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ENHANCING SEED PROTEIN ACCUMULATION IN SOYBEAN UNDER CADMIUM STRESS BY USING AM FUNGI

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ABSTRACT:

The present study was aimed at understanding alleviation of cadmium (Cd) stress using arbuscular mycorrhizal (AM) fungi in soybean by studying its growth, yield and seed protein characteristics. Considering the legumes' tolerance range to Cd and its permissible limit in the soil, Cd (as CdCl2.H2O) was added in the soil-filled pots at three concentration levels, viz., 10, 20, and 30 mg/kg of soil. Besides inoculating the AM fungi Glomus mosseae and G. fasciculatum, (individually and collectively) in the pots, they were also used for seed priming (VAM powder, 25g/kg of seeds). Without AM fungi, all growth parameters, yield, and seed protein characteristics were adversely affected by Cd stress. Furthermore, the Cd-induced decrease in polypeptide intensity (on SDS gels) of seed proteins, computed using GelAnalyzer, was restored to some extents. Compared to G. mosseae, G. fasciculatum was found more effective in boosting plant growth and alleviating the effects of Cd at 10 and 20 mg concentrations. In contrast, at 30 mg, both Glomus species were found equally effective. Thus, given the role of AM fungi in optimizing seed protein quality and quantity in soybean, these two species of AM fungi can be recommended for use to improve the plant performance in Cd-affected soil.

Keywords: AM fungi, Cadmium, Glomus, Seed proteins, Soybean, SDS-gel.

1. INTRODUCTION

The soybean (*Glycine max* L.) is a valuable agricultural crop known for meeting humans seed-based protein and oil demands (Hamayun *et al.,* 2010). It is used in over half of the high-protein meals consumed worldwide (He and Chen, 2013). It is also utilized extensively in the food sector to make flour, baked goods, and herbal cheese (Singh and Shivakumar, 2010). However, various abiotic stresses imposed naturally or by anthropogenic activities negatively impact soybean global production. Of these stresses, Cd stress has been linked to stunted growth and development in a wide variety of crops, including rice (Jan *et al.*, 2019), chickpeas (Ghosh *et al.,* 2022), ryegrass (Wang *et al.*, 2020), tomatoes (Rahmatizadeh *et al.,* 2019), maize (Zhang *et al.*, 2019), etc.

Cadmium is a hazardous heavy metal that may accumulate in various human tissues and organs (Godt *et al.*, 2006), although it tends to concentrate most heavily in the roots of higher plants (Arif *et al.,* 2010). Studies have been conducted to understand how Cd affects higher plants' metabolic processes. It has been demonstrated that Cd disrupts the synthesis of chlorophyll and inhibits enzymes involved in the photosynthesis and Calvin cycle (Wang *et al.,* 2009). Additionally, it suppresses the activity of several other enzymes involved in nitrogen and sulphur assimilation, glycolysis, and the pentose phosphate pathway. Cd also has the potential to trigger the generation of reactive oxygen species (ROS), which damage the biomolecules and cell organelles and hamper the plants' overall growth and yield (El Rasafi *et al.,* 2022).

According to Aparicio *et al.* (2022), heavy metals can be eliminated from agricultural areas using various physiochemical and biological remediation strategies. However, these methods are time-consuming and costly. They may result in more complex secondary pollutants deposition, further diminishing soil fertility (Ashraf *et al.,* 2019). The use of AM fungi is an important biological approach for the bioremediation of heavy metal contaminated soil (Kumar, S. and Saxena, S., 2019). AM fungi-improved nitrogen absorption in plants is directly proportional to the total accumulated seed proteins. So, exposure of plants to AM fungi enhances the performance of the host plant under heavy metal stress (Zhan *et al.*, 2018). It has been observed that symbiosis with AM fungi can protect plants against Cd stress; however, the role of AM fungi in alleviating Cd harmful effects can vary depending on the plant and fungus involved, as well as the concentrations of Cd (Garg and Bhandari, 2014). Under high concentrations of Cd and Zn, *Glomus* species have been found to increase presymbiotic hyphal expansion, sporulation, and spore germination (Pawlowska and Charvat, 2004). There is a need to gain insights concerning the effect of *G. mosseae* and *G. fasciculatum* on the accumulation of seed proteins in soybean under Cd stress. So, the present work aimed to investigate the role of these two different AM fungi in mitigating the adverse effects of Cd on soybean.

2. METHODOLOGY

2.1 Location, pot and soil

The pot field experimental area used for this experiment belongs to department of Botany, of Kurukshetra University, Haryana state in the north of India. At N 29°.95903, E 76° 813125, soil was collected (clay and sandy soil) at a depth of 12-15 centimetres, air-dried, pulverised and analysed for their physical and chemical properties (Table 1). After sterilization, 10 kg of soil was filled in each polybag (recyclable, UV protected with 15x16 inches dimension).

2.2 Seed variety, inoculum and seed priming

Soybean seeds of variety PS 1347 were procured from NSC (National Seed Corporation

Ltd.), Kota, India. This cultivar is resistant to yellow mosaic virus, soybean mosaic virus, bacterial pustule, and bacterial leaf blight (BLB). It is also resistant to charcoal rot and Rhizoctonia aerial blight (RAB). Both the *Glomus mosseae* and *G. fasciculatum* (densely colonized mycorrhizal inoculum having soil, spores, mycelium, and maize root pieces) were obtained from laboratory of Plant Pathology, Department of Botany, Kurukshetra University, Haryana. After surface sterilisation with 5% sodium hypochlorite (v/v), seeds were washed thoroughly with distilled water to remove disinfectant residues. *G. mosseae* and *G. fasciculatum* were used singly and dually, and the seeds were uniformly coated with the VAM powder (25g/1kg of seeds).

