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Amelioration of Mercury Stress using Mercury Tolerant Bacterium by Studying Growth, Antioxidant Enzyme Activities, Lipid Peroxidation and Proline Content in Pigeon Pea

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A B S T R A C T

Heavy metal pollution of agricultural soils is a widespread environmental threat. Heavy metals induce deleterious effects on plants. These effects can be minimized by the application of PGPB on plants under stress. The main objective of this study is to assess the effect of PGPB inoculants of *Brevundimonas sp*. of bacterium on growth and antioxidant enzyme activities of pigeon pea seedlings grown under high mercury concentrations. The plant antioxidant response has been analyzed by quantifying the catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR) enzyme activity in pigeon pea seedlings exposed to mercury and different PGPB treatments. Our data indicated that the bacteria acted as a shield and protect the *Cajanus* plants from mercury toxicity and hence extent of lipid peroxidation and antioxidant enzymes (CAT, POX and SOD) were determined for all three treatments i.*e.,* control (C), untreated (mercury exposed) and treated (bacteria + mercury). The antioxidant enzyme activities, MDA content and proline content were increased in the mercury exposed pigeon pea and a parallel reduction was observed in *Brevundimonas* bacterium treated pigeon pea seedlings under mercury stress indicate that these plants faced less stress in presence of *Brevundimonas* bacterium.

***Key words*:** Heavy metal, Mercury resistant, Antioxidant enzymes

It's really alarming to note that usage of mercury is getting higher and higher. Reports [1] stated that in the 20th century, global mercury release increased remarkably due to the usage of mercury for industrial purposes. Among all the available forms, ionic mercury ($Hg2^{+}$) is the most prevailing form in soils and is very significant due to its bioavailability. Reports suggest that heavy metal pollution caused due to human activities has become most common in various parts of India [2]. Mercury reported to be released into the atmosphere mainly as untreated industrial waste [3]. Among all the heavy metals, mercury is more hazardous and non-degradable metal. Hence it stays in the polluted areas for many years. Plants especially agricultural crops growing in contaminated soils are under continuous stress. Exposure of plants to high mercury concentration cause adverse effects plant growth parameters, antioxidant enzyme production and DNA [4-5]. To prevent fungal diseases, use of mercury compounds for dressing of the seed coat has become common these days. This causes an undesirable increase in the concentration of mercury in agricultural land cause a serious threat to humans.

Study of the effect of mercury tolerant PGP bacteria on plants

Earlier findings of mercury toxicity were mainly concerned with the illustration of harmful effects induced by mercury on plants. Quite recently, considerable attention has been paid to the application of bacteria in the alleviation the heavy metal induced stress on plants. In view of the cost controlling aspects, effectiveness and endurance, bioremediation of heavy metals was proved to be less harmful to the environment when compared to the physical and chemical methods [6]. Microbial remediation is all about use of bacteria for converting harmful substances into substances that are less toxic to human health and the environment. Bacteria found to have several possible means to deal with adverse environmental conditions including physiological, biochemical and genetic.

Study of mercury tolerance of the bacterial isolates

It is obvious from the earlier reports that bacteria that tolerate high concentrations of mercury could serve to effectively remediate mercury pollution [7].

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Role of bacteria on oxidative stress

Moreover, it should not be forgotten that defensive responses, including the activities of antioxidant enzymes such as catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) activate under metal stress, are also under bacterial regulation [8] (Hou *et al.*, 2021). Hence, altered levels of antioxidant enzymes considered an important parameter in the evaluation of stress caused by heavy metals.

Production of reactive oxygen species (ROS)

Exposure of plants to heavy metals usually coupled with a direct response of production of reactive oxygen species (ROS) and biosynthesis of antioxidant enzymes to scavenge the ROS as an indirect response. The correlation between the production of reactive oxygen species (ROS) and mercury stress is reported by Sahu *et al*. [9]. The over production and accumulation of ROS lead to oxidative stress which cause severe damage to plant cells [10]. Plants thoroughly responded to Hg induced oxidative stress by changing the levels of their antioxidative enzymes.

ROS scavenging

To avoid menacing impacts caused by ROS, plants are provided with various adjustment mechanisms to counteract heavy metal toxicity [11]. The plant cells have an intricate antioxidant network capable of removing ROS. A close relation has been observed between the removal of ROS and tolerance to stress [12]. So, in this study the seeds were exposed to mercury and treated with mercury tolerant bacterium in ameliorating the mercury stress. It is obvious from the earlier reports that bacterial treatment effectively reduces the mercury stress and hence the corresponding generation of antioxidant enzymes.

MATERIALS AND METHODS

Effect of isolated mercury tolerant bacteria on growth

For evaluation of growth, pigeon pea seeds have been taken out from three separate treatments. From three sets of each treatment, 10 seeds were taken for study. After 6 days of treatment, the growth parameters of plants were recorded. The root length, shoot length, fresh and dry weights of both root and shoot, of each treatment namely control, untreated and treated were measured. Root and shoot were separated from the plant excluding the endosperm region and used to determine the length, fresh and dry weight. The dry weight (DW) was recorded after drying in an oven at 80 °C temperature for 48 hr to obtain constant dry weights.

Effect of isolated mercury tolerant bacteria on Antioxidant enzyme activities

Catalase

Permanganate method of Povolotskaya and Sedenka [13] and subsequently with little modification was used to evaluate catalase activity. Two hundred mg of fresh leaf sample of control (distilled water), untreated (exposed to $Hg(NO_3)_2$) and treated (treated with *Brevundimonas* bacterial isolate + $Hg(NO_3)_2$) were grounded in a pre-cooled glass mortar and cooled phosphate buffer pH. 7.0. The extract was filtered through glass wool and made up to 25 mL with the same buffer solution.

