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ORIGINAL PAPER

# The Potential Utilization of Mycorrhizal Fungi and Glycine Betaine to Boost the Fenugreek (*Trigonella foenum-graecum* L.) Tolerance to Chromium Toxicity

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## Abstract

Chromium (Cr) is one of heavy metals that extremely dangerous to the ecosystem, especially plants as it has a detrimental influence on numerous physiological and metabolic pathways, which in turn affects the plant growth, development, and nutrient-taking capacity. The application of arbuscular mycorrhizal (AM) fungi or glycine betaine (GB) was widely studied regarding their potential to alleviate Cr toxicity. However, limited information is documented regarding their combined use. A greenhouse experiment was conducted to study the ameliorative effects of AM application and GB (50 mM) foliar spray either individually or in combination on fenugreek (*Trigonella foenum-graecum* L.) plants upon 200 mg $kg^{-1}$ Cr stress. Results showed that the separate application of AM and GB markedly alleviated Cr toxicity on shoot height, root length, total fresh and dry weights and Cr-stress indices of fenugreek plants, with a more obvious alleviation with their dual applications. A significant increase in total chlorophyll (32.31, 14.51 and 44.06%) validated the effectiveness of AM, 50 mM GB and AM + GB in Cr-stressed plants as compared to stressed one. Cr stress significantly increased arbuscules colonization in the roots of trigonella by 43.16%. The extraordinary result was that 200 mg $kg^{-1}$ of Cr increased mycorrhizal dependency of trigonella plants (120.40%) compared to those that were not stressed (73.16%). The combined application of AM fungi and GB markedly reduced Cr-induced malondialdehyde, and increased the total Chl, growth attributes, proline, protein, free amino acids and sugars as compared to their respective controls. AM colonization with GB counteracted Cr-induced alterations of peroxidase and ascorbate peroxidase activities in fenugreek shoots. These findings demonstrated that, in comparison to their individual applications, the combination use of AM and GB is more effective and will provide novel perspectives for reducing Cr toxicity, and will boost plant tolerance.

**Keywords** Chromium toxicity · Glycine betaine · AM colonization · Antioxidant enzymes · Osmolytes · Trigonella · Glomalin

## Abbreviations

| | |
|---|---|
| AC | Arbuscules colonization |
| AM | Arbuscular mycorrhiza |
| APX | Ascorbate peroxidase |
| CAT | Catalase |
| EE-GRSP | Easily extractable glomalin-related soil proteins |
| EL | Electrolyte leakage |
| GB | Glycine betaine |
| MDA | Malondialdehyde |
| MD | Mycorrhizal dependency |
| POD | Peroxidase |
| RL | Root length |
| RLSTI | Root length stress tolerance index |
| HC | Hyphal colonization |
| ROS | Reactive oxygen species |
| SH | Shoot height |
| SHSTI | Shoot height stress tolerance index |
| Tfwt | Total fresh weight |
| Tdwt | Total dry weights |
| TFAA | Total free amino acids. |
| TSP | Total soluble protein |
| TSC | Total soluble carbohydrates |
| TAC | Total antioxidant capacity |

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T-GRSP Total glomalin-related soil proteins
VC Vesicles colonization

## 1 Introduction

One of the top seven toxic heavy metals is chromium (Cr), which has oxidation numbers ranging from Cr(II) to Cr(VI) and is released into the environment by a variety of industry sectors and Cr mines. It is ranked 21st among metals often found in the earth crust (Shahid et al. 2017; Wakeel et al. 2020; Hassan et al. 2024). The hexavalent form of Cr (VI) has increased water solubility, mobility, and bioavailability, making it the most hazardous form of Cr (Sultana et al. 2014), which accumulated on agricultural land and severely inhibited crop development (Farid et al. 2018; Wakeel et al. 2019). The uptake of Cr from the rhizosphere by plant roots is usually accompanied by water and nutrients uptake due to the poor specificity of some essential element transporters and channels (Gomes et al. 2017; Wani et al. 2022; Ahammed 2023; Hassan et al. 2024), resulting in many phytotoxic effects on morpho-physiological, and biochemical processes (Farid et al. 2018; Singh et al. 2020; Wakeel et al. 2020). Cervantes et al. (2001) revealed the active mechanism for the Cr transportation that occurs through different transporters participating in the in the transportation of sulfate and essential ions. Wallace et al. (1976) reported that because Cr has a structural similarity to phosphate and sulphate, it is absorbed by their transporters in plants and interferes with sulphur, phosphorous, and iron for carrier binding during transportation (De-Oliveira et al. 2016).

Recent studies described the impact of Cr phytotoxicity on morpho-physiological attributes such as shoot height (SH) and root length (RL), root and shoot biomass, chlorophyll contents, antioxidants, stress markers, and proline in *Sesbania sesban* (Din et al. 2020),*Cicer arietinum* (Singh et al. 2020), and *Spinacia oleracea* (Zaheer et al. 2020). Cr toxicity has also been shown to impact seed germination and mineral nutrition (López-Bucio et al. 2014; Singh et al. 2020; Wakeel et al. 2020). Dotaniya et al. (2014) found that increased Cr accumulation in plants lowered germination, root and shoot growth rate, which affected total biomass and, ultimately, plant production that is associated with reduced cell size and enlargement, as well as decreased cell divisions. Cr toxicity in plant systems and its physiological modulation are primarily determined by a plant's Cr absorption, mobilization, and subsequent accumulation in different tissues as it enters the plant through its roots and is partially transferred into the aerial plant parts, where it influences the growth of the stem and leaves, together with other nutrients (Hayat et al. 2012; Hassan et al. 2024). Furthermore, Cr accumulation at the cellular level causes the generation and accumulation of reactive oxygen species (ROS), including hydrogen peroxide ($H_2O_2$), superoxide anion ($O^{2-}$), singlet oxygen ($^1O_2$) and hydroxyl ion ($HO^-$). These ROS cause cellular and extracellular damage in various plant species such as *Brassica napus*, sunflower, *Arabidopsis thaliana*, and *Sorghum bicolor* (Gill et al. 2015; Farid et al. 2018; Wakeel et al. 2019; Kumar 2021). Besides, ROS accumulation causes oxidative damage and increased electrolyte leakage (EL) and malondialdehyde (MDA) formation (Yu et al. 2018; Zaheer et al. 2019; Metwally et al. 2024a), disrupting membrane structure and function and oxidizing proteins and nucleic acids (Zhang et al. 2019; Wakeel et al. 2019; Zaid and Wani 2019; Nasrallah et al. 2020).

As a result, plants have developed various mechanisms for detoxifying Cr, such as Cr compartmentalization in less sensitive cell sites, modification of Cr forms or valence state, and reduction of Cr uptake and/or transport from root to shoot (Sharma et al. 2020; Guo et al. 2021). Furthermore, several enzymatic and non-enzymatic antioxidant enzymes effectively scavenge ROS (Selem et al. 2018), whereas metal chelation reduces Cr-induced direct damage to cells and organelles (Wakeel et al. 2020). As a result, recent research has focused on increasing plant tolerance to Cr by strengthening plant detoxification mechanisms and limiting Cr transport through soil-plant systems using biological agents.

One of these biological agents is arbuscular mycorrhizal (AM) fungi which are beneficial soil fungi that aid plant roots in water and nutrients uptake from soils via their hyphal network, reducing the need for fertilizers, in exchange; plants provide the AM with carbohydrates and lipids in this symbiosis (Kakouridi et al. 2022; Metwally et al. 2022; Zhu et al. 2022). Most importantly, AM fungi occurs in highly disturbed habitats, such as soils contaminated with heavy metals, and also are recognized to potentially boost the tolerance of plants to heavy metal toxicity (Zhang and Yang 2019; Kumar 2021; Ahammed 2023). Kumar (2021) shown that AM can promote *S. bicolor* L. growth while concurrently lowering Cr levels in plants. Furthermore, AM fungi produced glomalin, a heat shock protein or fungal glycoprotein that was capable of binding and sequestering heavy metals such as Cu, Cd, Pb, and Zn. There is evidence that elevated heavy metal levels may boost glomalin production (Gonzalez-Chavez et al. 2004; Cornejo et al. 2008; Vodnik et al. 2008). Inoculating plants with AM fungal spores or applying AM-rich soil as an amendment can be effective techniques for reducing Cr toxicity (Wu et al. 2019; Ramzan et al. 2023).

Aside from AM fungi, glycine betaine (GB) is a zwitterionic nitrogenous compound (quaternary amine) that has two main functions in plants: osmotic adjustment and cellular compatibility (Liang et al. 1994; He et al. 2019), and its accumulation in plants is associated with abiotic stress

tolerance (Park et al. 2007; Yang et al. 2007; Chen and Murata 2011). Foliar spray of GB stimulates ROS scavenging antioxidant defense and reduces its accumulation and cell death in cultivated tobacco cells exposed to NaCl or Cd stress, as demonstrated by (Banu et al. 2009; He et al. 2019). Islam et al. (2009) discovered that exogenous proline and GB boost antioxidant enzyme activity and confer tolerance to Cd stress in tobacco. However, the mechanism*via* which GB confers tolerance to plants against Cr stress is still poorly understood.

AM fungi and GB have been researched separately for their impacts on heavy metal tolerance and remediation; however, their combined application, specifically targeting Cr toxicity in trigonella, is relatively unexplored. As a result, in our current study, GB and AM were used as ameliorating agents to investigate the possibility of their combined application to reduce the harmful effects of Cr by improving the morphological and physio-biochemical characteristics of trigonella plants, pointing to a potential strategy for preventing Cr toxicity in these plants.

## 2 Materials and methods

Plant materials and seed sterilization:

The seeds of fenugreek var. Giza 30 (*Trigonella foenum-graecum* L.) were obtained from the Giza Agricultural Research Centre in Egypt. For the investigation, similar-sized seeds were picked and sterilized with sodium hypochlorite (5%) for 10 min and being washed with distilled water. After sterilization and soaking the seeds in sterilized water for 6 h, the seeds were allowed to grow in a preliminary and a pot experiments in a greenhouse, with natural light, average temperatures of 23/14°C, and a relative humidity of 50–75%.

