stress. (Pan *et al.,* 2017).

*Pseudomonas aeruginosa* has proven to be resistant to both biotic and abiotic stress. Under alkaline and high salt conditions, *Pseudomonas putida* RS-198 enhanced the rate of $k^+$, $Mg^{2+}$, and $Ca^{2+}$ uptake and lowered the absorption of $Na^+$. This enhanced the germination rate and several growth metrics, such as plant height, fresh weight, and dry weight of cotton. When applied to basal plants under abiotic stress, *Pseudomonas Spp*. increases the amount of antioxidant and photosynthetic pigment in the plants and has a good impact on the growth of seedlings (Liddycoat *et al.*, 2009). There are multiple plant species in the *Amarantheceae* family, which includes the basic genus Amaranthus (Li *et al*., 2012). Even though several researches have demonstrated that bacteria resistant to heavy metals may enhance plants' ability to absorb metals (Chen *et al*., 2015). Certain Amaranthus species satisfy the requirements to be categorized as Cd hyperaccumulators when they collect more than 100 mg Cd $kg^{-1}$ in aboveground dry matter (Reeves and Baker, 2000). Microbe-assisted phytoextraction in heavy metal-contaminated soils by Amaranthus species has received little attention. Sunflower (*Helianthus annuus*) plant belongs *Asteraceae* family, it's a fast-growing high biomass crop (Zhuang *et al*., 2007). According to Turgut *et al*. (2004), it is one of the plants that has been investigated the most for heavy metal phytoremediation. In addition to several other metals, Sunflowers can accumulate substantial amounts of Cd (Liphadzi *et al*., 2006). There are numerous findings on the buildup of Cd in Sunflower (De Maria *et al*., 2013). Nevertheless, there haven't been many attempts to use PGPR to help *H. annuus* plants in Cd-contaminated media with phytoextraction and Cd uptake. So, the aim of this study was to determine the ability of *Bacillus halotolerance* DSM 8802 (as Cadmium (Cd)-resistant bacteria isolate) to enhance

Cd phytoextraction at Cd stress concentrations by *Helianthus annuus* and *Amaranthus tricolor* grown in a hydroponic culture experiment.

## MATERIALS AND METHODS

**Experimental design:** The study was conducted in the Agriculture Faculty's Plant Physiology Lab at Al-Azhar University in Cairo, Egypt. According to El-Abyad *et al.* (1993) surface-sterilized *A. tricolor and H. annuus* seeds. Then, according to Hoagland and Arnon (1950), seeds have germinated in a hydroponic system using a half-strong Hoagland's solution. uniform in length, robust, and healthy seedlings were selected at random in triplicate after 25 days of germination, and they were kept in agriculture plates to treat them with Cd (as $CdCl_2$. $H_2O$) concentrations and bacterial inoculum at eight different treatments included in three sets of replications: (1) control without Cd and bacterial inoculum, (2) present bacterial inoculum only and absence of Cd, (3) Cd with 30 mg/l concentration, (4) Cd with 40 mg/l concentration, and (5) 50 mg/l concentration of Cd; (6) 30 mg/l concentration of bacterial inoculum; (7) 40 mg/l concentration of bacterial inoculum; and (8) 50 mg/l concentration of bacterial inoculum. Next, for 27 days, 2 mL of *B. halotolrance*'s bacterial suspension was applied to each nutritional solution plate (6, 7, and 8 treatments). Two controls are included in the study's experiment: a positive control that contains Cd in any concentration and a negative control that does not include any bacterial inoculum. Weekly nutrition solution renewal occurred. Following a 27-day period of exposure to both bacterial inoculum and Cd, the seedlings were plucked and thoroughly rinsed with distilled water to eliminate any remaining Cd from their roots. Subsequently, physiological and biochemical parameters were assessed.

**Enzyme extraction**: Fresh leaf samples weighing 0.2 g were ground into powder in liquid $N_2$, and 4 ml of a homogenizing solution containing 1% (w/v) polyvinylpyrrolidone (pH 7.8) and 50 mM potassium phosphate buffer was homogenized in an ice bath. The homogenate was centrifuged for ten minutes at 4°C and 14,000 rpm. Enzyme tests were conducted using the leftover supernatant. To take the enzymes' activity into consideration, the cuvette is placed in the spectrophotometer, and readings are taken at the wavelength at specific time intervals (0 time - 60 seconds - one minute - two minutes). Subtracting the change in the activity values for each time from zero time of the activity at the moment (ΔA/min) that the enzyme recorded its peak activity.Aebi (1984) reported that the CAT (EC: 1.11.1.6) action was precise, Chance and Maehly (1955) established the POD (EC: 1.11.1.7) activity approach, and Duckworth and Coleman (1970) determined the PPO (EC: 1.10.3.1) activity.

