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Effectiveness of Cadmium on Biochemical Shift of Pea Plant Treated with Mycorrhiza and Putrescine

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ABSTRACT

Heavy metals like cadmium (Cd), mercury (Hg), bismuth (Bi), and arsenic (As) are potent and harmful poisonous sources that cause havoc on health conditions for the population of the world. However, the response of our crop species to these potent heavy metals-related toxicity is still left to be fully understood. It is a matter of great concern, as we are heavily dependent on crop species like rice, wheat, peas, etc. Our study here aims to learn about the defensive mechanism of *Pisum sativum* L. aided with putrescine and mycorrhiza against the stress created by Cd-related toxicity. We quantified physiological parameters such as the membrane-related injury and stability index. We further measured the total free proline content, lipid peroxidation content, and SOD activity. We executed our quantitative experiments on the stressed pea plants due to the exogenously applied Cd-toxicity in the presence and absence of mycorrhiza and putrescine. Insights of our significant results will improve the understanding of readers of the role of mycorrhiza and putrescine in improvising the tolerance level of a pea plant over Cd-related toxicity.

INTRODUCTION

Cadmium metal pollution has become a major issue all over the world. Cadmium has been released into the environment as a result of its mobilization from ores and subsequent processing for various applications (Glowacka et al. 2019). As the level of industrialization rises, causing disruptions in natural biological cycles, the problem of heavy metals in soil and water pollution becomes a major threat (El-Amier et al. 2019). Heavy metals, unlike biological substances, are largely non-biodegradable and accumulate in the environment. The accumulation of heavy metals in soils and waters endangers both human health and the environment. As living organisms progress from lower trophic levels to higher trophic levels, these elements accumulate, and their concentrations increase in their body tissues (a biomagnification phenomenon). Heavy metals in soil have toxicological effects on soil microbes, which may result in a decrease in their number and activity (Khan et al. 2010). Soil fungi in terrestrial plant mycorrhizae can form a symbiotic relationship with the host plant, providing nutrients and water in exchange for carbon for survival (Kumar & Dwivedi 2014, Kumar & Dwivedi 2018a, 2018b). According to various research studies, mycorrhizae have beneficial effects on host plant growth under various stresses such as drought, salinity, heavy metal stress, and so on (Emamverdian et al. 2015). Many more mechanisms that increase fungal tolerance and host plant mechanisms under heavy metal stress must be clarified (Kumar & Dwivedi 2018a, 2018b, 2018c, Beshamgan et al. 2019). Because of the fantastic ability of mycorrhizae in the morphological and physiological developing mechanism, the fungal mycorrhizal has improved effects on plant growth as well as on the environment under heavy metal stress (Kumar et al. 2016 a, b, Alzahrani & Raddy 2019). Heavy metal stress is one of the most important stresses that harm plant growth and the environment due to its high toxicity (Nahar et al. 2016, Kumar et al. 2018a, 2018b, 2018c, 2018d). The biological mechanism is one of the most important methods for mitigating heavy metal stress, in which soil microbes such as Arbuscular Mycorrhizal, endophytic bacteria, and plant growth-promoting rhizobacteria are used for mitigating the hostile effects of heavy metal stress on plant growth as well as in maintaining the environment (Pathak et al. 2017, Taie et al. 2019). The various bioremediation details

mechanisms in which fungal association with the host flora has the expression of stress genes, glomalin production, and the phylogeny of fungal in different parts of the mycorrhizal plant under heavy metal toxicity (Soudek et al. 2016). The presence of cadmium harms plants growth and the environment (Abdelhameed & Metwally 2019). Different microbes in the soil are the most effective tool for increasing plant efficiency and improving environmental conditions (Cui et al. 2019). Microbes such as mycorrhizae, endophytic bacteria, and plant growth-promoting rhizobacteria increase plant growth as well as environmental properties under various stresses (Glick 2003). Nowadays, heavy metal is a serious stress, in addition to all other stresses that reduce plant efficiency and the environment. Based on their density (>5 $g.cm^{-3}$), the 53 elements are classified as heavy metals in the periodic table Holleman and Wiberg (1985). The primary function of the essential heavy metal is enzymatic catabolization, electron transfer, and DNA and RNA metabolism (Zenk 1996, Jamal et al. 2002). Heavy metals such as lead, arsenic, chromium, and others are sources of contamination that have negative effects on plants and the environment. The high concentration of heavy metals has an impact on plant metabolism, soil microbes, ecosystems, and the food chain (Opik et al. 2006, Friedlova 2010). The main source of heavy metal concentration in the environment is the increasing rate of industrialization and urbanization, as well as improper waste disposal. Excess herbicide use, fertilization, and sludge are all anthropogenic activities that contribute to heavy metal contamination in the environment. Still, the mining process is the most common source of trace elements (Whitmore 2006). If the concentration of heavy metals exceeds their permissible limits, it has negative effects on microbial activities as well as in plants and has an impact on the environment (Miransari 2011). The presence of a high concentration of heavy metal alters the structure of enzymes, protein structure, and essential element substitutes. The structure of the plasma membrane is also altered, reducing its permeability and functionality. Cadmium (Cd), for example, is a harmful and severely toxic heavy metal; even at levels as low as 0.1 mg to 0.2 mg, Cd is ranked seventh in the world's crust as the most toxic element (Kumar et al. 2011a, 2011b). Many anthropogenic practices, such as the use of sewage water for agriculture and continuous phosphorus processing, increased the amount of cadmium in farm soil (Tai et al. 2016). Cadmium toxicity is also increasing in the agricultural sector as a result of the constant use of phosphorus (Kumar et al. 2013). Cadmium is bioavailable and, as a metalorganic complex, easily transported within plants. Cadmium competed for absorption sites, preventing plants from absorbing phosphorus and other mineral nutrients. Several studies concluded that mycorrhizal fungi affect the growth and development of plants grown in heavy metal-contaminated waste sites (Kumar et al. 2018a, 2018b). Mycoremediation is a sub-remediation technique under Bioremediation. Mycoremediation is composed of two words: Myco and Remediation, where Myco means fungus and remediation means to cure. Various fungi are used in the Mycoremediation process to clean the environment of pollution and other toxic effluents (Miransari 2011, 2016, Miransari et al. 2009). Various studies have explained the calming effect of Arbuscular Mycorrhiza (AM) on various forms of stress, such as heat, salinity, and heavy metals. Any of these pathways demonstrates how fungi mitigate the negative effects of stress on plant growth and the surrounding ecosystem: (1) an increase in nutrient and water consumption, (2) an increase in plant hormonal output, (3) other microbial interactions on the soil, (4) an increase in sodium absorption of heavy metals, and (5) modification of the roots of fungal-based plants (Miransari 2011, Kumar 2018).

