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ORIGINAL ARTICLE

Establishment of *Serendipita indica* colonies and enhancing the antioxidant enzyme activities within mustard plant under cadmium stress

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ABSTRACT
Cadmium (Cd) is a non-essential metal, highly hazardous ecological contaminant which leads to significant environmental and agricultural issues globally. Severe cadmium dosages are carcinogenic to human beings. The accumulation of cadmium in plant is affected by entry through the roots, sequestration in root vacuoles, translocation in the xylem and phloem. Serendipita indica is a fungal endophyte that colonizes and facilitates an extremely huge number of plants in terms of growth. The current study was performed to assess the antioxidant efficiency of Serendipita indica in Brassica juncea under cadmium (Cd) stress. The roots of 7-day-old B. juncea plant seedlings were inoculated with S. indica and grown to hazardous Cd dosages (0.5mg, 1mg, 1.5mg and 2mg $CdCl_2$). After confirmation of colonization, the seedlings were transferred into pots and tested for various enzymatic antioxidants such as ascorbate peroxidase, superoxide dismutase, glutathione reductase and catalase. Our findings revealed that plant growth was improved in S.indica inoculated Cd stressed seedlings. Overall, the level of all anti-oxidant enzymes like superoxidase dismutase, glutathione reductase, ascorbate peroxidase and catalase was boosted with S. indica inoculation as compared to non-inoculated ones. The present study may indicate that S. indica endophyte has a significant potential to mitigate the cadmium induced stress by increasing the growth and antioxidant enzyme level.
Keywords: *Serendipita indica, Antioxidant enzymes, Cadmium, Brassica juncea, Plants.*

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INTRODUCTION

Among the environmental pollutants, heavy metals (HMs) are considered the most hazardous because of their enduring and bio-accumulative characteristics; consequently, they pose a substantial threat against living organisms [4]. The harmful effects of cadmium (Cd) on plants can lead to various damages from the germination stage to the harvesting phase; nevertheless, the degree of damage is influenced by the concentration and duration of exposure [42]. The main causes of the decrease in plant development and seed germination are membrane damage and Cd interaction with enzymatic and photosynthetic processes [17]. Increased exposure to cadmium damages plants through oxidative stress and disrupts their water and nutrient relationships. Furthermore, structural modifications in the photosynthetic machinery brought on by Cd stress disrupt plant output [11]. The usage of fertilizers and insecticides including Cd compounds might generate Cd toxicity on agricultural soil. Cd replaces calcium in calmodulin, a calcium binding protein, and affects calcium-related biological activities [41]. It may interfere with mineral nutrition by preventing the intake and transport of essential minerals. Exposure to Cd causes plant cell antioxidant imbalances, resulting in the generation of species of reactive oxygen [36].

