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# Accumulation of cadmium in maize roots inoculated with root organ culture of *Rhizophagus irregularis* improving cadmium tolerance through activation of antioxidative defense enzymes

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**ABSTRACT**

Monoxenic culture of arbuscular mycorrhiza, *Rhizophagus irregularis* in root organ cultures, was formulated in a dextrin-based carrier. *R. irregularis* was coated in maize seeds (African tall composite) at the rate of 50 g $kg^{-1}$. *R. irregularis* was evaluated for heavy metal tolerance at 25, 50, and 75 ppm cadmium (Cd) in a pot culture experiment. The mean root colonization potential at 25 ppm Cd inoculated with *R. irregularis* was 48%, which had the highest probability to reach its maximum during 30 DAS. In the present study, Cd was accumulated to a tune of 22.2–38.3% in mycorrhizal roots, which was more than non-mycorrhizal roots. Cd addition at 25 and 50 ppm levels decreases its translocation to shoots to 28% in *R. irregularis* inoculated plants when compared to uninoculated treatments. The highest tolerance indices were observed in $T_6$ and $T_7$ with 100.59 and 98.34, respectively, showing its increased ability of cadmium to bear heavy metal up to a level of 50 ppm. *R. irregularis* inoculated maize adapted well at 25 ppm Cd and confirmed its significant role in reducing Cd accumulation toward the shoot system.

## 1. INTRODUCTION

Heavy metal toxicity is the most common problem faced by agricultural soil and plants in the contemporary agricultural world. Perseverance of metal in nature, non-degradability in soil, and toxicity in biological cells and tissues cause metal toxicity. Heavy metals in soil can be absorbed by crops and accumulated in foods, eventually creating a negative collision on living beings. Heavy metals have an adverse impact on plant growth and human health is threatened through food chain [1]. Cadmium (Cd), a naturally occurring non-nutritive trace element in earth's crust, is one of the major heavy metal contaminants in sewage sludge. Sewage sludge is an insoluble solid residue obtained after sewage treatment. It is a by-product of biological wastewater treatment process, which contains a wide range of non-essential heavy metals including Cd. Huge disposal of waste water and sewage sludge from industries and rainwater runoff enters into the drainage system and pollutes soil, water, and air [2]. The anthropogenic sources of Cd in urban areas of Coimbatore district, India, are likely to be derived from the gold making wastes that were channelized into sewage during disposal. Sewage water and solid waste are disposed for several years in nearby areas and the agricultural soil in such areas had a high cadmium content of 4.5–8.2 mg $kg^{-1}$ [3].

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Hence, this article presents an environmental containment to overcome the abovementioned menace. Washing agents, synthetic surfactants, and biosurfactants are used in separation methods that are utilized for removing heavy metals from the contaminated soil in most of the chemical and physical extractions. The process is expensive and the derivatives become secondary contaminants because of their toxicity and resistance to biodegradation [4]. Phytoremediation is the most cost-effective, environment-friendly, and practical approach in the remediation of heavy metals, which involves the removal, relocation, or reduction of metal contaminants using plants that hyperaccumulate these contaminants [5,6]. In the present study, maize is employed for phytoremediation of heavy metal pollution because of mixed pollutant removal ability of *Zea mays* L. [7] and high biomass production potential.

Arbuscular mycorrhiza (AM), which has a symbiotic relationship with most vascular plants, plays the most significant role of alleviating the toxic effects of heavy metals. The extended and highly branched mycelial network of AM fungi translocates the heavy metals along the hyphae to the intercellular structure and transfers them to the root at the symbiotic interface. The mechanisms involved are enhanced uptake by fungal transporters in mycorrhizal pathway, phytoextraction by increased biomass of mycorrhizal plants, heavy metal binding to the fungal cell wall, chelation by glomalin, heavy metal compartmentalization in the vacuoles, and activation of antioxidative defenses [8]. The fungal hyphae percolate in the cortical cells of plant

roots and form arbuscules, vesicles, and hyphae [9]. The fungal hyphae bind with heavy metals and render them immobile at the cortical region of roots, prevent their translocation towards aerial parts of the plant, and prevent leaf tissues from damage [10]. AM fungi reduce heavy metal stress through heavy metal immobilization in fungal structure, precipitation, chelation in the rhizosphere, sequestration in vacuoles, and activation of antioxidant mechanisms in plants [11].

Therefore, phytoremediation strategies, possible responses of cadmium accumulation in plants, and AM symbiosis are ecofriendly and these approaches can be considered to improve soil health and protect plants against such abiotic stress. The present study was conducted to investigate the potential benefits of monoxenic or pure culture of AM fungi, *Rhizophagus irregularis*, developed through *Agrobacterium rhizogenes* transformed root organ culture in maize plant at different levels of artificially spiked Cd in the experimental soil.

## 2. MATERIALS AND METHODS

The experiments were conducted in the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore (India), in 2019.

### 2.1. Steps in Establishment of Monoxenic Culture of *R. irregularis*

The monoxenic culture of arbuscular mycorrhizal fungi (AMF), *R. irregularis* strain MUCL 41833 obtained from the Department of Agricultural Microbiology, was multiplied for 4 months through root organ culture in MSR medium. When the spore size reached maturity, the spores were harvested using 10 mM sodium citrate (pH 6.0) and by allowing them to pass through 45 micron sieve. The AM spores were mixed in dextrin powder, a water-soluble carrier, under aseptic conditions and formulated. The population was maintained with a minimum of 5000 spores/50 g of inoculum (i.e., 100 spores $g^{-1}$ inoculum) [12,13].

### 2.2. Seed coating technique of AM inoculum

Maize seeds (Fodder maize – African tall composite) were obtained from the Department of Forage Crops, TNAU, and were surface sterilized with 0.1% $HgCl_2$ for 2 min, followed by 70% ethyl alcohol for 2 min, with intermittent washings using sterile distilled water. Seeds were coated with AM spores formulated in dextrin at the rate of 50 g $Kg^{-1}$of seeds. It was air-dried at room temperature (37°C).

