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Morphobiochemical response of hydroponically grown *Cicer arietinum* L. (*chickpea*) to hexavalent chromium stress and AMF *Claroideoglomus claroideum* inoculation

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A B S T R A C T

Chromium (Cr), a heavy metal, is used in industries like electroplating and steel making. The hexavalent (Cr^{+6}) form of Cr is highly toxic to living organisms. The symbiotic association of plant with arbuscular mycorrhizal fungi (AMF) plays a vital role in protecting the plants environmental stress. AMF forms the mutualistic relationship with 80% of vascular plants which has evolved as an adaptive mechanism to enhance the plants' ability to protect themselves from adverse environmental conditions. In the present study, *Cicer arietinum* (chickpea) was grown hydroponically to different concentrations of Cr^{+6} (0, 20, 40 and 60 μM) and without or with AMF *Claroideoglomus claroideum* inoculation. After 30 days of growth, morphological and biochemical parameters were assessed which showed increasing level of Cr^{+6} adversely affected the growth and biochemical parameters of *C. arietinum.* The mycorrhiza-inoculated (M) plants exhibit enhanced growth and higher biomass compared to non-inoculated plants (NM). Biochemical parameters such as photosynthetic pigments, protein, carbohydrate content and catalase activity were higher in M plants than NM plants. However, amino acid and proline content were highest in NM plants compared to M plants. The findings suggest the effectiveness of the *Claroideoglomus claroideum* in mitigating Cr^{+6} stresses in *C. arietinum*, emphasizing the potential of AMF in amelioration of Cr^{+6} stress.

1. Introduction

Food security is important for global development, but contamination of soil with toxic heavy metals, such as Pb, Cd, Cr and Hg is a serious concern (Huang *et al*., 2018). Heavy metals are metallic elements or metalloids with a high atomic density i.e. greater than 4g/cm^3. Rapid industrialization and urbanization introduce these metals into the environment. The increased demand for food due to a growing population leads to the rampant use of fertilizer, agrochemicals, wastewater etc. for agriculture results in contamination of heavy metals into agricultural system.

Chromium (Cr), the 21st most abundant element in earth is a heavy metal found in rocks and water. In nature, Cr exists in two stable forms such as the trivalent (Cr^{+3}) and hexavalent (Cr^{+6}), whereas latter reported to be highly toxic. The hexavalent (Cr^{+6}) form, is especially harmful due to its water solubility (Saha *et al*., 2011). Cr^{+6} contamination comes from both natural and human activities, including industrial processes and mining (Cheng*,* 2003). Cr^{+6} toxicity also affects the physiological and metabolic processes of plants, leading to changes in stomatal function, decreased water potential, reduced pigment content, disruption of water-mineral linkages, growth inhibition, chlorosis and necrosis (Jena *et al.*, 2016).

To address issues of heavy metal contamination in agricultural system, sustainable farming practices and innovations are necessary. Arbuscular mycorrhiza fungi (AMF) can play a role in mitigating heavy metal toxicity of plants (Hashem *et al*., 2016). Arbuscular mycorrhiza is the symbiotic relationship between fungi and roots of higher plants (Read, 2008). AMF form mutually beneficial

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relationships with plants, enhancing their ability to tolerate stress and preventing metal uptake (Augé, 2001). It also alters the transport of heavy metals within plants, specifically reducing toxicities of aluminum (Al) and manganese (Mn) in acidic soil conditions (Cakmak, 2000). *Cicer arietinum* L. (chickpea) is an important legume crop of the world and is a rich source of vegan protein along with minerals. The present study is designed to study the morpho biochemical response of *C. arietinum* to different levels of Cr^{+6} without and with AMF *Claroideoglomus claroideum* inoculation grown in hydroponics.

2. Materials and methods

2.1 Seed collection and surface sterilization

Seeds of *Cicer arietinum* L. (chickpea) were collected from the Odisha University of Agriculture and Technology (OUAT), Bhubaneswar, Odisha. The collected healthy seeds were surface sterilized using $HgCl_2$ (0.1%) for 5 minutes, followed by washing multiple times with sterile distilled water to remove any residues. Surface sterilized seeds were further subjected to germination.