Elevated ROS level increases the level of defence-related genes and transcription factors, affecting the plant defence system (both enzymatic and non-enzymatic) [26]. Antioxidant enzymes, such as Catalase (CAT), Superoxide Dismutase (SOD), Glutathione Reductase (GR) and Ascorbate Peroxidase (APX), interact to protect the Ascorbate (Asc)-Glutathione (GSH) pool and prevent cadmium toxicity [50]. As cadmium penetrates plant cells, it negatively affects the morphological, physiological, and biochemical characteristics of plants, as well as damages their reproductive and vegetative systems [24]. Cd toxicity affects morphological parameters such as: root length, shoot length, leaf surface area and germination of various plants such as *Oryza sativa* [17] *Cicer arietinum* [10], *Glycine max* [27], *Helianthus annuus* [30], *Medicago sativa* [55] and *Nicotiana tabacum* [49]. Plant cells develop ROS in responses to Cd stress, which damage proteins, carbohydrates and DNA and eventually cause cell death [7]. Furthermore, ROS leads to the degradation of poly-unsaturated lipids in the membranes of cells, generating malondialdehyde (MDA) formation, which can be used as a bio marker to determine the extent of oxidative stress [45]. *Brassica juncea* (L.) belongs to Brassicaceae family. This family includes various species viz., Indian mustard (*B. juncea*), Brown Sarson (*B. rapa*), Yellow Sarson (*B. campestris* ssp. *Oleifera*). It is India's second largest oilseed crop after soybean. Mustard is hyper-accumulator of heavy metals like, cadmium, lead (Pb) and arsenic (As) [31]. Besides other environmental stress, agricultural fields are facing heavy metal contamination especially by Cd, Pb and As. It grows quickly, and produces a large amount of biomass even in heavy metal-polluted soils [3]. It contains great source of anti-oxidants as indoles, carotenes, flavonoids, zeaxanthin and lutein [23]. *Brassica* is a hyper-accumulator of toxic metals and its accumulation makes plant to be sensitive for metals and causes serious damage to pigments, growth and yield [29]. The *Serendipita indica* (formerly known as *Piriformospora indica*) is a root-colonizing endophytic fungus that was identified in 1992 from the roots of woody shrubs *Zizyphus nummularia* and *Prosopsis juliflora* in the Thar Desert, India [52]. In 2016, German and Italian scientists classified the fungus as *Serendipita indica* and established a new own family of descendants, namely, Serendipitaceae [53]. *Serendipita indica* belongs to the Basidiomycota division and Sebacinaceae family [54] such as arbuscular mycorrhizal fungi (AMF) invade the roots of over 200 plant species, enhancing plant development and resilience to biotic and abiotic stressors [8]. It may evolve to unfavourable circumstances including, soil nutrient deficiencies, extreme temperatures, high level of pH, and high amounts of heavy metal [56]. The fungus additionally controls stress-responsive genes and nutrient intake is to assist a host plant life under adverse conditions [19]. [43] Reported that *S.indica* enhances the plant vegetative growth such as plant height, root - shoot growth, photosynthetic pigments and phyto-hormone synthesis. *S. indica* plays a significant role in agricultural sustainability by enhancing the absorption of micronutrients, macronutrients, and water, as well as improving the nutrient content of plants, boosts various plant characteristics such as raising the amount of dry matter of roots and shoots, stem diameter, and leaf surface area [16]. Inoculating plants with *S. indica* enhances the level of photosynthetic pigments and the magnesium (Mg) absorption a key element of the chlorophyll molecule, which eventually boosts the efficiency of the plant's photosynthetic system [38]. Under various stressors, *S. indica* stimulates antioxidant enzyme system such as superoxide dismutase (SOD), Dehydroascorbate reductase (DHAR), Monodehydroascorbate reductase (MDAR), and Glutathione reductase (GR), reducing reactive oxygen species (ROS) in plants [8]. *S. indica* may also increase the levels of secondary metabolites and proline in plants as well as minimize oxidative stress produced by various stressors [18]. The present investigation studied the potential of *S. indica* to reduce cadmium induced oxidative damage in the mustard plants. We suggest that inoculated *S. indica* may strengthen mustard plants' antioxidant defence mechanism, lowering oxidative damage induced by cadmium exposure and limiting plant development under stress circumstances. The outcomes of this study may further help to understand the mechanisms by which *S. indica* defend plants from cadmium-induced oxidative damage and provide vital insights into its potential.

MATERIAL AND METHODS

Selection of plant material:

Mustard (*B.juncea* L.) plant was selected as the experimental plant. A known variety of mustard (PM-25) was procured from the Indian Agricultural Research Institute, New Delhi.

Rising of plants:

Mustard seeds were planted with ten seeds in each petri-dish as shown in Table-1. After germination, the seven day seedlings were transferred to the germinating sheets and treated with *S.indica*. The seedlings were transferred to the cadmium solution in beaker for further growth. After confirmation of root colonization, the plants were transferred into pots and tested for the biochemical assay on fifteen, thirty, and forty fifth day after treatment of $CdCl_2$.