### 2.3. Pot Culture Study

The experimental soil was an alfisol, red sandy loam in texture, neutral in pH (7.2), and free from salinity (0.02 $dSm^{-1}$). The mixture of soil and sand (2:1) was filled in earthen pots of 5 kg capacity (20 cm diameter and 20 cm height) and used for the experiment. The experimental soil was sterilized and spiked with hemi-pentahydrate ($CdCl_2.2.5.H_2O$) (Sigma-Aldrich CAS no. 202908) at different concentrations of 25, 50, and 75 ppm (mg $kg^{-1}$) and incubated for about 24 h before sowing. The following treatments were imposed and seeds were sown in Cd-spiked soils to test the tolerance of varying levels of Cd in pot culture soils. Two maize plants were grown with and without inoculation of AM fungi (*R. irregularis*) at varying levels of Cd spiked in soil. The duration of study was 70 days. The treatments were: $T_1$ – Uninoculated control, $T_2$ – Inoculated with *R. irregularis* (without Cd), $T_3$ – Uninoculated with *R. irregularis* (25 ppm Cd), $T_4$ – Uninoculated (50 ppm Cd), $T_5$ – Uninoculated (75 ppm Cd), $T_6$ – *R. irregularis* + 25 ppm Cd, $T_7$ – *R. irregularis* + 50 ppm Cd, and $T_8$ – *R. irregularis*+ 75 ppm Cd.

### 2.4. Root and Shoot Dry Weight

The roots were removed from soil in the pots and the adhering soil was washed and cleaned with water. The roots were shade dried for 15 min to remove water. Plant samples were air-dried at sampling periods 45 and 70 DAS and then kept in an oven at 60–70°C until a constant weight was obtained. The weight of the dried root and shoot samples was recorded and expressed as g $plant^{-1}$.

### 2.5. Total Chlorophyll Content

Total chlorophyll was measured by following the procedure of Sadasivam and Manickam [14]. One gram of fresh leaf sample was weighed and cut into fine pieces and the representative sample of 0.5 g was ground to a fine pulp in a chilled mortar with an addition of 20 ml of 80% acetone. The contents were centrifuged at 5000 rpm for 5 min. The supernatant was transferred to a 50 ml conical flask. The residue was ground again with 20 ml of 80% acetone and the supernatant was transferred to the same conical flask. The process was repeated until the residue turned colorless. The volume was made up to 50 ml with 80% acetone. Finally, absorbance of the solution was read at 645 and 663 nm against the solvent (80% acetone) in the spectrophotometer. The chlorophyll content was calculated at 45 and 60 DAS as per the formula given below.

$$\text{Total chlorophyll}\left(\text{mg g}^{-1}\text{tissue}\right)=\frac{20.2\left(A_{645}\right)+8.02\left(A_{663}\right)\times V}{1000\times W}$$

Where, A =Absorbance of specific wavelengths
V = Final volume of chlorophyll extract in 80% acetone
W = Fresh weight of tissue powdered

### 2.6. Assessment of AM Colonization

Root colonization was estimated during 30 and 45 DAS, as per the method described by Phillips and Hayman [15]. AM fungal spores were estimated from rhizosphere soil of maize (during 70 DAS) using wet sieving and decanting techniques [16].

### 2.7. Catalase and Peroxidase Activity

Catalase activities in the leaf and root samples were determined by quantitative measurement of interacted $H_2O_2$ with the titration method [17]. Five hundred milligrams of plant tissue were macerated with 10 ml of 0.1 M phosphate buffer and centrifuged at 3000 rpm for 10 min to collect the supernatant. Five beakers labeled 0–4 were taken with 5 ml of 1.5% sodium per borate, 1.5 ml of phosphate buffer, and 1 ml of supernatant. After $0^{th}$, $1^{st}$, $2^{nd}$, $3^{rd}$, and $4^{th}$ min, 10 ml of 0.2 N $H_2SO_4$ was added and titrated against 0.5 N $KMnO_4$. The reactive oxygen species (ROS) $H_2O_2$ was determined at 45 and 70 DAS in shoots and roots as per the formula given below. Lesser $H_2O_2$ production implies greater catalase activity and it is expressed as:

$$Ti=\frac{\begin{array}{c}\textit{Dry wt of the plant}\\ \textit{at polluted soil}\end{array}}{\begin{array}{c}\textit{Dry wt of the plant at non -}\\ \textit{polluted soil of the same treatment}\end{array}}\times 100$$

Peroxidase is determined through spectrophotometric determination on measuring the rate of increase of absorbance at 430 nm, according to the protocol adopted by Sadasivam and Manickam [14]. Five hundred milligrams of plant tissues were macerated with 10 ml 0.1M phosphate buffer that was centrifuged at 3000 rpm for 10 min to collect the supernatant. A 3 ml pyrogallol was added to 0.1 ml of supernatant

and adjusted to zero reading as blank. A 0.5 ml of $H_2O_2$ was added to the same solution and read at 430 nm at an interval of 30 s for a period of 3 min. The peroxidase activity was measured at 45 and 70 DAS in shoots and roots, and it was expressed as per the formula given below:

$$\text{Peroxidase activity } \left(\text{change in OD min}^{-1}\text{g}^{-1}\right) = \frac{\text{Absorbance value} \times 60 \times 10 \times 1000}{1 \times 30 \times 500}$$

### 2.8. Acid and Alkaline Phosphatase

The acid phosphatase enzyme activity was estimated as per the method followed by Tabatabai and Bremner [18]. It was expressed in µg of *p*-nitrophenol released $g^{-1}$ of soil $h^{-1}$ on dry weight basis at 35°C at pH 6.5.

### 2.9. Alkaline Phosphatase

The alkaline phosphatase enzyme activity was estimated as per the method followed by Tabatabai and Bremner [18]. It was expressed in µg of *p*-nitrophenol released per g of soil per h on dry weight basis at 35°C at pH 11.0

### 2.10. Soluble Proteins and Total Phenols

Soluble proteins were estimated in maize shoots and roots at 45 and 70 DAS and were expressed as mg protein $g^{-1}$ plant tissue [19]. Total phenols were estimated by the Folin–Ciocalteu reagent method in maize shoots and roots at 45 and 70 DAS and were expressed as mg of catechol produced $g^{-1}$ plant material [20].

### 2.11. Cd uptake in Shoot and Root Samples

Ten milliliters of concentrated nitric acid were added to 500 mg of plant samples and transferred to microwave digester. The temperature was maintained at 100°C for 1–2 h until it turned into a thick slurry form. It was cooled for another 15–30 min after which 2 ml of distilled water was added. The sample was filtered through Whatman No.1 filter paper and the volume was made up to 25 ml. Cd concentrations were determined using atomic absorption spectrophotometer (PerkinElmer) in the same conditions against Cd standards (5–20 ppm) [21].