**Anthocyanin content** was estimated using the method proposed by Alberto and Owen (1984).
**Proline** (μ moles /g FW) levels were established in accordance with Bates *et al*. (1973).
**For anatomical structures**, using a rotating microtome, sections were cut and stained with safranin and light green. Subsequently, the colored slices were mounted on slides using Canada balsam as a permanent preparation (Nassar and El-Sahhar, 1998). With a Carl Zeiss Jena microscope and a Nikon camera, all photos were taken. A stage micrometer was used for calibration, and the objective lens magnification was set to 20x (5μm = 100μm ÷20 {Magnified Pitch Width 10mm/100 divisions/pitch 0.1mm}). Following 52 days of germination under research circumstances, 0.2g dry weight of plant martials were removed and cleaned in distilled water. Representative sections were then wet digested using a modified method of (Kaiser *et.al*, 1972) in order to determine the amount of Cd in different tissues of plants.
**The concentration of Cd:** Shakoor *et al.* (2014) employed the following approach to measure the concentration of Cd in plant roots, stems, and leaves. The modified method of Kaiser *et al*. (1972) was used to measure the content of Cd (mg/g. DW) in the root and shoot of *H. annuus* and *A. tricolor*. The solution was then aspirated to an Atomic Absorption spectrophotometer AAS (Perkin Elmer-2280), using an air/acetylene flame for Cd estimation. The concentration of

Cd in plant roots, stems, and leaves was measured using the method described below: Cd concentration (mg/g. DW) = reading of AAS x (dilution factor/DW of plant organ) according to Shakoor *et al.* (2014).

The results of Cd concentration were shown as table 1.

Table 1: Cadmium concentration (mg/g. DW) of *H. annuus* and *A. tricolor* plants.

| Characteristics / Treatments | Cd concentration (mg/g. DW) | | | |
|---|---|---|---|---|
| | Root | | shoot | |
| | *H. annuus* | *A. tricolor* | *H. annuus* | *A. tricolor* |
| Cont. | 141.21 | 194.00 | 132.40 | 97.00 |
| 30 mg Cd | 667.54 | 213.00 | 310.17 | 209.25 |
| 40 mg Cd | 928.30 | 237.00 | 505.92 | 223.25 |
| 50 mg Cd | 1196.20 | 242.50 | 583.82 | 255.50 |
| 30 mg Cd + Inocu. | 683.10 | 216.75 | 302.17 | 231.50 |
| 40 mg Cd + Inocu. | 1007.70 | 304.75 | 497.97 | 253.50 |
| 50 mg Cd +Inocu. | 1336.90 | 319.00 | 574.31 | 266.00 |

Where: Cont: control – Inocu: *Bacillus halotolerance* inoculum- 30 mg/l Cd – 40 mg/l Cd – 50 mg/l Cd - 30 mg/l Cd + *Bacillus halotolerance* inoculum - 40 mg/l Cd + *Bacillus halotolerance* inoculum - 50 mg/l Cd + *Bacillus halotolerance* inoculum, *H. annuus Helianthus annuus, A. tricolor: Amaranthus tricolor*.

With few adjustments, Wilkins' (1978) method of calculating Cd tolerance indexes (TI) of plant roots—which show the resistance of the plants to the heavy metal stress—was used.

**Statistical analysis:** The statistical tool SPSS for Window (v 23.0; IBM Corporation, Armonk, NY, USA) (IBM Corp., 2015) was used to analyze the data using ANOVA. Duncan's multiple range test was used to examine differences at the 0.05 level.

## RESULTS AND DISCUSSION

### Relative Enzymatic Activity (%) of *H. annuus* and *A. tricolor:*

CAT, POX and PPO were elevated in response to Cd stress at different concentrations used and combination with Cd- resistance *B. halotolerance* compared to the control of *H. annuus* and *A. tricolor* after 52 ds (Fig. 1). In general, the relative activity of enzymes CAT, POD and PPO in the leaves of plants increased with increasing of Cd concentrations as compared with control plants. And clearly, the highest significant increase in CAT and POX activities was recorded when both of plants Cd-stressed at 40 mg/l Cd concentration especially. Also, the relative enzymatic activity measured in *H. annuus* were greater than *A. tricolor* particularly at the moderate Cd concentration as compared with the plants without any additions. For example, maximum values of CAT and POX activities was increased as much as 168.39% and 752.01% in *H. annuus.* But, the highest value of PPO was recorded 369.44% in *A. tricolor* at 40 mg/l Cd as compared with control plants. At the high Cd concentration, CAT, POD and PPO enzymes activity in *A. tricolor* were increased by 132.51%, 253.82% and 338.89% respectively higher than *H. annuus* by 130.11%, 208.71% and 113.42% respectively at the Cd concentrations gradually. On the other hand, the inoculation of *B. halotolerance* inoculum positively impacted the relative enzymatic activity of CAT, POX and PPO in *H. annuus* and *A. tricolor* under Cd-contaminated solutions (Fig 10). *B. halotolerance* inoculum alleviated the negative impacts of Cd stress in plants by further inducing the activities of anti-oxidative enzymes and increase them in comparison with Cd stressed and un-inoculated plants. The *B. halotolerance* increased the relative CAT, POX and PPO activities by 100.84%, 174.78% and 151.47 % in *H. annuus* and 106.75%, 133.02% and 149.65% in *A. tricolor* with respective controls. Also, after inoculum application, the maximum activities of CAT, POX antioxidant enzymes in *H. annuus* were 155.44% and 614.73% higher than *A. tricolor* by 84.45% and 250.19% at Cd 50 mg/l, while, the highest relative activity of PPO was showed in

