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Effect of chromated copper arsenate on protein, carbohydrate, and chlorophyll content of tropical *Eucalyptus* and *Acacia* species

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Abstract. *Kumari BMR, Nagaraja N. 2023. Effect of chromated copper arsenate on protein, carbohydrate, and chlorophyll content of tropical* Eucalyptus *and* Acacia *species. Asian J Trop Biotechnol 20: 56-61.* Contamination of heavy metals could cause morphological, physiological, and biochemical changes in plants. Chromated Copper Arsenate (CCA) is a wood preservative that contains Cr, Cu, and As. Leaching these heavy metals into agricultural fields from moderate to higher levels causes a serious threat to the ecosystem. A pot experiment was conducted to evaluate the effect of various concentrations of CCA (250-2,500 mg kg^{-1} soil) on the protein, carbohydrate, and chlorophyll content of *Eucalyptus* and *Acacia* seedlings using commercially available C-type CCA with a proportion of Cr (CrO_3-47.5%), Cu (CuO-18.5%), and As (As_2O_5- 35%). The quantitative estimation of total carbohydrate, protein, and chlorophyll content in control and treated seedlings was carried out by spectrophotometric methods. Results showed that the high concentrations of CCA significantly ($p \leq 0.05$) reduced protein, carbohydrate, and chlorophyll content of *Eucalyptus* and *Acacia* seedlings. The lowest amount of total carbohydrates, proteins, and chlorophyll content found in *E. citriodora* Hook. and *E. tereticornis* Sm. were 0.97±0.05 and 1.70±0.17 mg g^{-1}, 0.42±0.08 and 0.45 ±0.02 mg g^{-1} and 0.11±0.04 and 0.07±0.03 mg g^{-1} at 2,500 mg kg^{-1} soil CCA respectively. Furthermore, the lowest total carbohydrates, proteins, and chlorophyll content in *A. mangium* seedlings were 1.91±0.43, 0.52±0.13, and 0.13±0.01 mg g^{-1} at 2,500 mg kg^{-1} soil CCA, respectively. The present study advocates that the higher concentrations of CCA affect the biochemical parameters of *Eucalyptus* and *Acacia* tree species.

Keywords: *Acacia*, biochemical parameters, CCA, *Eucalyptus* spp, heavy metals

INTRODUCTION

Heavy metal contamination of the environment is one of the main threats affecting fauna and flora worldwide (Briffa et al. 2020; Mitra et al. 2022). Heavy metals are non-biodegradable and remain in the soil for a long time, thus posing a long-term environmental threat (Suman et al. 2018). Chromated Copper Arsenate (CCA) is a wood preservative that protects timber from microbial decay and insect damage (see reviews by Morais et al. 2021). The CCA-treated woods are widely used as construction material, resulting in the release of its components viz Cr, Cu, and As into agricultural fields (Kumpiene et al. 2008), which raises concern about food safety (Saleem et al. 2020; Kumari 2022). Several methods have been adopted for the remediation of soils contaminated with heavy metals. Several biological approaches have proven promising remediation tools for their cost-effective and eco-friendly properties (Yan et al. 2020).

Phytoremediation effectively reduces heavy metal contamination in soil (Ashraf et al. 2019; Yan et al. 2020). However, phytoremediation efficiency is related to the nature of heavy metals found in the soil and the physiological characteristics of plants (Bhat et al. 2022). Plants for phytoremediation should produce relatively high biomass, be tolerant to metal toxicity, and have high metal absorption capacity (Kumari and Nagaraja 2023). *Eucalyptus citriodora* Hook. and *E. tereticornis* Sm. have a shallow root system and produce a high amount of biomass in short periods, even in dry conditions (Luo et al. 2016). Similarly, *Acacia mangium* Willd is a medium-sized, fast-growing tree across India. It is a potential candidate for producing high biomass and is well-adapted to poor soil conditions. Furthermore, these species can accumulate large amounts of heavy metals such as Cd, Cu, Pb, Ni, Zn, and Cr in their tissues (Motesharezadeh et al. 2017; Kumari and Nagaraja 2023). Since these plant species have better tolerance to heavy metal stress, they have the potential to be used to remediate CCA-contaminated areas (Usman et al. 2012).

