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Exogenous application of citric acid alleviates copper-induced stress in Withania coagulans (Dunal)

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Authors' Contribution	**Fawad, M. & K. R. Hakeem** conducted the physiological and biochemical studies of *Withania coagulans*, **H. S. Al-Zahrani** & **M. A. Ali** measured the agronomic traits i.e,, number of leaves, plant height and weight.
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ABSTRACT

The current study was aimed to determine the mitigating effect of citric acid (CA) on physiological and biochemical attributes in *Withania coagulans*, exposed to copper (Cu) stress. The plants were treated with 10mM and 15mM concentrations of Cu and/or with 05mM and 10mM CA. The higher dosage of Cu has significant toxic effects on plant growth and biomass. The agronomic attributes of the plant were significantly reduced number of leaves (24%), shoot length (22%), root length (7%), fresh (8%), and dry (38%) weight as compared to the control. Higher concentration of Cu was also found toxic to photosynthetic pigments and significantly reduced chl *a* (27%), chl *b* (6%), and carotenoids (35%) compared to the control. However, the application of CA under Cu stress significantly improved the physiological and biochemical attributes of *W. coagulans*. Based on the results, it could be concluded that CA can play a role in mitigating the toxic effect of higher dose of Cu in *W. coagulans.*

Keywords: Heavy metal; Copper; Phytoextraction; Citric acid; Photosynthetic pigments.

INTRODUCTION: Industrial progress leads to toxic chemical pollution of the environment, particularly with heavy metals (HMs) (Shahid *et al.*, 2014). The HMs persist in the dust and soil due to their non-degradable nature thereby resulting in a hazard to the ecosystem (Suman *et al.*, 2018). Some of the heavy metals such as copper (Cu), iron (Fe), zinc (Zn), and manganese (Mn), are indispensable microelements. They are essentially needed in optimum amounts by plants to sustain their biological and biochemical processes (Vinogradov and Zubkova, 2022). When present in excess proportions, important metals can lower soil microbial activity, soil fertility, and crop yields (Zhu *et al.*, 2020). Cu is frequently found in nature as an elemental metal or as the ions Cu^{+} or Cu^{2+}, while the oxidation states +3 and +4 are also present (Conry, 2011). Cu has a crucial biological role for all living things due to its redox characteristics at low concentrations, participating in many metabolic activities (Wintz *et al.*, 2002). Cu is an essential micronutrient that is required for normal growth and development in higher plants (Li *et al.*, 2015). It is also involved in mineral nutrition and electron transfer reactions that take place in critical processes like respiration and photosynthesis, the biosynthesis of chlorophyll and primary metabolites, or the scavenging of radicals (Adrees *et al.*, 2015). At higher concentrations, Cu can cause toxicity symptoms (Stanojkovic-Sebic *et al.*, 2015). Cu toxicity in plants can hinder biochemical processes, affect gas exchanges, and limit plant growth (Yruela, 2009). Reactive oxygen species (ROS) build up and cause oxidative stress in plants cultivated in Cu-polluted soils (Liu *et al.*, 2018), which triggers the activation of antioxidant enzymes and the manufacture of antioxidant molecules. Proline content being higher in plant tissues has also been linked to Cu toxicity (Ku *et al.*, 2012; Monteoliva *et al.*, 2014). The target plant species, the types of soil, and the environmental factors all affect a plant's capacity to extract heavy metals (HMs) from its roots to aerial parts (Ali *et al.*, 2015). The ability of certain plant species to store HMs in shoot sections and remove them from soil or water makes phytoextraction one of the successful phytoremediation approaches (Laghlimi *et al.*, 2015). In general, the low bioavailability of HMs in soil negatively affects the phytoextraction technique. However, the promising use of soil amendments (organic compounds) can enhance the solubilization of metals by forming complexes with target sites of metals (Smolinska and Szczodrowska, 2017). Numerous organic chelators, such as citric acid (CA) have been shown in prior research to interact efficiently with HMs, aiding their immobilization or mobilization and reducing or increasing their bioavailability depending on a variety of conditions (Farid *et al.*, 2017). Additionally, CA is well known for its effectiveness as a metal chelation agent and stress alleviator (Freitas *et al.*, 2013).