### 2.12. Translocation Factor (TF)

TF for heavy metal Cd within a plant was calculated using the following formula (22).

$$TF = \frac{Cd\,(Shoot)}{Cd\,(Root)}$$

### 2.13. Tolerance Indices (Ti)

Ti of AM and non-AM plants to heavy metal (Cd) stress in soil was determined using the following formula [22].

$$Ti = \frac{Dry\ wt\ of\ the\ plant\ at\ polluted\ soil}{Dry\ wt\ of\ the\ plant\ at\ non\text{-}polluted\ soil\ of\ the\ same\ treatment} \times 100$$

### 2.14. Statistical Analysis

The experimental design was completely a random one with three replications. All results were analyzed with ANOVA. The data were subjected to statistical analysis by variance ($P$ = 0.05) with mean separation, as per the standardized methods detailed by Panse and Sukhatme [23]. The data were critically analyzed using computational tools available in R statistical programming to correlate and interpret various parameters through graphical representations.

## 3. RESULTS AND DISCUSSION

### 3.1. Monoxenic culture of *R. irregularis* in Root Organ Culture (ROC) and its Coating Efficiency

The ROCs grew tremendously with more number of lateral roots along with excessive branching of hyphae and profuse sporulation, which eventually filled the Petri plate in 4 weeks. The dynamic production of new spores after 3–4 months recorded nearly 5000–9000 spores per Petri plate [Plates 1 and 2]. The seed coating polymeric suspension was used to treat the dextrin-based carrier inoculum and it had the best coating efficiency with a maximum of 68% root colonizing potential after 25 days of treatment. Earlier conventional vermiculite-based method was used for obtaining AM inoculum from root segments of plants hosting AM fungi and/or the infected soil containing AM propagules [24]. However, this vermiculite-based AMF inoculum is highly voluminous and contaminated [25]. In the present study, a novel thick spore inoculum, extracted and prepared from *in vitro* root organ and cultured through a dual culture technique, was used for seed coating in maize. Mycorrhizal spores in the inoculum can be entrapped into cavities created inside the carrier matrix. Spore viability improved with the best coating efficiency of 83.2% [26]. Thus, it can be inferred that the mycorrhizal load in soil can be restored efficiently by applying the seed coating method that colonizes young roots early in a plant's life, that is, immediately after the seed begins to sprout. Such a seed coating technique infects the emerging root system of inoculated plants.

During 45 DAS, the root dry weight was significantly increased in $T_2$ than in $T_1$ and it was significantly reduced with increasing concentrations of Cd at 45 DAS. During 70 DAS, a decrease was noticed in root dry weight with respect to increasing concentrations in nonmycorrhizal plants, but the reduction was significant only in $T_5$ (18.5%). However, T6 had a higher mean root dry weight (4.2 $g^{-1}$ $plant^{-1}$; interquartile range 1.18) than the other two mycorrhizal inoculated treatments ($T_7$ and $T_8$) during 45 DAS [Figure 1a]. Similarly, significant reduction of shoot dry weight was observed in both mycorrhizal and non-mycorrhizal plants at an increasing level of Cd at 45 and 70 DAS. $T_6$ had a maximum mean shoot dry weight at 70 DAS (14 $g^{-1}$ $plant^{-1}$; interquartile range 1.65) [Figure 1b].

Furthermore, Figure 2 shows a higher mean plant height in mycorrhizal inoculated plants compared to uninoculated ones at both stages. The mean plant height was significantly reduced at 75 ppm Cd in AM uninoculated plants at both stages. Both $T_7$ and $T_8$ had a significant variation over $T_4$ and $T_5$ with the higher values in $T_7$ at 70 DAS (82.2 cm; interquartile value 6.3 cm).

Similarly, the root length was increased at 6.14–22.20% level over non-mycorrhizal Cd applied plants under mycorrhizal inoculation with different levels of Cd. During 40 and 70 DAS, the mean chlorophyll content was the highest in AM inoculated control, followed by mycorrhizal inoculated treatments $T_6$, $T_7$, and $T_8$, with the possibility of reaching the maximum of the high mean level. All uninoculated treatments ($T_1$, $T_3$, $T_4$, and $T_5$) showed poor chlorophyll content with a possibility of reaching a much lower level than the uninoculated control [Figure 3]. The effect of a good AM infectivity potential on

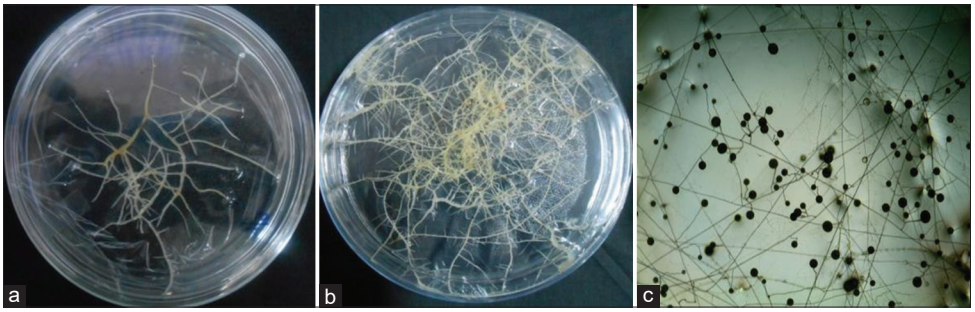


**Plate 1:** Growth of transformed roots and multiplication of *Glomus intraradices* spores. (a and b) Growth of transformed roots and (c) multiplication of Gi spores interlinked with extramycelial network.

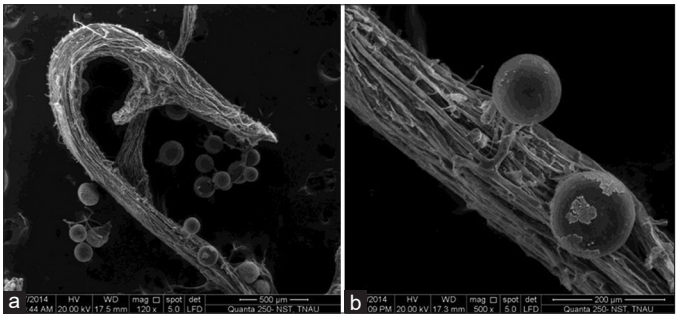


**Plate 2:** Scanning electron microscopic view of Gi spores in root organ culture. (a) Bunch of spores in AM colonized root apexes and (b) globose-shaped spores with size ranging from 115 to 145 µm.