### 2.1 AM Fungal Inoculum

The inoculum of AM fungi containing *Funneliformis mosseae*, *F. constrictum*, *Gigaspora margarita* and *Rhizophagus irregularis* was originally isolated from soils of Minia Al-Qamh, El-Sharkia Governorate, Egypt using the wet sieving technique (Gerdemann and Nicolson 1963), and carefully selected from our previously work (Metwally et al. 2024b). The detected AM fungal spores were multiplied twice for 5 months on Sudan grass (*Sorghum sudanense* Pers.) roots, which served as ideal trap plants. Mycorrhizal inoculum was made out of plant roots (85% colonization index) and a spore-containing soil combination.

### 2.2 Preliminary Experiment

A preliminary experiment was carried out in October 2023 at the Department of Botany and Microbiology, Faculty of Science, Zagazig, to demonstrate the effect of different levels of Cr and GB on the growth of trigonella seedlings. Sterilized trigonella seeds were germinated in in slotted polystyrene seedling trays filled with peat moss, and tap water was utilized for watering. After two weeks, potassium dichromate ($K_2Cr_2O_7$), a Cr (VI) source, was combined with water to create seven different levels of Cr solution (0, 10, 25, 50, 100, 200, and 400 mg $kg^{-1}$), which were applied three times per week during irrigation. The degree of respective stress was maintained by providing Cr solution in the respective slots at 7 or 14-day intervals. Separately, four GB (trimethyl glycine: $C_5H_{12}NO_2$) levels prepared by dissolving in distilled water and (0, 25, 50 and 100 mM) were applied by spraying the trigonella leaves twice in the early morning; tap water served as the control. The experiment was set up in a completely randomized way, with nine replicates for each treatment. After 7 and 14 days after Cr and GB administrations, when the visual differences between the treatments were visible, morphological parameters such as shoot height (SH), root length (RL), fresh (fwt), and dry weights (dwt) of the shoots and roots were measured. In addition, Cr and GB contents in trigonella seedlings were tested 14 days following their treatment.

### 2.3 Cr Accumulation in Trigonella Seedlings

The Cr content of trigonella seedlings at various Cr levels was measured by atomic absorption spectrometry at Zagazig University's Faculty of Veterinary Medicine. Initially, seedlings from various treatments were washed individually with water, dried at 60 °C for 48 h, and ground to fine powder. Then, using porcelain vessels, a known dwt of each sample was heated in a muffle furnace at 450–500˚C for 5 h to remove the organic components by thermal decomposition. The resultant ashes were then diluted in 10 mL of 2% $HNO_3$. The solutions were agitated at 100 rpm overnight, filtered, and diluted with deionized water to a volume of 25 mL (Ward 1960).

### 2.4 GB Determination in Trigonella Seedlings

The concentration of GB in trigonella seedlings was determined (Grieve and Grattan 1983), with GB serving as the standard. To extract the quaternary ammonium compounds, 1.5 mL of 2 N sulfuric acid was added to the lyophilized trigonella tissues and heated to 60 °C for 10 min. The mixture was centrifuged at 6,000 rpm for 10 min at room temperature. To precipitate GB as golden crystals, 50 µL of cool KI-$I_2$ was added to 125 µL of supernatant, kept at 0 to 4 °C for 16 h, then centrifuged at 14,000 rpm for 30 min at 0 °C. To dilute

the GB crystals, add 1.4 mL of 1,2-dichloroethane and read the absorbance at 356 nm.

## 2.5 Pot Experiment

Based on the pre-experimental results, Cr and GB conc. were chosen for the parallel pot experiment. In which 400 mg $kg^{-1}$ Cr caused severe damage to seedling growth, hence 200 mg $kg^{-1}$ was chosen. It was observed that 50 mM GB significantly increased trigonella development compared to higher and lower concentrations. As a result, a pot experiment was carried out to confirm the beneficial role of AM fungus and GB at 50 mM in alleviating Cr toxicity (200 mg $kg^{-1}$) in trigonella plants. The environmental conditions were comparable to those used in the trial experiment (Temp. 23 °C/14°C (day/night) and 50–75% relative humidity. Plastic pots (30 cm height, 24 cm in diameter) filled with 2.5 kg of clay soil were divided into two groups: AM-inoculated pots and non-AM pots. The soil was collected from Minia Al-Qamh in El-Sharkia Governorate, autoclaved for 1 h at 121 °C for three days, and then divided among the pots. Table 1 lists the experiment soil's initial physicochemical parameters and Cr content. The experiment was set up using a completely randomized design, with three replicates for each treatment.

AM fungal inoculum was applied slightly beneath the trigonella seeds during sowing at a rate of 25 g/pot. The non-AM pots received the same quantity of autoclaved inoculum and the AM inoculum filtrate, reintroducing a native microbial population free of AM propagules. Ten seeds were placed in each pot, watered, and the seedlings were thinned out six seedlings per pot once they emerged. After four weeks of growth and during the vegetative stage, the plants were treated with 200 mg $kg^{-1}$ Cr and 50 mM GB.

**Table 1** Physical and chemical properties of the collected soil

| Soil property | Value |
|---|---|
| Sand % | 9.7 |
| Silt % | 25.5 |
| Clay % | 62.8 |
| Texture | Clay |
| pH | 7.7 |
| Water holding capacity (%) | 169.4 |
| Moisture content | 3.15 |
| Electric conductivity (dS/m) | 1.93 |
| Saturation percentage (%) | 60 |
| Total nitrogen % | 0.58 |
| Organic matter % | 2.24 |
| Organic carbon% | 1.3 |
| Total P % | 0.66 |
| Total K % | 0.33 |
| Exchangeable $Ca^{2+}$ (Meq. $L^{-1}$ ) | 10.61 |
| Exchangeable $Mg^{2+}$ (Meq. $L^{-1}$ ) | 4.98 |
| Exchangeable $K^{+}$ (Meq. $L^{-1}$ ) | 0.42 |
| Exchangeable $Na^{+}$ (Meq. $L^{-1}$ ) | 4.70 |
| $CO_3^{2-}$ (Meq. $L^{-1}$ ) | – |
| $HCO_3^{-}$ (Meq. $L^{-1}$ ) | 5.66 |
| Cl (Meq. $L^{-1}$ ) | 8.47 |
| $SO_4^{-}$ (Meq. $L^{-1}$ ) | 6.57 |
| Cr (mg $kg^{-1}$ ) | 0 |

## 2.6 Evaluation of Growth Attributes of Trigonella

Trigonella plants were harvested 14 days after Cr and GB applications and separated into shoots and roots. The SH and RL of five randomly selected plants per treatment were measured in centimeters (cm). The total fresh weight of shoots and roots (tfwt) was measured in grams using an electronic scale before being placed in a hot air drying oven at 80 °C to determine their dry biomass (tdwt). Furthermore, the extent of trigonella plants' dependence on the presence of AM fungi under Cr stress was expressed as mycorrhizal dependency (MD) and estimated (Menge and Johnson 1978) as follows:

$$\text{Mycorrhizal dependency (MD)} = \frac{(\text{Total dwt of non} - \text{AM inoculated plants})}{(\text{Total dwt of AM} - \text{inoculated plants})} \times 100$$

Moreover, relative reduction of different growth parameters of trigonella plants in response to Cr toxicity was calculated as:

$$\text{Relative reduction (\%)} = \frac{(\text{values under optimal condition} - \text{values under Cr stress condition}}{\text{values under optimal condition}} \times 100$$

Stress tolerance indexes such as shoot height stress tolerance index (SHSTI), root length stress tolerance index (RLSTI), and total fresh and dry weight stress tolerance indices (FWSTI, DWSTI) were also calculated using following formulas:

$$\text{SHSTI (\%)} = \frac{\text{Shoot height of stressed plant}}{\text{Shoot height of control plants}} \times 100$$

$$\text{RLSTI}\,(\%) = \frac{\text{Root length of stressed plant}}{\text{Root length of control plants}} \times 100$$

$$\text{FWSTI}(\%) = \frac{\text{Total fwt of stressed plants}}{\text{total fwt of control plants}} \times 100$$

$$\text{DWSTI}(\%) = \frac{\text{Total dwt of stressed plants}}{\text{total dwt of control plants}} \times 100$$

### 2.7 Mycorrhizal Colonization

Trigonella roots were carefully cleaned to remove sticking soil particles, then cut into 1 cm lengths and preserved in a formalin/acetic acid/ethyl alcohol (5:5:90) solution until further processing. The roots were cleared with 10% KOH (90 °C for 7 min), acidified in 1 N HCl (3 min at room temperature), and dyed with 0.05% trypan blue in lacto-glycerol (90 °C for 7 min) based on Phillips and Hayman's (1970) method. The stained root segments were put on glass slides and examined using a light microscope. Each treatment brought about a total of five slides (10 segments each). The percentage of hyphal root colonization (HC%) was calculated as the proportion of root fragments colonized by hyphae (Trouvelot et al. 1986). Moreover, arbuscules (AC) and vesicles (VC) colonization were recorded.

$$\text{HC}(\%) = \frac{\text{Number of colonized roots}}{\text{Total number of roots}} \times 100$$

### 2.8 Evaluation of AM and GB Alleviation Capacity of Cr Toxicity on Total Chlorophyll, Electrolyte Leakage and Lipid Peroxidation

The total chlorophyll (Chl) content in 0.25 g of fresh trigonella leaves was measured using Arnon's (1949) method and expressed as mg $g^{-1}$ fwt. The Chl was extracted in 85% acetone solution and centrifuged at 6000 rpm for 10 min at 4˚C. The absorbance of the supernatant was recorded at 663 and 645 nm using a UV-visible spectrophotometer (RIGOL-Model Ultra-3660), and its content was calculated as follows:

$$\begin{aligned}\text{Total Chl}\,(\text{mg/g fwt}) = {} & 20.2\,(\text{OD at 663nm}) \\ & + 8.02(\text{OD at 645nm}) \\ & \times \frac{\text{total volume}}{1000 \times \text{fwt}}\end{aligned}$$

Electrolyte leakage (EL) was detected according to Valentovic et al (2006). Briefly, leaves from all treatments were chopped into small (5 mm) sections and placed to glass bottles containing 30 mL deionized water. The bottles were left at room temperature for 24 h to measure the initial electrical conductivity (EC1) and then incubated at 95˚C for 30 min to determine the final electrical conductivity (EC2). The EL was computed using the following equation:

$$\text{EL}\,(\%) = \frac{\text{EC1}}{\text{EC2}} \times 100$$

Malondialdehyde (MDA) contents in trigonella fresh shoots were measured according to Heath and Packer (1968) after being homogenized in 5 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 6000 rpm for 10 min. Subsequently, the supernatant (2mL) was added to 4 mL of 0.5% thiobarbituric acid in 20% TCA and heated in a hot water bath (95 ˚C) for 30 min. The reaction was stopped on ice, and absorbance was measured at 532 and 600 nm. The MDA content was calculated by subtracting the nonspecific absorption at 600 nm from the absorption at 532 nm, with an absorbance coefficient of extinction of 155 $mM^{-1}$ $cm^{-1}$.