2.2 Arbuscular mycorrhizal fungi (AMF)

The pure culture of AMF *Claroideoglomus claroideum* (CMCC/AM-2705) was procured from Centre for Mycorrhizal Culture Collection (CMCC), TERI, New Delhi, India. Mass propagation of the starter culture was carried out in pot culture with *Sorghum bicolor* as host plant. Substrate for pot culture constituted of dried soil and sand in the ratio 3:1 (v/v) which was mixed with organic manure at 2:1 ratio (v/v). The Potting mix was cleaned thoroughly by sieving in 2mm sieve and sterilized by autoclaving three times on alternate days at 120^{o}C in 15psi for 30 mins to kill any mycorrhizal spore in the substrate. In a 2 kg poly bag 5 seeds of host plants were sown and after 7 days of seedling growth *C. claroideum* spores were inoculated into the pots. The pots were irrigated with water on alternated days and host plants were allowed to grow for 4 months for AM root colonization and sporulation.

2.3 Preparation of Cr^{+6} treatment solutions

Potassium Dichromate ($K_2Cr_2O_7$) was used as the source of Cr^{+6}. To prepare 100 µM stock solution, 29.419 g of $K_2Cr_2O_7$ was added to 1000 ml of water. Appropriate dilutions were made to the stock solution to get different concentration of Cr^{+6} solutions (20, 40, 60, 80 and 100 µM).

2.4 Seed germination study and determination of LC 50

Ten numbers of surface-sterilized *C. arietinum* (chickpea) seeds were placed over cotton pads saturated with different concentrations of Cr^{+6} (0, 20, 40, 60, 80 and 100 µM) in sterilized petri plates. The seeds on petri plates were kept under dark at 25^{0}C for 5 days for germination. The percentage of germination was recorded and concentration of Cr^{+6} where only 50% of seed germination occurred was considered as LC 50.

2.5 Experimental design

The plant growth experiment was carried out hydroponically in modified Hoagland's solution (Hoagland and Arnon, 1950) after as described by Zeiger (2002). Experiment was randomized with 4 x 2 factorial designs consisting of four Cr^{+6} addition level such as 0 (Control), 20, 40 & 60 µM along with two AMF inoculation treatments such as without AMF inoculation the non-mycorrhizal (NM) and with AMF inoculation the mycorrhizal (M). AMF spore inoculums (Approx. 10 nos. per seed) was given to the overnight soaked *C. arietinum* seed placed on petriplates and after 5 days the germinated seeds were transferred to the hydroponic system for growth with different concentrations of Cr^{+6} (0, 20, 40 & 60 µM) in modified Hoagland's solution. For each treatment 3 replicates were prepared. After 30 days of plant growth different morphological and biochemical parameters were analyzed.

2.6 Growth and morphological parameters

Growth and morphology of *C. arietinum* under different treatments were studied in terms of shoot and root length, shoot and root fresh weight (FW), shoot and root dry weight (DW). The shoot and root length were measured using measuring scale and expressed in cm. The shoot and root fresh weight was determined by weighing in electric balance expressed in grams (g). The fresh biomass was dried in hot air oven at 80^{o}C for 3 hours and dry weight was measured and expressed in g.

2.7 Biochemical parameters

Biochemical parameters such as photosynthetic pigment content (Arnon, 1949), total carbohydrate content by the Anthrone reagent method (Hofreiter, 1962), reducing sugar content (Nelson, 1994), protein content by Lowry method (Lowry *et al.,* 1951), free amino acid (Moore and Stein, 1963) and proline content (Bates *et al*., 1973) in *C. arietinum* shoot were estimated. The antioxidative enzyme catalase (CAT) activity in shoot was estimated following the method of Aebi (1984).