Estimation

For 2 mL of the enzyme extract taken in a conical flask, 1 mL of 0.045 M hydrogen peroxide was added. After 300 sec, to stop the enzyme activity, 1 mL of 12% H_2SO_4 was added and titrated with 0.05 N potassium permanganate until the pink colour is formed in the solution which remains for 30 seconds. By substituting 2 mL of the enzyme extract with 2 mL phosphate buffer, a blank was run parallel to the above. The difference in the titer value between the blank and leaf enzyme extract gave the activity of catalase and was expressed as mg H_2O_2 destroyed /min/g fresh weight by using the following equation:

$$\text{Mg of } H_2O_2 \text{ destroyed/min/g} = 25/2 \times 0.85 \times V/W$$

Where;
V = difference between blank and leaf enzyme titre values
W = fresh weight in grams
2 = extract taken in mL.

The factor 0.85 represents 1 mL 0.05 N $KMnO_4$ is equal to 0.85 mg of H_2O_2.

A) *Peroxidase*

The protocol proposed by Kar and Mishra [14] was quantified to conduct the peroxidase activity.

Enzyme extraction

Each of 200 mg plant tissue samples of control (distilled water), untreated (exposed to $Hg(NO_3)_2$) and treated (treated with *Brevundimonas* bacterial isolate + $Hg(NO_3)_2$) were made into a paste with 10 mL of 0.1 M. phosphate buffer, pH was adjusted to 6.8 and centrifugation was done in a refrigerated centrifuge at 17, 000 x g. The top layer of liquid was taken as the enzyme extract.

Enzyme assay

The assay mixture for the peroxidase comprised of 1 mL of enzyme extract, 50 µ moles of pyrogallol, 125 µ moles of phosphate buffer (pH 6.8) and 50 µ moles of H_2O_2 and was incubated for 5 min at 25 °C. Optical density was measured at 420 nm in BIO- RAD smart spec plus UV visible spectrophotometer for purpurogallin formation. A similarly treated blank with only distilled water was used for zero setting. The enzyme activity was calculated as absorbance units (one unit of enzyme activity was considered as 0.1 differences in absorbance value) per mg protein. Method proposed by Lowry *et al*. [15] has been taken to read the protein content of the enzyme extract.

Superoxide dismutase activity

By following the method of Beauchamp and Fridovech [16], the amount of superoxide dismutase was measured by means of its ability to inhibit the photochemical reduction of nitro blue tetrazolium.

Preparation of enzyme extract

One gram of plant leaf material of control (distilled water), untreated (exposed to $Hg(NO_3)_2$) and treated (treated with *Brevundimonas* bacterial isolate + $Hg(NO_3)_2$) were collected and grounded by the gradual addition of 5 mL of 50 mM phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone. The grounded paste was filtered and centrifuged at 15000 x g for 10 min in a refrigerated centrifuge. The top layer of liquid obtained was used as an enzyme extract. All steps in the preparation of the enzyme extract were carried out at 0 to 4 °C. An aliquot of 0.1 mL of the enzyme extract was used for the determination of protein content as described earlier [15].

Enzyme assay

The 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM nitro blue

tetrazolium, 2 µM riboflavin, 0.1 mM EDTA and 0.1 mL of enzyme extract. Riboflavin was added last and the test tubes were shaken and well placed 30 cm below light blank consisting of two 15 W fluorescent lamps. The reaction was started by switching on the light. The reaction was allowed to take place for 30 min and was stopped by switching off the light. The tubes were covered with black cloth. The absorbance of the solution was measured at 560 nm in BIO-RAD Smart Spec plus UV visible spectrophotometer. The reaction mixture which was not exposed to the light did not develop colour and served as control. Log A_{560} was plotted as a function of the volume of enzyme extract used in the reaction mixture. From the resultant graph, the volume of the enzyme extract corresponding to 50% inhibition of the reaction was read and was considered as one enzyme unit.

Lipid peroxidation

The aim of this experiment was to estimate the extent of lipid peroxidation in pigeon pea seedlings. The procedure was the method adopted by Heath and Packer [17] with minor modifications.

Reagents

1. 0.5% TBA diluted in 20% (w/v) TCA
2. 0.1% w/v TCA

Procedure

Hundred mg of fresh leaf tissue of control (distilled water), untreated (exposed to $Hg(NO_3)_2$) and treated (treated with *Brevundimonas* bacterial isolate + $Hg(NO_3)_2$) was grounded in 2 mL of 80% ethyl alcohol using a mortar and pestle by the gradual addition of 0.5 mL of 0.1% (w/v) TCA. The grounded leaf paste was centrifuged at 15,000 x g, 40 °C for 10 min. The top layer of liquid was collected. 0.5 mL of supernatant was taken in Eppendorf tubes and 1.5 mL of 0.5% TBA diluted in 20% TCA was added to it. 4 mL of 20% TCA containing 0.5% thiobarbituric acid was added. The mixture was incubated in a water bath at 95 °C for 25 min. The mixture was heated at 95 °C for 30 min and then quickly cooled on an ice bath. Solutions were transferred to Eppendorf tubes containing 1 mL of 80% ethanol (for dilution). The solution was further centrifuged at 15000 rpm for 5 min (15000 x g, 4.0 °C) to make the solution clear. The absorbance of the supernatant was measured at 532 nm and the value for the non-specific absorption at 600 nm was subtracted. However, many plant tissues contain other chemical compounds which intervene with the absorption of MDA, that comprises of anthocyanins, phenolics, sugars and other intervening substances that absorb at 532 nm, leading to higher readings of MDA by up to 96.5% [18]. By this method absorbance at 532 nm of a solution was subtracted and corrected by taking reading without TBA from an identical solution containing TBA. The concentration of malondialdehyde is calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$. MDA concentration is calculated using the Lambert-Beer law with an extinction coefficient εM = 155 $mM^{-1}cm^{-1}$. Results are presented as micro mols MDA $g^{-1}FW$.