Cu is an essential metal required for plants at a certain level. However, it is toxic at higher concentrations and affects various biochemical processes in plants (Marastoni et al. 2019; Rather et al. 2020). A study by Feigl et al. (2015) showed a reduction in photosynthetic pigments in Indian mustard plants in increasing concentrations of Cu (10-50 μM). Cr is considered a non-essential metal for plant growth and development. Cr contamination in agricultural soils at a concentration of 350 mg kg^{-1} causes a serious threat to the ecosystem and induces oxidative stress in plants (Ertani et al. 2017). Cr interferes with photosynthetic mechanisms in plants (Bashir et al. 2021), and its toxicity highly depends on the duration of exposure and its concentration in plants (Christou et al. 2020).

The permissible arsenic (As) level in dry fodder was 4 mg kg^{-1} (Adamse et al. 2017). Arsenic (As) causes many morphological, physiological, and biochemical changes in plants (Abbas et al. 2018). It also affects the metabolic functions of the plant by inducing stress (Kalita et al. 2018). Limited studies have been conducted on the impact of different heavy metals on biochemical parameters. Little information is available on the effect of CCA compounds on the *Eucalyptus* and *Acacia* species (Kumari 2022). The objective of this study was to evaluate the impact of CCA on protein, carbohydrate, and chlorophyll content in the seedlings of *E. citriodora, E. tereticornis,* and *A. mangium* on various concentrations of CCA.

MATERIALS AND METHODS

Plant material and experimental design

Studies on the effect of components of chromated copper arsenate, namely Cr, Cu, and As, on biochemical parameters (protein, carbohydrate, and chlorophyll) were conducted using the seedlings of *E. citriodora* and *E. tereticornis* and *A. mangium* in Bengaluru, India (12.9716° N, 77.5946° E). Healthy seeds of *E. tereticornis* and *A. mangium* were procured from the Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore, India, and *E. citriodora* from CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Bengaluru, India. The seeds were surface sterilized with bavistin (50% WP), followed by a double wash with deionized water. After pre-germination treatment, seeds were placed in the Petri dish (9 cm diameter) and treated with 10 ml of five different concentrations (50,100, 500, 1,000, and 5,000 mg kg^{-1} soil) of CCA. Every treatment contained 20 seeds. CCA was prepared from commercially available C-type CCA with a proportion of Cr (CrO_3-47.5%), Cu (CuO-18.5%), and As (As_2O_5- 35%). Seeds were soaked in distilled water as a control treatment. Every treatment has four replications. The Petri dishes with different treatments were kept in a germination chamber. Seed germination rates were observed and recorded for 21 days (Kumari and Nagaraja 2023). Seeds with visible protruded radicals and plumules were considered germinated seeds.

Pot experiments

The healthy seedlings were transplanted to root trainer pots filled with sand, soil, and compost in the ratio of 2:1:2, followed by deoiled neem cake (10 kg/m^3) and single phosphate (4.5 kg/m^3) incorporated with fungicide (Indofil M-45), and pesticide (Phorate (0.25 kg/m^3 each) as a prophylactic measure. Furthermore, one-month-old seedlings (3 to 5 cm long) were transplanted in plastic pots (2,000 cc) filled with sufficient air-dried soil mixed with compost. All the pots were arranged in the greenhouse in four replications. Planting media was given at respective concentrations (250, 500, 750, 1,000, 1,250, 1,500, 2,000, and 2,500 mg kg^{-1} soil) of CCA separately based on the pilot experiments conducted on seed germination in Petri dishes. Regular management practices, including sufficient irrigation and weed control, were performed during the experiments.

Biochemical analysis (protein, carbohydrate, and chlorophyll content)

The seedlings of each species (6 months old) were harvested and washed thoroughly with deionized water. The spectrophotometric methods were used to estimate total carbohydrates, protein, and chlorophyll. The leaves from each treatment were cut into fine pieces and then ground with mortar and pestle. 0.5 g of fresh leaf powder from each treatment was homogenized with 5 µL of 80% acetone, incubated overnight at room temperature, and centrifuged at 5,000 rpm for 5 min. The supernatant was added with 80% acetone to 5 µL. The optical densities were measured at 645 and 663 nm wavelengths using a UV-VIS Spectrophotometer (Shimadzu, UV-1900, Kyoto, Japan). Total carbohydrates were analyzed using the Anthrone method (Hedge et al. 1962). Lowry's method (1951) determined the total protein of the seedlings. Total chlorophyll was estimated using Arnon's method (1949). The results of different treatments were compared with the control.