the inoculated *A. tricolor* up to 428.99% and 421.53% at 40 and 50 mg/l as compared with other treatments. Many studies have demonstrated that exposure to elevated concentrations of reactive metals decreases, rather than increases, anti-oxidative enzyme levels, despite the fact that plants can counteract the detrimental effects of heavy-metal stress through the presence of antioxidants activities (Schützendübel and Polle, 2002). Due to oxidative stress within the cells of the plant, Cd stress causes significant alterations in enzyme activity in plants (Hasanuzzaman *et al*., 2020; Gupta *et al*., 2019). Research has indicated that an excess of Cd leads to an overproduction of ROS in cells, including OH, $O_2$, and $H_2O_2$ (Meng *et al*., 2019). Many enzymes, such as peroxidase (POD), polyphenoloxidase (PPO), and catalase (CAT), become more active when free radicals are produced (Pandey and Dubey, 2019). In peroxisomes, CAT aids in the conversion of $H_2O_2$ to water and molecular oxygen (Noctor and Foyer, 1998). Furthermore, POD, which are present throughout the cell and have a far stronger affinity for $H_2O_2$ than do CAT, provide an additional mechanism of $H_2O_2$ elimination (Jimenez *et al*., 1997). Under heavy metal stress, PPO activity was seen in several plant species and was higher than in the control group (Saffar *et al*., 2009). The synthesis of phenolic compounds, which is crucial for the detoxification of heavy metals in plants, may be the cause of the induction of PPO activity (Ruiz *et al*., 1999). These findings suggest that variations in PPO activity may be a part of the licorice plant's defensive mechanism against Cd poisoning. According to Wang *et al*. (2012), PGPR strain inoculation increases plant enzyme activity, which reduces oxidative damage brought on by abiotic stressors. By reducing the generation of ROS, the inoculation of halotolerant bacteria from genera including Klebsiella, Pseudomonas, and Agrobacterium improved growth in salinized conditions. (Sharma *et al*., 2016).