Determination of proline

Proline content was estimated by the acid ninhydrin method of Troll and Lindsley [19] as modified by Tully *et al*. [20].

Preparation of ninhydrin reagent

The desired amount of the reagent was prepared using 125 mg of ninhydrin in 3 mL of glacial acetic acid and 2 mL of 6 M phosphoric acid in proportionate amounts and then heated to 70 °C. The reagent will remain stable for at least 24 hours.

Extraction

Initially, 200 mg of dried plant material and grounded in pistle and mortar by in 5 mL of water and heated at 100 °C for 30 min in sealed tubes, it was allowed to cool and the cooled content was centrifuged and the superficial layer of liquid was collected made up to a known volume.

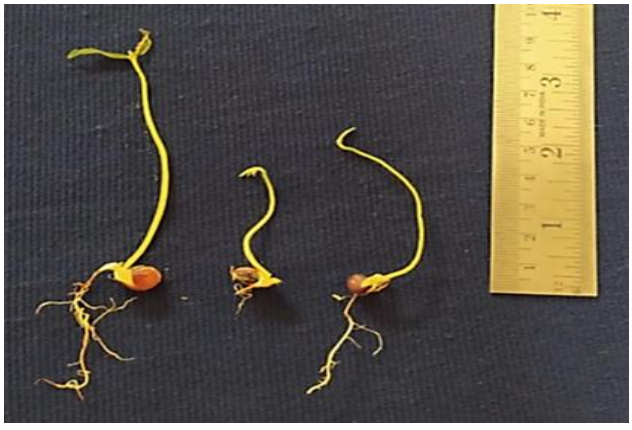
Procedure

For estimation of proline, 5 mL of sample extracts were taken. And 5 mL of glacial acetic acid and 5 mL of ninhydrin reagent were added to each sample extract and heated in a water bath for 1 hr in test tubes with plastic screw caps. The solutions were cooled to room temperature and the colour was extracted using 5 mL of toluene by shaking them vigorously for 5 min in separating funnels. The phases were allowed to separate and the toluene phases were transferred to colorimeter tubes and the absorbance was determined at 515 nm on BIO-RAD smart spec plus UV visible spectrophotometer. The amount of proline was determined from a standard curve in the range of 20 - 100 g/mL.

RESULTS AND DISCUSSION

Effect of bacterial treatment (Brevundimonas sp.) on pigeon pea seedling growth

The present study was therefore designed to understand the role of mercury resistant bacterial isolate in reducing stress by means of promoting plant growth and as a corrective measure of antioxidant enzyme activities. In this research, it was observed that the plant showed a significant reduction in growth at 150 ppm mercuric nitrate concentration. And at a further increase in mercury concentration, the plant showed drastic decelerated growth rates. Hence, 150 ppm $Hg(NO_3)_2$ was selected for this study as this concentration was found to be an effective concentration in terms of growth reduction and is thought to be improved by the use of mercury-tolerant bacteria. For this study, three treatments were selected namely control (pigeon pea seeds grown in distilled water), untreated (pigeon pea seeds exposed to 150 ppm mercury) and treated (pigeon pea seeds treated with bacteria and mercuric nitrate). Hence, it was assumed to promote growth.

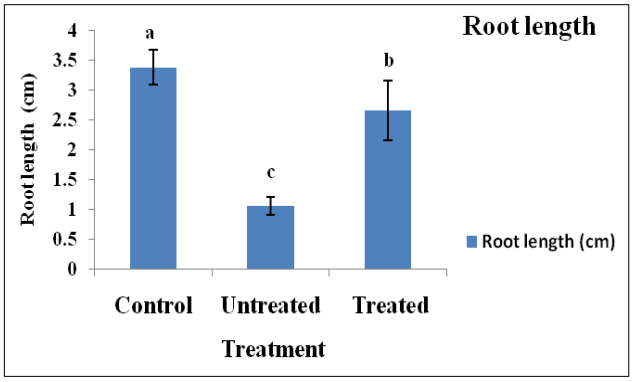


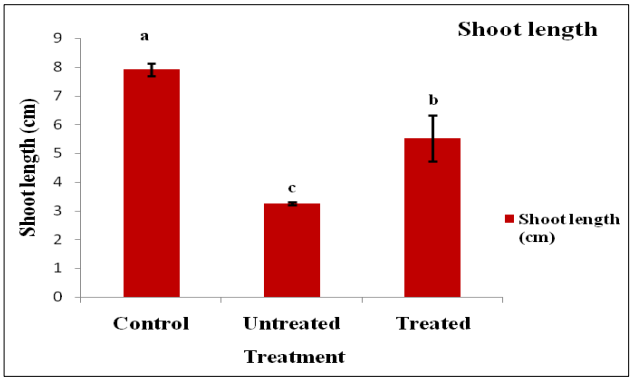
Control: Pigeon pea seedlings maintained after 168 hr in Petri plates at 28 ± 2 °C temperature and 60-5% humidity.
Untreated: Pigeon pea seedlings exposed to 150 ppm $Hg(NO_3)_2$ were considered as Untreated.
Treated: Pigeon pea seedlings treated with mercury tolerant bacterial isolate and exposed to 150 ppm $Hg(NO_3)_2$ were considered as treated