Table 1: Different treatment groups used in the study

S.No	Cd-treatment	No. of seeds per petri-dishes
1	Control (without Cd)	10
2	0.5mg $CdCl_2$	10
3	1mg $CdCl_2$	10
4	1.5mg $CdCl_2$	10
5	2mg $CdCl_2$	10
6	Positive control (*S.indica*)	10
7	0.5mg $CdCl_2$+ *S.indica*	10
8	1mg $CdCl_2$+ *S.indica*	10
9	1.5mg $CdCl_2$+ *S.indica*	10
10	2mg $CdCl_2$+ *S.indica*	10

Inoculation of fungal spores:

Freshly prepared culture of *Serendipita indica* was inoculated into the mustard roots. About 100µl of *S.indica* culture was inoculated within the root of each plant. One week after the treatment, roots were tested for colonization following the trypan blue staining method proposed by [39].

Plants extract preparation:

Fresh leaf tissue (200mg) were homogenized with an extraction solution comprising 100 mM K-phosphate buffer (pH 7.0), 0.5% Triton X-100, and 1% polyvinylpyrrolidone (PVP) using a cooled mortar and pestle. The entire mixture was centrifuged at 15,000x g for 20 minutes at 4 °C. The supernatant was used for the biochemical assays. The enzyme mixture was used to detect ascorbate peroxidase and superoxide dismutase.

Determination of Superoxide dismutase activity:

Among the enzymatic antioxidants, SOD is an essential aspect of a plant's anti-oxidative defence system. It causes the dismutation of free radicals by the creation of H_2O_2 [46]. The superoxide dismutase activity was estimated by following [12] and [13] protocol. The absorbance of the samples was read at 560nm. The SOD activity was expressed as enzyme unit (EU) mg^{-1} protein.

Ascorbate peroxidase activity:

It was estimated by the method used by [37]. Ascorbate peroxidase activity was determined by the decrease in absorbance of ascorbate at 290nm, due to its enzymatic breakdown. APX activity was determined as $EUmg^{-1}$protein.

Catalase activity:

It was determined according to [2]. The catalase activity was evaluated by monitoring the disappearance of H_2O_2 by measuring the absorbance at 240nm and expressed as $EUmg^{-1}$protein.

Glutathione reductase:

It was determined using the method employed by [14]. The absorbance was read at 340 nm for 2 minutes. The level of GR was measured using the extinction co-efficient of NADPH of 6.2 mM^{-1} cm^{-1} and expressed as EU mg^{-l} protein.

STATISTICAL ANALYSIS:

It was conducted with Graph Pad Prism v8, using a one-way ANOVA and Tukey's test ($p < 0.05$) for multiple comparisons. The error bar represents the standard deviation (SD ≤ 0.05). The stars above the bars mark a substantial difference.

RESULTS

Antioxidant enzyme activities:

Cadmium stress caused substantial changes in mustard plants' antioxidant defence mechanism. When mustard plants endured exposed to high doses of $CdCl_2$, their antioxidant enzyme activities (SOD, APX, CAT, and GR) increased significantly ($p \leq 0.05$).

Superoxide dismutase (SOD) is a vital antioxidant enzyme that defends plants against oxidative stress by catalysing the degradation of superoxide radicals from hydrogen peroxide and molecular oxygen. The current study examined the effect of $CdCl_2$ stress on SOD activity in mustard plants, both with and without *Serendipita indica* inoculation, on the fifteen, thirty and forty fifth day of treatment (Fig: 2). SOD activity increased significantly ($p \leq 0.05$) in responses to $CdCl_2$ stress at all periods of time (fifteen, thirty, and forty fifth days), showing an effective antioxidant defence mechanism in mustard plants. Application of the 0.5mg concentration of $CdCl_2$ resulted in a significant increase in SOD level on the thirty day and forty fifth day of treatment. The increase in SOD levels following $CdCl_2$ treatment is attributed to the generation of superoxide radicals, which is responsible for activating the plant's defence mechanism. Inoculation with *Serendipita indica* resulted in a moderate boost in SOD activity at lower $CdCl_2$ doses (0.5 mg and 1 mg) compared to non-inoculated (control), implying that the fungus has an increased protective response. However, at 1.5 mg and 2 mg, SOD activity in *S. indica* inoculated plants was less than in 0.5mg + *S. indica* and 1mg + *S. indica* plants, indicating that oxidative stress may be mitigated. A similar pattern was noticed on the thirty and forty fifth days of treatment, with plants treated with 0.5 mg of $CdCl_2$ showing the most substantial increase in SOD activity in inoculated plants. At higher $CdCl_2$ concentrations, *S. indica*-inoculated plants consistently demonstrated a more balanced SOD response, indicating lower oxidative stress than non-inoculated plants.