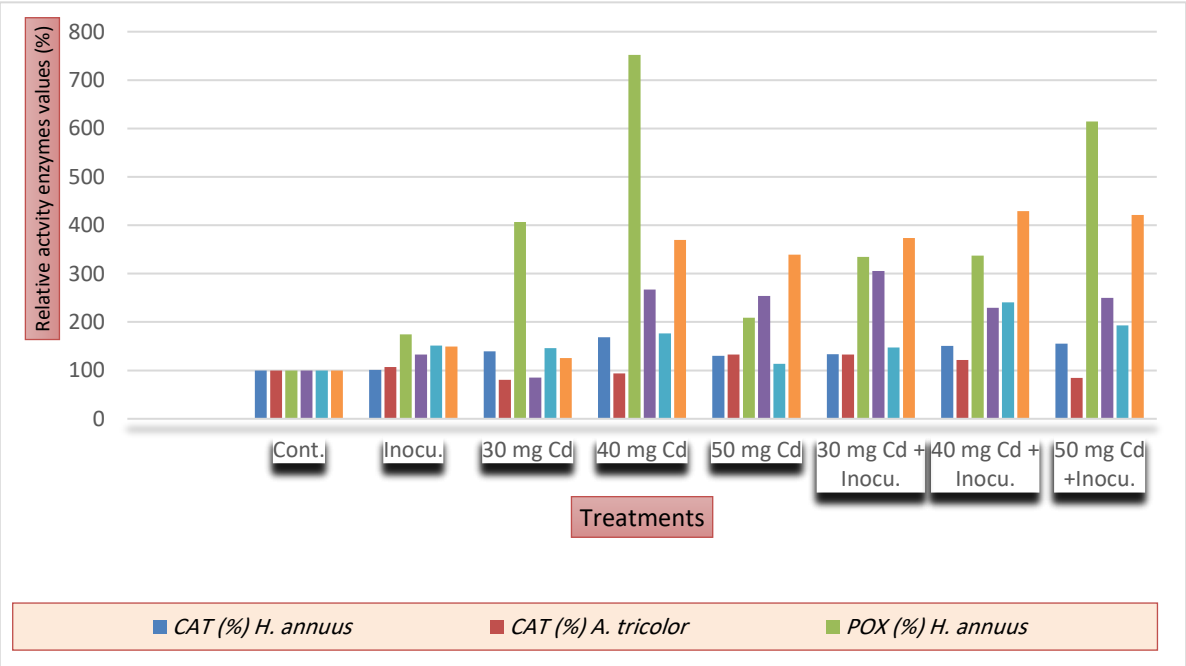


Fig.1: Relative Enzymatic Activity (%) of *H. annuus* and *A. tricolor*, Catalase (CAT), peroxidase (POX) and polyphenoloxidase (PPO) activities relative (%) of *H. annuus* and *A. tricolor*. Where, CAT: Catalase, POX: peroxidase, PPO: polyphenoloxidase, Cont: control – Inocu: *Bacillus halotolerance* inoculum- 30 mg/l Cd – 40 mg/l Cd – 50 mg/l Cd - 30 mg/l Cd + *Bacillus halotolerance* inoculum - 40 mg/l Cd + *Bacillus halotolerance* inoculum - 50 mg/l Cd + *Bacillus halotolerance* inoculum, *H. annuus Helianthus annuus, A. tricolor: Amaranthus tricolor.*

**Anthocyanin content and tolerance index TI (%) in *H. annuus* and *A. tricolor:***

Our results showed clearly that *H. annuus* and *A. tricolor* contaminated with Cd concentrations results in increased content anthocyanin in leaves of plants with increasing Cd concentrations (Fig 2-A). The given data revealed that the maximum anthocyanin content was recorded in *H. annuus* and *A. tricolor* plants treated with 40mg/l Cd concentration 0.087 and 0.134 respectively, where, the minimum values of anthocyanin was 0.079 at 50 mg/l Cd in *H. annuus* and 0.102 in *A. tricolor* at 30 mg/l Cd. The response of plants was different in anthocyanin content after *B. halotoleranc* inoculum treatment. Anthocyanin content in *H. annuus* inoculated increased by 11.93% at 50 mg/l Cd only as compared with the same Cd concentration in stressed and un-inoculated plants, while no increase was seen in anthocyanin content in inoculated *A. tricolor* except at 30 mg Cd by 14.70% as compared with the same Cd concentration in stressed and un-inoculated plants. On the other hand, the results of the present study indicate that increasing anthocyanin TI in *H. annuus* was higher than *A. tricolor* at the all-Cd concentrations (Fig 2-B). The maximum values of anthocyanin TI in *H. annuus* were 190.91% and 197.73% at 30 and 40 mg/l Cd respectively. Also, the obtained date declares that the significant influence application of *B. halotoleranc* inoculum with plants subjected with Cd concentrations used in comparison with the stressed and un-inoculated plants. *B. halotoleranc* inoculum recorded increase in TI by 200.00 in *H. annuus* at a high Cd concentration and *A. tricolor* respectively. These results indicated that *H. annuus* is supremacy of anthocyanin content from *A. tricolor* with all tested treatments. One of the main classes of secondary metabolites found in plants, anthocyanins are advantageous to both people and plants (Lee *et al*., 2016). Anthocyanin, one of the several phenolic chemicals found in leaves, is involved in the processes that lessen the metal's harmful effects (Hale *et al.*, 2001). Additionally, there is proof that when exposed to heavy metals, anthocyanin content increases (Chen *et al*., 2015). Plants rich in anthocyanins are more resilient to both biotic and abiotic stress situations because they have higher antioxidant levels, which can efficiently scavenge reactive oxygen species (ROS) (Nakabayashi *et al*., 2014). Furthermore, anthocyanins possess cellular antioxidant processes that are on par with or even stronger than those of other micronutrients, such vitamin E. Increased synthesis and decreased breakdown cause cellular proline in many plant species to accumulate; under normal conditions, this makes up around 5% of the amino acid pool; under stress, it can reach 20–80% (Kavi *et al*., 2005).