2.3 Field experimental setup

The experiment was carried out using a totally randomized block design. First set with one control (without any kind of seed or soil treatment), in second set, 3 controls (only with three different level of Cd) and in third set, 9 treatments (with single and dual *Glomus* species along with three Cd levels) represented in Table 2. So, there were total three set each with five replicates used in this experiment. After priming, seeds were sown in polybags and an extra 15 gm AM fungi (around 40 spores/g of soil) were added to each polybag after one week of germination. The plant thinning was done to maintain five plants per polybag.

2.4 Preparation of heavy hetal solutions and plant soil treatment

Cadmium salt as cadmium chloride ($CdCl_2.H_2O$) was used in three different concentration levels i.e., 10, 20 and 30 mg per kg of pot soil. The Cd solution was applied at three different times of plant growth and development: the first treatment was given during vegetative growth, the second at blooming stage, and the third before the pod formation. All the morpho-physiological parameters were studied and recorded. The crop took around 118 days to mature, producing a compact, determinate plant with tawny pubescence and bright yellow seeds. For further protein analysis, mature seeds were gathered, dried, and crushed into seed meals.

2.5 Morphological and physiological parameters analysis

Plant morphological characteristics, such as shoot length, were measured using a meter scale in centimeters. The number of branches and leaves/plant was counted manually from the base to the tip of the plant. The leaf area was determined by following the method given by Weirsma and Bailey (1975), using the equation: $A = 0.411 + 2.008\ LW$, where A = trifoliate leaf area, L and W are the maximum length and width of the terminal leaflet of a trifoliate leaf, respectively, and 0.411 and 2.008 are constants. The chlorophyll and carotenoid content were determined using the methodology as described by Arnon (1949) and Holden (1965) at 663, 645, 510, and 480 nm wavelengths.

2.6 Yield attributes

Yield attributes were recorded as per Hussain *et al.* (2014) methods. The number of pods/plant was determined by randomly counting the number of pods (containing at least one seed) from 10 plants (selected from each treatment) and then average number of pods/plant was calculated. The number of seeds/pod (randomly selected 25 pods from each treatment), the number of seeds/plant (randomly selected 10 plants from each treatment), seed weight (each replicate's pods were cleaned and crushed, and the seed weight/plant was calculated in gm/plant) and 100 seeds were then selected randomly from each set of treatments and weighed to determine 100 seeds weight; pod weight by randomly selecting 25 pods from each treatment, and average weight/pod was determined.

2.7 Defatting of seed meal

The finely crushed seed meal was defatted with hexane (1g/10ml) at 4ºC for two hours. After centrifugation for 10 minutes, the supernatant was discarded, and the process was repeated. The seed meal present in the pellet was then vacuum-dried (Singh and Matta, 2008) used for various protein characterization studies.

2.8 Protein quantification

The semi-micro Kjeldahl method was used to estimate the total seed protein content (Welcher, 1963). First the seed meal was digested with sulfuric acid in the presence of a mixture having copper sulphate, selenium dioxide, and potassium dichromate (1:2:1). Then Markham's distillation assembly was used to heat the digest with 40% NaOH and the resulting ammonia was fixed in boric acid that was then volumetrically titrated against N/40 HCl to estimate the nitrogen content. Finally, the protein content was calculated by multiplying nitrogen content with 6.25. The methods given by Thanh and Shibasaki's (1976) were used separate 7S and 11S globulin sub-fractions. The Bradford (1976) method was used to determine the protein concentration in the isolated sub-fractions.

2.9 Preparation of total seed protein extract

30 mg of defatted seed meal was extracted in 1 ml of 0.025M Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulphate (SDS). The suspension was heated on a water bath at 80ºC for 40 minutes. The content was centrifuged at 12,000g for 10 minutes, and the separated supernatant was then mixed with 10% (v/v) glycerol and 2% 2-mercaptoethanol (reducing conditions) and heated at 95°C for 10 minutes before loading on gels (Singh *et al.*, 2021).

2.10 SDS-polyacrylamide gel electrophoresis

Electrophoretic separation of total seed proteins extract was carried out following the discontinuous system established by Davis (1964) and Ornstein (1964) and formulation provided by Laemmli (1970) on a 14% polyacrylamide SDS-gel under reducing conditions. A direct current of 18 mA and 32 mA was used in the stacking and separation gel, respectively. Protein bands were visualized following staining with Coomassie Brilliant Blue R-250, and their molecular weights were calculated using standard marker proteins run on the same gel.

2.11 Densitometric scanning of gel

The densitometry of polypeptides was performed by using GelAnalyzer software. It analyzes gel images that have been scanned, taken with a camera, or recorded digitally. It enables visual control of band identification and comparative analysis, considering the comparative electrophoretic mobility (Rf) of bands. Additionally, it permits the evaluation of bands that are not very well resolved. The GelAnalyzer tool computes normalised coordinates of bands, analyses their spectra to identify similarities or changes in their components, and visually shows the results to provide a quantitative evaluation and analysis of bands. To simplify our data and graphs, we divided all collected raw values by a thousand in our computations.

2.12 Statistical analysis

The averages of five replicates for each set of data were displayed, followed by the standard error of the means. A statistical application, SPSS 17.0, was used to evaluate the gathered data.

3. RESULTS AND DISCUSSION

3.1 Growth and developmental variables

It was observed that using AM fungi *G. mosseae* and *G. fasciculatum* with different concentration levels of Cd, improved shoot length, the number of branches/plant and the number of leaves/plant of soybean plants compared to control (Table 3). In our first set, with 10 mg of Cd, the average shoot height, the number of branches/plant and the number of leaves/plant were better with *G. fasciculatum* compared to control. In the second set with Cd concentration 20 mg, the highest shoot height (55.6 cm) was noted again with the application *G. fasciculatum* as compared to the control (49.4 cm). The highest number of branches/plant and leaves/plant was also reported with the same *G. fasciculatum.* In the last level of Cd, i.e., 30 mg, the 52.4 cm average shoot height in plants inoculated with both AM fungi was significantly higher than the control (47.6 cm). Whereas the number of branches/plant was higher (7.9) with *G. mosseae* compared to the control (6.6). The number of leaves/plant was the highest (47.5) when both *Glomus* species were supplied together, compared to the control (40.6). A reduction in leaf area was observed with an increase in Cd concentration from 10 to 30 mg. However, these negative effects on the leaf were minimized with the application of *Glomus* species.