OBJECTIVES: The current study objectives was to evaluate how *W. coagulans* can effectively treat soil that has been contaminated with copper while being assisted along by CA. It was hypothesized that *W. coagulans* plants physiological and biochemical status would improve with CA application under Cu-induced stress.

MATERIALS AND METHODS: **Research description:** *Withania coagulans* Dunal (*P. coagulans* Stocks), was chosen as the test plant species for this study. The study was conducted in Department of Biology, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia. The seeds were adequately cleaned with double distilled water (DDW) and then submerged for 3 min. in 0.1% mercuric chloride ($Hgcl_2$). The pots with 500g of peat moss soil were designed in a complete randomized manner. Three viable seeds were planted in each pot, and irrigated 30 mL with DDW. Hoagland's solution was given to the young plants throughout the study period (28 days). The plants were treated with 2 concentrations (10mM and 15mM) of heavy metal of Cu ($CuSO_4.5H_2O$) while 05mM and 10mM doses of CA were also given. After 14 days of treatment, all plants of each treatment were separately harvested and packed for further analysis. The corresponding triplicate of each treatment was pooled together to obtain a composite sample of each treatment.

Assessment of agronomic traits: Plant agronomic attributes (number of leaves, lengths of root and shoots, and fresh and dry weights) of the plant were computed (Yuniarti *et al.*, 2022).

Estimation of photosynthetic pigments: Crushed fresh leaves (0.5 g) with pestle and mortal from each treatment to calculate the amounts of chlorophyll-*a*, *b*, and carotenoids. About 10 mL of acetone (80%) was added in the mixture and centrifuge at 6000 rpm for 10 min. A spectrophotometer (UV-1900) was used to measure the absorbance at 663, 645, and 470 nm for the relevant pigments (Lichtenthaler and Wellburn, 1983).

Estimation of proline concentration: The approach was used to estimate the proline content (Bates *et al.*, 1973). The fresh leaf (0.5g) were crushed in 10 mL of liquid nitrogen-free sulfosalicylic acid (3%), before being used. The sample was centrifuged for 15 min. at 11,500 g, incubated at 100°C for 60 min., and ninhydrin (2mL), glacial acetic acid (2 mL) and toluene (4 mL) were added. The OD was recorded at 520 nm and a standard curve was used to quantify the proline content, which was calculated as µg/g FW.

Estimation of total phenol concentration (TPC): The TPC was determined using the procedure JE and KI, (1977). A 50 µL of the methanolic residue was blended with Folin-Ciocalteu reagent (100 µL), and methanol (850 µL), and incubated for 5 min. at room temperature. The step was followed by the addition of 20% sodium carbonate (500 µL) and incubated at room temperature for 30 min. The absorbance was estimated at 750 nm. TPC was assessed using the standard curve produced by measuring the ODs of known gallic acid concentrations. The TPC was calculated as g/kg FW equivalent gallic acid.

Estimation of total flavonoids concentration (TFC): The method was used to estimate the TFC (Zhishen and Jianming, 1999). About 250 µL of the methanolic residue, 1.25 mL of deionized water, and 75 µL of the $NaNO_2$ (5%) solution were combined. The mixture was then held for 6 min., and combined with 150 µL of aluminum chloride (10%) solution and 0.5 mL of sodium hydroxide (1 M). After 5 min., 275 µL of deionized water was added to the mixture. At 510 nm, the solution's absorbance was measured. The flavonoid content was taken as g kg FW catechin equivalent, and the TFC was determined using a standard curve of known catechin concentrations.

Crude extract: A mixture was created by crushing 2g of leaf treated with Tris-HCl buffer (20 mM, pH 7.2). The mixture was centrifuged for 10 min. at10,000 rpm at 4°C. For a test using antioxidant enzymes, the supernatant was maintained at -20 °C.