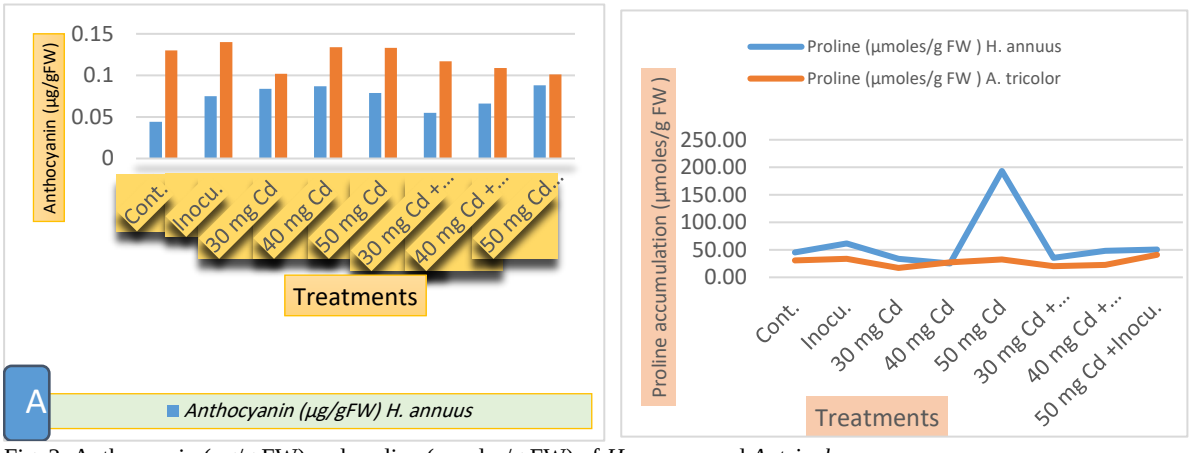


Fig. 2: Anthocyanin (µg/g FW) and proline (µmoles/g FW) of *H. annuus* and *A. tricolor*.
Where: Cont: control – Inocu: *Bacillus halotolerance* inoculum- 30 mg/l Cd – 40 mg/l Cd – 50 mg/l Cd - 30 mg/l Cd + *Bacillus halotolerance* inoculum - 40 mg/l Cd + *Bacillus halotolerance* inoculum - 50 mg/l Cd + *Bacillus halotolerance* inoculum, *H. annuus Helianthus annuus*, *A. tricolor: Amaranthus tricolor*.

By reducing damage from reactive oxygen species, this strategy increases plant tolerance. Proline and sugars may help stabilize proteins and cell structures, particularly in cases where stress is severe or persists for long periods of time. Many investigations conducted in the presence of abiotic stress revealed increased proline production for a variety of plant species infected with various PGPR (Hoque *et al*., 2007). Additionally, Ramon *et al.* (2014) discovered that following the application of three *P. putida* strains, anthocyanin varied very little in comparison to the control group of *Euphorbia pulcherrima* cultivars (Prestige and Sonora Marble). Our research supports the findings of previous researches 352 were out by Xu *et al.* (2018).

**Proline content (µmoles/g FW) and tolerance index TI (%) in *H. annuus* and *A. tricolor:***

Effect of Cd stress at concentrations used and *B. halotolerance* inoculum on proline content (µmoles/g FW) and proline TI (%) of plants documented in Fig. 3. The results indicated that Cd caused a marked high increase in proline content at high Cd concentration (50mg/l) only by 325.14% and by 5.74% in *H. annuus* and *A. tricolor* respectively over control. Inoculation of plants with *B. halotolerance* inoculum had an additive effect on proline accumulation and enhanced the biosynthesis of osmolytes as compared with control plants (Table 7). The accumulation of proline content increased after inoculum addition by 35.20% and 10.19% in *H. annuus* and *A. tricolor* respectively over control plants. A similar trend was also noticed after *B. halotolerance* inoculum addition within Cd concentrations as compared within Cd only. In *H. annuus* and *A. tricolor* inoculated, maximum values of proline content accumulated by 11.20% and 33.12% at 50 mg/l Cd concentration as compared with the stressed plants and without inoculum. TI of proline was calculated to both *H. annuus* and *A. tricolor* in Fig. 3.