Putrescine (Put) is a polyamine (PA) that influences the growth and development of plants, especially stress responses, as well as apoptosis and programmed death in plants (Kumar et al. 2012, Kumar et al. 2019). At optimal and cellular pH levels, PAs are small, positively charged aliphatic amines that bind negatively charged molecules such as reactive oxygen species and provide the shielding impact in the cell (Kumar & Dwivedi 2018, Kumar et al. 2018a, 2018b, 2018c). Given the history of heavy metal contamination in medicinal plants, there is an urgent need for systematic control of toxic heavy metals in plants used as economic crops (Kumar & Pathak 2019).

Our systematic approach to understanding the defense mechanism of *Pisum sativum* L. against Cd-toxicity aimed to learn a few key inherent physiological factors that are traceable in a stressed pea plant. Plants have a well-established tolerance mechanism of antioxidant enzyme systems that can tolerate the potential toxicity of ROS to cope with ROS-induced oxidative stress. An antioxidant defense system involving the sequential and simultaneous action of several enzymes is one of the protection mechanisms adopted by plants. Those noticeable factors are total free proline, membrane stability index, membrane injury index, lipid peroxidation, and Superoxide dismutase activity. We discussed our significant findings regarding these key factors in the presence and absence of mycorrhiza and putrescine, which works. We established here in favor of improvising tolerance level for pea plant.

MATERIALS AND METHODS

The pot experiment was performed with a genotype of a pea, Arkel, in our well-designed shared polyhouse. Disease-free

and healthy, bold seeds of Pea and Arkel were obtained from the Punjab Agriculture University, Ludhiana. The endomycorrhiza *Glomus mosseae* was obtained from the Tata Energical Research Institute, New Delhi. The cadmium in the form of cadmium nitrate ($Cd(NO_3)_2$) was obtained from the LPU block 26-401 laboratory and purchased from Merck Life Science, a vibrant science technology company. Similarly, Putrescinehas have been obtained from the LPU block 26-401 laboratory of Crop Physiology.

A few seeds of *Pisum sativum* L. were sowed in each pot containing enriched soil with a capacity of 14 kg. Targeted pots were inoculated with Endomycorrhiza *Glomus* species, and followed by heavy metal stress in the plant was created by exogenously applied cadmium nitrate in the soil of those pots. One best concentration of cadmium nitrate based on initial screening was selected, i.e., 100 ppm per pot of soil. Furthermore, putrescine was added to a foliar sample at an interval of 15 days at an optimized concentration of 10 ppm. The temporal observations were made after sowing seeds at three stages such as 30, 60, and 90 days after sowing (DAS). Ten treatments, including a control, were executed. For each treatment, three replicas were carried out. The detailed plan of treatments was: T1= Control; T2: Cadmium nitrate (100ppm); T3: Control + Mycorrhiza (*Glomus* sp., AMF); T4: Control + Putrescine (5 ppm); T5: Control + Putrescine (10 ppm); T6: Cadmium nitrate (100 ppm) + Putrescine (5ppm); T7: Cadmium nitrate (100 ppm) + Putrescine (10ppm); T8: Cadmium nitrate (100 ppm) + Mycorrhiza (*Glomus* sp., AMF); T9: Cadmium nitrate + Mycorrhiza (*Glomus* sp., AMF) + Putrescine (5 ppm); T10 : Cadmium nitrate (100 ppm) + Mycorrhiza (*Glomus* sp., AMF) + Putrescine (10 ppm).

Estimation of Free Proline ($\mu g.mL^{-1}$)

The estimation of free proline was measured as suggested by Bates et al. (1973). In short, tissue was removed with sulphosalicylic acid, and proteins were precipitated as a protein-sulphosalicylic acid system. Under the acidic environment, the extracted proline was allowed to react with ninhydrin to create a red color. The 100 mg plant test content was homogenized with mortar and pestle in 10 mL of sulphosalicylic acid (3%). The mixture was centrifuged at 6000 rpm for 10 min, and the supernatant was collected. 2.0 mL of the extract was mixed with 2 mL of glacial acetic acid and ninhydrin reagent in the test tube. The reaction mixture was kept for ~30 min in a water bath at 100°C until the brick red color developed, and then it was allowed to cool down. 5 mL of toluene was added to this reaction mixture, and afterward, it was transferred to the separation funnel. The absorbance of the separated content was shown at 520 nm using a toluene-free spectrophotometer. Proline (10 mg) was dissolved in 3% aqueous sulphosalicylic acid and finally diluted up to 100 mL afterward. The aliquots of 0.2, 0.4, 0.6, 0.8, and 1.0 mL were taken into various test tubes for the spectra measurement, and the volume increased to make up to 2 mL by adding 3% aqueous sulphosalicylic acid.