Thus, the introduction of AM fungi has been found to significantly enhance soybeans' growth and morphological characteristics, including shoot length, number of branches and leaves per plant, as well as leaf area. However, the increased supply of Cd in the soil negatively impacted growth and developmental parameters, particularly at higher concentrations. Numerous studies shown the beneficial impact of AM fungi on plant growth characteristics. It has been demonstrated that AM fungi can improve the growth and yield parameters of cotton and soybean (Cely *et al.,* 2016), wheat and faba bean (Ingraffia *et al.,* 2019), chickpea (Sohrabi *et al.,* 2019), tomato (Bona *et al.,* 2017), carrot (Kim *et al.,* 2017), etc. In addition, inoculation with AM fungi has been demonstrated to increase specific leaf area and root volume in plants (Tian *et al.*, 2013; Zhu *et al.*, 2014) compared to non-inoculated plants. Arbuscular mycorrhizal (AM) fungi have been shown to enhance plant growth and yields under heavy metal stress by facilitating nutrient uptake (Aloui *et al.,* 2011). AM fungi are crucial bioagents that produce fungal structures, such as arbuscules, which facilitate the exchange of inorganic chemicals and minerals and serve as a biological filter for heavy metals, aiding in their control (Li *et al.,* 2023). By improving water and mineral intake, rate of photosynthesis, AM fungi support the plant's ability to grow actively, even under heavy metal stress (Dhalaria *et al.,* 2020). Studies conducted by Diagne *et al.* (2020) have highlighted the potential of AM fungi in reducing heavy metal toxicity and enhancing plant growth and morphology.

3.2 Photosynthetic pigments

The effect of *G. mosseae* and *G. fasciculatum* on chlorophyll-a, b and carotenoid content under varying concentrations of Cd stress (Figure 2 A, B, C and D) revealed that both the *Glomus* species helped plants thrive well under heavy metal stress. In our first set with 10 mg of Cd, the highest content of chlorophyll a, b and total chlorophyll was recorded with *G. fasciculatum*, while carotenoids were maximum in *G. mosseae* treated plants. On the other hand, at 20 mg of Cd, chlorophyll a, total chlorophyll, and carotenoids were maximum in plants co-inoculated with the *Glomus* species. Without AM fungi, the content of photosynthetic pigments (chlorophyll a, b, total chlorophyll and carotenoids) was much more reduced under Cd stress. The photosynthetic pigments were significantly reduced at the highest level of Cd stress (30 mg), indicating more severe damage to plants' photosynthetic activity. However, plants recovered from this damage when they were dually inoculated with

Glomus species, suggesting that AM fungi can effectively mitigate the negative effects of heavy metal stress on plant growth and yield (Leyval *et al.*, 2002). Such positive enhancement in photosynthesis with AM fungi has also been reported in many different crops like wheat (Ibrahim *et al.*, 2011), rice (Porcel *et al.*, 2015), barley (Rezvani *et al.*, 2015), maize (Ghorchiani *et al.*, 2018), etc. In contrast to non- mycorrhizal plants, arbuscular mycorrhizal symbiosis protects photosynthetic pigments from photoinhibition and photo-destruction by reactive oxygen species, which are very frequent under abiotic stress (Asrar *et al.*, 2012).

Thus, our study reveals the potential benefits of AM fungi in improving crop production under Cd stress. Moreover, the different AM fungi species may have varying effects on plant growth and yield under heavy metal stress, highlighting the need for further research in this area. However, it is important to note that the optimal species and application method of AM fungi may vary depending on the plant species and environmental conditions.

3.3 Yield parameters

The yield parameters, including the number of pods/plant, number of seeds/pod, number of seeds/plant, seed weight (g), 100 seed weight (g), and pod weight (g), were evaluated at the time of harvesting. In the control sets, an increase in Cd concentrations from 10 to 30 mg had detrimental effects on all yield parameters (Table 4). However, at 10 mg Cd concentration, *G. fasciculatum* was found to be better than *G. mosseae* in alleviating the Cd stress and improving the yield parameters. In the second set with a Cd concentration of 20 mg, the highest number of pods/plant (18.1), the number of seeds/pod (2.3), and the number of seeds/plant (41.6) were reported when both *Glomus* species were applied to plants, as compared to control. However, the seed weight and pod weight were maximum with *G. fasciculatum*. In the last level of Cd, i.e., 30 mg, a sharp reduction in the number of pods/plant, the number of seeds/pod, and the number of seeds/plant was observed. However, the seed weight and pod weight were comparatively similar under individual supply of each *Glomus* species. We also recorded all these growth and yield parameters in plants grown in Cd and AM fungi soil (Table 3 and 4) and was found that plant grew and flourish well with enhanced yield parameters.

Our results demonstrated the harmful effects of Cd on soybean yield parameters. This is in agreement with studies of Andresen and Küpper (2013) and Goyal *et al.* (2020) that have reported the negative effects of Cd on plant growth and yield. The negative effects of Cd on soybean yield parameters can be attributed to the adverse effects of Cd on plant metabolism, including photosynthesis, respiration, and nutrient uptake (Silva *et al.*, 2014). Our findings are consistent with earlier studies that reported arbuscular mycorrhizal fungi's beneficial effects on plant growth under heavy metal stress (Jahromi *et al.*, 2008; Kanwal *et al.*, 2015). In a recent study by Adeyemi *et al.* (2021), soybean plants grown in copper, zinc, and lead-heavy metal contaminated areas showed improved growth and better seed yield when inoculated with AM fungi. The positive effect of *G. fasciculatum* on yield parameters were evident even at high Cd concentrations (20 mg), suggesting that this species is more tolerant to heavy metal stress than *G. mosseae*. Thus, our finding will have important implications for sustainable production of soybean in Cd-polluted soils.