2.9 Estimation of mycorrhizal root colonization

The roots of *C. arietinum* from different treatments were washed thoroughly and were cut into small pieces (1cm), cleared in 10% KOH, bleached in H_2O_2 for 5 min, acidified with 2% HCl and stained in trypan blue (0.05%) as per Phillips and Hayman (1970). The AMF colonization in each root segment was measured by following the method of Giovannetti and Mosse (1980) which involved gentle squashing of stained root segments on placed glass slide and covered with a cover slip. The squashed root segments were observed under microscope for AMF colonization. The percentage of AMF colonization in root was estimated by following formula:

$$\text{Mycorrhizal colonization (\%)} = \frac{\text{No. of root colonized with AM}}{\text{Total no. of roots inspected}} \times 100$$

2.10 *Statistical analysis*

The significant difference between parameters by the level of Cr^{+6} addition and AMF inoculation was statistically analyzed by two-way analysis of variance (ANOVA) at $P< 0.05$ using MS excel.

3. Results and discussion

3.1 *Germination study*

The study of seed germination (%) of *C. arietinum* under different concentrations of Cr^{+6} is presented in Figure 1. At control condition (0 µM) the rate of germination was highest (100%). However, as the concentration of Cr^{+6} increased, there was a decline in the rate of germination. At 80 µM of Cr^{+6}, the seed germination rate was 50%, hence it was considered LC50 where 50% of seeds failed to germinate. The presented data emphasizes the sensitivity of *C. arietinum* seeds to higher levels of Cr^{+6}, indicating the potential toxic impact of Cr^{+6} concentrations on seed germination. Similar findings of Mohanty *et al.* (2015) in *Sesbania sesban* supplemented with different concentrations of Cr^{+6} (5-10000 mg L^{-1}) showed decline in germination of *S. sesban* seeds with increase in Cr^{+6} concentration where LC 50 was at 300 mg L^{-1} Cr^{+6}, which was due to Cr^{+6} toxicity.

3.2 *Mycorrhizal root colonization*

The AMF root colonization (%) of *C. arietinum* was observed to be decreased with increase in concentration of Cr^{+6} (Figure 2). The highest percentage of root colonization was recorded at Cr^{+6} control (100%) followed by 20, 40 and 60 µM (Cr^{+6}) with 94, 83 and 61% of root colonization respectively. According to the findings of Zhan *et al*. (2017) the root colonization (%) in maize inoculated with AMF *Glomus intraradices* and grown in heavy metal cadmium (Cd) contaminated soil was shown to be maximum under control conditions which was observed to be declined at with increased of levels of Cd (3 and 6 mg/kg soil) suggesting toxicity of heavy metal which can be correlated with present findings.

3.3 *Growth and morphological parameters*

The plant growth experiment of *C. arietinum* was carried out in hydroponic cups in modified Hoagland's solution with different chromium treatment and AMF inoculation (Figure 3). The various growth parameters studied in the present study like shoot and root length, fresh weight (FW) of shoot and root, dry weight (DW) of shoot and root in both non-mycorrhizal (NM) and mycorrhizal (M) plants of *C. arientinum* showed gradual decrease with increasing Cr^{+6} concentrations (Table 1). The highest values for all parameters were observed at control while the lowest values were recorded at 60 µM. Similar adverse effects of Cr on root and shoot length, as well as biomass yield have been reported in lemongrass grown in soils with chromium contamination (Patra *et al*., 2018; 2019). The report on negative impact of Cr^{+6} on plant growth and development, leading to stunted growth of vegetables crops was also available (Zayed and Terry, 1998). These observations of declining growth and biomass with increasing level Cr^{+6} indicate inverse relationship between Cr^{+6} concentration and decreasing growth in *C. arientinum*.

However, the AMF inoculated plants (M) showed higher growth and biomass than non-inoculated (NM) plants. The findings of Diaz *et al*., (1996) showed increased shoot growth in *Anthyllis cytisoides* inoculated with the arbuscular mycorrhizal fungus (AMF) *Glomus macrocarpum* under Pb and Zn stress respectively. Present results are consistent with Garg and Cheema (2021) where AMF *Claroideoglomus claroideum* effectively mitigated the negative effects of As (V) and As (III) in *C. arientinum* by enhancing root and shoot biomass. The higher growth and biomass in M plants can be positively correlated to mycorrhizal root colonization in M plants of *C. arientinum,* where with increasing concentration of Cr^{+6} there was decline in root colonization. Thus, higher the root colonization, higher the alleviation of Cr^{+6} toxicity as observed in the present study. Hence, present study emphasizes the efficacy of AMF inoculation and root colonization in reducing the adverse effects of Cr^{+6}, enhancing plant resilience under metal stress.