reducing the toxic effect of Cd in polluted soil needs to be essentially studied and the present study has served this purpose. Heavy metal stress affects plant growth, but the inoculation of AM fungi in Cd contaminated soil decreases the reduction of overall plant growth in the present investigation, showing an increase in root and shoot length as well as dry weight at both stages of observation, as presented in Figure 1. The reduction observed at all levels of artificial Cd contamination, irrespective of the comparison between mycorrhizal inoculation and the inoculated control ($T_2$), is possibly due to the reduction in the profuse hyphal elongation and branching, which ultimately causes a decrease in prime nutrient uptake like phosphorus. The principal component analysis (PCA) biplot in Figure 4 depicts the positive Eigen vector values for alkaline and acid phosphatase and other parameters such as plant biomass production, root spread and root volume, root colonization potential, and chlorophyll content. This proves the massive sporulation and the growth of Gi in soil has a great impact on the growth of plant. This is well supported by a study on mycorrhiza-Cd interaction on plant growth, nutrients and Cd accumulation, and AMF root colonization [27]. The researchers posited that mycorrhiza promotes plant growth whereas Cd addition reduces plant biomass production. The PCA biplot [Figure 4] implies positive correlation among the variables, such as total chlorophyll content and root colonization, suggesting minimum variation among them. In addition, the correlogram [Figure 5] shows a highly significant positive correlation (0.97***) among the same variables.

The correlation plot [Figure 5] depicts a significant correlation between the AM root colonization and different growth parameters such as chlorophyll content (0.97***), shoot dry weight (0.95***), root dry weight (0.89**), root spread (0.87**), volume (0.87**), and alkaline phosphatase (0.85**). The root volume was positively correlated with root colonization in PCA biplot [Figure 4] and it was found increased in AM (+) plants than AM (-) plants at all levels of Cd [Figure 6]. The overall mean values depicted that the decrease in the root volume was more in 75 ppm Cd level ($T_8$). No significant changes were observed in root spread at all Cd levels during both stages. The mean percent root colonization in AM (+) and Cd treatments was high in $T_6$ (48.0%; interquartile value 17.3), which had the highest probability to reach its maximum with inoculated control during 30 DAS. Increasing Cd level in $T_7$ and $T_8$ decreased the mean root colonization percent at 45 DAS. However, uninoculated treatments revealed poor infectivity percent at all spiked levels in both stages of observation [Figure 7].

Heavy metal contamination in soil is associated with iron deficiency. Low iron content results in chlorosis, since it inhibits both chloroplast development and chlorophyll biosynthesis [28]. Cd has a direct effect on the structure, composition, and functioning of photo system II domains in the thylakoid membrane of plants [29]. The results of the present study indicate an increase of 19.2–25.7% in the chlorophyll content in AM fungi inoculated plants over uninoculated plants at all Cd spiked levels. This finding was supported by Rivera-Becerril *et al*. [30] who reported an increase in plant photosynthetic activity by AM inoculation in Cd stress conditions. The increase in chlorophyll content is due to great phosphorous availability and other macro- and micronutrients imported to the plant by the extraordinary multiplication of mycorrhizal spores, high-speed elongation, and profuse branching of extracellular hyphal structures, seen in the *in vitro* pure inoculum of Gi. The box and whisker plot analysis [Figure 7] shows that mean root infection of Gi at 25 ppm Cd was significantly higher (48%) than at 50 and 75 ppm Cd (28%) during 30 DAS. However, the probability of further increase was not observed on continuous exposure of the same Cd level after 45 DAS. The results suggest that root infectivity varies in accordance with the period of exposure and concentrations of Cd were artificially contaminated in the experimental soil, as stated by Liao *et al*. [31]. Further, beyond a critical concentration, the plant system might be recalcitrant to heavy metal due to the activation of *de nova* metabolic pathways and triggering of antioxidant defense system.

### 3.2. Impact on Antioxidative Enzymes

The production of ROS was significantly less in all uninoculated and inoculated controls ($T_1$ and $T_2$) without Cd spiking. However, $H_2O_2$ production was high at 75 ppm Cd ($T_5$) level in roots (6.80 µg $H_2O_2$ $g^{-1}$ $min^{-1}$) and shoots (6.377 µg $H_2O_2$ $g^{-1}$ $min^{-1}$) during 45 DAS and 70 DAS, respectively. However, treatments inoculated with mycorrhiza showed a relative decrease in ROS, thus confirming the increased catalase activity [Table 1]. A relative increase in the peroxidase activity with increasing Cd levels due to AM inoculation was observed in both roots and shoots with maximum in roots (6.12 change in OD $min^{-1}$ $g^{-1}$).

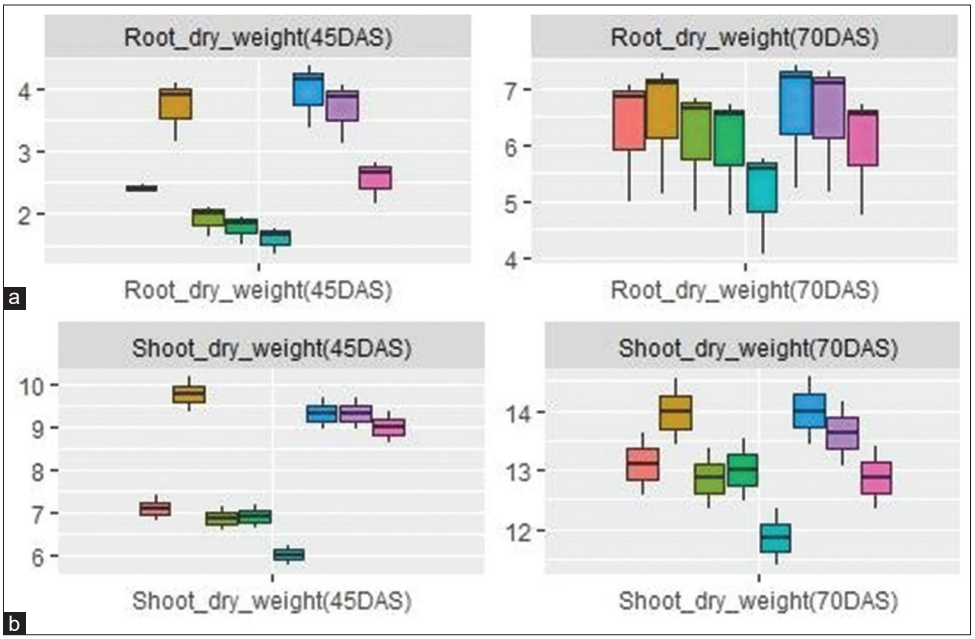


**Figure 1:** Box and whisker plot showing root (a) and shoot (b) dry weight in maize in mycorrhiza inoculated and uninoculated treatments at different levels of Cd. Box and whisker plots represent minimum, lower quartile, median, upper quartile, and maximum values.