3.4 Seed Protein content

Various concentrations of Cd have contrasting effects on seed protein content in soybean treated with either one or both the *Glomus* species (Figure 3 and Table 4). There was a negative correlation between the level of Cd stress and the amount of seed proteins accumulated. The *Glomus*-treated soybean plants showed higher seed protein content compared to non-treated plants under Cd stress. However, *G. fasciculatum* was more effective in alleviating Cd levels 1 and 2. However, at Cd concentration level 3, i.e., 30 mg/kg, the collective use of both *Glomus* species was found better, with a higher total seed protein content (41.56%, 13 T-3) compared to the control plants (38.50%, 10 C-3). Without Cd or any AM fungi treatment, total seed protein content was higher (43.43%, 1 C-0).

It has been found that Cd, Pb, Cr, Hg, Mn, and Co decreases dry matter, nitrogen content, seed protein content and seed production (Ghani, 2010). Cd interacts with protein functional groups such as carboxyl, sulfhydryl, and amine, which are essential for protein stability and function. This interaction cause protein damage or misfolding that eventually resulted in decreased seed protein accumulation (Tan *et al.*, 2010). Furthermore, soil contamination with heavy metals disrupts symbiotic nitrogen fixation and hence a reduction in synthesis of seed storage proteins (Ahmad *et al.*, 2012).

Mycorrhizal symbiosis helps plants absorb adequate nitrogen and phosphorus which are essential for producing proteins and enzymes (Meena *et al.*, 2018). In soybean, Marro *et al.* (2020) reported that AM fungi increases oil and seed protein content. Likewise, Alam *et al.* (2019) found that AM fungi improve biomass, nitrogen and phosphorus contents and seed proteins in mung bean plants treated with AM fungi compared to non-treated plants. Similarly, Zaidi and Khan (2007) reported that rhizotrophic microbes, particularly AM fungi, led to better yield and seed protein content in chickpea. Like previous studies, we have also found that soybean plants inoculated with *Glomus* species have higher levels of total seed proteins than non-inoculated plants under Cd stress. Furthermore, AM fungi symbiosis is known to enhance Cd tolerance in plants through various mechanisms, such as decreased Cd absorption and accumulation in roots, and enhanced phosphorus uptake by AM fungi. Huang *et al.* (2018) and Zhang *et al.* (2018) reported that AM fungi increases Cd accumulation in soybean plants. However, it has been found to decreases Cd accumulation in maize plants (Li *et al.*, 2016).

Content of globulin subfractions

The percentage of β-conglycinin and glycinin varied under different Cd concentrations and with a single or dual inoculation of *Glomus* species (Figure 4). In the first set of experiments with a Cd dosage of 10 mg/kg, the β-conglycinin fraction ranged from 22.5% (control) to 26.8% (AM fungi), while glycinin varied between 23.1% (control) and 24.9% (AM fungi). β-conglycinin was highest (26.8%) with *G. fasciculatum* whereas glycinin was maximum (24.9%) with dual AM fungi treatments.

In the second set of experiments with a Cd concentration of 20 mg/kg, the β- conglycinin and glycinin content was 19.5% and 20.9% (control) respectively, whereas they were recorded highest in *G. fasciculatum* treated plants. In the last set of experiments with a Cd concentration of 30 mg/kg, the β-conglycinin and glycinin content was found highest in plants with both fungi as compared to control. In the zero sets (1C-0) without any Cd or AM fungi treatment, β-conglycinin and glycinin content were found to be 28.1 and 26.5%, respectively, as shown in Figure 4.

Abiotic stresses, including heavy metal toxicity, have been found to negatively impact seed development, resulting in reduced biomass, yield, seed protein content and its major subfractions (Juhász *et al.*, 2018; Luo *et al.*, 2018; Nagy-Réder *et al.*, 2022). The variations in β-conglycinin and glycinin content with Cd concentration suggest that Cd affects the synthesis and accumulation of seed proteins in soybean. Similar findings were reported in earlier studies, where Cd exposure was found to alter the expression of genes related to seed storage proteins in soybean (Bashir *et al.*, 2019). A study on pea plants revealed that Cd exposure led to a significant decrease in globulin and albumin content in seeds, while the content of vicilin and legumin increased (Metwally *et al.*, 2005). Nadgórska-Socha *et al.* (2013) revealed that Cd stress in faba bean increases the vicilin and legumin content but decreases convicilin and albumin content. AM fungi have been found to enhance the accumulation of seed protein fractions in soybean, under Cd stress (Molina *et al.*, 2020). Similarly, in *Cajanus cajan*, AM fungi have been reported to increase the content of albumin and globulin fractions under Cd stress (Garg and Chandel, 2012). A decrease in the accumulation of β- conglycinin during soybean seed development under heavy metal polluted sites has been reported in several studies (Danchenko *et al.*, 2009; Klubicova *et al.*, 2012). The profiles of glycinin proteins were also analyzed in plants grown under heavy metal stress, although the difference was not statistically significant.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was run to observe the qualitative and quantitative changes in the polypeptide pattern of total proteins extracted from seeds of plants grown under varying levels of Cd concentrations along with and without AM fungi. We differentiated the two sub-fractions of globulins, β- conglycinin and glycinin, based on their molecular weights on the gel, consistent with earlier studies (Liu *et al.*, 2007; Ochnio *et al.*, 2018). The intensities of β-conglycinin and glycinin subfractions varied with increased Cd concentration.