Estimation of Membrane Stability Index and Injury Index (%)

Membrane stability index (MSI) and membrane injury index (MII) were quantified using the methodology defined by Premchandra et al. (1990). Solute leakage (electrolyte leakage) assessment from cells and the measurement of MSI can indirectly help us to measure the damage to the membrane. The stimulating effect of stress on the leakage of electrolytes could be attributed to plasma membrane injury, too. The MSI and MII were measured by putting 200 mg of leaves in two sets of 10 mL of double-distilled water. One set was heated in a warm water bath at 40°C for 30 min and measured electrical conductivity (C1). The second set was boiled at 100°C for 10 min in a boiling water bath, and the electrical conductivity (C2) was measured. The electrical conductivity in both cases was measured using a conductivity meter (ME977-C, Max Electronics, India). The equations for the measurement of MSI and MII are mentioned below;

$$MSI = 100\left[1 - \frac{C1}{C2}\right]$$

$$MII = 100\left[\frac{C1}{C2}\right]$$

Estimation of Lipid Peroxidation [Malondialdehyde (MDA) Content]

Heath and Packer (1968) originally developed the estimation of Lipid Peroxidation. By measuring the amount of malondialdehyde (MDA) content, lipid peroxidation was estimated. A 5 mL solution of 5% trichloroacetic acid (TCA) was applied to decrease the volume of collected and processed leaf tissues into small pieces. The homogenates were then moved to fresh tubes, which were centrifuged for 15 min at 12,000 rpm at room temperature. Applied in a 20% TCA solution, the same amounts of supernatant, and 0.5% thiobarbituric acid (TBA) to a fresh tube and boiled at 100°C for 25 min. The tubes were placed in the ice bath and then centrifuged for 5 minutes at 10,000 rpm at room temperature. The supernatant absorption was measured at 532 nm, and the absorption was removed at 600 nm for non-specific turbidity, while 0.5% TBA was used blank in a 20 percent solution for TCA. The sum of the MDA-TBA complex (red pigment) was measured as 155 M^{-1} cm^{-1} from the extinction coefficient. Measurements were taken from

the values of MDA material. The results were reported as MDA g^{-1} fresh weight (FW) µmoles.

Estimation of Superoxide Dismutase (SOD) Activity

Superoxide dismutase (SOD) enzyme activity was calculated as exactly described in Dhindsa et al. (1981). SOD's assay is based on inhibiting EDTA, L-methionine, and nitro-blue tetrazolium formazan formation. Leaf extracts (100 mg) are homogenized with an extraction buffer of 5 mL (0.1 M phosphate buffers, 0.5 mM EDTA pH 7.5). In a cooling centrifuge (REMI, C-24), the homogenate was centrifuged at 10,000 g for 10 min. The supernatant was collected after centrifugation, and this supernatant was used as a source of enzymes. The sample pipelines for each particular enzyme contain three mL of a reaction mixture, which should be 0.1 M of 1.5 M sodium carbonate, 0.2 mL of 200 mm methionine, 0.1 mL of 2.25 mM NBT, 0.1 metric mL of 3 mm EDTA, 1.5 mL of 100 mm potassium phosphate solution, 1 ml of distilled water and 0.1 mL of enzyme extract. Two tubes have been taken as a monitor without removing the enzyme. The reaction was initiated by adding 0.1 mL of riboflavin (60 µM) to all test tube sets and placing the 2 tube sets (one in which enzyme was added and the other in which enzyme was not added) below a light source of two 15 min fluorescent lamps. A black cloth switching off the light and covering the tube sets halted the reaction. The set of tubes developed the maximum color without an enzyme extract. A non-irradiated set in which a light source was not supplied, but enzyme extract was kept in the dark, and therefore, color did not develop and served as blank. Using a spectrophotometer (Elico, SL 196), the absorption of all test tube sets was measured at 560 nm. Enzyme unit (EU) was calculated as per the formula given below:

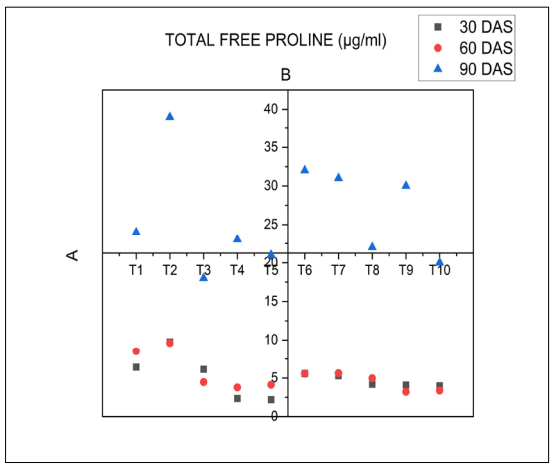

Fig. 1: Scatter central plot of Total Stability Index.

EU = Absorbance without enzyme in light - (Absorbance with an enzyme in light - Absorbance in Dark)/Absorbance without enzyme in light/2

The EU was expressed on a per gm fresh weight basis as well as based on per mg protein (specific activity).

Statistical Analysis

The mean values were calculated from three replicates. The data were analyzed statistically by applying the technique of analysis of variance for a completely randomized design and significance to be tested by using the Duncan Multiple Range Test (DMRT) with the help of Statistical Package for the Social Sciences (SPSS). Critical difference for treatments means-tested at a 5% level of significance.

RESULTS AND DISCUSSION

Estimation of Total Free Proline

In Arkel, a pea variety, under cadmium imparted stress, the total free proline [EU g^{-1} Fresh Weight] was measured (Table 1, Fig. 1). The average total free proline content was observed to increase significantly from 34.28% to 11.52%, and 38.46% at intervals of 30, 60, and 90 days after sowed (DAS) respectively when the pea plant was subjected to heavy metal-related stress applied in 2nd set of experimental condition (T2) relative to the controlled experiment (T1) (Table 1, Fig. 1). Exogenous application of endomycorrhiza in soil (T3) demonstrated the mitigating impact by reducing

Table 1: Total free proline of pea after different treatments.

Treatments	30DAS	60DAS	90DAS
T1	$6.42^{b} \pm 0.13$	$8.52^{b} \pm 0.23$	$24^{d} \pm 0.39$
T2	$9.77^{a} \pm 0.11$	$9.63^{a} \pm 0.09$	$39^{a} \pm 0.48$
T3	$6.14^{b} \pm 0.03$	$4.47^{de} \pm 0.28$	$18^{h} \pm 0.09$
T4	$2.34^{e} \pm 0.10$	$3.78^{fg} \pm 0.03$	$23^{de} \pm 0.07$
T5	$2.19^{e} \pm 0.11$	$4.12^{ef} \pm 0.09$	$21^{fg} \pm 0.13$
T6	$5.58^{c} \pm 0.17$	$5.57^{c} \pm 0.16$	$32^{b} \pm 0.17$
T7	$5.29^{c} \pm 0.05$	$5.63^{c} \pm 0.07$	$31^{bc} \pm 0.28$
T8	$4.18^{d} \pm 0.10$	$4.97^{d} \pm 0.15$	$22^{ef} \pm 0.06$
T9	$4.09^{d} \pm 0.05$	$3.21^{h} \pm 0.10$	$30^{c} \pm 0.74$
T10	$4.01^{d} \pm 0.09$	$3.38^{gh} \pm 0.04$	$20^{g} \pm 0.42$