Statistical analysis

The experimental data on changes in total carbohydrates, proteins, and chlorophyll content of tree species on exposure to different CCA concentrations (250-2,500 mg kg^{-1} soil) were statistically analyzed by one-way analysis of variance (ANOVA). Biochemical parameters of CCA treatments were compared to the control and analyzed statistically for significant differences at $p \leq 0.05$ by Duncan's multiple Range Test (DMRT).

RESULTS AND DISCUSSION

The effect of CCA on the biochemical parameters of *Eucalyptus* seedlings

Nutrient sufficiency is required for the healthy growth and development of plants. However, soil contaminated with toxic heavy metals may affect morphological and biochemical parameters. The effect of different concentrations of CCA (250-2,500 mg kg^{-1} soil) on the biochemical parameters of the seedlings of *E. citriodora* and *E. tereticornis* is presented in Table 1. The results showed a gradual decrease in total carbohydrate, protein, and chlorophyll content with increased CCA concentrations. Lower concentrations (250-500 mg kg^{-1}) of CCA had no significant effect. Total carbohydrates were significantly ($p \leq 0.05$) decreased in CCA concentrations of ≥2,000 mg kg^{-1} soil. The total carbohydrates in the seedlings of *E. citriodora* in the control treatment and treatment of 2,500 mg kg^{-1} soil were 2.71±0.15 mg g^{-1} and 0.97±0.05 mg $g\text{-}^{1}$, respectively. The treatment of CCA ≥750 mg kg^{-1} soil significantly ($p \leq 0.05$) reduced the protein and chlorophyll content in *E. citriodora.* The lowest and highest amounts of proteins and chlorophyll content were in the control, and the treatment of 2,500 mg CCA kg^{-1} soil were 1. 6±0.12, and 0.42±0.08 mg g^{-1} and 0.21±0.02 and 0.11±0.04 mg g^{-1}, respectively.

Table 1. Effect of Chromated Copper Arsenate (CCA) on total carbohydrate, protein and chlorophyll content of seedlings of *Eucalyptus citriodora* and *Eucalyptus teretonicornis* at six-months age

Treatments	***E. citriodora***			***E. teretoricornis***			
(CCA in mg kg-^{1} soil)	**Carbohydrate (mg g^{-1})**	**Protein (mg g^{-1})**	**Chlorophyll (mg g^{-1})**	**Dry Weight (g)**	**Carbohydrate (mg g^{-1})**	**Protein (mg g^{-1})**	**Chlorophyll (mg g^{-1})**
Control	2.71±0.15^{a}	1.16±0.12^{a}	0.21±0.02^{a}	10.84±1.23^{ab}	2.76±0.17^{a}	1.66±0.03^{a}	0.24±0.01^{a}
250	2.72±0.16^{a}	1.15±0.11^{a}	0.20±0.01^{a}	11.88±1.30^{a}	2.73±0.18^{ab}	1.54±0.05^{a}	0.25±0.02^{a}
500	2.62±0.13^{a}	1.05±0.11^{a}	0.19±0.03^{ab}	10.15±1.02^{bc}	2.53±0.15^{abc}	1.41±0.06^{a}	0.23±0.01^{ab}
750	2.60±0.11^{a}	0.97±0.09^{b}	0.17±0.02^{b}	10.03±1.29^{bcd}	2.48±0.13^{abc}	1.25±0.04^{ab}	0.22±0.02b
1000	2.58±0.09^{a}	0.92±0.10^{b}	0.16±0.03^{b}	9.37±0.90^{cd}	2.42±0.14^{abc}	0.99±0.05^{ab}	0.17±0.03^{c}
1250	2.39±0.10^{a}	0.87±0.08^{c}	0.15±0.02^{b}	8.74±0.81^{de}	2.39±0.16^{bc}	0.94±0.03^{ab}	0.16±0.02^{cd}
1500	2.15±0.07^{ab}	0.85±0.09^{c}	0.14±0.01^{bc}	7.96±0.78^{ef}	2.23±0.19^{cd}	0.68±0.02^{ab}	0.15±0.04^{d}
2000	1.78±0.09^{b}	0.78±0.07^{d}	0.12±0.02^{c}	7.00±0.75^{fg}	1.97±0.20^{de}	0.49±0.01^{bc}	0.10±0.01^{e}
2500	0.97±0.05^{c}	0.42±0.08^{e}	0.11±0.04^{c}	5.79±0.70^{g}	1.70±0.17^{e}	0.45±0.02^{c}	0.07±0.03^{f}