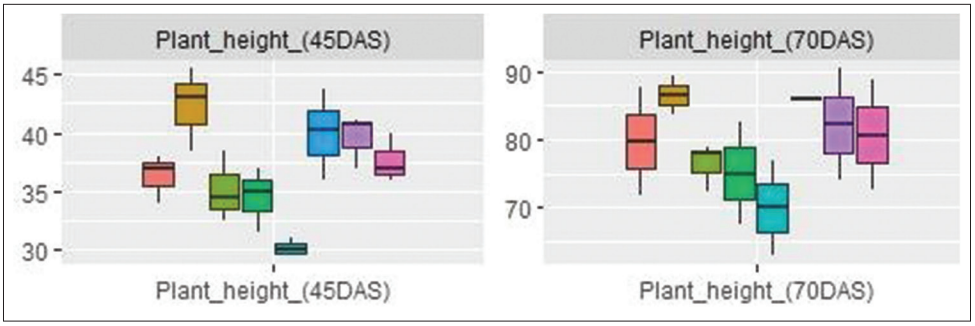


**Figure 2:** Box and whisker plot showing plant height in maize in mycorrhiza inoculated and uninoculated treatments at different levels of Cd. Box and whisker plots represent minimum, lower quartile, median, upper quartile, and maximum values.

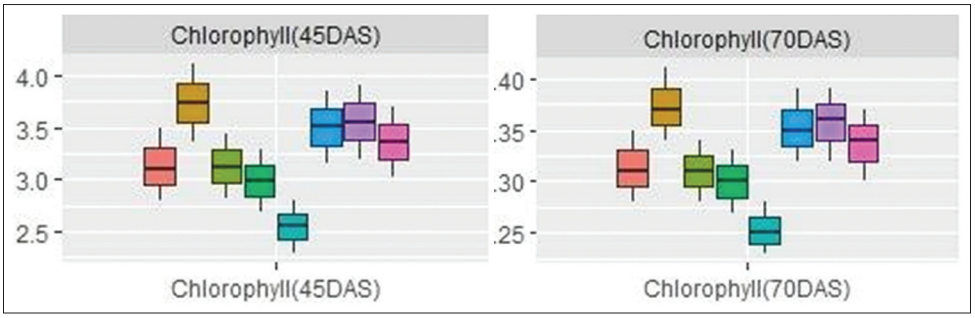


**Figure 3:** Box and whisker plot showing chlorophyll content in maize in mycorrhiza inoculated and uninoculated treatments at different levels of Cd. Box and whisker plots represent minimum, lower quartile, median, upper quartile, and maximum values.

PCA was performed using all attributes among different treatments. Out of the seven PCs, the first three axes with Eigen value of more than 1.0 evidenced that the identified attributes within the axes exhibited a significant influence among the treatments. The PCA biplot [Figure 4] showed $T_8$ and $T_5$ (75 ppm Cd with and without mycorrhizal inoculation) in the positive coordinates for antioxidants in both roots and shoots, whereas other parameters were not influenced significantly. $PC_1$ and $PC_2$ showed variances of 68.6% and 24.7%, respectively. Cd is an important abiotic stress element, which endorses the creation of ROS through the inhibition of electron transport, especially the primary events in Photo system-II [32]. It is a well-known fact that ROS would damage lipids, DNA, RNA, and proteins involved in the constructive metabolism of plant growth. Catalase is an ever-present tetrameric heme-containing enzyme that catalyzes the dismutation of

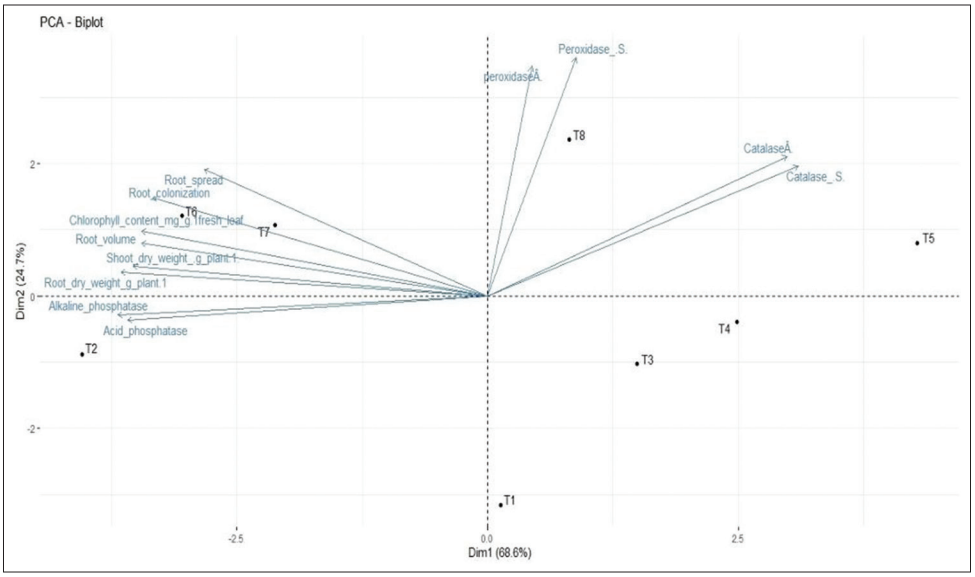


**Figure 4:** PCA biplot representing correlation of growth parameters, root colonization, and antioxidative enzymes at different levels of Cd in maize.