### 2.9 Determination of Shoot Proline, Total Free Amino Acids (TFAA), Total Soluble Carbohydrates (TSC) and Glomalin-Related Soil Proteins (GRSP) Contents

The proline level in 0.25 g fwt of trigonella shoot was determined using the Bates et al. (1973) technique at 520 nm after homogenizing it in 3% aqueous sulphosalicylic acid. Proline concentration was given as µM $g^{-1}$fwt. In addition, the TFAA level was determined in trigonella shoots using the method established by Lee and Takahashi (1966). The level of TSC was assessed using the Dubois et al. (1956) technique. Total and easily extractable glomalin-related soil proteins (T- and EE-GRSP) were extracted from 2 g rhizosphere soil using Wright and Upadhyaya's (1996) method, and their levels were quantified using the Coomassie brilliant blue colorimetric method (Bradford 1976).

### 2.10 Total Soluble Protein (TSP) and Enzymatic Antioxidants Extraction and Estimation

One g of trigonella shoot fwt was homogenized with 10 mL of 0.1 M potassium phosphate buffer (PPB) (pH: 7.0). The homogenate was centrifuged at 6000 rpm for15 min. The supernatant was collected as a crude extract and refrigerated for total soluble protein (TSP) and antioxidant enzymes assay at the same time. The method of Lowry et al. (1951) was adopted to determine TSP content.

#### 2.10.1 Catalase (CAT)

To determine CAT activity, a 3 mL reaction mixture containing 2.5 mL of 0.1 M PPB (pH 7.0), 0.2 mL supernatant, and 0.3 mL of 100 mM $H_2O_2$ were added sequentially, and the absorbance was promptly measured at 240 nm (Aebi 1984).

#### 2.10.2 Ascorbate Peroxidase Activity (APX)

APX was measured (Nakano and Asada 1981) in a reaction mixture containing 2.5 mL of 0.1 M PPB (pH 7.0), 0.1 mL L-ascorbate, and 0.15 mL $H_2O_2$. The reaction was started by adding 250 µL of enzyme extract. A decrease in absorbance at 290 nm was measured spectrophotometrically compared to a blank. The enzyme activity was determined based on ascorbate's molar extinction coefficient of 2.8 $mM^{-1}$ $cm^{-1}$. One enzyme unit is equivalent to the amount necessary to oxidize 1 nmol of ascorbic acid/min.

#### 2.10.3 Peroxidase (POD)

POD activity was assayed by Chance and Maehly (1955). The reactant mixture included 50 mM PPB (pH 7.0), 1% guaiacol, 0.4% $H_2O_2$ and 100 µL enzyme extract. The absorbance was measured at 470 nm.

### 2.11 Total Antioxidant Capacity (TAC) in Trigonella Under Cr Stress

TAC content in trigonella extract was expressed as µg /g fwt after the methanolic extract was combined with a reagent solution containing 4 mM ammonium molybdate, 0.6 M sulfuric acid, and 28 mM sodium phosphate, as described by Prieto et al. (1999). After 90 min of incubation in a boiling water bath, the tubes were cooled, and the absorbance was measured at 695 nm.

### 2.12 Statistical Analyses

The morpho-physiological and biochemical parameters of trigonella plants under Cr stress were affected differently by GB and AM treatments. The acquired data were compared using a one-way ANOVA. The graphical presentation was created using Origin. Pearson correlation coefficients were produced for trigonella's measured variables. Hierarchical clustering analysis and principal component analysis (PCA) graphs between different treatment used in this study and trigonella measured parameters were created using Past.

## 3 Results

### 3.1 Preliminary Experiment

#### 3.1.1 GB and Cr Effects on the Morphological Traits of Trigonella Seedlings

Tables 2 and 3 show the preliminary results of applying Cr and GB at different conc. on the morphological growth features of trigonella seedlings. The foliar spray of various levels of GB revealed that 50 mM GB was the most effective in enhancing trigonella growth when compared to other levels and the control. The results also showed that trigonella treated with 50 mM GB conc. had the highest seedling SH, RL, fwt, and dwt when compared to the higher conc. or control, implying that 50 mM GB is the appropriate one to alleviate Cr detrimental effects on trigonella growth and may be suitable to modulate the antioxidant defense system on trigonella in the greenhouse experiment. Besides, Fig. 1C showed the effect of GB on the morphological features of trigonella seedlings where these features were increased with increasing GB conc. up to 50 mM GB as compared to their respective controls.

Also, a declining trend in trigonella seedling growth attributes was seen at all Cr conc. (25, 50, 100, 200, and 400 mg $kg^{-1}$), with the greatest drop occurring at Cr 400 mg $kg^{-1}$ (Fig. 1A). Higher Cr conc. resulted in decreased root and shoot height, and biomass of trigonella seedlings (Fig. 1A and Table 2).

#### 3.1.2 Cr Uptake by Trigonella Seedlings

Figure 1B shows the Cr content in trigonella seedlings after 14 days of Cr treatment. Significant increases in Cr accumulation in trigonella seedlings were detected under various Cr conc. (0.3, 0.39, 0.587, 0.973, 1.397, and 2.19 µg $g^{-1}$ dwt) as compared to the control. The lowest Cr value was observed at 10 mg $kg^{-1}$ Cr. The varying levels of Cr content in trigonella seedlings under different Cr conc. indicated concentration-dependent performance of trigonella, which, in turn, resulted in decreased trigonella growth traits as shown in Fig. (1 A) and Table (2).

#### 3.1.3 GB Content in Trigonella Seedlings

Exogenous foliar spray of trigonella leaves with varying levels of GB (0, 25, 50, and 100 mM) increased endogenous GB content (8.18, 21.042, 41.67, and 75.40 mg/g fwt) in trigonella seedlings, and this induction was dose dependent (GB content increased in tandem with GB application level) (Fig. 1D and E). Trigonella seedlings treated with the

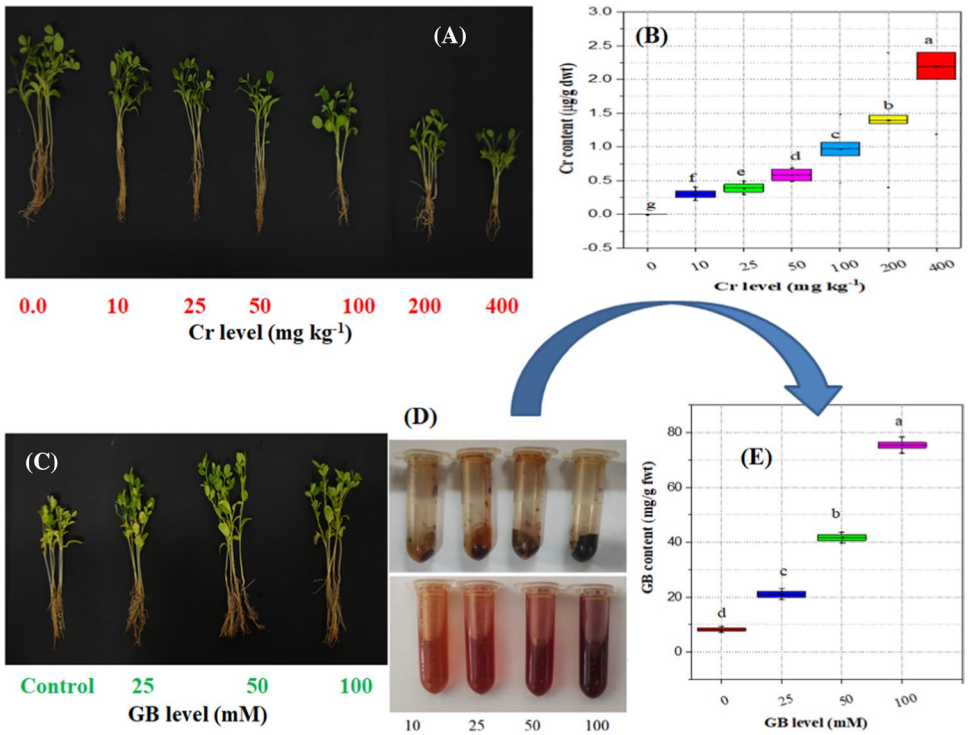



**Fig. 1** (**A**) represents trigonella seedlings after 7 days of Cr different levels application (ppm). (**B**) represents the Cr content (µg/g dwt) in trigonella seedlings after 14 days of Cr (mg $kg^{-1}$) different levels application. (**C**) displays the effect of GB (mM) on trigonella seedlings after 14 days of GB application. (**D**) and (**E**) show the GB content (mg/g fwt) in trigonella seedlings after foliar spraying of different levels of GB (mM)

highest GB level had a greater GB content than the control and other levels.

## 3.2 Greenhouse Experiment

### 3.2.1 Cr-Toxicity Inhibits the Morphological Traits of Trigonella Plants

In parallel, a pot experiment was carried out to investigate morphological changes such as SH, RL, tfwt, and tdwt (Fig. 2) in response to Cr-200 stress, as well as the alleviating impact of AM and GB (50 mM). Cr stress lowered these morphological features. At Cr 200 mg $kg^{-1}$, the percent reduction of SH, RL, tfwt, and tdwt was recorded as 36.15%, 18.75%, 27.30%, and 27.34%, respectively, whereas in plants with GB treatment, the drop was decreased (24.35%, 12.50%, 25.24%, and 25.32%), as compared with the control plants. However, a considerable rise in trigonella SH, RL, tfwt, tdwt, and stress indices following AM colonization under Cr toxicity verified the efficacy of AM fungi in comparison to control (Fig. 2). Interestingly, the treatment of AM or GB, either individually or in combination, reduced Cr toxicity, as seen by increases in SHSTI, RLSTI, FWSTI, and DWSTI values (Table 4). The results also showed that the highest trigonella SH and RL were found in GB-AM-inoculated plants under non-stress conditions. Statistics revealed that a combined application of AM and GB performed much better than their individual applications under control or Cr toxicity. On average, AM + GB combined application improved SH (34.28%) and tfwt (28.81%) of trigonella compared to Cr 200. It is clear that AM differed significantly from the control or GB treatment in non-stressed or stressed conditions for all evaluated growth metrics. The notable conclusion was that 200 mg $kg^{-1}$ Cr increased MD of trigonella plants (Table 4) compared to those that were not stressed, indicating that the improved development of trigonella under Cr stress is heavily reliant on AM fungi.