Estimation of PPO, POD and CAT activities: The method was used to calculate the PPO (Jiang *et al.*, 2002). Using catechol as a substrate, the PPO activity was calculated. About 0.2 mL of the extract was quickly added to 2.8 mL of the substrate (20 mM) solution already prepared in 0.01 M BPS (pH 6.8). The OD was taken at 400 nm and was noted for 3 min. utilizing a spectrophotometer. The enzyme activity was expressed as how much the enzyme causes a change of 0.1 in OD / min.

According to protocol, the POD activity was estimated (Miranda and Cascone, 1995). As a reaction mixture, 8 µL of 0.97 M H_2O_2, 80 µL of 0.5 M guaiacols, 250 8 µL of 0.2 M BPS (pH 5.5), and the smallest amount of catalyst were prepared. Using a spectrophotometer, the fluctuation in OD value at 470 nm was measured for 1 min. How many enzymes, under typical test conditions, changed the OD at a rate of 1.0 nm/min. was used to determine the enzyme activity. CAT activity was computed using the procedure of (Bergmeyer), 2012. About 2 mL of substrate solution was mixed wht 25 mM H_2O_2 in phosphate buffer solution (75 mM, pH 7.0) and 500 µL of the plant extract. Using a spectrophotometer for 1 min., the OD was measured at 240 nm. The other antioxidant enzymes' previous references were used to determine the enzyme activity.

Statistical analysis: Analysis of variance (ANOVA) was used to assess the experimental data in a randomized fashion with a triplicate of each treatment. For this, SAS (SAS Institute Inc., 2000, Cary, NC, USA), a statistical programming program, was used. The Tukey's test was used to compare the mean values, specifying the P value as $P \leq 5\%$.

RESULTS AND DISCUSSION: The effect of CA on agronomic traits of dunal under Cu-stress: The morphological traits of *W. coagulans* were significantly decreased with higher concentrations of CU (TCu_2): 24% (number of leaves), 22% (shoot length), 7% (root length), 8% (fresh weight), and 38% (dry weight) as compared to control. Contrarily, the use of CA significantly improved the morphological characteristics of dunal and reduced the detrimental effects of TCu_2 on agronomic traits. For instance, as compared to the control, the addition of CA along TCu_2 significantly improved 66% (number of leaves), 31% (shoot length), 87% (root length), 43% (fresh weight), and 78% (dry weight). The effects of a lower copper concentration (TCu_1) have significantly enhanced the agronomic parameters of the studied plant: 36% (number of leaves), 21% (shoot length), 42% (root length), 26% (fresh weight), and 36% (dry weight). TCu_1 with CA increased the morphological traits by 60–90% (number of leaves), 41–86% (shoot length), 70–103% (root length), 40–60% (fresh weight), and 86-128% (dry weight) more significantly than control (figure 1a-e). The current study evaluates the impact of a high concentration of Cu, while also determining the effectiveness of *Withania coagulans* when exposed to organic amendments, such as CA. Previously, heavy metal intake reduces plant growth by preventing the uptake of nutrients and obstructing all metabolic processes (Afshan *et al.*, 2015). In our study, it was observed that *W. coagulans* growth was severely hampered by a high concentration of Cu-induced stress. However, the addition of CA reduced the stress brought on by Cu. The application of CA as an organic amendment significantly alleviated Cr-induced inhibition in plant growth (Farid *et al.*, 2017). Similar findings from the previous study confirmed that CA effectively mitigates the negative effects of heavy metals in *Sorghum bicolor*, *Helianthus annus*, *Brassica napus*, *Oryza sativa*, and especially in tall fescue and Kentucky bluegrass (Ehsan *et al.*, 2014; Wang *et al.*, 2017; Farid *et al.*, 2018; Bao *et al.*, 2019; Farid *et al.*, 2019).