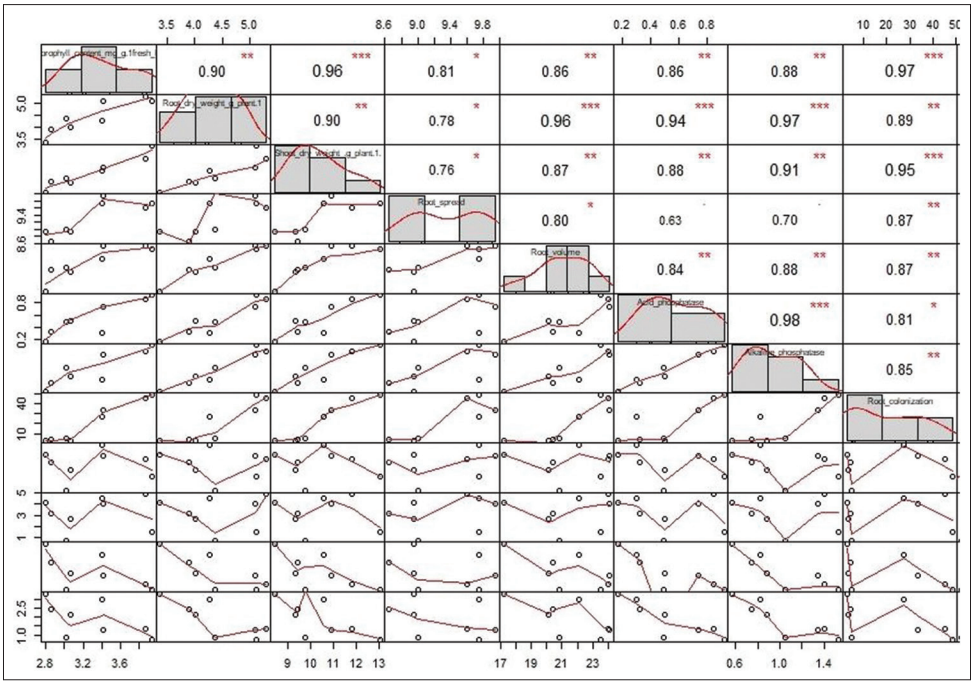


**Figure 5:** Correlogram showing the summarized data of AM colonization, growth parameters, and enzyme studies at different levels of Cd in maize.

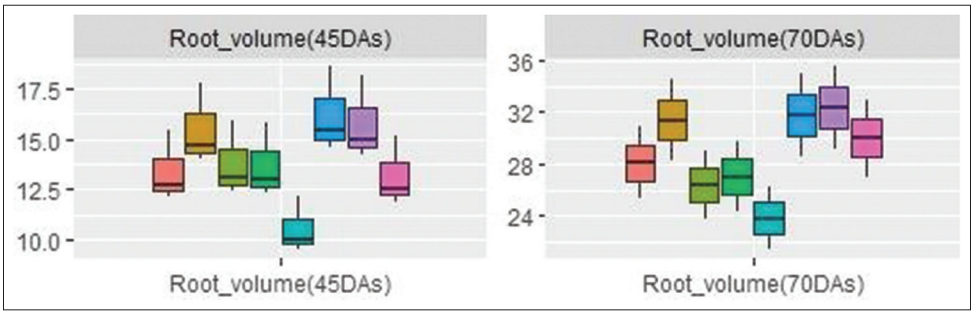


**Figure 6:** Box and whisker plot showing maize root volume in mycorrhiza inoculated and uninoculated treatments at different levels of Cd. Box and whisker plots represent minimum, lower quartile, median, upper quartile, and maximum values.

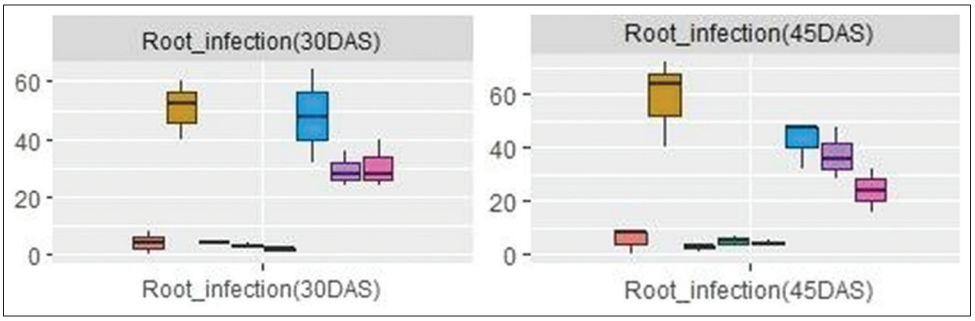


**Figure 7:** Box and whisker plot showing AM root colonization percent in maize in mycorrhiza inoculated and uninoculated treatments at different levels of Cd. Box and whisker plots represent minimum, lower quartile, median, upper quartile, and maximum values.

**Table 1**: Effect of *Glomus intraradices* inoculation on antioxidative enzymes, total phenols, and soluble proteins of maize grown in Cd-spiked soil.

| Treatments | Catalase (µg H2O2 $g^{-1}$ $min^{-1}$) | | | | Peroxidase (change in OD $min^{-1}$ $g^{-1}$) | | | | Total phenols (mg of catechol $g^{-1}$) | | | | Soluble proteins (mg $g^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Shoot | | Root | | Shoot | | Root | | Shoot | | Root | | Shoot | | Root | |
| | 45 d | 70 d | 45 d | 70 d | 45 d | 70 d | 45 d | 70 d | 45 d | 70 d | 45 d | 70 d | 45 d | 70 d | 45 d | 70 d |
| T1 | 1.27 | 2.12 | 0.65 | 0.85 | 0.09 | 0.31 | 0.61 | 0.81 | 1.38 | 1.74 | 1.40 | 1.80 | 6.81 | 10.51 | 0.56 | 1.17 |
| T2 | 0.43 | 0.70 | 0.87 | 0.72 | 1.25 | 1.44 | 1.89 | 1.10 | 1.59 | 1.80 | 1.69 | 1.83 | 9.53 | 13.68 | 0.66 | 1.54 |
| T3 | 2.12 | 2.97 | 2.55 | 2.13 | 1.73 | 2.15 | 2.19 | 3.12 | 1.79 | 2.02 | 2.25 | 2.67 | 6.62 | 10.34 | 0.45 | 1.16 |
| T4 | 2.97 | 3.40 | 4.25 | 2.45 | 3.05 | 2.21 | 3.45 | 2.89 | 1.81 | 2.14 | 2.70 | 2.80 | 5.60 | 8.01 | 0.36 | 1.08 |
| T5 | 4.25 | 6.37 | 6.80 | 3.31 | 2.88 | 3.63 | 4.87 | 3.34 | 2.40 | 2.94 | 2.95 | 2.97 | 6.01 | 8.27 | 0.28 | 0.83 |
| T6 | 1.70 | 2.12 | 1.27 | 1.34 | 2.79 | 2.97 | 6.12 | 3.67 | 1.90 | 2.40 | 3.10 | 2.80 | 10.51 | 12.39 | 0.64 | 1.88 |
| T7 | 2.40 | 2.55 | 2.97 | 1.27 | 4.19 | 2.11 | 4.15 | 4.02 | 2.45 | 3.10 | 3.46 | 3.28 | 10.82 | 13.00 | 0.56 | 1.54 |
| T8 | 3.53 | 3.82 | 5.10 | 2.97 | 4.29 | 3.88 | 4.68 | 4.31 | 2.34 | 3.00 | 2.71 | 2.92 | 5.79 | 6.81 | 0.50 | 1.71 |
| Mean | 2.37 | 3.00 | 3.05 | 1.88 | 2.53 | 2.33 | 3.50 | 2.91 | 1.96 | 2.39 | 2.53 | 2.63 | 7.71 | 10.38 | 0.50 | 1.36 |
| SEd | 0.22 | 0.28 | 0.30 | 0.17 | 0.42 | 0.37 | 0.64 | 0.51 | 0.19 | 0.23 | 0.09 | 0.26 | 0.51 | 0.87 | 0.04 | 0.12 |
| CD (0.05) | 0.46 | 0.59 | 0.64 | 0.36 | 0.89 | 0.80 | 1.35 | 1.08 | 0.40 | 0.48 | 0.20 | 0.56 | 1.08 | 1.84 | 0.09 | 0.25 |