### 3.2.2 Mycorrhizal Hyphal Colonization (HC%)

Cr stress has no effect on HC percentage of colonized trigonella roots as shown in Fig. 3A. All trigonella roots either applied with Cr or GB were colonized with AM fungi. Different AM structures were detected in the AM colonized trigonella roots (Fig. 3D–E) as arbuscules, vesicles and intraradical hypha in cortical cells. Also, there was a significant effect on vesicles (VC%, Fig. 3B) and arbuscules colonization (AC%, Fig. 3C) of trigonella roots with GB (50 mM) and Cr applications as compared to non-stressed ones. Comparing with the non-stressed roots, VC and AC rates increased significantly with Cr which reached to 83.32, and 43.16%. The increase of AC% was at its maximum rate (59.77%) with GB application under Cr-stressed conditions as it increases the growth of mycorrhiza and speed up its colonization as compared with the control ones.

**Table 2** Morphological parameters of trigonella seedling after 7 and 14 days of Cr application

| Treatments Cr conc. (mg $kg^{-1}$) | Length (cm/plant) | | | | Fwt (g/plant) | | | | Dwt (g/plant) | | | | Leaves number | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Shoot | | Root | | Shoot | | Root | | Shoot | | Root | | | |
| | 7 days | 14 days | 7 days | 14 days | 7 days | 14 days | 7 days | 14 days | 7 days | 14 days | 7 days | 14 days | 7 days | 14 days |
| 0 | 8.1 ± 0.21a | 9 ± 0.23a | 5.7 ± 0.15a | 7.3 ± 0.19a | 0.292 ± 0.007a | 0.3578 ± 0.009a | 0.12 ± 0.0032a | 0.2028 ± 0.0053a | 0.0246 ± 0.00065a | 0.03012 ± 0.00079a | 0.00752 ± 0.00019a | 0.01278 ± 0.00034a | 6 ± 0.158a | 7 ± 0.185a |
| 10 | 7.3 ± 0.19b | 8.4 ± 0.22b | 5.4 ± 0.14a | 6.8 ± 0.18b | 0.252 ± 0.006b | 0.2672 ± 0.007b | 0.112 ± 0.0029b | 0.1708 ± 0.0045b | 0.0179 ± 0.00047b | 0.0285 ± 0.00075ab | 0.00628 ± 0.00016b | 0.01082 ± 0.00028b | 5 ± 0.132b | 7 ± 0.185a |
| 25 | 6.8 ± 0.18b | 7 ± 0.18c | 5 ± 0.13b | 6.2 ± 0.16c | 0.224 ± 0.006b | 0.2578 ± 0.007c | 0.074 ± 0.0019c | 0.1558 ± 0.0041c | 0.0158 ± 0.00042c | 0.02728 ± 0.00072b | 0.00564 ± 0.00015c | 0.01066 ± 0.00028b | 5 ± 0.132b | 7 ± 0.185a |
| 50 | 6 ± 0.16c | 6.5 ± 0.17 cd | 4.4 ± 0.12c | 5.9 ± 0.16c | 0.252 ± 0.006c | 0.2574 ± 0.007c | 0.07 ± 0.0018c | 0.1011 ± 0.0027d | 0.0148 ± 0.00039 cd | 0.02448 ± 0.00065c | 0.00478 ± 0.00013d | 0.0089 ± 0.00023c | 5 ± 0.132b | 6 ± 0.159b |
| 100 | 5.5 ± 0.14 cd | 6 ± 0.16de | 4 ± 0.11d | 5.4 ± 0.14d | 0.211 ± 0.005c | 0.2534 ± 0.007c | 0.068 ± 0.0018c | 0.0788 ± 0.0021e | 0.0138 ± 0.00037d | 0.02364 ± 0.00062c | 0.00438 ± 0.00011d | 0.00654 ± 0.00017d | 5 ± 0.132b | 6 ± 0.158b |
| 200 | 5 ± 0.13d | 5.8 ± 0.15e | 3.2 ± 0.08e | 5 ± 0.13d | 0.191 ± 0.005d | 0.216 ± 0.008d | 0.048 ± 0.0013d | 0.0576 ± 0.0015f | 0.0136 ± 0.00036d | 0.02092 ± 0.00055d | 0.00338 ± 0.00009e | 0.00488 ± 0.00013e | 5 ± 0.132b | 6 ± 0.159b |
| 400 | 4.2 ± 0.11e | 4.6 ± 0.12f | 2.9 ± 0.07e | 3.7 ± 0.09e | 0.171 ± 0.004e | 0.198 ± 0.005e | 0.032 ± 0.0008e | 0.0338 ± 0.00089 g | 0.0104 ± 0.00027e | 0.01972 ± 0.00052d | 0.00296 ± 0.00008f | 0.00398 ± 0.00011f | 4 ± 0.0106c | 5 ± 0.132c |

The data in the table was mean ± standard error of the means ($N = 6$). Letters indicate statistical significance at $p \leq 0.05$ level where different small letters inside each column (a, b, c, d…) indicate significant difference between treatments, according to a Duncan multiple range as a post hoc test

### 3.2.3 Changes in Total Chl Content

To investigate the photosynthetic changes in response to Cr, the total Chl content of trigonella leaves was measured. Cr stress reduced total Chl (31.30%) in trigonella leaves compared to the control group (Fig. 4A). However, AM and GB applications improved their content whether under controlled or stressed situations. Under Cr 200 mg $kg^{-1}$, decreases in its level were 9.10, 12.32, and 1.02% in plants treated with AM, GB, or AM + GB, respectively, as compared to non-treated control plants.

### 3.2.4 Oxidative Stress Markers in Trigonella in Response to Cr Stress

The level of oxidative stress generated by Cr toxicity in trigonella shoots was confirmed by comparing oxidative stress markers (EL as well as MDA levels) to the control (Fig. 4B and C). Cr promotes excessive ROS generation, leading in alterations in antioxidant enzyme activity and increased oxidative stress. Moreover, Fig. (4B and C) show that GB and AM fungal treatments improve membrane biochemical properties and reduce oxidative stress in response to Cr stress. Under 200 mg $kg^{-1}$ Cr stress, the most significant increase in MDA and EL levels was seen as compared to controls. At this level, MDA and EL conc. increased by 75.84 and 33.92%, respectively, compared to the control group. However, GB and AM administered singly or in combination considerably reduced these oxidative stress indicators (MDA and EL) and produced the lowest levels as compared to Cr-stressed one. Thus, 50 mM GB combined with AM was found to be the most effective at lowering oxidative stress parameters.

### 3.2.5 Effect of GB and AM treatments on non-enzymatic antioxidants (Proline, TSP, TSC and TFAA) Contents in Trigonella Under Cr Stress

It was discerned that Cr increased ROS formation, which then activated enzymatic and non-enzymatic antioxidant components as seen in Table (5) and Fig. (5); the quantity of these antioxidants indicates the extent of the stress. The effects of GB and AM on Cr toxicity in trigonella were investigated using proline, TSP, TSC, and TFAA. All of these metabolites rose significantly under Cr stress, with the exception of TFAA, which decreased with either AM or GB (Table 5). A further rise in proline and TSP levels was found with GB exogenous application or AM application, either alone or together. Table (5) shows that at 200 mg $kg^{-1}$ Cr, the highest increase in proline and TSP conc. (93.49 and 47.95%) was found in shoots of trigonella

**Table 3** Effect of GB different conc. On trigonella seedling parameters after 14 days of GB spray

| GB conc. (mM) | Shoot length (cm/plant) | Root length (cm/plant) | Fwt (g/plant) | | Dwt (g/plant) | | leaf no./plant |
|---|---|---|---|---|---|---|---|
| | | | shoot | root | shoot | root | |
| 0 | 6.30 ± 0.167c | 6.10 ± 0.161c | 0.265 ± 0.007d | 0.083 ± 0.0022c | 0.024 ± 0.0006c | 0.0060 ± 0.00016d | 6.00 ± 0.159c |
| 25 | 8.50 ± 0.225b | 6.70 ± 0.177b | 0.301 ± 0.0079c | 0.106 ± 0.0028d | 0.027 ± 0.0007b | 0.0077 ± 0.0002c | 7.00 ± 0.185b |
| 50 | 9.70 ± 0.257a | 7.70 ± 0.204a | 0.421 ± 0.011a | 0.155 ± 0.0041a | 0.037 ± 0.00098a | 0.0105 ± 0.00028a | 8.00 ± 0.212a |
| 100 | 9.00 ± 0.238ab | 7.00 ± 0.185b | 0.363 ± 0.0096b | 0.135 ± 0.0036b | 0.036 ± 0.00095a | 0.0086 ± 0.00023b | 7.50 ± 0.198ab |

The data in the table was mean ± standard error of the means ($N = 6$). Letters indicate statistical significance at $p \leq 0.05$ level where different small letters inside each column (a, b, c, d…) indicate significant difference between treatments, according to a Duncan multiple range as a post hoc test