3.4 *Biochemical Parameters*

3.4.1 *Photosynthetic pigment content*

The photosynthetic pigments, Chlorophyll a (Chl a), Chlorophyll b (Chl b) and total chlorophyll in both non-mycorrhizal (NM) and mycorrhizal (M) plants showed decreasing trend with increasing Cr^{+6} concentrations (Table 2). The highest values for all parameters were observed at control while the lowest values were recorded at 60 µM. The present study can be correlated with the findings of Mohanty and Patra (2012), where a decline in total chlorophyll

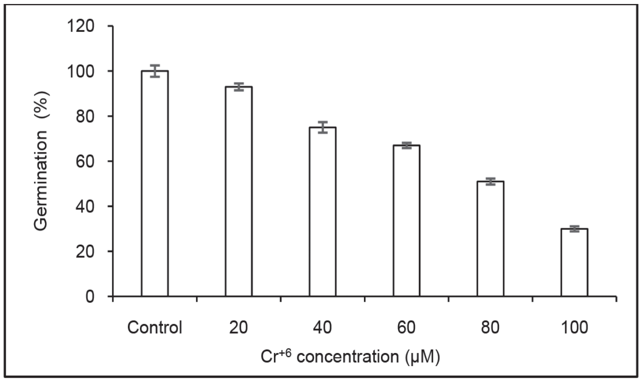

Figure 1. Seed germination under different concentration of Cr^{+6}

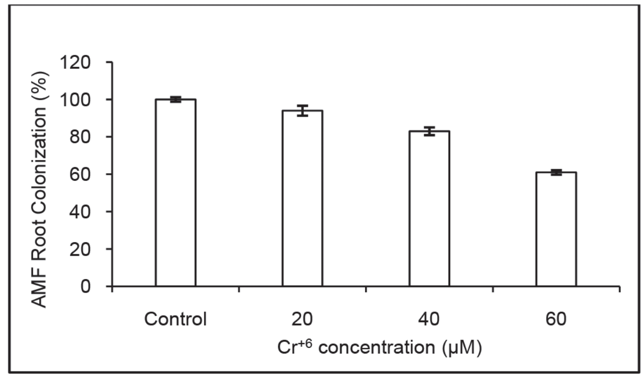

Figure 2. AMF root colonization (%) in *C. arientinum* under different concentration of Cr^{+6}.