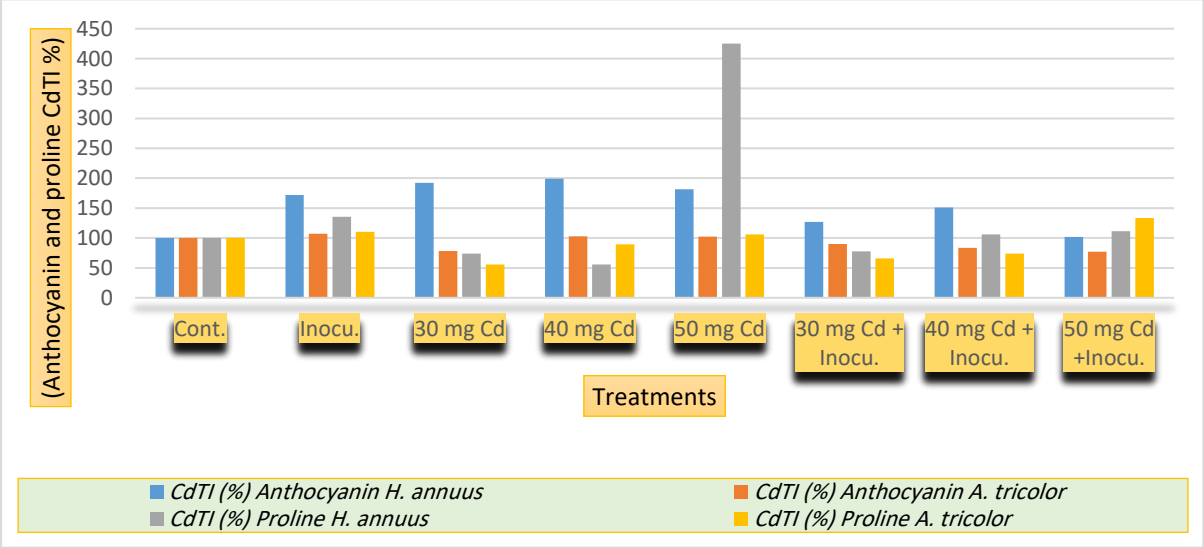


Fig. 3: Tolerance index TI (%) of anthocyanin and proline in *H. annuus* and *A. tricolor*
Where: Cont: control – Inocu: *Bacillus halotolerance* inoculum- 30 mg/l Cd – 40 mg/l Cd – 50 mg/l Cd - 30 mg/l Cd + *Bacillus halotolerance* inoculum - 40 mg/l Cd + *Bacillus halotolerance* inoculum - 50 mg/l Cd + *Bacillus halotolerance* inoculum, *H. annuus Helianthus annuus*, *A. tricolor: Amaranthus tricolor.*

In general, proline TI of *H. annuus* was higher than *A. tricolor* after *B. halotolerance* inoculum addition. Proline TI of *H. annuus* increased by 25.14% and 105.75% in *A. tricolor* at high Cd concentration (50 mg/l). Similarity, proline TI of *H. annuus* was 77.87% and 106.03% after *B. halotolerance* inoculum application higher than *A. tricolor* 65.61% and 73.89% at 30 and 40 mg/l Cd respectively. But, proline TI of *A. tricolor* increased more than *H. annuus* by 133.12% and 111.21% and respectively at high Cd concentration (50 mg/l). It is well known that proline acts as an osmoregulatory molecule, adjusting the osmotic pressure to keep cells from drying

out. Furthermore, it might interact with essential cell macromolecules to preserve their biological activity in stressful situations. Many plant species have been shown to exhibit elevated proline content as a result of Cd exposure. Among these are Hackberry (*Celtis australis*) (Hatamian *et al*., 2020), Cucumber (Semida *et al.*, 2018), *Arachis hypogaea* (Dinakar *et al*., 2008), and *Solanum nigrum* (Sun *et al*., 2007). There has also been a noticeable increase in both the roots and leaves of Olive plants (*Olea europaea*) (Zouari *et al*., 2016). Plants have been observed to accumulate proline as a means of reducing oxidative stress, altering osmotic potential, and stabilizing membrane structures in response to Cd stress (Semida *et al*., 2018; Ryan *et al*., 2019). Proline is an essential metabolite for plant adaptation, protection, and tolerance to Cd stress. Proline also serves as a source of C and N, activates the antioxidant system, and forms a nontoxic Cd-proline complex, among other defensive functions (Zouari *et al*., 2016). Proline has the ability to directly react with hydroxyl radicals or physically quench singlet oxygen (Heidari and Golpayegani, 2012). In many plant species, greater synthesis and decreased breakdown lead to the accumulation of cellular proline, which increases from around 5% of the amino acid pool under normal conditions to 20–80% under stress (Kavi *et al*., 2005). This process improves plant tolerance by lowering damage from reactive oxygen species. Proline and sugars may aid in the stabilization of proteins and cell structures, especially if the stress is severe or lasts for extended periods of time. Numerous studies under abiotic stress conditions showed enhanced proline production for diverse plant species inoculated with different PGPR (Hoque *et al*., 2007). According to Heidari and Golpayegani (2012), under stressful circumstances, PGPR inoculation increased the proline of basil (*Ocimum basilicum* L.).

**The anatomical structure in stem of *H. annuus* and *A. tricolor:***

Cd-stressed plants showed reduction in stem dimension (St. di. µm), epidermis thickness (Ep. th. µm), cortex thickness (Co. th. µm), endodermis thickness (En. th. µm), xylem vessels dimension (Xy. V. di. µm) and number of vascular bundles (No. of V. B.). A cross section of stem showed that variation in anatomy of stem in *H. annuus* was (3444x1107, 20.5, 192.7, 20.5and 32.8 µm) in St. di., Ep. th., Co. th., En. th. and Xy. V. di at 50 mg Cd where, the same characters of *A, tricolor* were lower by (1599x 820, 12.3, 61.5, 12.4 and 20.5 µm) at the same Cd concentration. Application of bacteria strain used was ameliorated in those stem characters in *H. annuus* by (1845x 4920, 24.6, 221, 20.5 and 32.8 µm) and in *A. tricolor* by (1845x1558, 16.4, 123, 16.4 and 24.6 µm) of St. di., Ep. th., Co. th., En. th. and Xy. V. di respectively at 50 mg Cd within bacteria strain used compared with Cd concentration without inoculum bacterial (Fig. 4-5). Numerous plants undergo multiple anatomical changes to fortify their structures and limit the absorption of metals. The most common changes are cell wall modifications and secondary metabolite impregnation, especially in peripheral tissues (exodermis and endodermis) that come into direct contact with the pollutants. (Yadav *et al.*, 2021). This entails adjusting the cellular and organ-level anatomy and morphology of plants; additionally, Cd can reduce the widths of xylem vessels and the thicknesses of the endodermis, epidermis, and exodermis (Li *et al.*, 2019a).