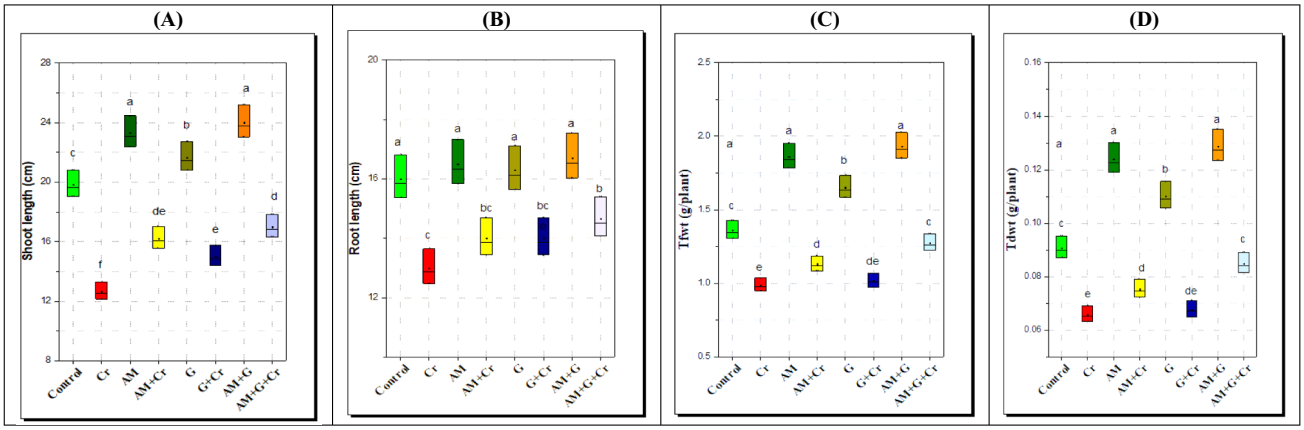



**Fig. 2** Effect of GB and AM on shoot length (**A**), root length (**B**), total fresh weight (tfwt; **C**) and total dry weight (tdwt; **D**) of trigonella plants under Cr stress. The data was the mean ± standard error ($N = 6$). Letters indicate statistical significance at $p \leq 0.05$ level where different small letters above column (a, b, c, d…) indicate significant difference between treatments, according to a Duncan multiple range as a post hoc test

**Table 4** Mycorrhizal dependency (MD), relative reduction, shoot height stress tolerance index (SHSTI), root length stress tolerance index (RLSTI), fresh weight stress tolerance index (FWSI) and dry weight stress tolerance index (DWSI) of trigonella plants under different treatments

| Treatments | MD (%) | | Relative reduction (%) | SHSTI (%) | RLSTI (%) | FWSTI (%) | DWSTI (%) |
|---|---|---|---|---|---|---|---|
| | tdwt | Shoot height | | | | | |
| Control | – | – | – | – | – | – | – |
| Cr | – | – | 36.45 ± 0.964a | 63.84 ± 1.689c | 81.25 ± 2.15b | 72.69 ± 1.92c | 72.69 ± 1.923c |
| AM | 73.16 ± 1.93c | 85.10 ± 2.25b | – | – | – | – | – |
| AM + Cr | 120.40 ± 3.18a | 122.40 ± 3.23a | 18.30 ± 0.484c | 81.69 ± 2.16ab | 87.50 ± 2.315ab | 83.13 ± 2.19b | 83.05 ± 2.197b |
| GB | – | – | – | – | – | – | – |
| GB + Cr | – | – | 24.35 ± 0.644b | 75.64 ± 2.00b | 87.50 ± 2.315ab | 74.72 ± 1.97c | 74.67 ± 1.975c |
| AM + GB | 70.49 ± 1.86c | 82.62 ± 2.18b | – | – | – | – | – |
| AM + GB + Cr | 106.83 ± 2.86b | 116.64 ± 3.08a | 14.27 ± 0.377d | 85.72 ± 2.27a | 91.62 ± 2.42a | 93.64 ± 2.47a | 93.60 ± 2.476a |

The data in the table was mean ± standard error of the means ($N = 6$). Letters indicate statistical significance at $p \leq 0.05$ level where different small letters inside each column (a, b, c, d…) indicate significant difference between treatments, according to a Duncan multiple range as a post hoc test

plants dually treated with 50 mM GB and AM compared to the control. However, after AM fungal application, TSC content dropped (Table 5), which could be attributed to the mitigation of Cr toxicity caused by AM fungi's elevation of morphological features (Fig. 2).

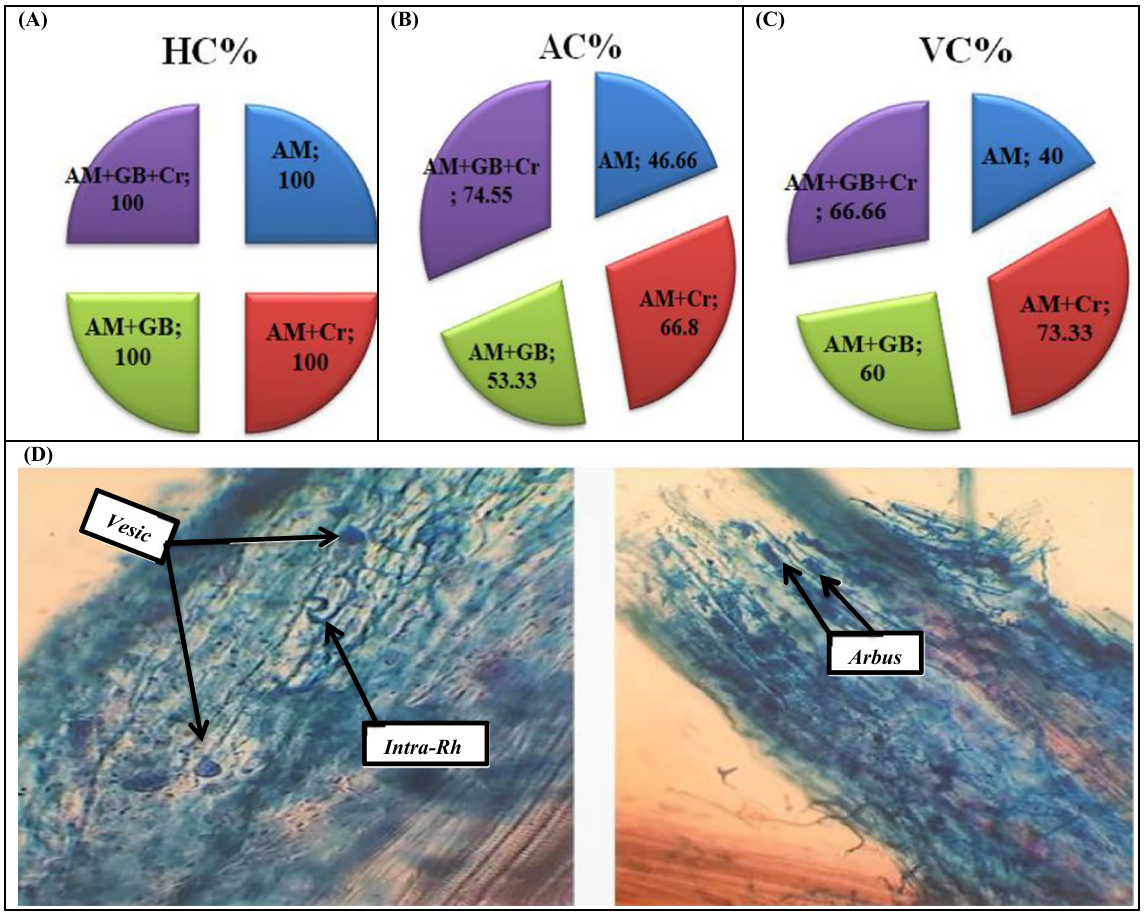



**Fig. 3** AM colonization status of trigonella with AM fungi. (**A–C**) represents the effect of GB and 200 ppm Cr on mycorrhizal levels (HC%: hyphal, AC%: arbuscules and VC%: vesicles colonization) of trigonella plant roots. (**D**) represents colonized trigonella root showing AM fungal structures. Where, *Intra-Rh*: intra-radical hyphae, *Vesic*: vesicles and *Arbus*: arbuscules

#### 3.2.6 Effects of Cr Stress and AM Inoculation on T- and EE-GRSP

Under Cr treatment, both T-GRSP and EE-GRSP levels rose in comparison to the control (Table 5). AM fungal colonization significantly ($p < 0.05$) increases their contents under control or Cr-stressed conditions, while GB produces a small rise. Furthermore, the correlation data (Fig. 5) showed that Cr stress and AM inoculation had a substantial interaction effect on T-GRSP and EE-GRSP.

#### 3.2.7 Effect of GB and AM on the Anti-Oxidative System in Trigonella Under Cr Toxicity

The antioxidant enzyme activities (CAT, APX, and POD) and TAC conc. in trigonella shoots were measured to investigate Cr's harmful effects and the involvement of AM or GB in alleviating them. TAC content, APX, and POD activities increased in trigonella shoots after Cr stress treatment, regardless of GB or AM treatments (Fig. 6B, C and D). At 200 mg $kg^{-1}$ Cr stress, TAC, APX, and POD activity elevated by 10, 24.30, and 7.37%, respectively. However, there was no significant difference in CAT activity between control and stressful circumstances (Fig. 6A). GB and AM, either separately or in combination, increased POD and APX activity in both control and Cr-stressed trigonella shoots (Fig. 6B and C). AM-trigonella treated with 50 mM GB showed the greatest increase in APX activity and TAC content (35.1 and 39.52%, respectively), indicating that GB and AM reduce Cr-induced toxicity, as evidenced by lower EL and MDA levels, implying that both AM and GB have superior stress tolerance capacity (Fig. 4B and C).

#### 3.2.8 Understanding Relations between Different Treatments and Measured Parameters through Pearson Correlation, Hierarchical Clustering Analysis and PCA-Based Biplot

Pearson correlation, Hierarchical clustering analysis and PCA were drawn to assess the data variability and better