Note: DAS = Days after sowing. Data are in the form of mean± SEM. Significance at $P \leq 0.05$ using SPSS ver. 22. T1= Control; T2: Cadmium nitrate (100ppm); T3: Control + Mycorrhiza (*Glomus* sp., AMF); T4: Control + Putrescine (5 ppm); T5: Control + Putrescine (10 ppm); T6: Cadmium nitrate (100 ppm) + Putrescine (5ppm); T7: Cadmium nitrate (100 ppm) + Putrescine (10ppm); T8: Cadmium nitrate (100 ppm) + Mycorrhiza (*Glomus* sp., AMF); T9: Cadmium nitrate + Mycorrhiza (*Glomus* sp., AMF) + Putrescine (5 ppm); T10 : Cadmium nitrate (100 ppm) + Mycorrhiza (*Glomus* sp., AMF) + Putrescine (10 ppm)

the total free proline content by 37.15%, 53.58% and 53.84% relative to the condition of T2 at 30, 60 and 90 DAS, respectively (Table 1, Fig. 1). When it was compared to T2, the exogenous application of putrescine (T6) showed a mitigating effect by decreasing total free proline by 42.88%, 42.15%, and 17.94% on proposed DAS, respectively. Significantly, the average total free proline content was noticed a reduction by 77.58%, 57.21%, and 46.15% at 30, 60 and 90 DAS, respectively, when a higher dose of putrescine (T5) was applied compared to the experimental condition of T2 (Table 1, Fig. 1). Similarly, the total free proline output was observed with a substantial decline of 45.85%, 41.53%, and 20.51% at the proposed DAS when we compared the data between T7 and T2. Moreover, the average total free proline content was noticed to decrease significantly by 57.21%, 48.39%, and 43.58% in comparison to that of T2 when we administered a higher dose of putrescine in T8 (Table 1, Fig. 1). Interestingly, the combination of putrescine and mycorrhiza in the treatment of T9 showed a decreasing level of the average total free proline content by 58.13%, 66.66% and 23.07% at 30, 60, and 90 DAS treatment, respectively. The substantial total free proline content was decreased by 58.95%, 64.90%, and 48.71% in T10, concerning T2, respectively. The total free proline content in the treatment of T4 was observed to decrease significantly at the suggested DAS by 76.04%, 60.74%, and 41.02%, respectively, in comparison to that of T2. The putrescine impact (5ppm) was more active at 30 DAS, and the mycorrhiza + putrescine (5ppm) was more successful at 60 DAS. Therefore, it is clear that proline synthesis in the plant may be a result of any kind of injury, either through heavy metal. Biosynthesis of proline accelerated when the plants are treated with toxic heavy metal content. Exposure to cadmium causes toxicity within the plant (a kind of stress); consequently, proline is synthesized and helps to protect the plant. The external application of putrescine with 5 ppm was found to be a potent mitigating agent to combat the effect of cadmium toxicity (Zengia & Munzuroglu 2005). Mycorrhiza may reduce the transfer of heavy metals to the target plant or the end of the aerial component from roots. The resistance to heavy metals improves with enhanced nutritional intake and P. The roots of the colonized target plant may store heavy metals in the root and the fungal hyphae, preventing them from spreading to the plant tip or aerial portion. A ceasing movement of heavier metals in the fungal hyphae emerges from the very critical process of the fungus that protects the main plant from the bad impact of heavy metals. The vesicles, arbuscular, and vacuoles may be the source of the aggregation of heavy metals. It prevents the entry of cadmium into the plants. The research has shown the rise in the concentration of Cu in the vacuoles of spores and the accumulation of Cd vacuoles of the hyphae of *Glomus intraradices* under the stress of heavy metals. This type of storage structure can develop tolerance towards the stress in the target plant fungi. The cell wall of the fungi is a perfect site for heavy metal binding as it has carboxyl, hydroxyl, and free amino acids for heavy metal absorption. The appearance of the cell wall and thickness can observe this type of absorption ability. As an example, the species of fungus, which belongs to the family Glomeraceae, does not possess thick hyphae because their diameter lies between the range of 0.8 to 4.5 µm and the hyphae cell wall is equal to 1.2 µmto the maximum (Kumar & Dwivedi 2018a, 2018b, 2018c).

Membrane Stability Index [MSI] [%]

In pea variety Arkel under cadmium stress, the impact of polyamine (putrescine), mycorrhiza, and their combination on MSI (%) was examined. Table 2 documented the MSI.

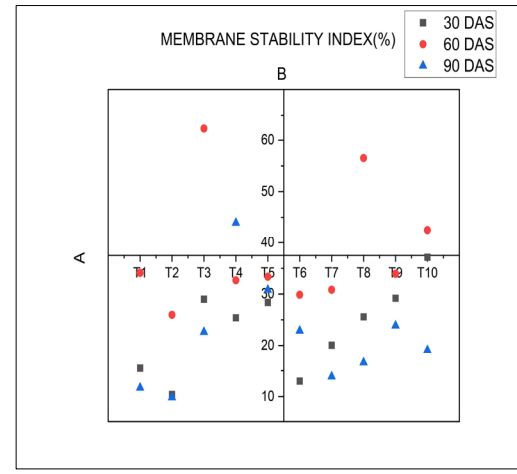

Fig. 2: Scatter central plot of Membrane Free Proline.

Table 2: Membrane Stability Index of pea after different treatments.