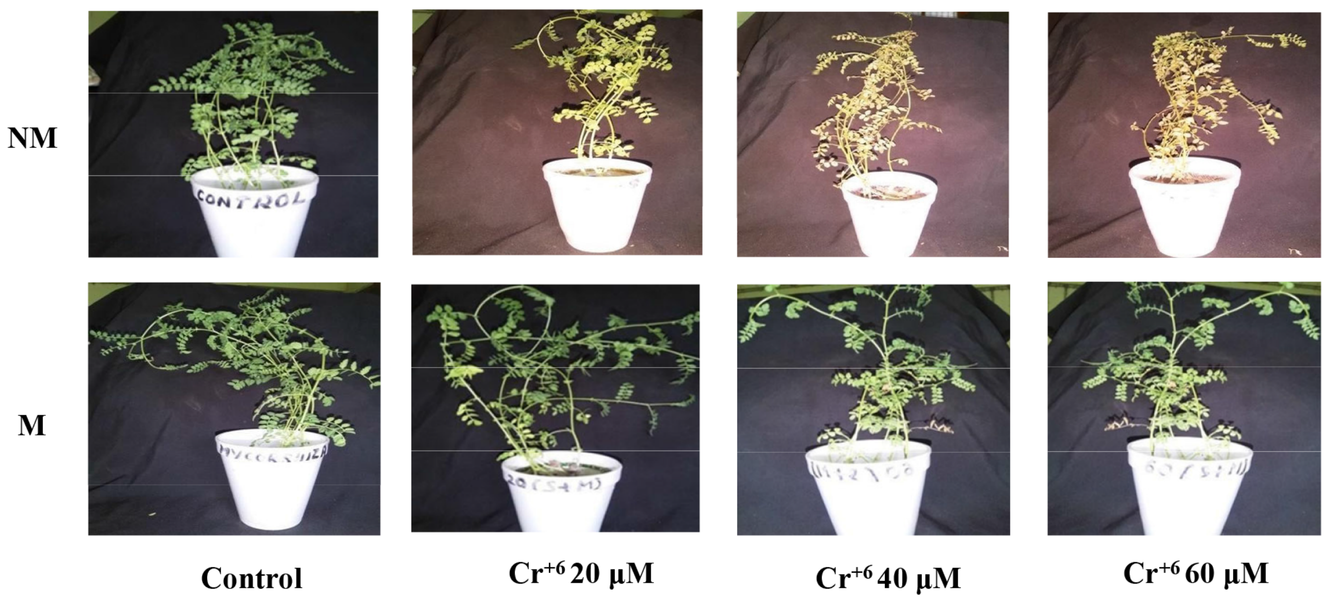

Figure 3: Plant growth of *C. arietinum* in hydroponic cups with modified Hoagland's solution at different concentration of Cr^{+6} in non-mycorrhizal (NM) and mycorrhizal (M) treatments.

Table 1.

Growth parameters of *C. arientinum* under different concentration of Cr^{+6} and AMF treatments.

Cr^{+6} concentration (μM)	Control		20		40		60	
	NM	M	NM	M	NM	M	NM	M
Shoot Length(cm)	20.67±1.20	25.00±1.15	12.13±0.47	19.21±1.67	8.05±0.03	5.09±0.01	7.00±0.29	13.69±0.31
Root Length (cm)	16.66±1.45	19.33±1.45	11.00±1.15	17.33±1.09	6.67±0.33	15.67±1.01	5.17±0.67	9.60±0.31
Shoot FW(g)	1.55±0.02	1.92±0.05	0.74±0.02	0.92±0.02	0.57±0.01	0.83±0.02	0.46±0.01	0.68±0.02
Root FW(g)	0.11±0.01	0.14±0.00	0.06±0.01	0.08±0.04	0.04±0.02	0.05±0.00	0.02±0.001	0.03±0.001
Shoot DW (g)	0.110±0.01	0.183±0.00	0.092±0.001	0.107±0.009	0.073±0.002	0.087±0.002	0.064±0.001	0.079±0.01
Root DW(g)	0.0196 ±0.0001	0.0219 ±0.0001	0.0094 ±0.0002	0.0122 ±0.0002	0.0084 ±0.0002	0.0098 ±0.0002	0.0056 ±0.0001	0.0079 ±0.0001

content in rice and wheat seedlings with increasing Cr^{+6} concentration in hydroponic culture. Wani and Khan (2010) also reported the toxic effects of Cr^{+6} in *C. arietinum* and observed a linear decrease in chlorophyll content with the increase in Cr^{+6} concentration (34, 68 and 136 mg/kg Cr in soil) after a 90-day exposure period.

The M plants showed higher Chl a, Chl b and total chlorophyll content compared to NM plants at different Cr^{+6} treatments. Such observation is similar to the findings of Kullu *et al.*, (2020) in *Brachiaria mutica*, where plants inoculated with *Rhizophagus irregularis* showed increased chlorophyll levels under different concentrations of hexavalent chromium compared to the non-mycorrhizal (NM) one. The study suggests a potential protective effect of mycorrhizal associations in Cr^{+6} stress on plant photosynthetic pigments.