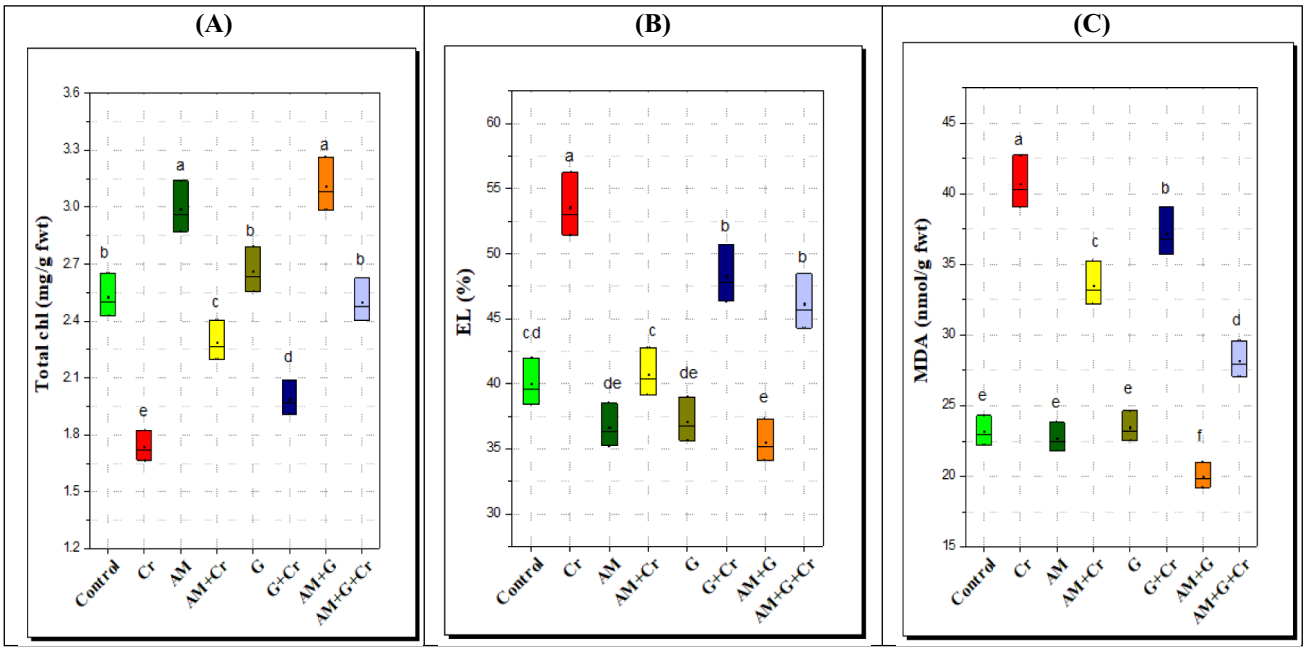



**Fig. 4** Effect of GB and AM on total chlorophyll (total Chl; **A**), electrolyte leakage (EL; **B**) and malondialdehyde (MDA; **C**) of trigonella plants under Cr stress. The data was the mean ± standard error ($N = 6$). Letters indicate statistical significance at $p \leq 0.05$ level where different small letters above column (a, b, c, d…) indicate significant difference between treatments, according to a Duncan multiple range as a post hoc test

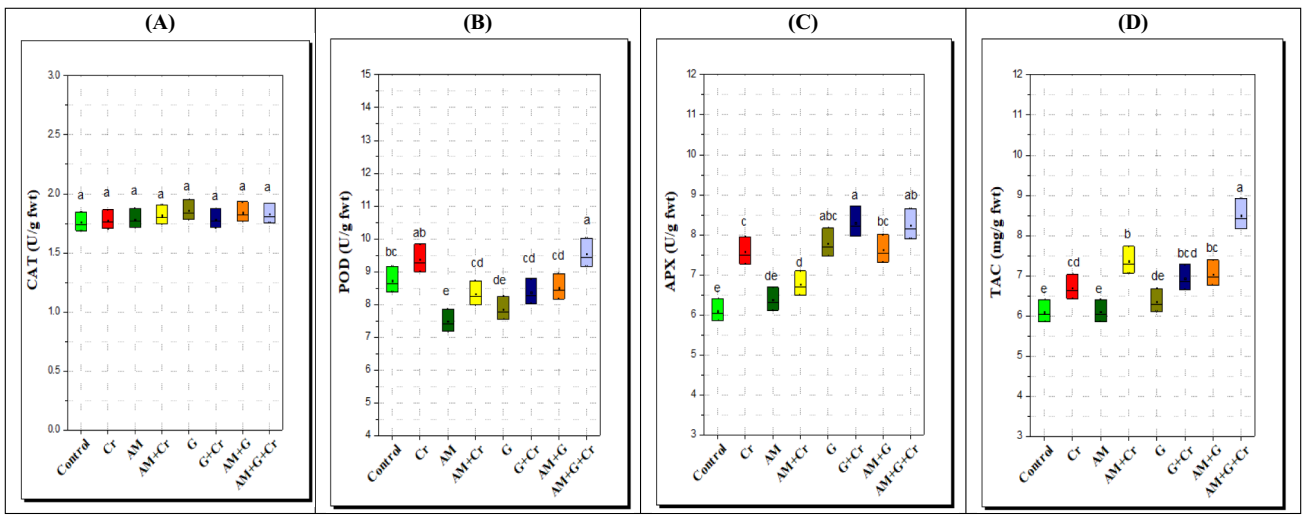



**Fig. 5** Effect of GB and AM on the activity of catalase (CAT; **A**), peroxidase (POD; **B**), ascorbate peroxidase (APX; **C**) and total antioxidant capacity (TAC; **D**) of trigonella plants under Cr stress. The data was the mean ± standard error (N= 6). Letters indicate statistical significance at $p \leq 0.05$ level where different small letters above column (a, b, c, d…) indicate significant difference between treatments, according to a Duncan multiple range as a post hoc test

understand the results (Figs. 5 and 7). Growth attributes (SL, RL, tfwt, and tdwt), and physio-biochemical parameters all had varying Pearson's correlations (Fig. 5A). This figure showed a strong positive correlation between growth parameters, total Chl, carbohydrates and TFAA, on contrary, a significant negative correlation was found between stress markers (MDA and EL) and most of the measured parameters. The Hierarchical clustering analysis (Fig. 5B), which verified the differences among treatments, revealed the clustering between treatments of AM and AM + GB followed

**Table 5** Effect of GB and AM on the content of proline, total soluble carbohydrates (TSC), total soluble protein (TSP) and total free amino acids (TFAA) of trigonella plants and easily extractable glomalin related soil protein (EE-GRSP) and total glomalin related soil protein (T-GRSP) under cr stress

| Treatments | Proline (µmols/g fwt) | TSC (mg/g dwt) | TSP (mg/g fwt) | TFAA (mg/g fwt) | EE-GRSP (mg/g soil) | T-GRSP (mg/g soil) |
|---|---|---|---|---|---|---|
| Control | 93.753 ± 2.48e | 245 ± 6.48de | 29.563 ± 0.782e | 9.10 ± 0.241b | 0.523 ± 0.0138e | 1.445 ± 0.038e |
| Cr | 138.42 ± 3.66c | 287.16 ± 7.59b | 33.191 ± 0.878 cd | 7.87 ± 0.208d | 0.539 ± 0.0143de | 1.753 ± 0.046d |
| AM | 114.40 ± 3.03d | 264.5 ± 6.99 cd | 34.193 ± 0.905c | 10.25 ± 0.711a | 0.803 ± 0.0212c | 2.682 ± 0.071c |
| AM + Cr | 163.10 ± 4.3b | 217.66 ± 5.76f | 37.679 ± 0.996b | 8.35 ± 0.221bcd | 1.019 ± 0.0269ab | 3.606 ± 0.095a |
| GB | 90.68 ± 2.39e | 330 ± 8.73a | 31.075 ± 0.822de | 9.02 ± 0.238bc | 0.571 ± 0.0151de | 1.775 ± 0.047d |
| GB + Cr | 124.14 ± 3.28d | 143.5 ± 3.79 g | 33.995 ± 0.899 cd | 8.31 ± 0.219 cd | 0.603 ± 0.0159d | 1.841 ± 0.048d |
| AM+GB | 121.41 ± 3.21d | 267.66 ± 7.08bc | 32.707 ± 0.865 cd | 9.94 ± 0.263a | 0.971 ± 0.0256b | 2.721 ± 0.072c |
| AM + GB + Cr | 181.41 ± 4.79a | 237.5 ± 6.28ef | 43.739 ± 1.157a | 8.59 ± 0.227bcd | 1.079 ± 0.0285a | 3.095 ± 0.082b |

The data in the table was mean ± standard error of the means ($N = 6$). Letters indicate statistical significance at $p \leq 0.05$ level where different small letters inside each column (a, b, c, d…) indicate significant difference between treatments, according to a Duncan multiple range as a post hoc test

by control and GB treatment. This indicates the role of AM and/ or GB in enhancing the plant growth and performance. On the PCA-associated scatter plot, we observed a strong separation between the treatments in terms of PC1 (91.58%) and PC2 (7.63%) (Fig. 7). Furthermore, the PCA-associated loading plot indicated a negative link of Cr treatment to the trigonella plant where stress markers (MDA and EL) were positively associated with Cr treatment. On contrast, treatments of AM, AM + GB, GB and control treatment were positively grouped with and all growth attributes, including RL, SL, tfwt and total Chl, while negatively connected with MDA and EL. Other treatments of AM + Cr, AM + GB + Cr were greatly linked with the protein, proline and antioxidant. The PCA results were agreed with the Hierarchical clustering analysis and overall, these findings suggested that combining AM and GB could be a viable method for increasing trigonella tolerance while avoiding Cr toxicity.

## 4 Discussions

The foliar spray of various levels of GB revealed that 50 mM GB was the most effective at enhancing trigonella growth when compared to other levels and the control. Many researchers reported that GB had positive impacts on the growth and development of plants under control or stressed conditions (Shahbaz et al. 2011; Bharwana et al. 2014; Ahmad et al. 2020). However, a higher amount of GB (100 mM) had no greater influence on growth than 50 mM GB, and in some cases produced a response similar to 25 mM GB. This could be due to the phytotoxicity of GB (Mickelbart et al. 2003; Hu et al. 2012). Our findings are consistent with Lou et al.'s (Lou et al. 2015; Singh et al. 2020) findings on perennial ryegrass. Moreover, higher Cr conc. resulted in decreased root and shoot height, and biomass of trigonella seedlings; similar Cr toxicity effects were seen in *C. arietinum* (Singh et al. 2020) and mung beans (Jabeen et al. 2016). These findings suggest that Cr ions may interact with some critical mineral elements in soil, reducing their uptake by plants (del Real et al. 2013). Cr stress caused a decrease in seedling RL, which could be due to Cr accumulation in root cells or damage to root tip cells, whereas the decrease in shoot length could be due to ultrastructural damage in leaf mesophyl cells, resulting in decreased shoot development (Kumar 2021). Singh et al. (2021) also noted that Cr accumulation had a significant impact on root seedlings growth due to its immobilization in root cells.

Significant increases in Cr accumulation in trigonella seedlings were detected under various Cr conc., which, in turn, resulted in decreased trigonella growth traits, as higher Cr concentrations resulted in enhanced Cr uptake by plants. Our findings on Cr accumulation are consistent with those of Anjum et al. (2017) and Singh et al. (2020) in *Zea mays* and *C. arietinum.* Moreover, exogenous foliar spray of trigonella leaves with varying levels of GB increased endogenous GB content in trigonella seedlings. These findings are consistent with those of Mahmood et al. (2009) and Lou et al. (2015), who found that exogenous GB treatment increased GB content in wheat and perennial ryegrass plants during drought and Cd stress conditions.