Furthermore, single and dual AM fungi regimes positively affected seed protein accumulation, resulting in intense and dark bands (Figure 5). GelAnalyzer software was used to measure the peak intensity of each β- conglycinin α', α, and β band and glycinin acidic and basic bands. It was observed that co-inoculation of both AM fungi improves the globulin sub-fractions. The band intensities of α and α′ (of β-conglycinin) increased from 2.22 and 2.79, respectively, in control plants (2C-1) to 3.24(α) and 4.21(α′) at 4T-2, and 3.27(α) and 4.52(α′) at 5T-3 levels of *Glomus* species. Likewise, the intensity of β of β-conglycinin increased from 3.81 (2C-1) in control to 5.05 (4T-2), and 5.25 (5T-3) under single and dual inoculation of both *Glomus* species. The band intensity of acidic and basic subunits of glycinin fraction was found to be 3.35 and 7.0 in control plants. It increased to 3.54, 4.22, and 5.05 (acidic) and 6.46, 9.14, and 8.80 (basic) with AM fungi (3T-1, 4T-2, 5T-3) treatment. These findings indicate that even low levels of Cd exposure, such as 10 mg/kg, significantly impact soybean seed protein fractions and their polypeptide intensities.

Under the influence of 20 mg/kg concentration of Cd (6 C-2) on two sub-fractions and their counter effect by *Glomus* species. The AM fungi species, *G. fasciculatum,* was observed to be better in alleviating Cd stress than *G. mosseae* and their collective inoculation. In control plants, the band intensities of α', α and β of 7S β-conglycinin were observed as 2.14, 2.74 and 3.52, respectively. With *Glomus* species treatments (*G. mosseae*, *G. fasciculatum* and their simultaneous supply), α' values were 2.92, 3.17, 3.33, α values as 3.68, 4.18, 4.10, and β values were 4.18, 5.39, 4.92. Glycinin, acidic and basic subunits band intensities in Cd-stressed plants were 3.79 and 7.10, respectively. With *G. mosseae* and *G. fasciculatum* individual treatments, these intensity values increased to 3.95 and 7.81 and 8.57, respectively. With dual inoculation of both *Glomus* species, these acidic and basic subunit intensities were noted as 4.77 and 8.37. The gel picture (Figure 5) depicts the apparent loss in band thickness of 6 C-2, as compared to 7 T-1, 8 T-2, and 9 T-3.

In the third set with 30 mg/kg Cd (10 C-3) both *Glomus* species showed similar improvement in seed protein characteristics. However, alleviation was slightly lesser than in the previous two sets (10 and 20 mg Cd). Both the subfractions (11S and 7S) showed almost similar degrees of enhancement in band intensity with AM fungi treatment. The band intensities of the 7S α', α and β under control conditions were 1.98, 2.69, and 3.8, respectively. In contrast, these band intensities were altered with *G. mosseae* treatment to 2.85, 3.62, 4.5 (11 T-1), with *G. fasciculatum* treatment to 2.71, 3.31, 4.84 (12 T-2), and with their co-inoculation to 2.88, 4.01, 4.56 (13 T-3). In this last set, the acidic and basic glycinin subunits under control plants were found to be 3.13 and 7.37, respectively. With AM fungi treatments, the peak

intensity values of both glycinin subunits changed to 4.18 and 8.06 (*G. mosseae*), 3.79 and 7.88 (*G. fasciculatum*), and with co-inoculation to 4.45 and 8.06.
SDS-PAGE analysis using GelAnalyzer indicates that the impact of Cd supply on seed protein characteristics was more pronounced in the last set, despite the addition of AM fungi. Numerous studies have reported similar effects of heavy metals and AM fungi on seed proteins. Repetto *et al.* (2013) observed changes in the protein profile in pea under Cd stress. Likewise, the qualitative and quantitative alterations in soybean (Amit and Kumar, 2023) and chickpea seed protein fractions were seen under Cd stress (Ghosh *et al.*, 2022). Ahsan *et al.* (2007) observed Cd-induced changes in seed protein patterns on SDS- PAGE, with variations in protein patterns between molecular weights 116 and 45 kDa and 25 and 14 kDa, indicating that these are the major metal-binding proteins in rice. Mycorrhizal exposed plants exhibited a significant downregulation of these Cd stress-induced bands, indicating their role in reducing the effects of Cd stress. Massa *et al.* (2020) demonstrated the tight regulation of total nitrogen content in common bean by AM fungi and *Rhizobia*, either alone or in combination, and the highest total seed protein content was detected in the seeds of plants inoculated with both microorganisms. Stress-responsive proteins could be those polypeptides that were either newly detected in protein fractions or whose levels changed in response to a stress (Kosová *et al.*, 2023). Heavy metal stress is known to cause the upregulation of several proteases, which could explain why some polypeptides, which are degraded by these proteases, become less noticeable (Seneviratne *et al.*, 2019). AM fungi have also been reported to enhance the accumulations of Mg, K, Zn, Mn, and starch, resulting in increased plant resistance against abiotic stress and improved plant growth and physiology, ultimately leading to increased yield and seed protein content (Chen *et al.*, 2023).

4. CONCLUSION

We studied the effects of cadmium in soybean in the presence of two *Glomus* species. The results highlight the deleterious impact of cadmium on soybean growth, physiology, yield, and seed protein characteristics. Heavy metal stress resulted in reduced biomass and photosynthetic activity, ultimately decreasing overall growth and yield. However, inoculation with arbuscular mycorrhizal fungi (AMF) ameliorated the Cd-induced toxicity and improved soybean growth and seed protein content. Therefore, the inoculation with both *Glomus* species could be a crucial strategy to enhance soybean performance in Cd-contaminated agricultural soils. Future research should focus on optimizing the use of AM fungi species in crop plants based on the level and type of heavy metals in specific regions, given the crucial role of AM fungi in enhancing legume yields and metal detoxification.

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Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables and figures

Table 1. Chemical composition of soil.