The carotenoid content and chl a/b ratio of *C. arientinum*, in both non-mycorrhizal (NM) and mycorrhizal (M) plants showed enhancement with increasing level of Cr^{+6}. The highest values were observed at 60 µM while the lowest values were recorded at control. However, in M plants the carotenoid content and chl a/b ratio were found to be lower compared to NM plant at all the concentration. The current findings can be compared with the reports of Dhali *et al.,* (2020), which showed an increase in carotenoid content with the increase in concentration (25 to 200 µM) in response to Cr^{+6} in *Macrotyloma uniflorum* and different growth stages (15, 30 and 45 days) in hydroponics. Such findings indicated that a rise in carotenoid content in *C. arientinum* with increasing Cr^{+6} concentrations, suggesting an adaptive photo-protective strategy due to the toxic effects of Cr^{+6} and potential photo-oxidation.

3.4.2 Total carbohydrate, reducing sugar and protein content

The total carbohydrate, reducing sugar and protein content in *C. arientinum* shoots under different Cr^{+6} concentrations for both non-mycorrhizal (NM) and mycorrhizal (M) plants showed declining trend with increasing Cr^{+6} concentrations (Figure 4). The highest value was observed at 0 µM Cr^{+6} while the lowest levels were recorded at 60 µM Cr^{+6}. Raklami *et al*., (2020) reported that in *Medicago sativa*, total sugar content decreased with increasing concentrations of heavy metal Cd and Zn (300 and 600 mg/kg). The present study also correlated with the findings of Patra *et al*., (2020) where *Sesbania sesban* in Cr^{+6} rich environments showed a decrease in protein content with rising of Cr^{+6} which was concentrations, attributed to an increased rate of protein denaturation.

The current study also revealed that M plants showed enhanced total carbohydrate, reducing sugar and protein content compared to NM plants at all Cr^{+6} concentrations. Similar report by Panigrahy *et al*., (2019) in *Eleusine coracana* with *Rhizophagus irregularis* and their findings indicating improved primary metabolite content, supports the idea that AMF inoculation has a positive effect on the nutritional content of plants. Garg and Cheema (2021) reported that the inoculation with different AMF (*Rhizoglomus intraradices*, *Funneliformis mosseae* and *Claroideoglomus claroideum*) in *C.arietinum* has enhanced sugar synthesis under arsenic (As) stress which supports the positive role of AMF symbiosis in plant stress response. Thus, present results suggest that AMF inoculation improves the nutritional status of plants and helps them cope with the toxicity of Cr^{+6} under different concentrations.

3.4.3 Free amino acid and proline content

In both non-mycorrhizal (NM) and mycorrhizal (M) plants, the total free amino acid and proline content in shoot of *C. arientinum* were increased with the increase Cr^{+6} concentrations (Figure 4). The lowest value was observed at control while the highest value was recorded at 60 µM Cr^{+6}. The present results are comparable with the reports of Dhali *et al.,*(2020) where an augmentation in total free amino acid and proline content in *Macrotyloma uniflorum* with rising concentrations of Cr^{+6} (25 to 200 µM) across various growth stages (15, 30 and 45 days) in hydroponic culture. High level of free amino acid indicates protein degradation and as proline is an osmolyte, its concentration increases in plants under stress which maintains the osmotic balance.

The free amino acid and proline content were observed to be lowered in M plants compared to NM plants at all the concentration. Chaturvedi *et al*. (2018) observed elevated proline levels in non-mycorrhizal *Solanum melongena* plants compared to mycorrhizal, grown in Pb and Cd contaminated soils (25 to 100 mg/kg). The study by Ma *et al.,* (2019) reported reduction in proline content in *Helianthus annuus* leaves inoculated with *Claroideoglomus claroideum* under heavy metal stress, indicating potential regulation of osmotic balance and maintenance of cell bioenergetics. Hence, findings of the current study suggest that amelioration of Cr^{+6} toxicity in AMF inoculated plants resulting decrease in proline content of *C. arientinum* compared to non-mycorrhizal plants at different Cr^{+6} treatments.