Treatments	30DAS	60DAS	90DAS
T1	$15.49^{e} \pm 0.40$	$34.1^{d} \pm 0.41$	$11.71^{fg} \pm 0.38$
T2	$10.41^{g} \pm 1.18$	$25.97^{g} \pm 0.48$	$9.85^{g} \pm 0.80$
T3	$28.98^{b} \pm 0.16$	$62.24^{a} \pm 0.95$	$22.63^{c} \pm 0.46$
T4	$25.4^{c} \pm 0.21$	$32.66^{de} \pm 0.29$	$43.96^{a} \pm 0.37$
T5	$28.35^{b} \pm 0.46$	$33.34^{d} \pm 0.511$	$30.80^{b} \pm 1.27$
T6	$13.01^{f} \pm 0.34$	$29.84^{f} \pm 0.04$	$22.88^{c} \pm 0.70$
T7	$20.08^{d} \pm 1.86$	$30.83^{ef} \pm 0.53$	$13.87^{f} \pm 0.54$
T8	$25.58^{b} \pm 0.38$	$56.52^{b} \pm 1.25$	$16.57^{e} \pm 1.41$
T9	$29.19^{b} \pm 0.16$	$33.91^{d} \pm 0.47$	$23.88^{c} \pm 0.69$
T10	$37.13^{a} \pm 0.31$	$42.54^{c} \pm 0.61$	$19.1^{d} \pm 0.46$

Note: Symbols as per Table 1.

It is clear that, when subjected to heavy metal stress (T2) at 30, 60 and 90 DAS intervals, the mean MSI decreased significantly by 32.79%, 23.84%, and 15.88% relative to control (T1) (Table 2, Fig. 2). Exogenous application of endomycorrhiza in soil (T3) shows the mitigation impact by growing MSI by 64.07%, 58.27%, and 56.47% relative to T2 at 30, 60, and 90 DAS (Table 2, Fig. 2). The exogenous application of putrescine (T6) shows the increasing MSI by 19.98%, 12.96%, and 56.94% at 30, 60, and 90 DAS. Similarly, when T5 was compared with T2, the mean MSI increased significantly by 63.28%, 22.10%, and 68.02% (Table 2, Fig. 2). Likewise, the MSI increased significantly to 48.15%, 15.76%, and 28.98% at the suggested DAS (T7) (Table 2, Fig. 2). The average MSI increased significantly compared to T8 by 63.57%, 54.05%, and 40.55% when treated with a higher dose of putrescine compared to T2. Combining putrescine and mycorrhiza in treatment, T9 significantly increases the MSI at the planned DAS by 64.33%, 23.41%, and 58.75%, respectively. Compared with T2, T10 improved substantial MSI by 71.96%, 38.96%, and 48.42%, respectively. The MSI was observed to rise significantly in T4 by 59.01%, 20.48%, and 77.59%, respectively, relative to T2 in the original DAS (Table 2, Fig. 2). Therefore, putrescine's effect on 30 DAS at 10 ppm + mycorrhiza, just mycorrhiza at 60 and 90 DAS, Putrescine (5 ppm) shows the best-mitigating impact in improved MSI words. AMF increases anti-oxidant activity and has often helped reduce exposure to oxidative stress. The thickness of the hyphae of fungus from the family of Gigasporaceae is higher than 20 µm of the average diameter. The research work has shown that a greater amount of heavy metals can be maintained by the thinner hyphae if compared with the hyphae with a greater thickness. This is due to the greater surface area in the thinner hyphae. Research work has shown that in mycorrhiza plants, the cortex of the root retains a much greater amount of heavy metals, which include Fe, Zn, and Niand. Various species of mycorrhizal fungus also have the variable ability to absorb heavy metals (Cabral et al. 2015). The important mechanism that can convert mycorrhizal plants into heavy metal-absorbing plants is the larger production of glutathione-derived peptides and phytochelatins; only higher plants create these. As a result, these plants will have the ability to chelate metalloids and heavy metals (Garg & Pandey 2015)

From all the parameters that help in determining the mitigating effects of the heavy metal stress of mycorrhizal fungi, the ability of AM fungus to mitigate heavy metals from the soils that are polluted is unique. Some of the mycorrhizal fungi can be better than other species of fungus when compared (Kumar et al. 2018d). Heavy metal allocation to various parts of the target plant by AM fungi also varies because some heavy metals can be accumulated at the aerial parts while some are at the roots of the plant (Kumar et al. 2018).

Membrane Injury Index [MII] [%]

It is evident (Table 3, Fig. 3) that when exposed to heavy metal stress (T2) at 30, 60, and 90 DAS intervals, the average MII was significantly increased by 5.67%, 10.98%, and 2.06% compared to the control (T1). Exogenous application of endomycorrhiza in soil (T3) demonstrated the impact of mitigation by reducing the MII by 20.72%, 48.99%, and 14.17% relative to T2 at 30, 60 and 90 DAS. Relative to T2, putrescine (T6) exogenous application had a beneficial impact on potential DAS by reducing MII by 2.90%, 5.22%, and 14.45% (Table 3, Fig. 3). The average MII was significantly reduced when treated with a higher dose of putrescine (T5) compared to T2 by 20.02%, 9.95%, and 23.24%. Similarly, the MII declined dramatically to

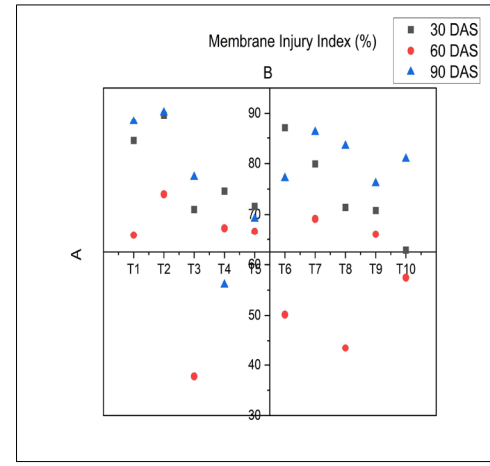

Fig. 3: Scatter central plot of Membrane Injury Index.

Table 3: Membrane Injury Index of pea after different treatments.