Identification of root colonization:

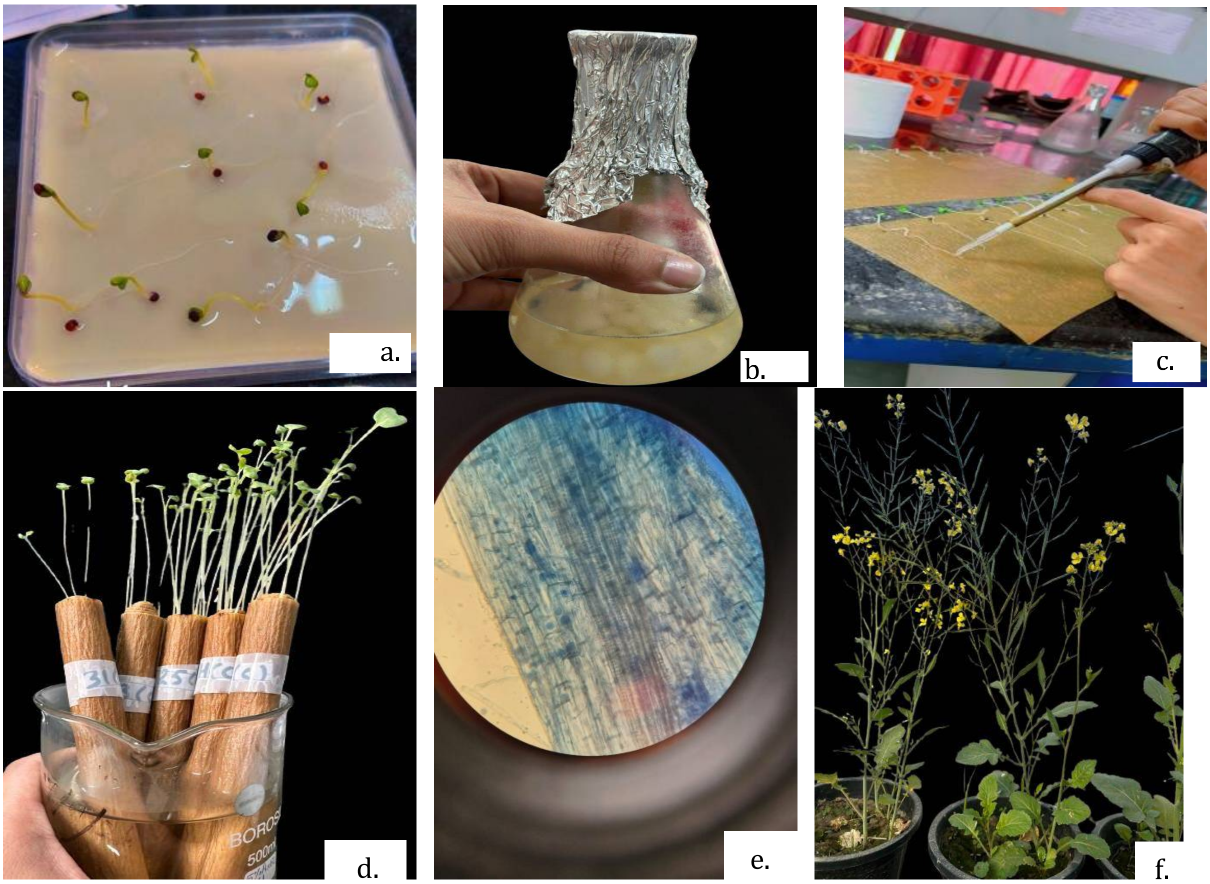

Fig. 1: Inoculation and colonization of *Serendipita indica* within mustard plant roots. A.) Healthy mustard seedlings. B.) *Serendipita indica* culture. C.) Inoculation of culture in plant roots. D.) Fungi inoculated plantlets. E.) Identification of *Serendipita indica* colonies *via*. Trypan blue staining. F.) *Serendipita indica* inoculated plants in pots.