Additionally, according to Ronzan *et al*. (2018), Cddecreases cell division, disrupts cell organization in the lateral roots, and increases the lignification of vascular tissues. Common bean plants exposed to Cd showed no discernible change in stem diameter; nevertheless, the number and size of xylem vessels were reduced and the stem cortex appeared to have enlarged. In Cd-treated *Triticum foenum* graecum, Ahmad *et al*. (2005) observed a rise in the fraction of cortex and a significant decrease in vascular density, size, and xylem fiber length. According to Abdo *et al*. (2012), the thickness of the midvein and lamina decreased by 37.9% and 8.6%, respectively, in soybean plants treated with Cd. PGPR, the administration of PGPR significantly enhanced anatomical features in both Cd and non-stressed plants. El-Afry *et al*. (2012) presented comparable findings. Since Cd inhibits cambium cell division, elongation, and differentiation, Barcelo *et al.* (1988a) hypothesized that decreased vessel radius and number are the causes of reduced water circulation.

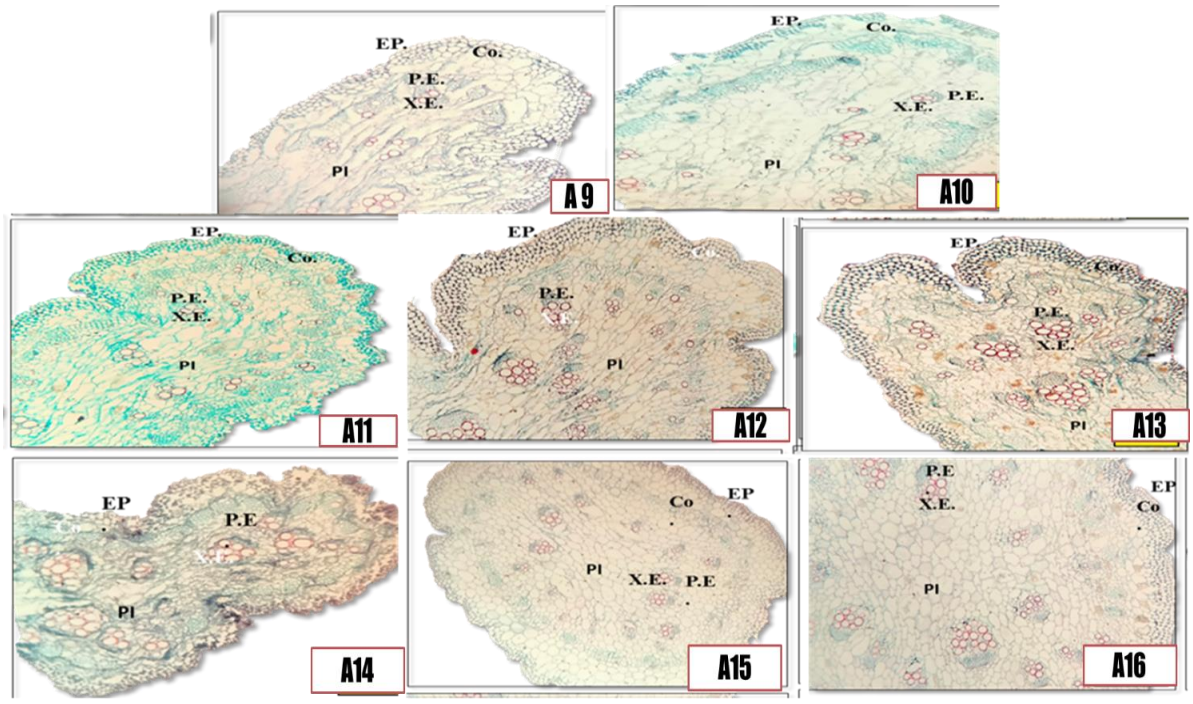


Fig. 4: A cross section of stem shows variation in anatomy of stem in *H. annuus.*

Cross section in stem of *H. annuus*, X= 20, H9: control – H10: *Bacillus halotolerance* inoculum- H11: 30 mg/l Cd – H12: 40 mg/l Cd – H13: 50 mg/l Cd – H14: 30 mg/l Cd + *Bacillus halotolerance* inoculum – H15: 40 mg/l Cd + *Bacillus halotolerance* inoculum – H16: 50 mg/l Cd + *Bacillus halotolerance* inoculum, cortex (Co.), stem dimension (St. di. µm), epidermis thickness (Ep. th. µm), endodermis thickness (En. th. µm), xylem vessels dimension (Xy. V. di. µm).