3.4.4 Anti-oxidative enzyme activity

The present study showed that the anti-oxidative enzyme CAT activity in shoot of *C. arientinum* was decreased with the increase Cr^{+6} concentrations in both non-mycorrhizal (NM) and mycorrhizal (M) plants (Figure 4). The highest value was observed at control while the lowest value was recorded at 60 µM Cr^{+6}. The present results showed similarity with the reports of Rath and Das (2021) where the anti-oxidative enzyme CAT in hydroponically grown *Vigna mungo* decreased with rising concentrations of Cr^{+6} (100, 150,200, 250 and 300 µM) across various growth stages (15, 30 and 45 days) which lead to ROS mediated oxidative stress in subcellular compartments.

Findings of the present study also revealed that anti-oxidative enzyme CAT activity increased in M plants than NM plant in all the treatments which is similar to the findings of Kullu *et al.* (2020), where the anti-oxidative enzyme activities in *Brachiaria mutica* plants inoculated with *Rhizophagus irregularis* were significantly higher than non-inoculated (NM) plants in all the treatment. Such findings suggest the increase in the rate of scavenging free radicals formed in the plant cell in response Cr^{+6} stress and enhancement of protection against oxidative stress with AMF inoculation resulting in higher stress tolerance potential than the mycorrhiza non-inoculated *C. arientinum.*

3.4.5 Two-way analysis of variance

The two-way analysis of variance (ANOVA) of parameters assessed in *C. arientinum* showed significant variations influenced by different treatments of Cr^{+6} and AMF inoculation. The analysis indicated significant statistical variations between and within the treatments (Table 3).