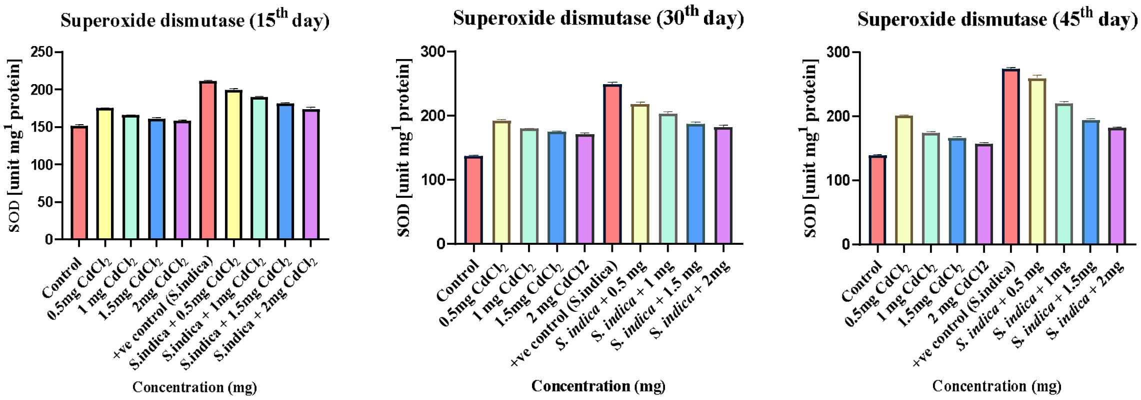

Fig. 2: The effect of $CdCl_2$ and *Serendipita indica* on root superoxide dismutase level in mustard seedlings. Each bar indicates the mean of three independent replicates and a significant difference ($p \leq 0.05$) as determined by one-way ANOVA with Least Significant Difference (LSD) and Tukey's test.

Similarly, $CdCl_2$ treatment and fungal inoculation had a substantial effect on ascorbate peroxidase (APX) activity at all intervals (fifteen, thirty, and forty fifth days), as shown in Fig. 3, expressing the impact of oxidative stress in mustard plants. APX activity raised in response to the $CdCl_2$ treatment, with its highest values found at 0.5 and 1 mg $CdCl_2$ concentration. However, reduction was observed at 1.5mg and 2mg, indicating an inability to improve antioxidant defence at high concentration of Cd. The inoculation with *Serendipita indica* increased APX activity when compared to their non-inoculated counterparts, especially at elevated $CdCl_2$ concentrations, demonstrating that the fungus reduces oxidative stress. A Similar result was found on the thirty and forty fifth days after Cd treatment, with increasing APX activity with increasing concentration of 0.5 and 1 mg $CdCl_2$ concentration, then a slight decrease was observed at 1.5mg and 2 mg $CdCl_2$.

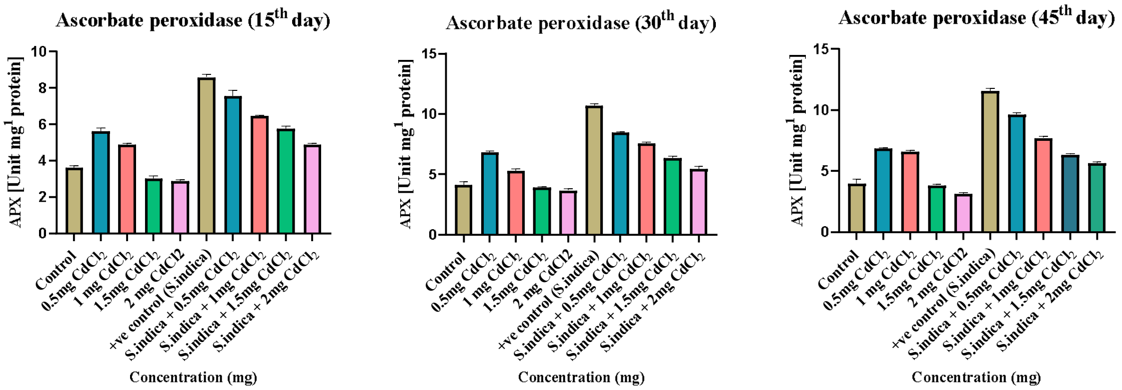

Fig. 3: The effect of $CdCl_2$ and *Serendipita indica* on root ascorbate peroxidase levels in mustard seedlings. Each bar indicates the mean of three independent replicates and a significant difference ($p \leq 0.05$) as determined by one-way ANOVA with Least Significant Difference (LSD) and Tukey's test.