Soil type	Sandy loam soil
pH	7.60
EC (ds/m)	0.55
Organic carbon (%)	0.52
Available N (ppm)	62.0
Available P (ppm)	0.65
Available K (ppm)	56.0
Available S (ppm)	74.0

Table 2. Experiment treatment allocations (G1= *Glomus mosseae*, G2= *G. fasciculatum*)

1 C-0	Control	• Without any amendments (no Cd, no AM fungi)
2 C-1 6 C-2 10 C-3	Only with Cd treatments	• Cd (10, 20 and 30 mg/kg)
3 T-1 4 T-2 5 T-3	With AM fungi treatments	• Cd 10mg + G1 • Cd 10mg + G2 • Cd 10mg +G1+G2
7 T-1 8 T-2 9 T-3		• Cd 20mg + G1 • Cd 20mg + G2 • Cd 20mg + G1 + G2
11 T-1 12 T-2 13 T-3		• Cd 30mg + G1 • Cd 30mg + G2 • Cd 30mg + G1 + G2

Table 3. Representing the average mean value of morphological parameters of soybean plants attributes grown with two *Glomus* species and with different Cd concentrations.

Lane	Heavy metal treatment Cadmium	VAM Treatment	Shoot length (cm)	No. of branches/plant	No. of leaves/plant
1C-0	NA	NA	56.5 ± 1.70	9.9 ± 2.10	57.5 ± 1.10
2C-1	Cd level 1	None	52.2 ± 1.86	7.8 ± 0.96	48.3 ± 1.98
3T-1	Cd level 1	*G. mosseae*	56.3 ± 1.75	9.8 ± 1.51	55.9 ± 1.66
4T-2	Cd level 1	*G. fasciculatum*	57.8 ± 1.53	10.3 ± 1.90	58.6 ± 1.50
5T-3	Cd level 1	Both	57.1 ± 1.68	10.1 ± 1.84	57.7 ± 1.61
6C-2	Cd level 2	None	49.4 ± 1.36	7.0 ± 1.06	45.2 ± 1.71
7T-1	Cd level 2	*G. mosseae*	54.2 ± 1.52	8.8 ± 1.16	52.5 ± 1.91
8T-2	Cd level 2	*G. fasciculatum*	55.6 ± 1.52	9.4 ± 1.35	55.4 ± 2.23
9T-3	Cd level 2	Both	54.4 ± 1.74	8.8 ± 1.10	53.0 ± 2.32
10C-3	Cd level 3	None	47.6 ± 1.40	6.6 ± 0.82	40.6 ± 2.52
11T-1	Cd level 3	*G. mosseae*	51.9 ± 2.07	7.9 ± 1.10	45.7 ± 2.01
12T-2	Cd level 3	*G. fasciculatum*	52.3 ± 1.85	7.5 ± 0.79	46.1 ± 1.80
13T-3	Cd level 3	Both	52.4 ± 2.52	7.7 ± 0.97	47.5 ± 1.63

Each value is a mean of five replicates, ± SE and means followed by same letter/s are not significantly different at $P \leq 0.05$.

Table 4. Showing various average mean value of yield attributes of soybean plant grown under Cd stress with two *Glomus* species inoculated seeds.

Lane	No. of Pods/plant)	No. of seeds/pod	No. of seeds/plant	Seed Weight (g)	100 seed weight (g)	Pod weight (g)	Nitrogen Content %
1C-0	19.8 ± 2.11	2.6 ± 0.39	52.4 ± 4.39	0.16 ± 0.03	16.5 ± 3.07	0.51 ± 0.05	6.95
2C-1	16.2 ± 1.68	2.2 ± 0.23	36.6 ± 4.50	0.12 ± 0.02	12.6 ± 2.33	0.41 ± 0.09	6.65
3T-1	18.1 ± 1.47	2.4 ± 0.20	44 ± 5.47	0.14 ± 0.02	14.2 ± 2.62	0.44 ± 0.14	6.75
4T-2	19.5 ± 1.61	2.6 ± 0.20	50.6 ± 7.82	0.15 ± 0.02	15.1 ± 2.39	0.47 ± 0.13	6.92
5T-3	19.5 ± 1.59	2.5 ± 0.24	48.7 ± 6.45	0.14 ± 0.03	14.6 ± 3.00	0.45 ± 0.16	6.88
6C-2	14.7 ± 1.39	1.9 ± 0.29	28 ± 5.83	0.11 ± 0.02	11.8 ± 3.01	0.35 ± 0.08	6.35
7T-1	17.5 ± 1.58	2.2 ± 0.31	38.6 ± 6.42	0.13 ± 0.02	13.8 ± 2.31	0.39 ± 0.09	6.70
8T-2	17.9 ± 1.43	2.3 ± 0.33	41.2 ± 7.69	0.13 ± 0.02	13.7 ± 2.54	0.4 ± 0.09	6.85
9T-3	18.1 ± 1.94	2.3 ± 0.31	41.6 ± 9.78	0.13 ± 0.03	13.6 ± 3.60	0.39 ± 0.11	6.82
10 C-3	12.5 ± 1.51	1.7 ± 0.30	21.2 ± 3.49	0.1 ± 0.02	10.9 ± 2.73	0.28 ± 0.05	6.16
11T-1	14.6 ± 1.02	1.8 ± 0.20	26.4 ± 5.72	0.11 ± 0.02	11.5 ± 2.37	0.33 ± 0.04	6.60
12T-2	14.7 ± 1.59	1.8 ± 0.20	26.6 ± 7.40	0.11 ± 0.02	11.5 ± 2.56	0.35 ± 0.07	6.55
13T-3	14.9 ± 1.62	1.8 ± 0.33	26.8 ± 7.75	0.12 ± 0.02	11.8 ± 2.59	0.36 ± 0.07	6.65

Each value is a mean of five replicates, ± SE and means followed by same letter/s are not significantly different at $P \leq 0.05$.