Treatments	30DAS	60DAS	90DAS
T1	$84.51^{c} \pm 0.40$	$65.9^{ab} \pm 0.41$	$88.29^{ab} \pm 0.38$
T2	$89.59^{a} \pm 1.19$	$74^{a} \pm 0.48$	$90.15^{a} \pm 0.80$
T3	$71.02^{f} \pm 0.17$	$37.76^{c} \pm 0.98$	$77.37^{e} \pm 0.47$
T4	$74.6^{e} \pm 0.21$	$67.34^{ab} \pm 0.29$	$56.04^{g} \pm 0.38$
T5	$71.65^{f} \pm 0.45$	$66.66^{ab} \pm 0.48$	$69.19^{f} \pm 1.21$
T6	$86.99^{b} \pm 0.35$	$50.16^{bc} \pm 16.85$	$77.12^{e} \pm 0.74$
T7	$79.92^{d} \pm 1.91$	$69.17^{ab} \pm 0.54$	$86.13^{b} \pm 0.54$
T8	$71.42^{f} \pm 0.39$	$43.48^{c} \pm 1.22$	$83.43^{c} \pm 1.43$
T9	$70.81^{f} \pm 0.17$	$66.09^{ab} \pm 0.47$	$76.12^{e} \pm 0.70$
T10	$62.87^{g} \pm 0.32$	$57.45^{abc} \pm 0.59$	$80.9^{d} \pm 0.47$

Note: Symbols as per Table 1.

10.79%, 6.56%, and 4.45% at the suggested DAS in T7 (Table 3, Fig. 3). The median MII decreased dramatically when administered with a higher dose of putrescine compared to T8 by 20.28%, 41.26%, and 7.45% relative to T2 (Table 3, Fig. 3). Combining putrescine and mycorrhiza in treatment T9 by decreasing MII at the proposed DAS by 20.96%, 10.72%, and 15.56%(Table 3, Fig. 3). The T10was compared with the T2 treatment, and significant MII decreased by 29.82%, 22.39%, and 10.26%, respectively (Table 3, Fig. 3). The MII was observed to decrease significantly in this therapy (T4) by 16.73%, 9.03%, and 37.83%, respectively, relative to T2 in the original DAS (Table 3, Fig. 3). In terms of enhanced MII, the effect of putrescine at 10 ppm + mycorrhiza on 30 DAS, only mycorrhiza at 60 and 90 DAS, Putrescine (5 ppm) shows the best mitigating effect. Some fungal species are not tolerant under heavy metal stress. Still, one of the most important fungal species, named Glomeraceae, is the dominant species, which can tolerate and be used in the inoculation of the host plant under heavy stresses due to some special morphological as well as physiological potential of fungal species having the fast growth to produce the spores (Lenoir et al. 2016). In the contaminated soil, the fungal can prevent the plants from absorbing the heavy metal as well as by restricting the interaction of host plants with heavy metals. The fungal grows even in contaminated soil and can have a positive interaction with terrestrial plants, which are used for bioremediation (Cabaral et al. 2015). When the stress conditions, germination spore stages, colonization, hyphal growth, and sporulation are harmfully affected by the stress conditions, it results in the reduction of fungi as well as the growth of roots of host plants (Zhu et al. 2012). Under heavy metal stress, there is the production of reactive oxygen species hydrogen peroxide, radical of hydroxyl, and superoxide anion radicals due to the induction of activities of antioxidants, which affects the cellular structure and their activities (Apel & Hirt 2004, Fobert & Despres 2005). From the different research, it was reported that the Glomus species are the best and able to mitigate the effect of various stresses like salinity as well as drought (Sanchez-Castro et al. 2012). The fungal growth is very fast, and they colonize rapidly around the roots of host plants and provide resistance to the host plant to withstand the heavy metal stress (Mohammad et al. 2003). The growing pollutants harm the lipids of the mycorrhizal fungi membrane, which is essential for the establishment of symbiotic relations with host plants (Calonne et al. 2012).

Under the stress of copper, fungi overexpressed glomalin production, and fungi were protected from the negative effect of arsenic and helped to fix the proteins of fungus (Ferrol et al. 2009).

Under the conditions of stress, growing the mycorrhizal pant by indigenous isolation was an appropriate method and producing stress-tolerant fungi in stressful ecosystems such as toxic areas and saline stress conditions. The fungi increase the environmental quality and act as bioindicators as well as help transfer pollutants by making an interface between soil and plants (Lenoir et al. 2016). In detail, these mechanisms of mycorrhizal fungi are used for avoiding the stresses, i.e., (1) various gene expressions, (2) morphological transformation, (3) formation of various molecules and proteins like chaperone, trehalose, and antioxidants, etc., (4) compartmentalization, and (5) transportation of contaminants.

Lipid Peroxidation [MDA]

It is clear that (Table 4, Fig. 4) when subjected to heavy metal stress (T2) at 30, 60, and 90 DAS periods, the mean MDA content increased significantly by 69.45 percent, 40.86 percent, and 42.76 percent relative to control (T1) (Table 4,

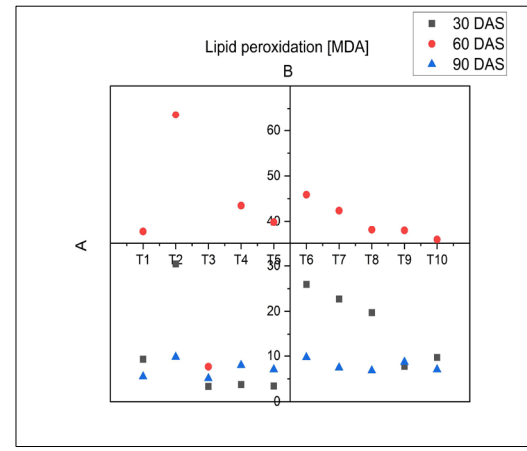

Fig. 4: Scatter central plot of Lipid Peroxidation (MDA Content).

Table 4: MDA content of pea after different treatments.

Treatments	30DAS	60DAS	90DAS
T1	$9.3^{a} \pm 0.14$	$37.58^{ef} \pm 0.09$	$5.5^{f} \pm 0.17$
T2	$30.45^{a} \pm 0.36$	$63.55^{a} \pm 0.82$	$9.78^{ab} \pm 0.15$
T3	$3.35^{g} \pm 0.23$	$7.66^{g} \pm 0.31$	$5.11^{f} \pm 0.11$
T4	$3.76^{g} \pm 0.26$	$43.47^{c} \pm 0.49$	$7.98^{cd} \pm 0.39$
T5	$3.45^{g} \pm 0.23$	$39.83^{d} \pm 0.57$	$7.05^{de} \pm 0.45$
T6	$25.94^{b} \pm 0.76$	$45.88^{b} \pm 0.40$	$9.72^{a} \pm 0.25$
T7	$22.73^{c} \pm 0.13$	$42.37^{c} \pm 0.47$	$7.45^{de} \pm 0.18$
T8	$19.73^{d} \pm 0.36$	$37.97^{e} \pm 0.39$	$6.82^{e} \pm 0.32$
T9	$7.75^{f} \pm 0.28$	$37.82^{e} \pm 0.66$	$8.67^{bc} \pm 0.30$
T10	$9.68^{e} \pm 0.19$	$35.82^{f} \pm 0.40$	$7.06^{de} \pm 0.28$

Note: Symbols as per Table 1.