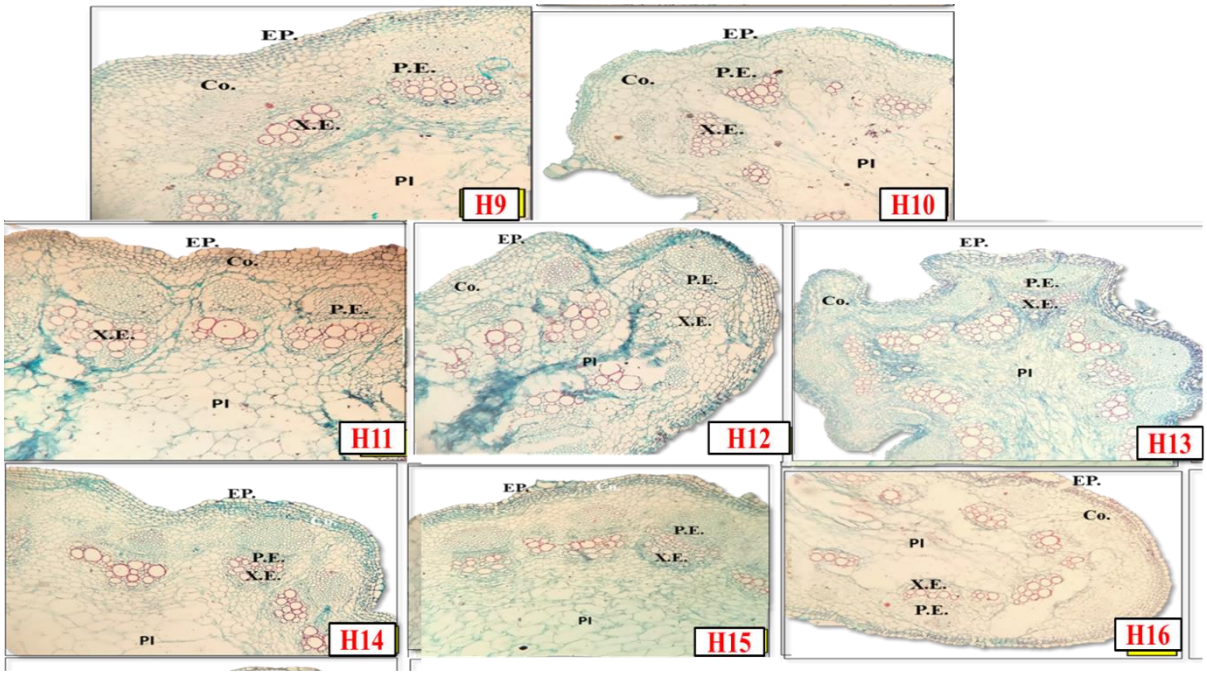


Fig. 5: A cross section of stem shows variation in anatomy of stem in *A. tricolor*

Cross section in stem of *A. tricolor*, X= 20, A9: control – A10: *Bacillus halotolerance* inoculum- A11: 30 mg/l Cd – A12: 40 mg/l Cd – A13: 50 mg/l Cd – A14: 30 mg/l Cd + *Bacillus halotolerance* inoculum – A15: 40 mg/l Cd + *Bacillus halotolerance* inoculum – A16: 50 mg/l Cd + *Bacillus halotolerance* inoculum, cortex (Co.), stem dimension (St. di. µm), epidermis thickness (Ep. th. µm), endodermis thickness (En. th. µm), xylem vessels dimension (Xy. V. di. µm).

## Conclusion

Our results indicated that *H. annuus* and *A. tricolo* under study were different in physiological and biochemical defense mechanisms as response against Cd. This study demonstrated that *H. annuus* was higher than *A. tricolor* and it has different tolerance and defense mechanics against Cd concentrations stress. And the bacterial inoculum used improved *H. annuus* more than *A. tricolor* stressed by Cd and increase removal, accumulation and phytoextraction Cd efficiency after *B. halotolerance* applied and succeed in enhancing of phytoremediation combination with PGPB system. Generally, its showed that anatomical modifications in structures of leaves in *H. annuus* was lower than *A. tricolor* in different treatments. A cross section of stem showed that variation in anatomy of stem in *H. annuus* was lower than *A, tricolor*. Application of bacteria strain used was ameliorated in those stem characters specially in *H. annuus* from *A. tricolor* compared with Cd concentration without inoculum bacterial.

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