Cr stress lowered the morphological development features whereas in plants with GB treatment, the drop was decreased as compared with the control plants. Our findings are consistent with Danish et al. (2019), who found that Cr inhibits plant development by inducing Chl content degradation and poor nutrient uptake. Additionally, Alam et al. (2022) revealed that the carotenoids level, total Chl, shoot and root lengths, Chl a, and Chl b, were all reduced in Faba bean plants treated with 300 µM Cr. Higher Cr intake also leads to decreased root growth and chlorosis (Lopez-Luna

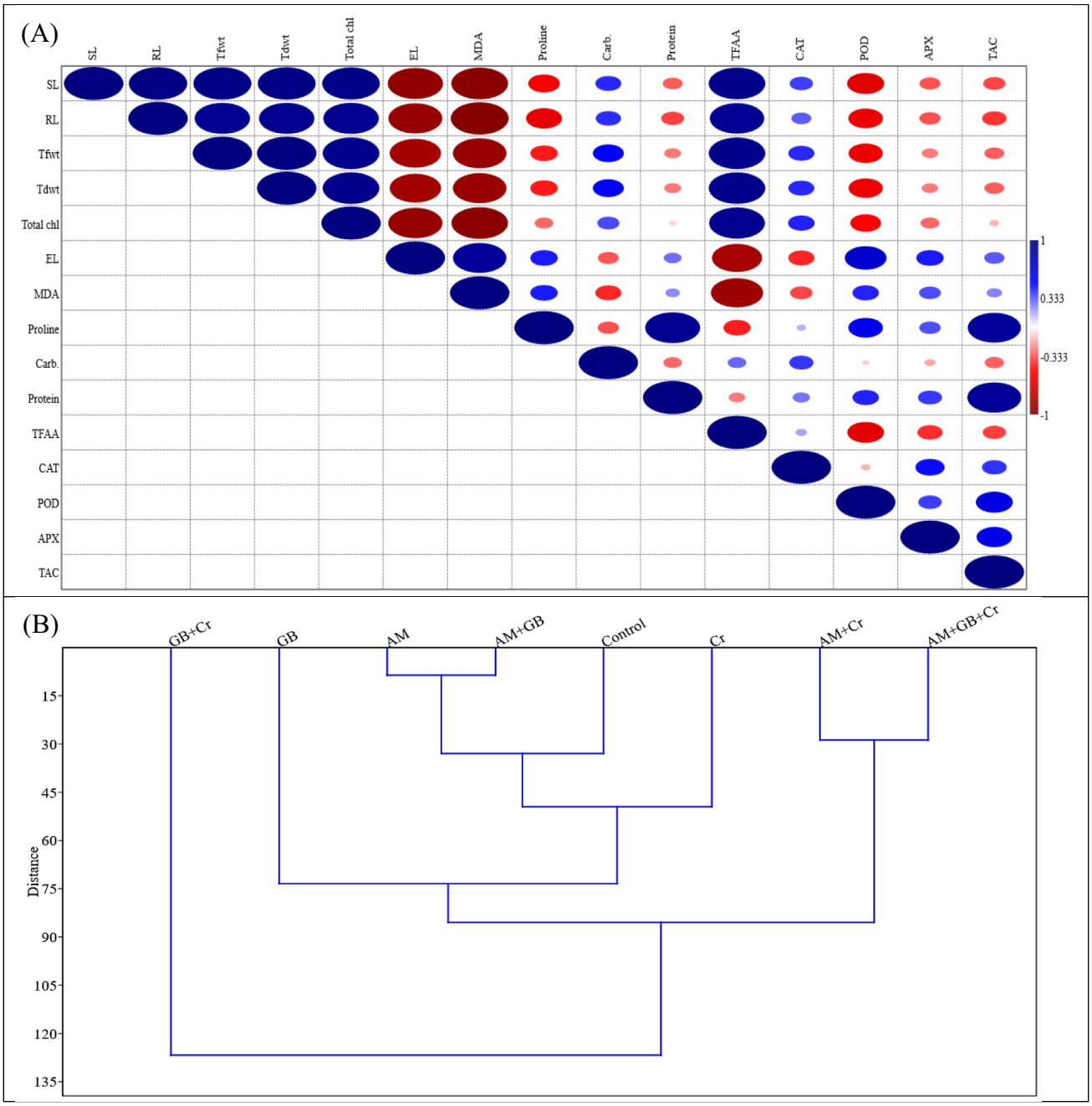



**Fig. 6** (**A**) Pearson correlation between different measured parameters and (**B**) Hierarchical clustering analysis (HCA) between different treatments. (SL: shoot length, RL: root length, Tfwt: total fresh weight, Tdwt: total dry weight, total chl: total chlorophyll, MDA: malondialdehyde, EL: electrolyte leakage, carbo: carbohydrates, TFAA: total free amino acids, TAC: total antioxidant capacity, CAT: catalase, APX: ascorbate peroxidase, POD: peroxidase)

et al. 2009; Sundaramoorthy et al. 2010). Furthermore, Panda and Patra (2000)reported a similar result in terms of reduced root elongation due to inhibitition of cell division under Cr toxicity. Interestingly, the treatment of AM or GB, either individually or in combination, reduced Cr toxicity, as seen by increases in morphological parameters, SHSI, SLSI, FWSI, and DWSI values. This rise in morphological features could be attributed to improved nutritional intake. Also, the increased MD of trigonella plants under 200 mg $kg^{-1}$Cr compared to those that were not stressed, indicating its heavily reliant on AM fungi under Cr stress. These findings are consistent with Metwally and Abdelhameed (2018); Abdelhameed and Metwally (2022) under salt stress, and they support our argument that better nutrient absorption by

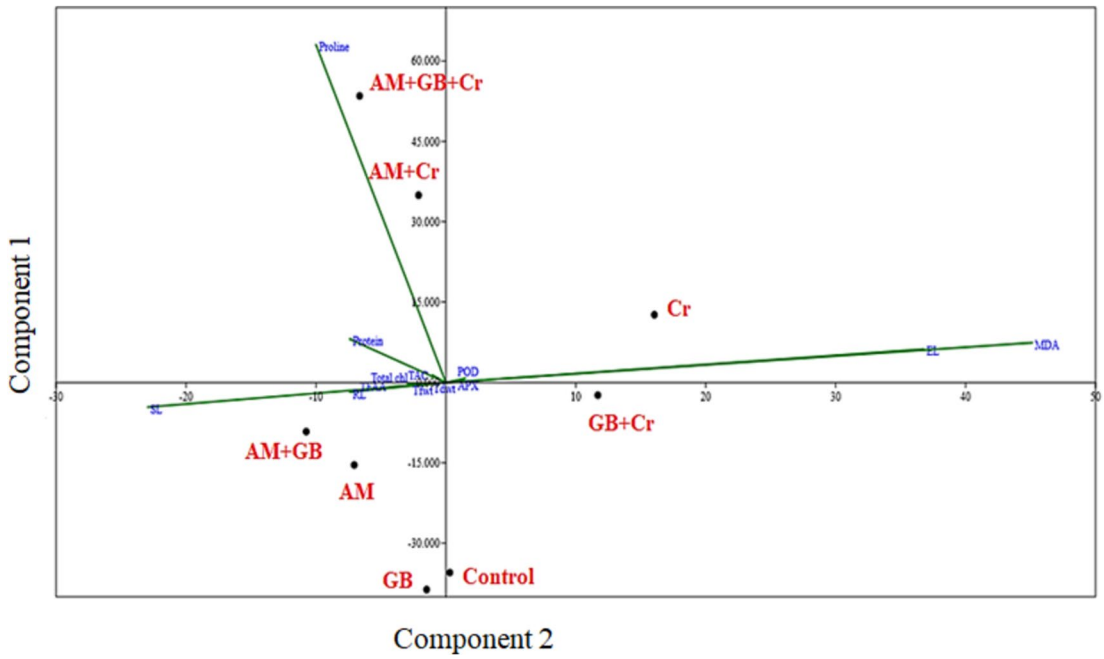



**Fig. 7** Ordination diagram (biplot) of principal component analysis (PCA), showing interaction between different treatment (red color) and parameters (blue color) under study (SL shoot length, RL root length, MDA malondialdehyde, EL electrolyte leakage, TAC total antioxidant capacity, APX, ascorbate peroxidase, POD peroxidase)

AM fungi can improve the tfwt and tdwt of trigonella plants. Significant improvement in RL is crucial for enhanced nitrogen uptake in plants (Abdelhameed and Metwally 2022). Vivas et al. (2006) and Kumar (2021)proved AM's extraordinary ability to boost host plant growth and identified AM fungi as a potential biological agent that enhances the host plant's ability to endure heavy metal stress.

In addition, Hasanuzzaman et al. (2014) and Tisarum et al. (2019) revealed the influence of GB in stress tolerance in rice seedlings and sweet potato under salt and drought stresses. Furthermore, Ahmad et al. (2020) reported that GB effectively reduced Cr toxicity in cauliflower by decreasing its uptake while maintaining higher plant growth and photosynthetic properties compared to their respective controls. Similarly, GB alleviated Pb stress in cotton plants (Bharwana et al. 2014) and Cd stress in tobacco and rice by increasing growth characteristics (Islam et al. 2009; Cao et al. 2013). Trigonella's increased plant growth could be attributed to GB's ability to improve nitrogen uptake in stressed plants. Another possible explanation is that GB protects the cell membrane from destruction, resulting in a minimal amount of Cr entering the cytoplasm (Giri 2011).

Cr stress has no effect on HC percentage of AM-colonized trigonella roots. Although, comparing with the non-stressed roots, VC and AC rates increased significantly with Cr. The fungal vesicles in AM plants are analogous to the plant vacuoles, which are concerned with toxic compounds accumulation. Also, the external mycelium of roots has a chief role on metals immobilization in the stressed soil because of its wider surface area, which makes them more efficient than the plant roots for absorbing metals. This retention mechanism by AM fungi decreases the translocation of metals and protects leaf tissues from injury by retaining them in roots, therefore contributing to phytostabilization (Dhalaria et al. 2020). Also, the increase of AC% was at its maximum rate with GB application under Cr-stressed conditions as it increases the growth of mycorrhiza and speed up its colonization. The acceleration in AC and VC% due to Cr stress was concomitant with Audet and Charest (2006) who reported comparable results where the colonization of *Rhizophagus intraradices* to wild tobacco were increased with increased Zn levels due to the capacity of the AM fungi to inhabit contaminated soils (Huang et al. 2018). This was also in accordance with Kuang et al. (2023) indicated that the low Cd conc. significantly increased the colonization compared with non-stressed plants. The reason for this may be the vesicles and hyphae of AM are used for chelating heavy metals after the addition of Cd. However, Sheikh-Assadi et al. (2015) and Zhang et al. (2019) observed that spore germination, root colonization, and hyphal development decreased when AM fungi were subjected to heavy metals.

