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Protective effects of the exogenous application of salicylic acid and chitosan on chromium-induced photosynthetic capacity and osmotic adjustment in *Aconitum napellus*

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Abstract

Chitosan (CTS) is recognized for enhancing a plant's resilience to various environmental stresses, such as salinity and drought. Moreover, salicylic acid (SA) is acknowledged as a growth regulator involved in addressing metal toxicity. However, the effectiveness of both compounds in mitigating Cr-induced stress has remained relatively unexplored, especially in the case of *Aconitum napellus*, a medicinally and floricultural important plant. Therefore, the primary objective of this study was to investigate the potential of CTS and SA in alleviating chromium (Cr)-induced stress in *A. napellus*. To address these research questions, we conducted a controlled experiment using potted plants to evaluate the individual and combined impacts of CTS and SA on plants exposed to Cr stress. Foliar application of CTS (0.4 g/L) or SA (0.25 mmol/L) led to significant improvements in the growth, chlorophyll content, fluorescence, and photosynthetic traits of *A. napellus* plants under Cr stress. The most notable effects were observed with the combined application of CTS and SA, resulting in increases in various morphological parameters, such as shoot length (2.89% and 7.02%) and root length (27.75% and 3.36%) under the Cr 1 and Cr 2 treatments, respectively. Additionally, several physiological parameters, such as chlorophyll a (762.5% and 145.56%), chlorophyll b (762.5% and 145.56%), carotenoid (17.03% and 28.57%), and anthocyanin (112.01% and 47.96%) contents, were notably improved under the Cr 1 and Cr 2 treatments, respectively. Moreover, the combined treatment of CTS and SA improved the fluorescence parameters while decreasing the levels of enzymatic antioxidants such as catalase (27.59% and 43.79%, respectively). The application also notably increased osmoprotectant parameters, such as the total protein content (54.11% and 20.07%) and the total soluble sugar content (78.17% and 49.82%) in the leaves of *A. napellus* in the Cr 1 and 2 treatments, respectively. In summary, these results strongly suggest that the simultaneous use of exogenous CTS and SA is an effective strategy for alleviating the detrimental effects of

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Cr stress on *A. napellus*. This integrated approach opens promising avenues for further exploration and potential implementation within agricultural production systems.

Key message

This explains the interactive role of chitosan and salicylic acid in alleviating chromium stress in *Aconitum napellus*.

Keywords *Aconitum napellus*, Antioxidant defense system, Heavy metal, Osmoprotectant, Photosynthesis

Introduction

Abiotic stress refers to the adverse effects that nonliving elements have on living things within their surroundings [1]. One of the most significant issues facing agriculture today is abiotic stress. It lowers planted acreage and results in major losses in agricultural output across the globe. This situation becomes more complicated as the population grows and the climate changes. Global food production is expected to increase by 60–110% by 2050, as the world's population is expected to increase from 7 to 10 billion people [2].

Owing to the negative impacts of heavy metals (HMs) on crop development, soil health, food safety, and marketability, their contamination has become a significant concern in sustainable agriculture [3]. When agricultural soil ecosystems are exposed to heavy metals (HMs) via natural or artificial processes, plants may suffer significant harm [4]. Several metals, such as lead (Pb), mercury (Hg), arsenic (As), cadmium (Cd), chromium (Cr), and cobalt (Co), are present [5–7]. Environmental pollution from industry and anthropogenic sources, such as chromium (Cr) contamination, harms biochemical processes, plant growth, and production [8–10]. Among heavy metals, Cr has the highest potential to pollute ecosystems, with a density of 7.19 g/cm³ [11]. Cr^{+VI} is more hazardous than Cr^{+III} because of its greater oxidation capability, water solubility, and increased ability to pass through cell membranes [12].

Research has revealed multiple harmful effects of Cr on agricultural physiological and biochemical mechanisms [13–15]. There is also a significant quantity of Cr present in sludge and wastewater from industrial sectors, such as tanneries, distilleries, and pulp paper [16, 17]. Chromium (Cr) is one of the most widespread heavy metals in wastewater from industry outputs and has caused tremendous public concern because of its wide distribution and redox sensitivity [18, 19]. Chlorosis, stunted development, browning of roots, and occasionally even death are among the apparent signs that plants grow in heavy metal-contaminated soils [20, 21].

Additionally, disruption of the cell membrane structure and photosynthesis by Cr reduces the photosynthetic and gas exchange characteristics of plants [22, 23]. Elevated levels of Cr in plants result in ultrastructural changes [24], oxidative stress, and elevated concentrations of electrolyte leakage (EL) and malondialdehyde

(MDA). Additionally, they alter the activities of antioxidant enzymes, including catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD) [25–27]. Therefore, protecting plants against Cr toxicity is crucial to combat the effects of phytotoxicity and oxidative damage caused by plant ingestion of Cr [28].