Plate 1 Effect of *Brevundimonas* bacterial treatment on the growth of pigeon pea under mercury exposure

The effect of bacterial treatment (*Brevundimonas sp*. (HG 2)) on growth characteristics of 168 hr old *Cajanus cajan*

seedlings under mercury stress was assessed in terms of root length, shoot length and fresh weight of the seedlings. From the results (Plate 1, Fig 2-3), it was observed that mercury stress resulted in the reduction of all the growth parameters of seedlings. A considerable reduction in root and shoot length was noticed in mercury treated seedlings. The root length was decreased from 3.38 ± 0.31 to 1.05 ± 0.15 in seedlings exposed to 150 ppm mercury which showed a 68.93% decrease. And a similar reduction in growth was observed in shoot growth in mercury exposure and recorded 58.83% reduction in growth. It was noticed an improvement in growth by the treatment of growth promoting, mercury resistant bacterial isolate. The growth was significantly increased from 1.05±0.15 to 2.66±0.5 by the application of bacteria in pigeon pea accounted 153.3% increase in root length and 69.32% increase in shoot length when compared to seeds under mercury exposure (Fig 1-2).

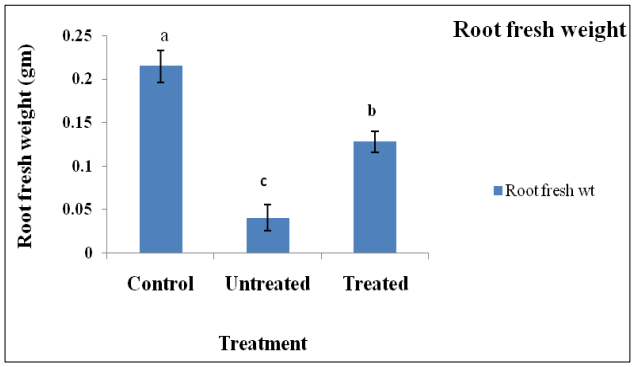




Control: Pigeon pea seedlings maintained after 168 hr in Petri plates at 28 ± 2 °C temperature and 60-65% humidity.
Untreated: Pigeon pea seedlings exposed to 150 ppm $Hg(NO_3)_2$ were considered as Untreated.
Treated: Pigeon pea seedlings treated with mercury tolerant bacterial isolate and exposed to 150 ppm $Hg(NO_3)_2$ were considered as Treated. Bars represent root length. Error bars represent standard deviation. Vertical lines represent the standard deviation

Fig 1 Effect of *Brevundimonas* bacterial treatment on root growth of pigeon pea seeds exposed to mercury

Fig 2 Effect of *Brevundimonas* bacterial treatment on shoot growth of pigeon pea seeds exposed to mercury



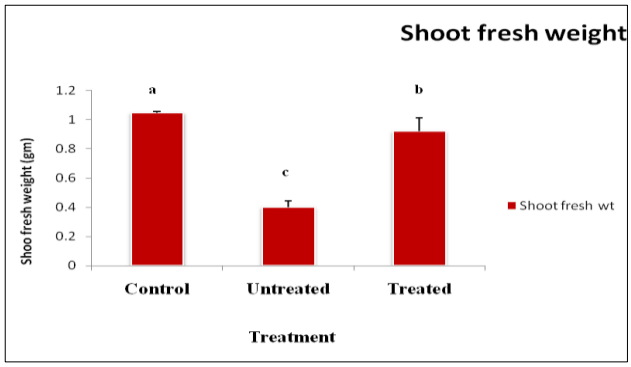


Fig 3 Effect of *Brevundimonas* bacterial treatment on root fresh weight of pigeon pea seeds exposed to mercury

Fig 4 Effect of *Brevundimonas* bacterial treatment on shoot fresh weight of pigeon pea seeds exposed to mercury

Fresh weight of root and shoot

The root fresh weight was more affected and showed a reduction of fresh biomass from 0.2148 ± 0.0182 to 0.0405 ± 0.0151 by 81.14% following the mercury exposure. However, root fresh weight increased from 0.0405 ± 0.0151 to 0.1281 ± 0.012 which recorded an increase of 216.2% in the presence of *Brevundimonas sp*. (HG 2) was observed when compared to the seedlings exposed to mercury. The results showed that shoots similarly affected and showed a significant decrease (61%) in shoot fresh weight by mercury exposure as the shoot fresh weight decreased from 1.05 ± 0.008 to 0.403 ± 0.04. And an increase in shoot fresh weight from 0.403 ± 0.04 to 0.923 ± 0.0086 was noticed which showed 129.03% increase following the bacterial treatment when compared to pigeon pea exposed to mercury stress (Fig 3-4).

The dry weight of root and shoot

The results of the present study showed an increase in terms of the dry weight of root and shoot of pigeon pea treated with bacterium and exposed to mercury. In pigeon pea, root dry weights of seedlings were decreased from 0.0372 ± 0.0013 to 0.0104 ± 0.0036 which recorded 72.04% reduction in the mercury exposure compared to the control seedlings. When seedlings under Hg stress, when treated with mercury tolerant bacteria, the dry weight of root was enhanced from 0.0104 ± 0.0036 to 0.0291 ± 0.0018 showing an increase by 179% in when compared to seedlings subjected to mercury stress. Coming to dry weight of shoot, the same results were obtained i.e., shoot dry weight reduced from 0.171 ± 0.005 to 0.065 ± 0.003 under mercury stress (61.98%) when compared to control and this was significantly increased from 0.065 ± 0.003 to 0.101 ± in dry weight by bacterial treatment was showed 55.38% increase over the mercury exposed (Fig 5-6).