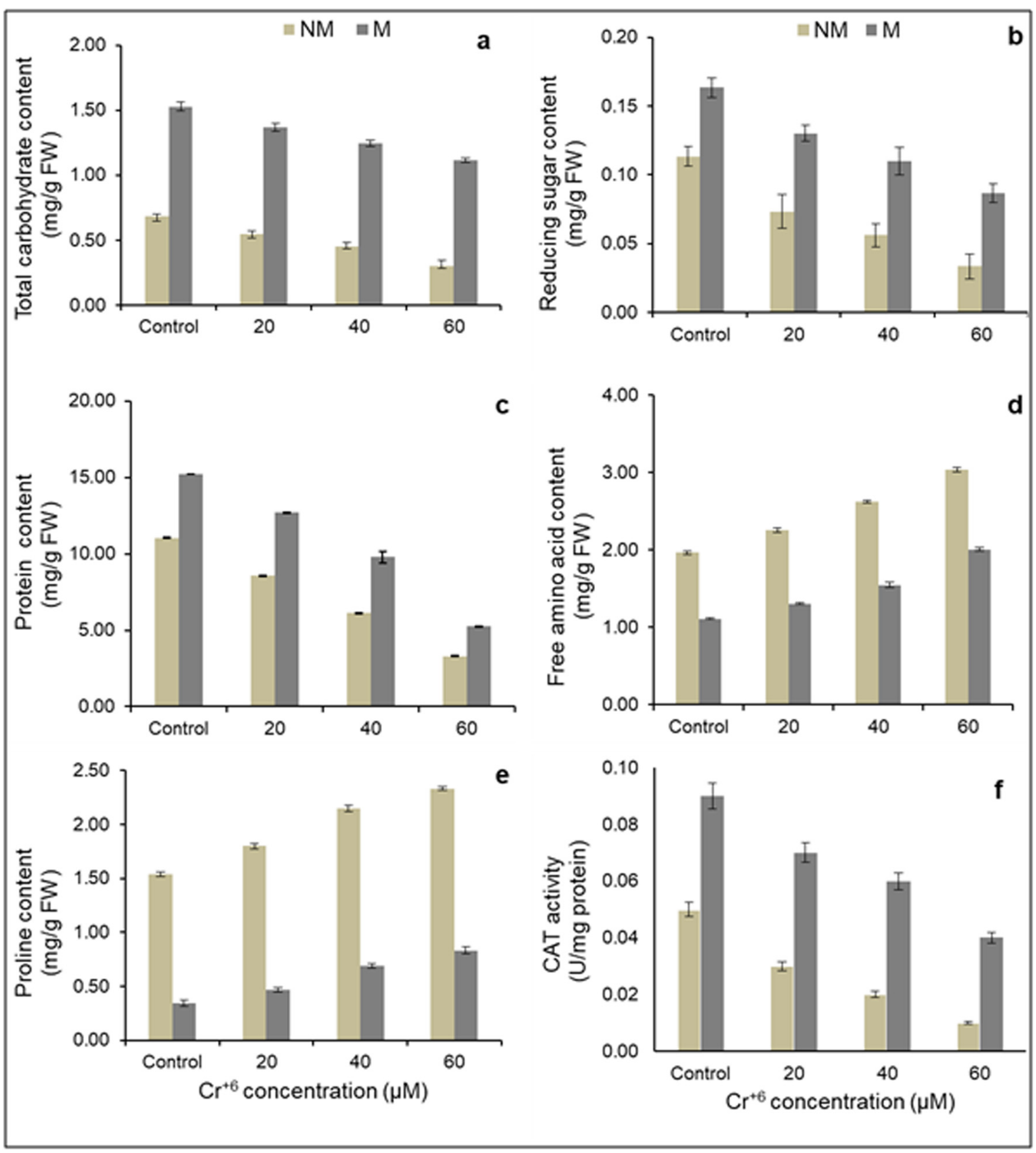

Figure 3. Biochemical parameters a. Total carbohydrate content b. reducing sugar c. protein content d. free amino acid content e. proline content and f. CAT activity in shoot of *C. arientinum* under different concentration of Cr^{+6} and AMF treatments.

Table 3:

F values for two-way ANOVA of different parameters analyzed in *C. arientinum* influneced by various concentrations of Cr^{+6} and AMF treatment.

Parameter	Cr^{+6}	M	Cr^{+6} x M
Shoot length	84.971*	105.758*	1.152
Root length	31.129*	48.266*	2.821
Shoot Fresh weight	947.390*	218.995*	5.706*
Root Fresh weight	335.850*	84.114*	6.393*
Shoot Dry weight	134.518*	103.143*	25.922*
Root Dry weight	3789.655*	487.972*	9.131*
Chlorophyll a	1201.749*	7524.113*	151.537*
Chlorophyll b	335.528*	2695.712*	6.849*
Total Chlorophyll	1659.761*	11622.285*	114.675*
Chlorophyll a:b	62.498*	460.293*	10.817*
Carotenoid	269.867*	190.865*	0.3498
Total carbohydrate	62.701*	1492.761*	0.270
Reducing sugar content	28.070*	73.286*	0.048
Protein content	1773.675*	1485.637*	34.231*
Free amino acid content	590.400*	3136*	8.054*
Proline content	236.792*	5418.632*	13.389*
Catalase activity	12.186*	62.883*	1.674

Asterisk symbol (*) indicate statistically significant *F* value at $p = 0.05$ (n=3),

Cr^{+6}: Cr^{+6} Concentration effect, M: AMF inoculation effect, Cr^{+6} x M: Variable interaction effect

4. Conclusion

In the context of Cr^{+6}stress, AMF *C. claroideum* inoculation exhibits a positive impact on the growth and physiology of *C. arietinum* with enhanced biomass accumulation and favorable alterations in photosynthetic pigments, carbohydrates, proteins and reducing sugar content. The observed augmentation in antioxidative enzyme activities and proline accumulation in mycorrhizal plants suggests a contribution to reactive oxygen species (ROS) scavenging activity. The stress-ameliorative effect of AMF association is more noticeable at lower concentrations of Cr^{+6} (20 µM) compared to higher concentrations (60 µM), exhibiting a positive correlation with the percentage of root association. The findings indicate that AMF association enhances *C. arietinum* tolerance to Cr^{+6} stress, with the effectiveness decline at higher stress levels, possibly inhibiting AMF colonization. Hence, it was concluded that AMF *C. claroideum* inoculation can mitigate low level Cr^{+6} stress in *C. arietinum*.

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