Cr stress reduced total Chl in trigonella leaves, implying that Chl pigments may signal the former to Cr stress (Ahmad et al. 2020). However, AM and GB applications improved their content whether under controlled or stressed situations. Matile et al. (1997) argued that greater ethylene biosynthesis

and accumulation owing to abiotic stress breaking down lipids in the cell wall and stimulating the chlorophyllase gene, which initiates Chl degradation and causes chlorosis in plants. Asgher et al. (2018) found that Cr reduces photosynthesis by increasing $H_2O_2$, $O^{2-}$ generation, and lipid peroxidation in Mustard. Furthermore, increased Cr intake disorganizes the ultrastructure of the chloroplast and inhibits the electron transport chain, diverting electrons from PSI to Cr(IV) and greatly reducing photosynthesis (Danish et al. 2019). Gill et al. (2017) reported that rapeseed plants treated with Cr exhibited enormous black plastoglobules with a greater size and lots of starch granules. According to Eleftheriou et al. (2015), Cr toxicity also caused *Phaseolus vulgaris'* thylakoid membranes to burst and increasing the number of thylakoid leaf cells and basal granules. These factors could explain why trigonella's Chl content decreased during Cr stress.

Previously, it was revealed that AM colonization protects photosynthetic pigments from damage caused by salinity and Cd stress in trigonella (Metwally and Abdelhameed 2018; Abdelhameed and Metwally 2019). The improvement in Chl content in trigonella plants with AM fungi could be attributed to the greater function played by AM fungi in antagonizing Cr stress. Abd-Elghany et al. (2021) found that AM treatment improved photosynthetic pigments during drought stress by increasing Mg absorption by their hyphae (Nazeri et al. 2014). Furthermore, AM plants improve Mg absorption and store more Zn via extraradical mycorrhizal hyphal structure. Both of these nutrients are essential for photosynthesis and increase Chl concentration, which increases photosynthate production (Evelin et al. 2019; Abdelhameed and Metwally 2022). Furthermore, foliar application of GB played a vital function in the removal of accumulated Cr in leaves and increased total Chl, as Nomura et al. (1998) observed that its application increased photosynthetic rate and Rubisco activity under drought stress.

Cr promotes excessive ROS generation, which may impair trigonella defense mechanisms, leading in alterations in antioxidant enzyme activity and increased oxidative stress. As a result, the balance between the prooxidative and antioxidant defense systems may be disrupted (Lou et al. 2015; Zaid and Wani 2019; Adhikari 2020). Furthermore, Alam et al. (2022) and Abdelhameed et al. (2024) reported that in *Vicia faba*, Cr and Pb increased the levels of MDA, EL, and hydrogen peroxide. However, GB and AM fungal treatments improve membrane biochemical properties and reduce oxidative stress in response to Cr stress. Membrane lipids are more susceptible to ROS harm, making them reliable indicators of oxidative stress in plants. Our findings revealed that oxidative stress was more apparent in Cr-stressed trigonella plants due to a considerable rise in EL and MDA levels in their shoots when compared to AM or GB-treated plants. Increased MDA and EL levels in trigonella are thought to indicate oxidative damage caused by Cr exposure, which disrupted the balance between ROS production and the anti-oxidative defense mechanism. Although ROS formation caused oxidative stress in all Cr-stressed trigonella plants, whether AM or GB treated or untreated, the detrimental effects were less in AM or GB treated plants, showing that AM and GB can remove ROS better and tolerate Cr toxicity. Similar findings have been reported for oilseed rape (Gill et al. 2015), cauliflower (Ahmad et al. 2020) *S. bicolor* (Kumar 2021).

Cr increased ROS formation as its ions work a signaling molecular which initiates the plant's antioxidant defense system to counter ROS (Hassan et al. 2024), therefore it activated both enzymatic and non-enzymatic antioxidant components and the quantity of these antioxidants indicates the extent of the stress. Non-enzymatic antioxidants such as proline, TSP, TSC, and TFAA are important for plant defense against oxidative stress because they act as antioxidant buffers. All of these metabolites rose significantly under Cr stress, with the exception of TFAA, which decreased with either AM or GB. According to Andrade et al. (2010), elevated Cu and Zn concentrations resulted in a reduction in amino acid content in *Coffea arabica* seedling leaves. Furthermore, Zaid and Mohammad (2018) reported that in *Mentha arvensis* plants, 150 mg $kg^{-1}$Cd enhanced the activities of antioxidant enzymes and raised the content of osmolytes and1 µM methyl jasmonate reversed its negative effects when was applied to the stressed plants. A further rise in proline and TSP levels was found with GB exogenous application or AM application, either alone or together compared to the control. These findings demonstrate the significance of GB and AM in regulating membrane integrity and ROS production in cells under Cr stress conditions. Tisarum et al. (2019) found that GB exogenous application regulated physiological adaptations in sweet potatoes under water deficit conditions by accumulating soluble sugar. Kumar et al. (2019) and Singh et al. 2021) found that *S. bicolor* and Mungbean had considerably higher proline content during Cr stress. Similar results have been reported for *C. arietinum* (Singh et al. 2020), Z. *mays* (Adhikari 2020), and *Helianthus annuus* (Qadir et al. 2020).

Furthermore, according to Kumar (2021), the use of GB and AM preserves the integrity of cell membranes and protects cells from injury as a result of a drop in Cr content in plant samples, which limits the quantity of Cr that may enter the cell. Plants also showed decreased Cr absorption following GB application, which could be attributed to GB's capacity to protect cell membranes, reducing Cr transfer into cells. Similar findings on GB's protective activity in maintaining membrane integrity have been reported in mung beans (Hossain and Fujita 2010) and rice (Cao et al. 2013) under salinity and Cd stress. Karagiannidis and Hadjisavva (1998) found that AM inoculation improved nutrient uptake

while suppressing Cr, Mn, Fe, Co, Ni, and Pb absorption in duram wheat. It was postulated that competition between nutrients and Cr for entrance into cells could be another factor contributing to decreased Cr absorption with AM and GB treatment.

AM fungal colonization significantly increases both T-GRSP and EE-GRSP under control or Cr-stressed conditions. These findings are consistent with those of Gao et al. (2022), who found an increase in T-GRSP and EE-GRSP levels in alfalfa rhizospheric soil after Cd exposure and increased temperature. Glomalin release into the soil has several impacts, including increased soil aggregate stability, decreased soil erosion, improved soil quality, and the creation of protein–metal complexes, which reduces the amount of metal ions in the soil. In addition, the glomalin protein structure contains functional groups that enhance heavy metal binding (Gonzalez-Chavez et al. 2004; Dhalaria et al. 2020).

Yang et al. (2017) and Li et al. (2022) found that both T-GRSP and EE-GRSP contents increased significantly with AM inoculation, but declined with increasing Pb concentration. Furthermore, Cornejo et al. (2008) and Gao et al. (2022) found that metal toxicity may increase glomalin synthesis by AM fungi. According to Gonzalez-Chavez et al. (2004), glomalin in the soil has the potential to sequester toxic metals via hyphae, colonized roots, and glomalin deposition in the soil. Furthermore, in a soil severely contaminated with Cu and Zn, Cornejo et al. (2008) discovered that GRSP bind to around 27% Cu and 5.8% Zn. The aforementioned studies show that AM fungi play major roles in heavy metal biostabilization by generating glomalin.

Enhanced antioxidant activity (CAT, APX, and POD) and TAC conc. in trigonella shoots during Cr toxicity resulted in a significant increase in plant defense response activation, as documented in perennial ryegrass and maize under Cd and Cr stress (Lou et al. 2015; Adhikari et al. 2020). Because of the increase in heavy metal ions in plants, the cell's oxidant level increased, activating antioxidant enzymes to counteract their negative effects. According to a study conducted in mung beans and*S. bicolor*under Cr toxicity by Jabeen et al. (2016) and Kumar (2021), the cause of GB and AM's antioxidant activity promotion could be their inhibition of Cr absorption and increased nutritional absorption. Furthermore, AM fungi promote the buildup of GB, which functions as an osmoprotectant and combats oxidative stress by boosting the amounts of antioxidant enzymes. Our findings are also consistent with those reported for*Triticum aestivum* (Ali et al. 2015) *B. oleracea* (Ahmad et al. 2020). Hasanuzzaman et al. (2014), Hisyam et al. (2017), and Tisarum et al. (2019) have also observed increased antioxidant activities with exogenous GB application as a means of mitigating salt and drought stress in rice and sweet potato plants because GB operates as compatible solutes (Genchi et al. 2021). Wang et al. (2014) had a similar viewpoint, claiming that GB acts as an osmoprotectant in wheat, sheltering plant cells from osmotic stress. Raza et al. (2014) and Gill et al. (2015) reported similar results on GB exogenous application in wheat and brassica under Cr toxicity.

## 5 Conclusion

The widespread use of Cr in several manufacturing industries has caused serious environmental damage. As a result, our findings showed its deleterious effects on trigonella growth features and physio-biochemical characteristics such as total Chl content and total free amino acids levels. Cr stress increases malondialdehyde and electrolyte leakage as oxidative stress indicators. However, ascorbate peroxidase and peroxidase levels increased in trigonella shoots under Cr stress, but this was insufficient to resist its toxicity. Surprisingly, GB and AM enhanced stress tolerance due to increased ascorbate peroxidase and peroxidase enzyme activity, as well as defense system metabolites (total soluble protein and proline). As a result, their combination treatment significantly improved the growth characteristics of trigonella plants and was the most effective one for reducing the toxicity of Cr. Therefore, our findings may provide breeders with useful methods to reduce the Cr toxicity in agricultural crops, assuring both environmental sustainability and food security. However, further study is needed to test these pathways in field conditions, which is becoming a significant challenge in agricultural systems, in order to have a thorough understanding of Cr toxicity tolerance mechanisms.

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**Data Availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Competing Interests** The authors declare no competing interests.

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