Catalases are key antioxidant enzymes responsible for helping plants to withstand oxidative stress by scavenging excess stress-induced H_2O_2. Catalase level was increased enormously in response to $CdCl_2$ stress at all stages of time (fifteen, thirty, and forty fifth days) (Fig.4). Catalase levels were significantly higher in plants treated with $CdCl_2$, with a gradual decrease evident with progressive increase in concentration from 0.5mg to 2mg. The maximum catalase accumulation was seen in plants treated with 0.5mg of $CdCl_2$, showing the least stress conditions. Inoculation with *S. indica* resulted in improved catalase levels, particularly at the highest $CdCl_2$ doses (1.5mg and 2mg) as compared to non-inoculated Cd stressed seedlings. However, at 1.5mg and 2mg $CdCl_2$ concentrations, catalase level declined to a lesser extent, indicating that *S. indica* provided only partial stress mitigation under severe $CdCl_2$ exposure. A similar pattern was observed on the thirty and forty fifth day, where catalase content continued to decline with increasing $CdCl_2$ concentration. The inclusion of *S. indica* alleviates stress at all four $CdCl_2$ concentrations by reducing the need for excessive catalase accumulation.

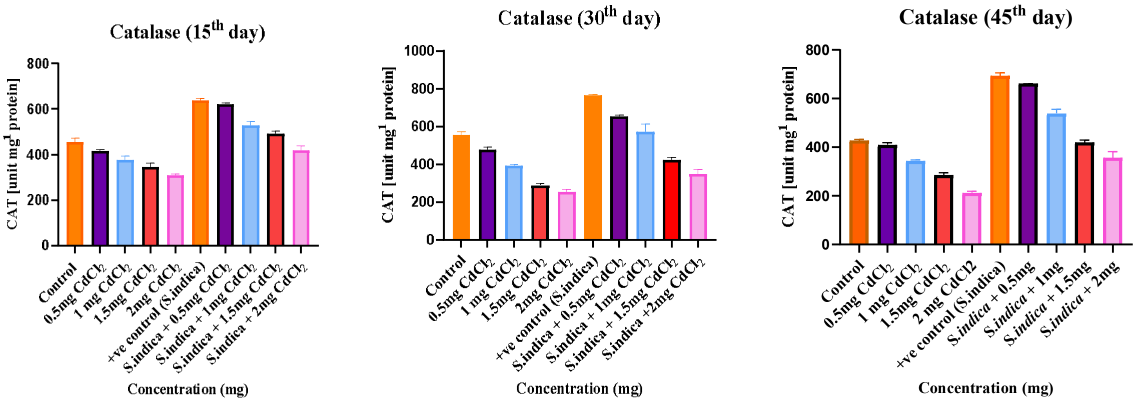

Fig. 4: The effect of $CdCl_2$ and *Serendipita indica* on root catalase levels in mustard seedlings. Each bar indicates the mean of three independent replicates and a significant difference ($p \leq 0.05$) as determined by one-way ANOVA with Least Significant Difference (LSD) and Tukey's test.