In previous studies, the supportive role of CA is reported, which showed the increase in biomass production and growth of plants in *B. napus L.* under Pb and Cu stress after treatment with CA (Zaheer *et al.*, 2015; Al Mahmud *et al.*, 2018; Han *et al.*, 2018). The supportive effect of CA on plants under metal stress can also increase the availability of nutrients to plants, such as Fe, Zn, and Mn (Afshan *et al.*, 2015; Ahmad *et al.*, 2020).

The effect of CA on photosynthetic pigments in leaves of *W. coagulans* under Cu-stress: Chl *a* (27%), Chl *b* (6%), and carotenoids (35%), which are photosynthetic pigments, significantly decreased under TCu_2 as compared to control, whilst significant improvement in photosynthetic pigments was observed under TCu_1 as compared to control: Chl *a* (23%), Chl *b* (47%), and carotenoids (65%).

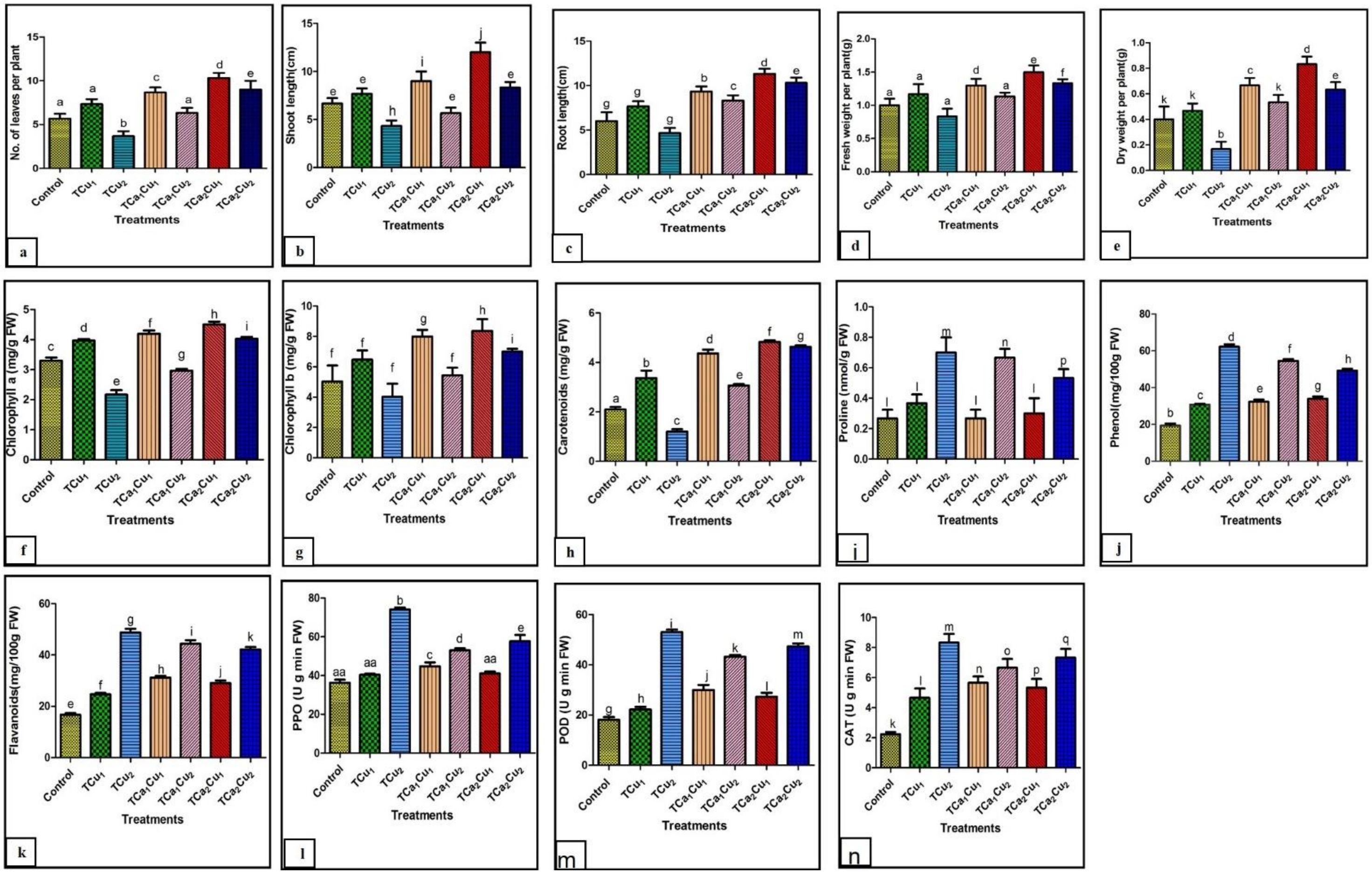

Figure 1: The effect of citric acid on the agronomic traits of *W. coagulans* under Cu-stress. The number of leaves (a), shoot length (b), root length (c), fresh weight per plant (d), dry weight per plant (e), chlorophyll *a* (f), chlorophyll *b* (g), carotenoids (h), the proline content (i), phenol (j), flavonoids (k), PPO (l), POD (m) and CAT (n).

Vertical bars represent ± SD of means for three replicates and values followed by different letters are significantly different according to the Tukey test at $P \leq 0.05$. T: treatment, Cu: copper, Ca: citric acid, Cu_1: first conc. of copper (10mM), Cu_2: second conc. of copper (15mM), Ca_1: first conc. of citric acid (05mM), Ca_2: second conc. citric acid (10mM).

Chl *a* (25%), Chl *b* (58%), and carotenoids (125%) showed a significant improvement in photosynthetic pigments under CA with TCu_2 compared to control. Additionally, TCu_1 with CA significantly increased Chl *a* (30-39%), Chl *b* (78-85%), and Carotenoids (112-135%). In our findings, a rise in CA concentration significantly increased the concentration of photosynthetic pigments and alleviated copper-induced stress (figure 1f-h). The damage to chlorophyll pigments caused by heavy metal stress frequently results in leaf chlorosis (Zong *et al.