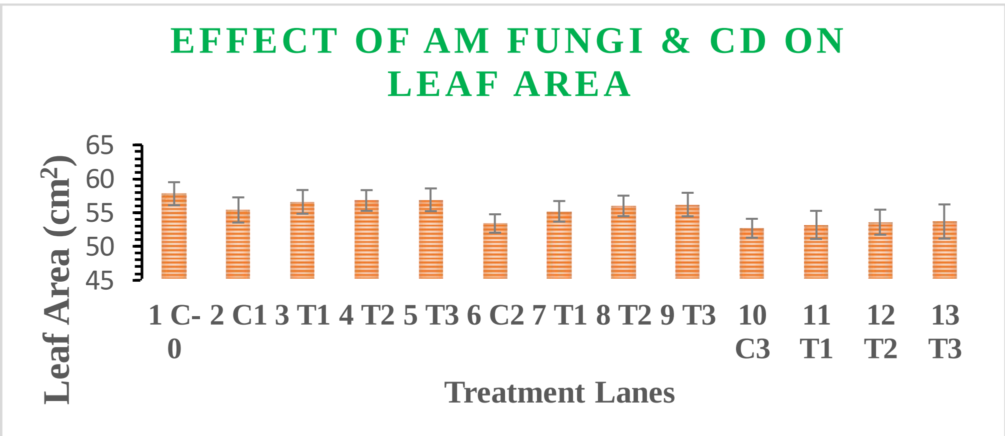

Figure 1. The effects of Cd and AM fungi various treatments on soybean leaf area (cm^2).

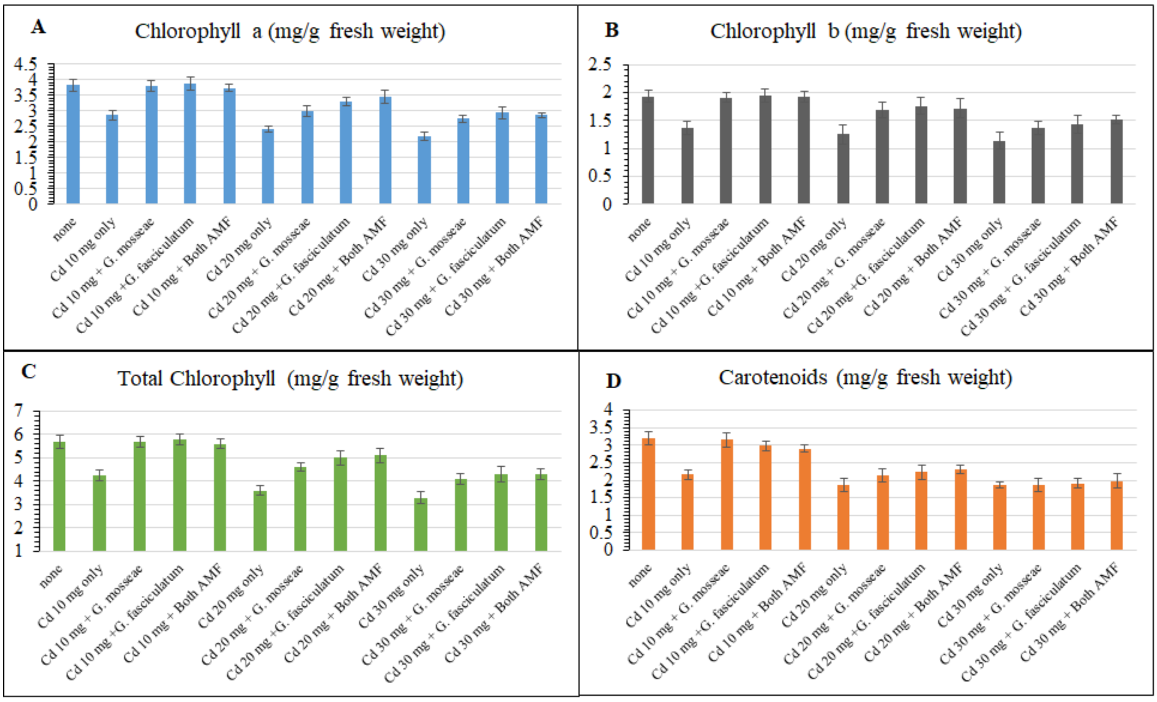

Figure 2. The effects of *Glomus* species on soybean plants grown under different Cd levels.

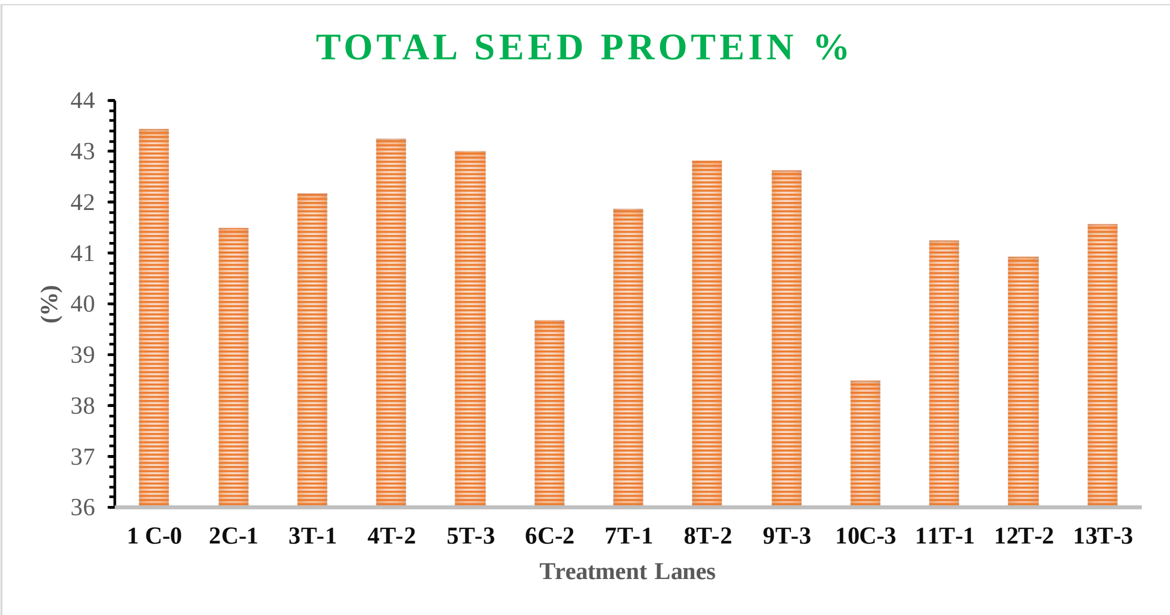

Figure 3. The seed protein content (%) under different concentration of Cd and with different treatment with AM Fungi.