Values represent mean of three replications, T1 – UIC, T2 – Gi seed coated, T3 – T1+25 ppm Cd, T4 – T1+50 ppm Cd, T5 – T1+75 ppm Cd, T6 – T2+25 ppm Cd, T7 – T2+50 ppm Cd, T8 – T2+75 ppm Cd. DAS – days after sowing, AMF - arbuscular mycorrhizal fungi, ROS – reactive oxygen species

two molecules of $H_2O_2$ into $H_2O$ and $O_2$, and protects the cell against membrane lipid peroxidation through ROS [33,34].

The present study shows a significant reduction in $H_2O_2$ production in roots and shoots when Gi is inoculated at both stages (45 and 75 DAS) with increasing levels of Cd in soil. This steady increase of catalase activity in mycorrhizal roots under Cd stress is due to the decreased oxidative damage to rhizosphere, because AMF plants had consistently better plant biomass production [Figure 1]. A relative increase was observed in the peroxidase activity with increasing Cd levels due to the inoculation of Gi, and inoculum of root organ culture was observed in both roots and shoots. A positive correlation between the two enzymes prevented the negative effects of ROS during increased stress levels. PCA results evidenced the activation of both CAT and peroxidase activity in roots and shoots of AM primed maize seedlings. This study revealed that stress reaction is not perceived in mycorrhizal roots grown in artificial Cd polluted soil. Such less oxidative damage in AM seedlings might be due to the AM associations, conferring better defense mechanism and higher antioxidative defense systems [35]. Sensitivity of plants to heavy metals is determined by an interrelated network of physiological and molecular mechanisms [36] such as uptake and accumulation of metals through binding to extracellular exudates and cell wall, complexation of ions inside the cell by various substances (for example, organic acids, amino acids, ferritins, phytochelatins, and metallothioneins), general biochemical stress defense responses such as induction of antioxidative enzymes, activation, or modification of plant metabolism, and rapid repair of damaged cell structures.

### 3.3. Impact on Soluble Proteins and Total Phenols

The roots were generally observed with very low soluble proteins than shoots with an overall mean value ranging from 0.50 mg $g^{-1}$ to 1.36 mg $g^{-1}$ with the maximum in $T_6$ (25 ppm Cd, AM inoculated), which was found on par with $T_2$, $T_7$, and $T_8$ (AM inoculated). Mycorrhizal Cd applied plants registered higher protein concentration than non-mycorrhizal Cd plants to the tune of 42.2–78.6% on 45 DAS and 42.5–103% on 70 DAS. Obviously, the soluble protein content was decreased more profoundly in mycorrhizal plants at high level Cd. An increase in total phenol content was recorded in all plants exposed to the heavy metal Cd, irrespective of mycorrhizal inoculation. The total phenols were recorded at 45 and 70 DAS in maize shoots and roots and were expressed as mg of catechol produced $g^{-1}$ plant tissue [Table 1]. The maximum value was recorded in $T_7$ (shoot – 3.10; root – 3.28 mg of catechol $g^{-1}$ plant tissue) during 70 DAS, where AM was inoculated at 50 ppm Cd level, which was significantly higher than $T_1$ and $T_2$. Furthermore, our study revealed a significant increase in soluble protein content in shoots and roots at both stages of observation when mycorrhiza (Gi) was treated in seeds in $T_6$ and $T_7$. However, when artificial level of contamination was increased at

75 ppm, a steep decrease in soluble protein was noticed in shoots even in mycorrhiza inoculated treatments, possibly due to the toxic negative impact of Cd on the hyphal proliferation and massive sporulation of Gi. In addition, Cd could induce DNA damage, such as single- and double-strand breaks, and modified bases that could lead to a decline in protein synthesis. Numerous studies have shown that genotoxicity of Cd is directly related to its effect on structure and function of DNA [37,38]. The total soluble protein increases as the mycorrhizal infectivity increases [27]. It is obvious to note a significant increase in total phenols at 50 ppm Cd in AM inoculated $T_7$ in comparison to uninoculated $T_4$. The phenolic compounds have the capacity of scavenging free radicals and donating hydrogen atoms. The electrons or chelate metal ions thus contribute to the antioxidant activity [39]. However, the increase in concentration of total phenols in Gi inoculated treatments might be caused by the increase in precursors of these compounds that are optimized through mycorrhizal inoculation [40]. In contrast, the profound decrease in soluble protein at high level of Cd (75 ppm) in mycorrhiza inoculated maize was due to severe reduction in intra/extra radical mycorrhizal colonization.

### 3.4. Cd Uptake in AM (+) and AM (−) Maize

In the present study, Cd was accumulated to a tune of 22.2–38.3% in mycorrhizal roots that were more than the accumulation in non-mycorrhizal roots [Table 2]. Moreover, Cd addition in mycorrhizal plants did not affect Cd accumulation in shoots, which was decreased significantly to a level of 28.0% in mycorrhizal plants amended with 25 and 50 ppm Cd. Therefore, the uptake in shoot increased even in mycorrhizal plants at higher concentrations of Cd, beyond a certain limit. TF is the ratio of metal concentration in shoot to root, which indicated the internal metal transportation [22]. TF between mycorrhizal and non-mycorrhizal treatments is apparent to show the reduced translocation of Cd in $T_5$, $T_6$, and $T_7$ with the lowest factor (0.14) at 50 ppm Cd and the highest Ti of 100.59 and 98.34 in T6 and T7, respectively. The correlogram [Figure 8] represents the relationship among dry weights, Cd content, and its uptake in maize roots and shoots at different levels of artificial Cd spiking in soil. An increase in shoot or root cadmium content causes a decrease in shoot or root dry weight (negative correlation). Even though the correlation plot shows an overall negative correlation with the total biomass of the plant, tolerance index of Cd was 100.59 in $T_6$. This represented that dry weight of the plant did not reduce due to artificial spiking of 25 ppm Cd when inoculated with AM fungi, *R. irregularis*, whereas it was positively correlated with Cd uptake in shoot, in spite of showing comparatively less values in $T_6$ over $T_3$ and in $T_7$ over $T_4$, respectively. Table 2 shows that the root dry weight was significantly increased in Gi inoculated Cd levels ($T_6$ and $T_7$) than in uninoculated Cd levels. Our study registered higher Ti in AM inoculated maize plants.