Note: Data are means ± SE of four replications in each experiment. Data with the same letter are not significantly different. Different letters indicate significant differences at p≤0.05 level according to the Duncan's multiple range test

Table 2. Effect of Chromated Copper Arsenate (CCA) on total carbohydrate, protein and chlorophyll content of seedlings of *Acacia mangium* at six months age

Treatments (CCA in mg kg-^{1} soil)	**Carbohydrate (mg g^{-1})**	**Protein (mg g^{-1})**	**Chlorophyll (mg g-^{1})**
Control	3.87±0.90^{a}	1.56±0.40^{a}	0.31±0.02^{a}
250	3.57±0.87^{a}	1.48±0.30^{a}	0.27±0.03^{ab}
500	3.25±0.75^{ab}	1.40±0.51^{ab}	0.27±0.02^{ab}
750	2.95±0.68^{ab}	1.21±0.62^{bc}	0.25±0.03^{ab}
1000	2.90±0.65^{bc}	1.15b±0.41^{c}	0.22±0.02^{bc}
1250	2.70±0.51^{cd}	1.03±0.32^{cd}	0.21±0.03^{c}
1500	2.57±0.49^{a}	0.78±0.34^{d}	0.20±0.02^{cd}
2000	2.30±0.36^{a}	0.72±0.22^{de}	0.15±0.01^{d}
2500	1.91±0.43^{b}	0.52±0.13^{e}	0.13±0.01^{d}

Note: Data are mean ± SE of four replications in each experiment. Data with the same letter are not significantly different. Different letters indicate significant differences at p≤0.05 level according to the Duncan's multiple range test

The amount of total carbohydrates, proteins, and chlorophyll content was significantly different in the seedlings of *E. tereticornis* treated with various concentrations of CCA (Table 1). The results showed a significant (p ≤ 0.05) decrease in the total carbohydrates with increased CCA concentrations at ≥1,250 mg kg^{-1} soil. Total carbohydrate content in the treatment of 2,500 mg CCA kg^{-1} soil and control were 1.70±0.17 mg g^{-1} and 2.76±0.17 mg g^{-1}, respectively. Protein content in the leaf at various concentrations of CCA differed significantly. Protein content was decreased significantly (p≤ 0.05) in the treatment of CCA concentrations ≥2,000 mg kg^{-1} soil (0.45±0.02 mg g^{-1}), and protein content in control was 1.66±0.03 mg g^{-1}. Chlorophyll content in the leaves decreased with increasing CCA concentration compared to the control. Chlorophyll content in control and treatment of 2,500 mg CCA were 0.24±0.01 mg g-^{1} and 0.07±0.03 mg g^{-1}, respectively.

Toxicity of CCA on biochemical parameters of *Acacia* seedlings

The biochemical parameters of the seedlings of *A. mangium* treated with CCA at various concentrations (250-2,500 mg kg^{-1} soil) were presented in Table 2. The carbohydrate content of the seedlings was significantly (p ≤0.05) decreased in the treatment of 2,500 mg kg^{-1} soil. The highest carbohydrate was in control (3.87±0.90 mg g^{-1} and the lowest was in the treatment of 2,500 mg CCA kg-^{1} (1.91±0.43 mg g^{-1}). It is apparent that the CCA concentrations of ≥1,000 mg kg^{-1} in soil significantly reduced the protein and chlorophyll content in the seedlings of *A. mangium*. The highest and lowest protein and chlorophyll content were in control (1.56±0.40, 0.31±0.01 mg g^{-1}) and 2,500 mg CCA kg^{-1} soil (0.52±0.13 mg g^{-1}, 0.13±0.02 mg g^{-1}).

Discussion

Heavy metals and metalloids are essential in plant development for metabolic processes and as micronutrients (Rahman and Singh 2019). Nevertheless, heavy metals that exceed the threshold are toxic to plants. Despite plants having developed several biochemical and molecular strategies to cope with heavy metal stress (Naila et al. 2019, Kapoor et al. 2019), exceeding heavy metals can impair plants' morphological, biochemical, and physiological processes (Thakur et al. 2016). This study showed that CCA consisting of Cu, Cr, and As affected the carbohydrate, protein, and chlorophyll of *Eucalyptus* and *Acacia* seedlings due to their toxicity.