Fig. 4). Exogenous application of endomycorrhiza in soil (T3) demonstrated the mitigating impact by reducing the MDA content by 88.98%, 87.94%, and 46.82% relative to T2 at 30, 60, and 90 DAS (Table 4, Fig. 4). Compared to T2, putrescine (T6) exogenous application showed a mitigating effect on the proposed DAS by decreasing MDA by 14.81%, 27.80%, and 3.22% (Table 4, Fig. 4). Across 88.66%, 37.32%, and 26.63%, when administered with a higher dose of putrescine (T5) relative to T2, the mean MDA concentration was significantly reduced (Table 4, Fig. 4). Furthermore, the MDA amount decreased significantly to 25.35%, 33.32%, and 22.47% at the suggested DAS was associated with T7 (Table 4, Fig. 4). The average MDA content decreased significantly when treated with a higher dose of putrescine compared to T8 by 35.20%, 40.25%, and 29.03% compared to T2 (Table 4, Fig. 4). The use of putrescine and mycorrhiza in T9 treatment decreased by 74.54%, 40.48%, and 9.71%, respectively (Table 4, Fig. 4). As T10 was associated with T2 therapy, substantial MDA content decreased by 68.21%, 43.63%, and 26.53%, respectively (Table 4, Fig. 4). The amount of MDA in this therapy (T4) was observed to decrease significantly by 87.65%, 31.59%, and 16.96%, respectively, relative to T2 in the original DAS (Table 4, Fig. 4). ROS is potentially harmful to cell membranes, resulting in membrane lipid oxidation (lipid peroxidation). Malondialdehyde (MDA) is one of the lipid peroxidation end breakdown products and can be used as an in vivo lipid peroxidation predictor. To defend against oxidative stress, a network of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) has evolved multiple scavenging mechanisms to regulate the rate of ROS. Scientists studied the impact on *Brassica rapa* of treatment with selenium dioxide, putrescine, and cadmium chloride. The experiment showed that the treatment of selenium dioxide and cadmium chloride produced stress in turnip plants, thus inducing enhanced MDA and anthocyanin content into maximized development of ROS and inhibiting chlorophyll biosynthesis. The application of SeO_2 and putrescine improved antioxidant function and fostered better growth of crops.

Fungus of mycorrhiza is responsible for the production of a suitable environment for the activities of microbes present in soil and their growth by raising the amount of organic matter in soil and increasing the activities and growth of roots (Silva et al. 2006; Khan et al. 2000). Hence, the use of mycorrhiza fungi was an appropriate technique for soil bioremediation. If mycorrhiza fungi were used with growth hormones, then bioremediation would work with greater effectiveness. The single spores of fungi help to produce and grow the vast hyphae network. If hosted plants existed. Bioremediation of polluted areas is possible by a single use of mycorrhizal fungi (Cabral et al. 2010, Declerck et al. 2005, Mugnier & Mosse 1987)

There is two most important mechanisms upon which the mycorrhizal fungal works to mitigate the effect of heavy metal under the contaminated soil named as phytostabilization and phytoextraction. When the mycorrhizal fungi uptake the heavy metal from the contaminated soil it has a couple of hyphae physiological mechanism, which includes protein expression like metallothionein as well as glomalin, heavy metal retention by the spores of fungal, in cellular membrane heavy metal complexation, molecular gene expression which results in the phytostabilization activation process (Behra 2014, Yang et al. 2014). When the symbiosis occurs between the mycorrhizal fungi and the hyperaccumulating plants, it results in increasing the absorption and the translocation of heavy metals, which leads to phytoextraction. Different plant parts, like herbaceous as well as corn plants, undergo the process of phytostabilization, and phytoextraction has been

Table 5: SOD activity of pea after different treatments.

Treatments	30DAS	60DAS	90DAS
T1	$0.57^{c} \pm 0.025$	$0.16^{de} \pm 0.004$	$0.39^{e} \pm 0.019$
T2	$0.85^{a} \pm 0.012$	$0.74^{a} \pm 0.025$	$1.35^{a} \pm 0.060$
T3	$0.3^{g} \pm 0.012$	$0.03^{f} \pm 0.002$	$0.37^{e} \pm 0.037$
T4	$0.58^{c} \pm 0.008$	$0.33^{c} \pm 0.004$	$0.78^{b} \pm 0.021$
T5	$0.4^{f} \pm 0.025$	$0.44^{b} \pm 0.016$	$0.55^{cd} \pm 0.002$
T6	$0.4^{f} \pm 0.025$	$0.35^{c} \pm 0.009$	$0.82^{b} \pm 0.026$
T7	$0.51^{cd} \pm 0.016$	$0.41^{b} \pm 0.014$	$0.61^{c} \pm 0.023$
T8	$0.72^{b} \pm 0.029$	$0.18^{d} \pm 0.008$	$0.59^{cd} \pm 0.025$
T9	$0.48^{de} \pm 0.021$	$0.13^{e} \pm 0.004$	$0.52^{cd} \pm 0.004$
T10	$0.43^{ef} \pm 0.012$	$0.15^{de} \pm 0.012$	$0.50^{d} \pm 0.004$

Note: Symbols as per Table 1.