*, 2017). Previously, these results were confirmed by Falusi *et al.* (2016), Iqbal *et al.* (2015) and Pimple (2017), that the concentration of chl *a* and chl *b* in leaves is decreased in the contamination, showed that the chl *a* and chl *b* content of the leaf were significantly higher in the tested plant grown with less contamination than in higher concentrations of contamination. Additionally, the heavy metals have been linked to reduced the photosynthetic capacity in the plants growing in the polluted environments, which results in the depletion of carotenoids and chlorophyll in the plant leaves (Chauhan and Joshi, 2008).

A plant grown with a higher concentration of Cu had lower carotenoid levels in its leaves. The carotenoid content of leaves was higher with a value of 0.36 mg/g FW and significantly decreased in contaminated Uniben woodland forest with a value of 0.006 mg/g FW (Agbaire *et al.*, 2017). A rise in CA concentration alleviated the stress caused by Cu, which then led to an increase in the concentration of pigments involved in photosynthesis (figure 1f-h). The CA has ability to enhance plant defensive systems against metals toxicity and supports the normal functioning of chloroplast and stomata under metals stress (Kaur *et al.*, 2017). Previously, prominent CA role in facilitating the photosynthesis process under metals stress has been confirmed (Han *et al.*, 2018; Farid *et al.*, 2019).

The effect of CA on the proline content of *W. coagulans* under Cu-stress: Non significant between TCu_1 alone, with CA, and control was recorded. TCu_2 significantly increased the concentration of proline content in plant by 181% as compared to the control (figure 1i). The examined plant had significantly more proline concentrations in its leaves because it had been cultivated in the soil that contained more copper. Metal-induced stress led to greater proline concentrations in *Eucalyptus sp.* and *M. indica* (Assadi *et al.*, 2011; Nyoki and Ndakidemi, 2016). Additionally, a much-increased proline concentration in plant species' leaves indicates the defense mechanism of plants growing under the stress of heavy metals (Agbaire, 2016).