The present study primarily focused on the role of mycorrhiza in overcoming Cd stress in contaminated soil, which is possible only by its symbiotic association with the selected plant species. The bizarre bountiful extracellular hyphal branching of the root organ culture of *R. irregularis* improved the accumulation of Cd tied in AM structures surrounded by the root system and restricted its movement toward the shoot or economical part of the plant. The results warrant that AM fungi might have some other mechanisms that act as a barrier for the movement of Cd in the plant system through xylem and phloem vessels. AM produces a type of insoluble protein called glycoprotein, which binds heavy metals beyond the plant rhizosphere [41]. Passive adsorption of this protein to the hyphae leads to a binding of up to 0.5 mg Cd $g^{-1}$ dry biomass [42].

The capacity of plants to tolerate different heavy metals is indicated by the Ti [43] which is important for sinking the toxic stratum of heavy metal from the polluted soil. Our results show that mycorrhiza reduced the transport of Cd from root to shoot and it can reduce metal accumulation in the above ground parts of the plant, thus increasing the tolerance level. Mycorrhizal roots act as a blockade against Cd transport, tumbling its transfer from roots to shoots. Even if the plant tolerance to abiotic heavy metal stress is improved by its mutualistic relationship with AM fungi, the overall performance in heavy polluted soils highly depends on the efficient fungal isolate, nature of plant selected, and the type of heavy metal involved [8]. The distinct strategies involved in molecular mechanisms of plant cells include sequestration of non-essential heavy metals such as cadmium, mercury, and arsenic in root vacuoles. It involves vesicular trafficking in heavy metal detoxification [44].

**Table 2**: Bioaccumulation of cadmium in maize.

| Treatments | Root | | | Shoot | | | Translocation factor (b/a) | Tolerance indices (%) |
|---|---|---|---|---|---|---|---|---|
| | Cadmium content (µg $g^{-1}$) | Dry weight (g $pot^{-1}$)* | Cadmium uptake (mg $pot^{-1}$) [a] | Cadmium content (µg $g^{-1}$) | Dry weight (g $pot^{-1}$)* | Cadmium uptake (mg $pot^{-1}$) [b] | | |
| T1 | 0 | 12.60 | 0 | 0 | 26.20 | 0 | - | - |
| T2 | 0 | 13.00 | 0 | 0 | 27.96 | 0 | - | - |
| T3 | 14.40 | 12.20 | 0.176 | 2.10 | 25.72 | 0.054 | 0.31 | 97.73 |
| T4 | 24.50 | 12.00 | 0.294 | 3.20 | 26.00 | 0.083 | 0.28 | 97.94 |
| T5 | 29.00 | 10.26 | 0.298 | 3.70 | 23.74 | 0.088 | 0.29 | 87.63 |
| T6 | 17.60 | 13.20 | 0.232 | 1.50 | 28.00 | 0.042 | 0.18 | 100.59 |
| T7 | 33.90 | 13.04 | 0.442 | 2.30 | 27.24 | 0.063 | 0.14 | 98.34 |
| T8 | 39.00 | 12.60 | 0.491 | 3.90 | 25.74 | 0.100 | 0.19 | 93.60 |
| Mean | 28.1 | 12.37 | 0.346 | 2.78 | 26.34 | 0.071 | 0.23 | 95.97 |
| SEd | 4.91 | 0.50 | 0.076 | 0.12 | 1.08 | 0.008 | | |
| CD (0.05) | 10.69 | 1.07 | 0.165 | 0.26 | 2.29 | 0.019 | | |

Values represent mean of three replications, *dry weight of two plants per pot, T1 – UIC, T2 – Gi seed coated, T3 – T1+25 ppm Cd, T4 – T1+50 ppm Cd, T5 – T1+75 ppm Cd, T6 – T2+25 ppm Cd, T7 – T2+50 ppm Cd, T8 – T2+75 ppm Cd.

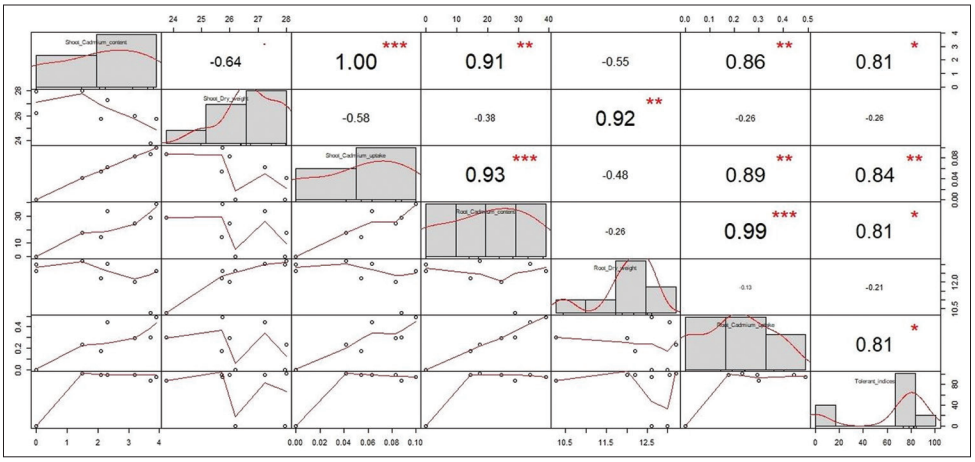


**Figure 8:** Correlation plot showing the summarized data of cadmium uptake in shoot and roots.

## 4. CONCLUSION

The present study on Cd tolerance of *R. irregularis* MUCL 41833 revealed mycorrhizal plants through seed treatment could be exploited in polluted soils. The seed coating technique is focused to evaluate the *in vitro* inoculum of *R. irregularis* developed through root organ culture. It plays a significant ecological role in the phytostabilization of Cd polluted soils through metal sequestration and translocation. Mycorrhizal plants can adapt well to Cd stress conditions at 25 ppm and tolerate up to 50 ppm reducing translocation of heavy metal from roots to shoots. This novel technique can be exploited for alleviation of other heavy metals using other hyperaccumulators.

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## 6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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## 8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## 9 ETHICAL APPROVALS

This study did not involve any animal studies.

## 10. DATA AVAILABILITY

All data are available in manuscript.

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