Glutathione reductase (GR) is an essential enzyme of the enzymatic antioxidant system that maintains the reduced status of GSH via the Ascorbate-Glutathione pathway, plays a significant role in the preservation of the sulfhydryl (-SH) group, and serves as a substrate for glutathione-S-transferases. The majority of GSH's metabolic actions require its oxidation to glutathione disulfide (GSSG). This enzyme exhibits varied reactions to cadmium exposure. At all-time points (fifteen, thirty, and forty five days), fungal inoculation and $CdCl_2$ treatment had a significant effect on GR activity shown in Fig: 5, reflecting the oxidative stress response in mustard plants. GR activity increased in response to $CdCl_2$ application, with the highest levels observed at 0.5 mg $CdCl_2$ concentration. However, decline was noted at 1.5mg and 2mg, suggesting an inability to further enhance antioxidant defence at excessive $CdCl_2$ levels. Inoculation with *Serendipita indica* resulted in enhanced GR activity as compared to their non-inoculated counterparts, particularly at higher $CdCl_2$ concentrations, indicating that the fungus mitigates oxidative stress. A similar pattern was observed on the thirty and forty fifth day, with GR activity increasing up to 1mg $CdCl_2$ concentration, followed by a slight decline at 1.5mg and 2mg $CdCl_2$.

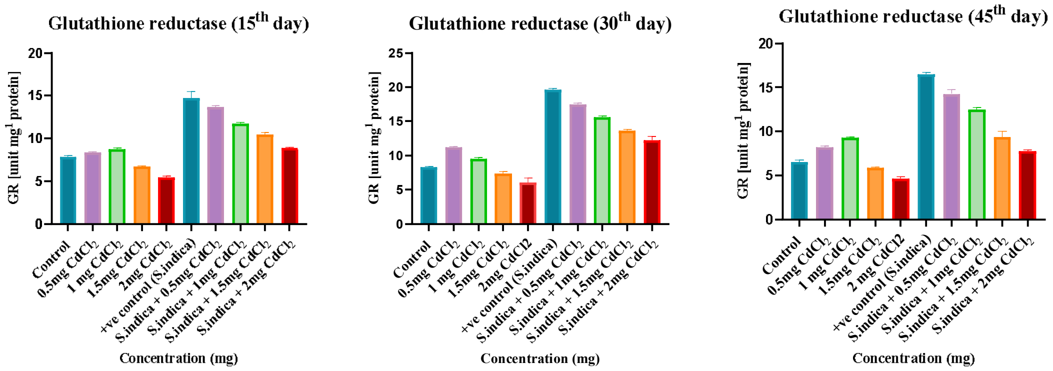

Fig. 5: The effect of $CdCl_2$ and *Serendipita indica* on root glutathione reductase levels in mustard seedlings. Each bar indicates the mean of three independent replicates and a significant difference ($p \leq 0.05$) as determined by one-way ANOVA with Least Significant Difference (LSD) and Tukey's test.