All the data obtained was mean of three replicates. The results obtained in the experiment were subjected to analysis of variance (ANOVA). The vertical lines represent the Standard deviation (SD). The mean followed by different letters represented on vertical lines of the figures were significantly different at $p \leq 0.01$.

Seedling growth

The ability of *Brevundimonas* bacterium to promote growth and biomass production was previously reported in potatoes [21]. Similar findings were reported in wheat and *Bt*-cotton when inoculated with *Brevundimonas* [22]. It was observed that mercury treatment severely affected the growth

of root and shoot. These deleterious effects were minimized by the application of bacteria. Results have shown that higher growth rates in pigeon pea seedlings exposed to Hg have observed in presence of *Brevundimonas sp*. bacterium when compared to pigeon pea seedlings cultured in mercury-containing medium and in the absence of bacterial treatment.

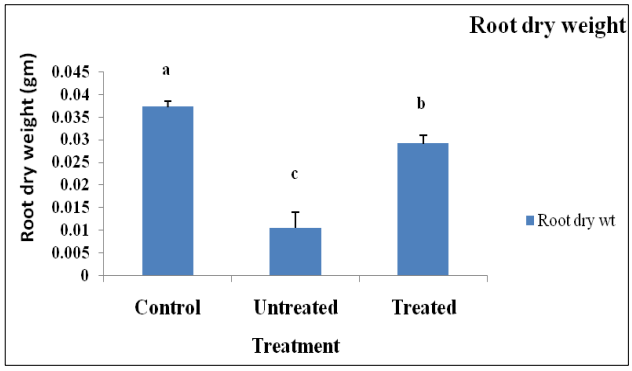


Fig 5 Effect of *Brevundimonas* bacterial treatment on root dry weight of pigeon pea seeds exposed to mercury

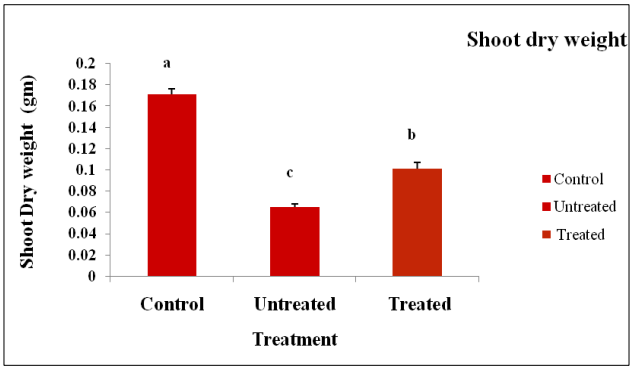


Fig 6 Effect of *Brevundimonas* bacterial treatment on shoot dry weight of pigeon pea seeds exposed to mercury

Biomass

These results lead to a similar conclusion that bacterial treatment significantly increases all growth parameters including biomass. In Comparison with 150 ppm mercuric nitrate treated seedlings of pigeon pea, bacterial treated seedlings under mercury stress showed enhancement in biomass production. This finding indicates that inoculation of metal-resistant bacterial isolate HG 2 has improved the biomass of the pigeon pea seedling. Our results are consistent with the previous reports which found the reduction in biomass by arsenic exposure and a subsequent rapid enhancement of it resulted in chickpea due to bacterial treatment [23].

It was widely believed that a decrease in growth by higher doses of mercury can be explained by the binding of mercury ion with the cell wall components [24] or due to a reduction in the cell division [25]. Inoculation with *Pseudomonas sp*. decreased the accumulation of chromium and enhanced the growth of the mustard plant [26]. Effect of mercury tolerant bacterium on antioxidant enzyme activities, lipid peroxidation and proline of pigeon pea exposed to mercury.

Catalase (CAT)

In the fourth day of treatment, a significant increase in catalase content was observed in mercury exposed (68.08%) over control. In the subsequent bacterial treatment, the content was decreased to 28.54% when compared to mercury exposed (Fig 7). An enhancement in CAT activity of 58.85% and 48.71% was observed when compared to the control seedlings on the sixth and eighth days of treatment respectively in mercury exposed. These percentages were decreased to 29.17% and 27.27% respectively on bacterial treatment (treated with bacteria and 150 ppm mercury) on the sixth and eighth days of treatment (Fig 7). CAT activity was significant for all treatments and all days at $p \leq 0.01$.