The effect of CA on the antioxidant content of leaves of *W. coagulans* under Cu-stress: The studied plant growing in TCu_2 had significantly more total phenol content (TPC) in its leaves than the control, which was 219% higher. When CA was applied along TCu_2, the TPC values were comparatively low (159%), indicating that the stress caused by copper had alleviated (figure 1j). *Withania coagulans* cultivated on TCu_2 soil had significantly more TFC in leaves (178%) than the control (figure 1k). In contrast, the inclusion of CA lessens the impact of TCu_2 and reduces TFC by 148% when compared to control. Plants cultivated in contaminated soil may have increased flavonoid content as protection against abiotic stressors. A significant difference was found between TCu_1 alone and with CA. *Triticum aestivum* subjected to heavy metal stress, along with CA chelate and *Bacillus sp*. increased the number of non-enzymatic antioxidants (Zhuang *et al.*, 2017). The increase in the plant's cellular TPC with a higher dose of copper is closely in line with those of Radwan *et al.* (2018). Test plants growing in soil contaminated with metals had TPC concentrations that were considerably higher. In our findings, plants cultivated in soil with added copper had much more TFC in their leaves than the control. Under the influence of increasing metal concentration in the growing medium, TFC content in plants is higher (Qayoom Mir *et al.*, 2009). TFC levels significantly increased in Erica andevalensis species leaves grown under stress caused by cadmium (Ibrahim *et al.*, 2017). A defense mechanism for plants against abiotic stresses may be the rise in flavonoid content of plants grown in polluted soil (Rezanejad, 2009).

The effect of CA on antioxidant enzymes activity of leaves of *W. coagulans* under Cu-stress: In our study, it was noticed that under TCu_2, the activities of antioxidant enzymes i.e., PPO, POD, and CAT were significantly increased by 110%, 200%, and 280%, respectively as compared to control, whereas the addition of CA with TCu_2, reduced significantly the activities of antioxidant enzymes as compared to TCu_2 alone, however significantly increased by 70%, 167%, and 245%, compared to control (figure 1l-n). Furthermore, TCu_1 alone and with CA has also improved the activities of antioxidant enzymes compared to control. A tested plant growing in a higher Cu concentration had antioxidant enzymes that were significantly higher. Reactive oxygen species (ROS) are produced as a result of heavy metal toxicity, which also promotes the plant's antioxidant defense mechanism (Ashraf *et al.*, 2017). Heavy metals cause the production of genes that code for proteins implicated in stress responses, like phytochelatins and metallothionein, as well as antioxidant enzyme activity to scavenge active oxygen species (Hasan *et al.*, 2017). In the current study, *W. coagulans* were able to activate these tolerance mechanisms when exposed to high Cu concentrations. According to the results of Polovnikova and Voskresenskaya (2008), the impact of heavy metal stress results in an increase in PPO activity in trifolium and meadow-fescue leaves. When mercury and cadmium concentrations are higher, the antioxidant enzyme activity in *Raphanus sp*. leaves increases (Sharma *et al.*, 2012). Heavy metal stress considerably increases CAT activity in plants (Zaimoglu *et al.*, 2011).

CONCLUSION: *Withania coagulans* morpho-physiological and biochemical characteristics are significantly reduced by higher Cu concentrations. Cu effects were more severe at higher doses than those of other treatment of Cu, although the combination of CA with Cu, alleviated the negative impact of Cu on *W. coagulans* via increasing the activities of antioxidant enzymes and plant biomass. The morphological properties of *W. coagulans* significantly improved as a result of the addition of CA. For instance, the addition of CA combined with a higher concentration of Cu greatly improved the morphology of *W. coagulans* 66% (number of leaves), 31% (shoot length), 87% (root length), 43% (fresh weight), and 78% (dry weight). As a method of Cu stress adaptation, CA is beneficial in enhancing the agronomical and biochemical state of this plant and also CA is a modest choice for lowering environmental concerns to assist plants in the extraction of heavy metals from the soil, as reported in previous studies. Our results fully embrace the benefits of the phyto-stabilization strategy using CA.

CONFLICT OF INTEREST: Authors have no conflict of interest.

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