DISCUSSION

Cadmium is a very toxic heavy metal that is damaging to biological systems through absorption and accumulation in phototrophs and subsequent trophic transmission [18]. According to the Agency for Toxic Substances and Disease Registry (ATSDR) (2024), it has been classified as a seventh element amongst the top twenty most dangerous substances due to its extraordinary potential health effects. It is classified as "Group-1 Human Carcinogen" by the International Agency for Research on Cancer (IARC) 2024. Cadmium exposure showed a significant alteration in the seed germination, stunted growth and short lateral root formation in *Brassica juncea* [47]. Another study observed that cadmium impacts morphological, physiological and biochemical characteristics, suppressing growth and decreased anti-oxidant enzyme level in *B.juncea* Cd-stressed plants [57]. Our results also demonstrated that when mustard plants were subjected with different concentration of cadmium, there was a significant retardation in plants growth and anti-oxidative enzymes activity. *S. indica* improves nutrient uptake, plant growth, tolerance to abiotic stressors, and disease resistance. It colonizes plant roots and has numerous beneficial impacts on its host [29]. *S. indica* possesses phyto-promotional potential and can considerably increase the host plant's resilience to environmental stress such as Cd contaminants [44]. The previous data revealed that *S. indica* inoculation alleviates the detrimental impacts of cadmium stress in *D. kotschyi* plants, potentially prolonging their survival under stressful conditions [33]. *S. indica* increases antioxidant responses in inoculated plants, which contribute in the reduction of ROS; this might account for the reduction in H_2O_2 [17]. Previous research revealed that inoculation of *S. indica* boosts antioxidant enzyme activity in *Oryza sativa* [16] and *Nicotiana tabacum* [20]. Improving antioxidant defence systems and modulating plant hormone biosynthesis are two putative routes by which the interaction between *S. indica* and the host plant alleviates the effects of oxidative stress in Cd-stressed plants [5]. Our findings suggest that *S.indica* activates antioxidant metabolism in cadmium induced plants at various concentrations. Superoxide dismutase is an enzyme which converts O_2^- to H_2O_2, by the reaction [15] $2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$. Plants treated with Cd had increased antioxidant enzyme activity to battle the ROS assaults and increased SOD activity appears to be the first line of defence [25]. [35] Reported that abscisic acid (ABA) is responsible to induce SODs, which scavenges ROS and defend plants against imbalances in the redox state of Cd stress [48]. Our study found that Cd stress significantly reduced SOD level in *Brassica juncea.* Interestingly, *S. indica* appears to reduce the negative effects of Cd stress by enhancing oxidative defense mechanisms and increasing SOD levels in mustard plants. Ascorbate peroxidase (APX) enzymes are essential for catalysing the conversion of H_2O_2 into H_2O by employing ascorbate as a particular electron donor. This process is a primary hydrogen peroxide detoxifying system in plant cells. It has similarities with the class I heme-peroxidases; similarly, working as a CAT, [40]. Plant cells are directly protected from unfavourable environmental circumstances by the APX responses [9]. The increase in the activity of antioxidant enzymes in the previous study indicates the important role of these enzymes in scavenging free radicals formed in Cd-stressed plants. Inoculation with *S. indica* significantly increases the activity of APX [33]. Our study revealed that *S.indica* boosts ascorbate peroxidase in mustard plants under different concentration of cadmium toxicity. Catalase (CAT) enzyme is a heme-containing tetrameric enzyme that catalyses the conversion of H_2O_2 into H_2O and O_2. CAT is an effective enzyme in degrading H_2O_2 under heavy metal stress [32]. This enzyme is usually found in peroxisomes as it participates in aerobic processes such as β-oxidation of fatty acids, photorespiration, purine catabolism, and oxidative stress [11]. Antioxidant enzymes, such as CAT, deal with increased ROS generation and may decrease cell death and defend against the negative effects of Cd stress [6]. Our findings suggest that enhanced CAT activity in inoculated plants may play a significant role in ROS scavenging. Inoculation with *S. indica* increased the activity of CAT in cadmium- induced mustard plants as respective to non-inoculated ones. GR is a flavo-enzyme that catalyses the NADPH-dependent reduction of glutathione disulfide (GSSG) to glutathione (GSH). This process maintains an appropriate GSH/GSSG ratio in cells [51]. In a study, Cd effect on *Vicia faba* plants, the results showed that GR activity was decreased in response to cadmium. The negative connection of anti-oxidative enzyme activity with the concentration of Cd was shown [1]. A prior study revealed that this is clearly related to an increase in GSH production and a quick decrease of GSSG by the enzyme GR following Cd treatment. The increase in activity of GR after Cd treatment in presence of *S.indica* was reported in leaves of *Ceratophyllum demersum* [34] and *B. juncea* [22]. The study determined that *S. indica* increases the level of glutathione reductase under Cd stress and show positive effects on plant growth and development.

CONCLUSION

The study investigated the efficiency of *S. indica* inoculation against different concentration of Cd toxicity in *B. juncea* seedlings. Our analysis revealed that the expression of anti-oxidant enzymes was up-regulated in the *S.indica* inoculated plants by the exposure of cadmium chloride at lower concentrations and improves plant growth parameters under Cd stress plants. The increased tolerance level of *B. juncea* to Cd stress aided in instilling tolerance without affecting development, implying that *S. indica* can be used as a microbial partner in both phytoremediation and bioremediation processes. Keeping these things in my mind, we may suggest that *S. indica* can effectively mitigate the detrimental effects of cadmium by bolstering the plant's antioxidant defence system. This study contributes to the expanding body of knowledge about the adverse effects of cadmium and the potential of beneficial microbes to ameliorate these effects. However, this work requires field experimental validations to found more effective results as compared to pots. The findings have significant implications for sustainable agriculture, as they provide viable techniques for lowering cadmium toxicity and encouraging ecologically friendly agricultural practices.

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