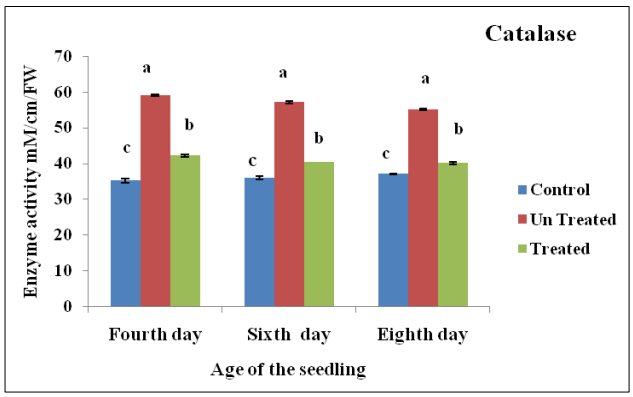


Fig 7 Effect of *Brevundimonas* bacterial treatment on catalase content of pigeon pea under mercury exposure

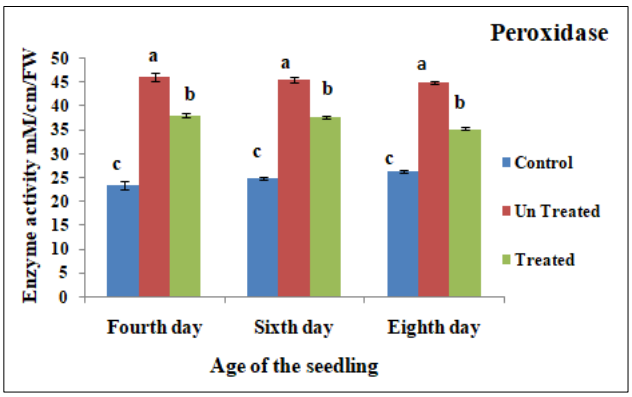


Fig 8 Effect of *Brevundimonas* bacterial treatment on peroxidase content of pigeon pea under mercury exposure

In the present study, a decrease in CAT levels by the bacterial treatment was observed. This can be assumed that bacterial treatment might cause a decrease in the intensity of mercury toxicity. Therefore, the seedlings showed lowered activities of CAT than control seedlings. In *Vicia faba* the bacterial treatment registered a significant reduction in catalase amounts when compared with the uninoculated control plants at all the increasing concentrations of heavy metal [27]. From the results, it is clear that catalase levels were increased under exposure to mercury when compared to the control seedlings. A steep rise in catalase activity was noticed with the increasing days of mercury exposure.

Peroxidase (POX)

In this investigation, mercury exposed seeds were treated with mercury resistant bacterial isolate (*Brevundimonas*) to study the bacteria's role in detoxification of mercury. Plants treated with *Brevundimonas* bacterium under Hg stress showed a significant reduction in POX content when compared to seedlings exposed to mercuric nitrate alone. An increase in activity in mercury exposed over control was significant on the fourth day of treatment which showed a 96.42% increase at $p \leq 0.01$. Peroxidase activity was increased with increasing days in control, while it was decreased in mercury exposed and bacteria treated under stress. In mercury exposed, the peroxidase activity

was shown to be increased over control by 96.42%, 82.07% and 70.68% for the fourth day, sixth day and eighth days respectively (Fig 8).

The content was decreased in bacteria treated under mercury exposure (treated with bacteria and 150 ppm mercury), compared to mercury treated by 17.19%, 17.07% and 21.61% respectively for the fourth, sixth and eighth days of treatment. The results have shown that a significant difference in peroxidase contents between untreated (seedlings exposed to mercury) and treated (bacterial treated under mercury stress) were observed.

The activity of peroxidase was found to be very high in mercury-treated seedlings of pigeon pea (untreated) and comparatively a decreased activity of peroxidase was observed in seedlings exposed to mercury and underwent bacterial treatment (treated). These results bear with the findings of Jain *et al*. [28] in maize seedlings treated with selected zinc metal tolerant bacterial isolates which showed lowered activity of POX seen as a way to minimize the stress induced by the test heavy metal. Increased peroxidase activity under heavy metals treatments may be considered as a stress tolerance mechanism evolved by the plant species. These results agree with the earlier reports of increased POX activity induced by Hg exposure in seedlings of *Sesbania grandiflora* [29].

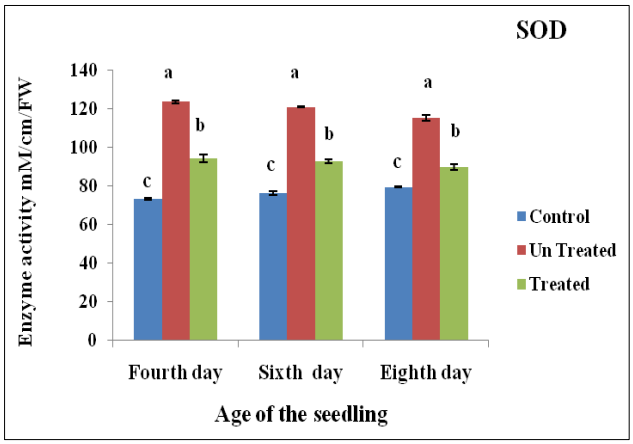


Fig 9 Effect of *Brevundimonas* bacterial treatment on superoxide dismutase (SOD) content of pigeon pea under mercury exposure

Superoxide dismutase (SOD)