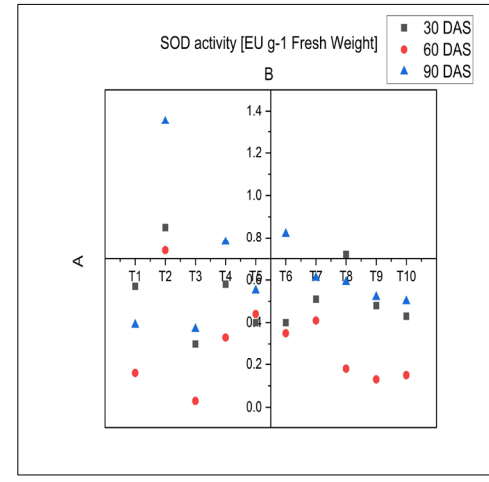

Fig. 5: Scatter central plot of SOD activity.

found in different research conducted by scientists (Soares & Siqueira 2008). The mechanism's efficiency or ability depends upon different parameters like the production of biomass, the growth rate of the plant, as well as the tolerance power of the plants to withstand the heavy metal stress even at higher concentration levels, and the heavy metal bioavailability.

SOD Activity [EU g^{-1} Fresh Weight]

The mean SOD output increased significantly at 32.94%, 78.37%, and 70.14% when subjected to heavy metal stress (T2) relative to control (T1) at 30, 60, and 90 DAS intervals (Table 5, Fig. 5). Exogenous application of endomycorrhiza in soil (T3) demonstrated the mitigation impact by minimizing SOD production by 64.70%, 94.59%, and 72.22% relative to T2 at 30, 60, and 90 DAS (Table 5, Fig. 5). Compared to T2, putrescine (T6) exogenous application showed a mitigating effect on the proposed DAS by decreasing SOD activity by 52.94%, 52.70%, and 40.74% (Table 5, Fig. 5). The median SOD output was significantly reduced by 52.94%, 40.54%, and 58.51% when administered with a higher dose of putrescine (T5) relative to (T2) compared to T2 (Table 5, Fig. 5). Likewise, the SOD behavior decreased significantly with 40.0%, 44.59%, and 54.81% at the proposed DAS in T7 (Table 5, Fig. 5). The median SOD output decreased dramatically when administered with a higher dose of putrescine relative to T8 by 15.29%, 75.67%, and 56.29% compared to T2 (Table 5, Fig. 5). The combination of putrescine and mycorrhiza in the T9 treatment decreased by 43.52%, 82.43%, and 61.48% in the proposed DAS treatment (Table 5, Fig. 5). Comparing T10 treatment with T2 treatment, significant SOD activity decreased by 49.41%, 79.72%, and 62.96%, respectively (Table 5, Fig. 5). The SOD activity was found to decrease significantly in this treatment (T4) by 31.76%, 55.40%, and 42.22%, respectively, compared to T2 in the proposed DAS (Table 5, Fig. 5). Mycorrhiza has thus been found to have a greater impact on SOD behavior with 30, 60 & 90 DAS (Table 5, Fig. 5). ROS is likely to damage the cell membranes, resulting in oxidative lipid degradation (lipid peroxidation). Superoxide dismutase (SOD) is a metal enzyme that catalyzes the oxygen and H_2O_2 dispersion of superoxide radicals. It is well known that various environmental stresses frequently lead to increased production of ROS since SOD was proposed to be important in the resistance of plant stress and provide the first line of defense from the toxic effects of high ROS concentrations. The experiment was carried out, suggesting that the growth of plant roots and shoots was decreased when *Pisum sativum* L. was treated with different Cd concentrations. The protein, which is produced by mycorrhizal fungi, is a glycoprotein, glomalin, that helps in soil particle binding, and hence, properties of the soil are improved and able for heavy metal binding in the soil (Wu et al. 2014). Wu et al. (2014), reported that the AM fungi glomalin protein (Wu et al. 2014) produces the absorption of heavy metals in nickel and lead in the soil by organic matter. As chromium is highly toxic both for human health and for plants, mycorrhizal fungi have less efficacy in mitigating the levels of Cr in the contaminated soil rhizosphere. Hence, the function of mycorrhizal fungi is not further studied in one of its remedial techniques. It requires more study of that specific metal (Gil-Cardeza et al. 2014). It is important to be aware that the effects of mycorrhizal fungal phylogenesis on heavy metals' biological remediation have been questioned (He et al. 2014). Under the stress of heavy metal, he conducted an experiment in which the effect of phylogenetics on plant growth of the host plant of Glomeraceae and in glomerate was evaluated. From the result, it was found that both the phylogenetic mycorrhizal fungi have positive effects on the growth of the host plants under heavy metal stress and the variation in different concentrations of heavy metal. Glomerate has also been reported to be more favorable for the host plant's growth under heavy metal stress, whereas non-Glomeraceae mycorrhizal fungi were better for host plants under no-stress conditions. The fungus response of glomeraceae depends on different plant species, as plant growth of legumes is better than that of nonlegumes under heavy metal stress. As a result, the use of legumes, in combination with glomeraceae, was indicated to have a more beneficial way of heavy metal bioremediation. When phosphorus is applied by the exogenous to improve the nutrition of host plants, the effects of heavy metal contamination using mycorrheatic fungi are mitigated (Wu et al. 2016a, 2016b). It was concluded that the mycorrhizal fungi could enhance the growth of a plant by increasing the absorption of nutrients such as phosphorus and by immobilizing chromium from the roots of the host plants with fungi. The test also showed that the chromium is stabilized and distributed in the roots of host plants. As a result, mycorrhiza fungal roots can adsorb chromium, which reduces the translocation of chromium to aerial parts of the host plant.

CONCLUSION

The use of putrescine and mycorrhiza outperformed SOD's antioxidant activity, as well as MDA, proline, and membrane injury. The impact of putrescine was more active at 30 DAS. In contrast, the application in combination with mycorrhiza was more active at 60 DAS for the possible reduction of toxicity through improved cell stability and reduced injury. In all days of intervals, mycorrhiza was found to have a greater impact on superoxide dismutase behavior. We learned that

polyamines such as putrescine as well as mycorrhiza glomus offer significant protective mechanisms against Cd-toxicity in peas through their protective role in plants, mediated by balancing the enzymatic and non-enzymatic antioxidants. This study gave us insights into the efficient and balancing role of putrescine and mycorrhiza glomus in minimizing the damage done by Cd contamination. Importantly, we are confident that this defensive work could be extended to other heavy metals-related toxicity, but that needs a serious assessment in future work.

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