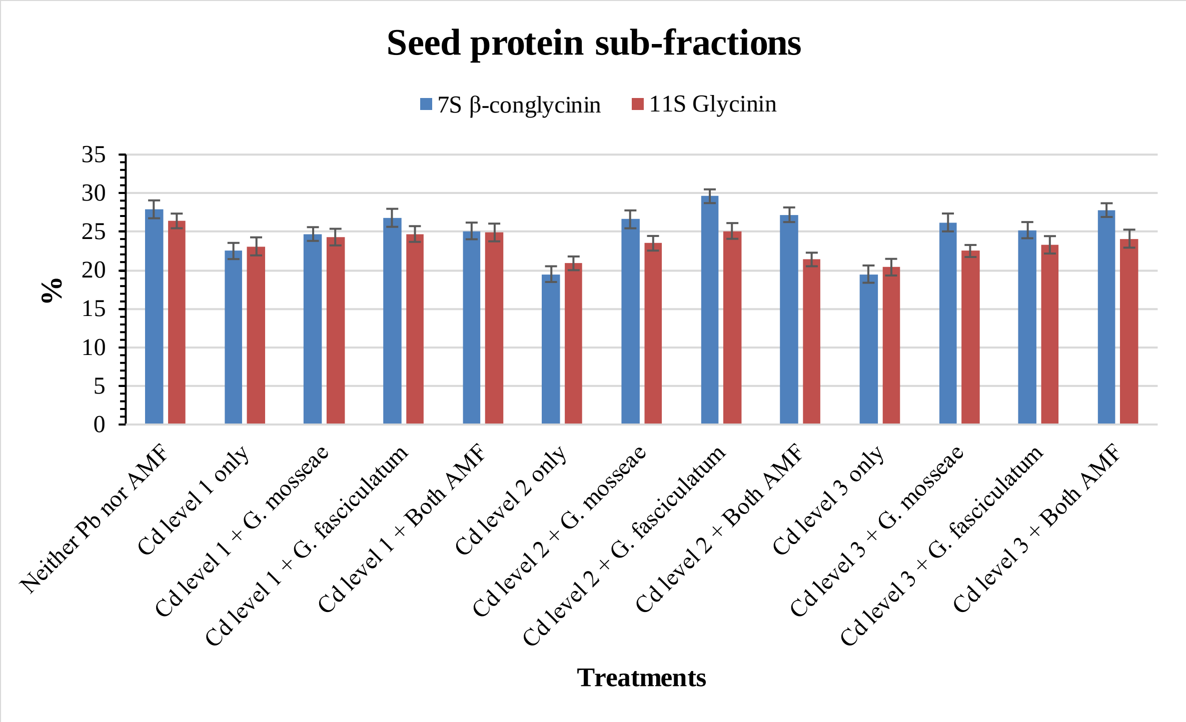

Figure 4. Effect of Cd toxicity on soybean major globulin seed protein fractions.

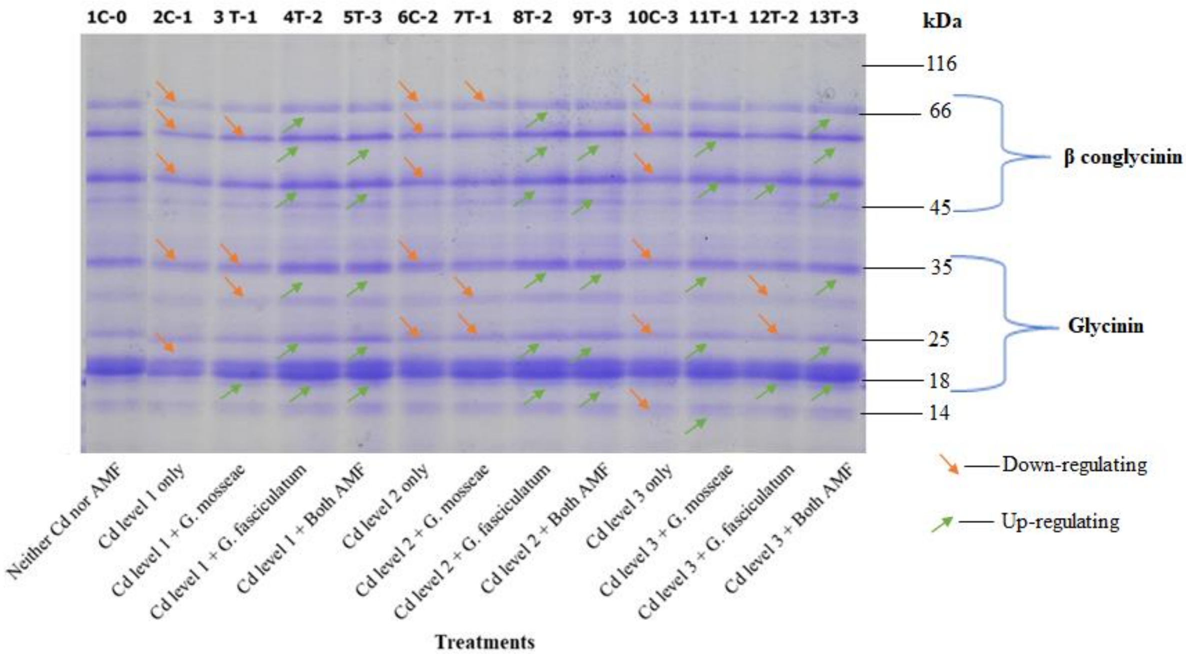

Figure 5. The SDS-gel of soybean seed protein and major fractions from plants grown at various Cd and *Glomus* species.