In the present study pigeon pea seeds were exposed to 150 ppm $Hg(NO_3)_2$ and seeds treated with both 150 ppm $Hg(NO_3)_2$ and bacteria were maintained for a total of eight days and SOD activity was measured after the 4th, 6th, and 8th day and the results were presented in (Fig 10). Control was maintained without the treatment of $Hg(NO_3)_2$ as well as the bacteria. In mercury treated (*Cajanus cajan*) seedlings, increased SOD activity was noticed in the Hg concentration of 150 ppm compared to normal control. It was observed that SOD activity increased by 68.71%, 58.89% and 44.75% under the treatment of mercury (150 ppm $Hg(NO_3)_2$) for the 4th, 6th, and 8th day respectively when compared to control. The results of the experiment found increased SOD activity in untreated mercury exposed seedlings and a decrease in SOD content was observed in bacterial treated under mercury stress. These observations can be interpreted as an effort to fight against the harmful free radicals that are produced during oxidative stress to balance the internal environment of the cell. It was widely noticed that plants usually respond to mercury-induced oxidative stress by altering the levels of their antioxidative enzymes. Reports suggested that an increase in SOD activity in response to heavy metal was commonly observed and the same was verified by [30]. SOD is a major superoxide scavenger and its enzymatic action brings about the conversion of superoxide radicals (O_2^-) to H_2O_2 and O_2 [31]. The enhanced levels of SOD with heavy metal exposures provide indirect evidence for the production of ROS [32]. SOD can eliminate O_2^-, which helps in decreasing the peroxidation of membrane lipids and maintain cell membrane stability. Increased SOD activities in plants under heavy metal stress indicate an effort to fight against the harmful free radicals that are produced during oxidative stress to balance the internal environment of the cell, while CAT and peroxidase enzymes are involved in scavenging H_2O_2.

It was observed that the SOD levels were found to decrease in treated seedlings (*Brevundimonas* bacterium + 150 ppm $(Hg\ NO_3)_2$). These results were very significant. And it can be understood that mercury tolerant bacterial treatment provides an approach to mitigate the stress caused by mercury exposure. Similar results were observed in *Spartina Maritima* where a significant reduction in SOD activity was observed in bacterial inoculated plants. Several authors opined that PGPR accomplishes the amelioration of abiotic stress by regulating the antioxidative enzyme activities [33]. But these contents recorded higher to those found in control suggest that the bacterium could not nullify the metal toxicity, but metal exposed seedlings encountered less stress in the presence of the bacterium. The role of *Brevundimonas* bacterium in the mitigation of stress indicates that it might be efficient for heavy metal removal, even at higher concentrations. There were different opinions on the mechanisms by which bacteria minimize reduce the production of antioxidant enzymes. One view expresses that bacterial inoculation may protect the plant from abiotic stress not by controlling the activity of antioxidant enzymes, but by reducing the heavy metal toxicity [34]. Contrary to the above view [35-36], expressed their view that the bacterial inoculants mitigate the metal toxicity by minimizing the production of ROS and there will be decreased production of antioxidant enzymes, which are actually produced to act upon the oxygen free radicals.

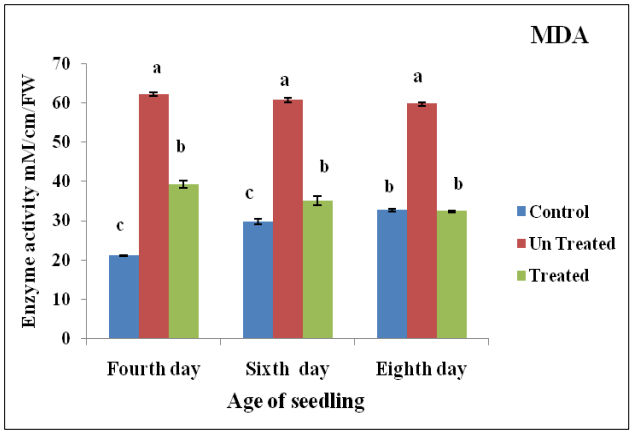


Fig 10 Effect of *Brevundimonas* bacterial treatment on MDA content of pigeon pea under mercury exposure

Lipid peroxidation

In the present study, the oxidative stress induced by mercury is measured in terms of MDA content was evaluated in all the treatments of pigeon pea i.e., the leaves of control the mercury exposed (150ppm) and seedlings treated with bacterial isolate (HG 2) and 50ppm $Hg(NO_3)_2$. The main aim of this study is to investigate the effect on selected mercury tolerant bacterium in alleviating the mercury induced oxidative damage on pigeon pea seedlings.

An increase in MDA content was observed in pigeon pea seedlings treated with mercury when compared to control in all days irrespective of days of exposure. Significant enhancement

of MDA content was observed on the fourth day of mercury treatment over control (Fig 10). The percent enhancement of MDA in seedlings treated with mercury over control was 195%, 103% and 82.5% respectively for the fourth, sixth and eighth days of treatment. The MDA contents showed decreasing trends in seedlings that are exposed to mercury and treated with bacteria. The decrease in MDA content in bacteria treated were 36%, 42% and 45.8% respectively for the fourth, sixth and eighth days of exposure, the decrease was significant on the eighth day of bacteria treatment. The MDA content showed an increasing tendency in control over increasing days of exposure while they were decreasing in mercury exposed and bacteria treated under mercury stress.

The results indicated that mercury induces oxidative stress by producing oxygen free radicals. ROS overproduction triggered by mercury would causes lipid peroxidation within the cell membranes of plants. Malondialdehyde (MDA) is generated as a metabolic product of lipid peroxidation. This study is carried out to investigate the effect of the tested bacteria in the alleviation of oxidative damage caused by mercury on the *Cajanus* plant.

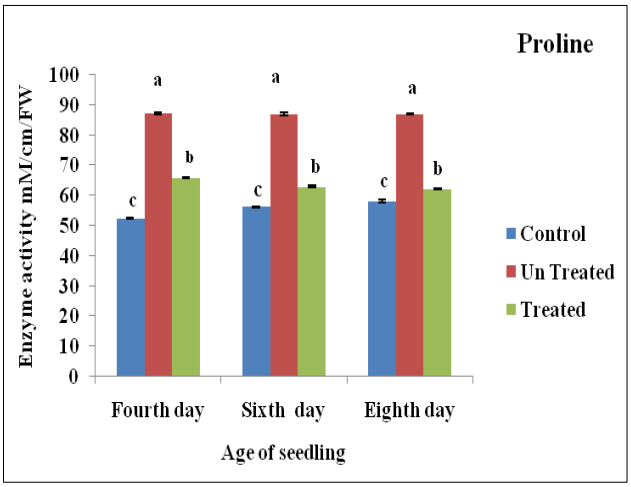


Fig 11 Effect of *Brevundimonas* bacterial treatment on proline content of pigeon pea under mercury exposure

Lipid peroxidation

The results demonstrate two things.