In excess, Cu inhibits respiration, affects nitrogen and protein metabolism, and causes a reduction of chlorophyll contents in plants (Chen et al. 2022). Cu may also destabilize membrane integrity, decrease photosynthesis, and alter enzyme activity (Shabbir et al. 2020). Oxidative stress caused by Cr inhibits plant development and ion liberation from cells, which initiates damage to proteins and nucleic acids (Pizzino et al. 2017), thereby inhibiting their metabolic processes. Similarly, arsenic (As) is one of the most potent non-metalloid environmental contaminants that damage plant growth.

Carbohydrates play an essential role in plant growth and immunity. Results of the study showed that carbohydrate content decreased with increased CCA concentrations in the seedlings of *Eucalyptus* and *Acacia* species due to stress induced by CCA components. Exposure to heavy metals in plants produces Reactive Oxygen Species (ROS) through oxidative stress that degrades carbohydrates in the plants (Yang et al. 2023). This study's results align with the findings of Jha and Dubey (2004), who reported a decreased ratio of reduced and non-reduced sugars in shoots of As-supplemented *Oryza sativa*. A previous study by Wiszniewska et al. (2019) showed that the soluble sugar content of *Aster tripolium* was significantly decreased under Cd and Pb stress. The results of this study showed that increasing chromium concentrations decreased the carbohydrate content of the seedlings. Correspondingly, Cr also reduced the lateral roots of alfalfa and white clover (Wakeel et al. 2018). A study by Finnegan and Chen (2012) reported that arsenic (As) stress may also lead to reduced water availability by hindering carbohydrate metabolism.

Proteins are essential components of the cell that are prone to environmental stress. Consequently, any changes in these compounds indicate oxidation stress (Pant and Tripathi 2014). Increasing concentrations of CCA reduced the protein content of the seedlings. The study's results confirm the findings of Tripathi and Tripathi (1999) that reduced protein content in *Albizia lebbeck* on exposure to heavy metal. Adekunle et al. (2019) also observed a decrease in protein content of up to 33 % in *Zea mays* exposed to Cd (44 mg/kg). Ugwu and Agunwamba (2020) showed that excessive Cr inhibits the cell cycle, enzyme activity, nitrogen assimilation, and other vital metabolic processes. Arsenic (As) in high concentration decreased total protein content in *Pteris vittata* (Singh et al. 2006) and *Vigna radiata* (Ismail 2012). The reactive oxygen species (ROS) cause severe changes in protein structure, altering the proteins' functions and subsequently affecting metabolic pathways (Gan et al. 2023).

Chlorophyll is sensitive to heavy metal stress and is used as an indicator of toxicity evaluation caused by different pollutants (Gan et al. 2023). Our study showed a decrease in the chlorophyll content in the seedlings exposed to various concentrations of CCA. Reduced chlorophyll synthesis in metal-stressed plants was possibly due to imbalanced ion homeostasis (Acemi et al. 2017). Excessive amounts of Cu caused damage to the chloroplast's ultrastructure, directly changing the thylakoid membrane composition (Rahman and Singh 2019). Feigl et al. (2015) noted reduced photosynthetic pigments in young rapeseed and Indian mustard plants due to exposure to higher Cu concentrations. In addition, increasing Cr concentration may lead to the deterioration of the chlorophyll in many plants (Sharma et al. 2020).

Plants exposed to Cr stress showed depleted chlorophyll contents that might be due to the disrupted chlorophyll biosynthesis (Muhammad et al. 2021). Cr-induced toxicity decreased chlorophyll in several plant species, such as *Pistia stratiotes* (Sinha et al. 2005), *Citrus limonia*, and *Citrus reshni* (Balal et al. 2017). The decrease in chlorophyll contents under Cr toxicity could be due to the impairment of chlorophyll biosynthesis enzymes, which are compromised under Cr toxicity (Singh et al. 2020). A reduction in chlorophyll synthesis was reported on exposure to arsenic (As) in *Zea mays* (Emamverdian et al. 2015) and *Trifolium pratense* (Hasanuzzaman et al. 2017). The proline amount in plant species increases under heavy metal stress by decreasing the chlorophyll concentration (Ahmed et al. 2021).

In conclusions, The findings suggest that high concentrations of CCA (≥ 1,000 mg kg^{-1} soil) reduced the total carbohydrate, protein, and chlorophyll content in *E. citriodora, E. tereticornis*, and *A. mangium* seedlings. Further research on the effect of CCA contamination of *Eucalyptus* and *Acacia* under long-term field conditions must be done.

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