Firstly, it indicates that mercury-induced oxidative stress causes lipid peroxidation. Lipid peroxidation occurs due to the removal of hydrogen ions from unsaturated fatty acids. Malondialdehyde (MDA) is generated as a metabolic product of lipid peroxidation. Consequently, it has become a major protocol to assess the extent of lipid peroxidation by measuring malondialdehyde, which in turn used as an indicator of oxidative injury in plants [37]. Several other studies obtained similar results of MDA enhancement under various heavy metal stressors such as Hg [38] and Ni and Cr [39] in different plant species.

Secondly, it was established that bacterial treatment limit the extent of lipid peroxidation, subsequently decreased levels of MDA observed in bacterial treated under mercury exposure. The decline in MDA content in bacterial treated plants is in line with the earlier studies which suggest the stress-tolerance mechanisms operated by bacteria affecting lipid peroxidation [40].

The results have showed that the bacterial strain which possess the highest tolerance to mercury was identified as *Brevundimonas* species. The mercury-resistant bacteria possess various mechanisms for remediation of heavy metals. These mechanisms help in converting hazardous metals to less toxic forms and arrest the entry of it into the food chain. In this way, it would lessen the toxicity of metals to affect the health of human beings and other organisms.

Proline (Pro)

Mercury exposed plants, when treated with mercury tolerant bacterial inoculant (*Brevundimonas*) had shown a significant decrease of proline levels (24.40%, 27.72% and 28.54%, respectively) when compared to the plants exposed to mercury. In the study, bacteria treated plants under $Hg(NO_3)_2$ stress showed low proline content compared to mercury treated plants, but the level was significantly higher than the control. These levels were decreased with days of incubation.

In the present study, it was found that proline content increased in seedlings exposed to mercury. In mercury treated seedlings, a significant increase in proline level was observed in plants treated with 150 ppm $Hg(NO_3)_2$ in the 4^{th}, 6^{th} and 8^{th} days of mercury exposure (66.26%, 54.87%, and 49.78% respectively) compared with control plants (Fig 11). The maximum increase (88% increment) was observed on the 4^{th} day of exposure to 150 ppm $Hg(NO_3)_2$ compared to control. However, Proline levels then decreased gradually at the increasing days of exposure (4^{th}, 6^{th} and 8^{th}) in seedlings irrespective of the treatment, but its level was still higher than the control. The proline content of the mercury exposed seedlings showed a 0.24% decrease and the mercury exposed seedlings treated with bacteria showed a 5.70% decrease with increasing days of exposure which are calculated on the 8^{th} day. Hg contamination caused a significant increase in proline and MDA contents in plant parts with the increasing days of contamination.

The results showed that levels of proline found to be declined in seedlings treated with mercury resistant bacterium when compared to the control seedlings and an increase in the proline levels was observed in plants exposed to mercury. From the results two conclusions can be drawn. One is the stimulation of the proline production in pigeon pea following mercury treatment and the other conclusion is the *Brevundimonas* bacteria treatment reduces the proline content significantly. Proline accumulation has also been documented in many plants under heavy metal stress and such reports explain its osmoregulatory role in the alleviation of heavy metal stress [41]. A variety of mechanisms have been suggested by which Proline accumulation increases the resistance of plants to HM toxicity. Among other mechanisms, the most important mechanism suggested by several authors is the accumulation of proline is to reduce oxidative stress [42]. Overproduction of proline in order to detoxify ROS generated during osmotic stress induced by heavy metal there by protecting the membranes [43].

The second conclusion can be explained that the *Brevundimonas* bacterium acts as a stress restraint and may reduce heavy metal stress by regulating proline biosynthesis which was significantly increased by mercury treatment. Our results are in accordance with results obtained by Gontia-Mishra *et al*. [44], which suggested that wheat plants treated with PGPR namely *Enterobacter ludwigii* and *Klebsiella pneumoniae*, showed low proline content under mercury stress. Hence it was established that bacteria could negate the mercury stress and reduced ROS production.

Study of role of mercury tolerant bacterium on Cajanus cajan plant

Control, untreated (mercury exposed) and treated (bacterial treated under mercury exposure) pigeon pea seeds were selected to study germination percentages, growth

parameters, biochemical responses and genomic studies. It was found that *Brevundimonas* bacterium had contributed to the highest germination rates and enhanced the growth of pigeon pea under mercury exposure.

CONCLUSION

To summarize the effects of treatment of pigeon pea seedlings with the selected bacterial isolate (*Brevundimonas sp*.), the detrimental effects of mercury exposure were minimized by the bacterial treatment and all the growth metrics including germination were enhanced in bacterial treated than in mercury exposed. It was also observed that bacterial treatment improved the levels of photosynthetic pigments in pigeon pea seedlings. Bacterial treated seedlings showed lower levels of proline than mercury treated seedlings but higher than control seedlings, suggesting that the *Brevundimonas* bacterium in pigeon pea seedlings lessen the magnitude of mercury stress.

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