Many physiological, morphological, and metabolic characteristics of plants are negatively impacted by Cr exposure, which eventually results in plant death. For example, Cr toxicity changes antioxidant activities, increases the formation of reactive oxygen species (ROS), and impacts photosynthesis, nutrient uptake, and plant development [29]. Because it affects the majority of agriculturally significant crops, including pulses, cereals, and vegetables, Cr toxicity has a significant effect on sustainable agriculture and food security [30, 31].

Salicylic acid (SA) is highly effective for plant growth under abiotic stress conditions [32, 33]. In particular, phenolic compounds such as the phytohormone SA regulate the growth and development of plants under both favorable and unfavorable conditions [34, 35]. SA alleviates stress-induced oxidative damage, decreased growth, and decreased photosynthetic efficiency in plants [36, 37]. SA controls plant growth and protects plants from environmental stresses such as salt stress [38].

Numerous studies have shown that the use of SA contributes to the maintenance of cell membrane stability by increasing the number of numerous antioxidants, including SOD, POD, CAT, and APX, and increasing the capacity of leaves for photosynthesis under a variety of stresses [39–41]. There have been observations indicating reductions in lipid peroxidation and electrolyte leakage after the use of SA [42]. Foliar sprays of plant growth regulators, such as salicylic acid, significantly improve rose growth, flowering quality, and vase life [42]. Salicylic acid plays a crucial role in regulating plant growth by affecting cell division and expansion, with potential applications in crop improvement [43]. Salicylic acid (SA) plays a crucial role in plant growth, metabolism, and defense systems, helping plants cope with environmental stresses and promoting development under various environmental conditions, including heavy metal stress [44, 45].

Chitosan is a naturally occurring biopolymer, amino polysaccharide, and biodegradable substance that is extracted from the exoskeletons of marine crustaceans for commercial use [46]. According to [47], it is known

to increase the levels of plant development regulators, which shield plants from oxidative stress. It may also promote the growth and development of different plant species in nonstressful environments. Research has revealed that chitosan enhances the production of rice (*Oryza sativa*) [48] and increases the inflorescence and floret sizes of rice [49]. Furthermore, chitosan enhances the ability of plant cells to tolerate drought by increasing the activity of cytoprotective enzymes that scavenge O₂ and H₂O₂ and reducing the formation of organic materials in wheatgrass (*Agropyron repens*) cultivars [50]. Many studies have shown that the use of CH has several benefits, including increased growth, better transpiration, chemical flexibility, nutrient availability, and carboxylation efficiency, when plants are exposed to abiotic stresses [51–53]. However, proline, hydrogen peroxide, malondialdehyde, total soluble protein, soluble carbohydrate, total antioxidant, and antioxidant enzyme activities all increased when the chitosan–salicylic acid nanocomposite was treated with varying NaCl concentrations [54]. Chitosan and its oligosaccharides enhance crop growth by promoting photosynthesis, regulating primary photochemistry, and preventing pathogen proliferation, offering a sustainable option for sustainable crop production [55]. Chitosan supports plant growth and development while protecting against pathogens, with the potential for crosstalk with phytohormones, antioxidants, and cellular signaling molecules [56]. Chitosan-based delivery systems for plants offer benefits such as protection from harsh environmental conditions and prolonged release of active ingredients, offering potential for future applications in agriculture, food, and health [57]. Copper(II) complex-based plant growth regulators accelerate corn root system development, with chitosan-coated calcium alginate microcapsules providing effective delivery to plants [58]. Chitosan stimulates plant growth, protects edible products, and induces stress tolerance in various horticultural commodities, with potential applications in sustainable crop productivity [59].

Aconitum napellus is a poisonous but valuable medicinal plant that is widely used in traditional Chinese medicine [60, 61]. It is used as a cardiotonic, analgesic, anti-inflammatory, and treatment for asthma, vomiting, and diarrhea. This perennial herbaceous plant is

indigenous to Europe's western and central regions [62]. Unani physicians use the root of *Aconitum napellus* as an antipyretic in conditions such as pneumonia and pleurisy, which is beneficial for black bile and phlegmatic diseases, nerve tonics, anesthetics, and antifebrile [63]. Aconitum extract assists in eliminating fat, while the root component is used to cure obesity, diarrhea, fever, and nervous system diseases [64]. *Aconitum napellus* L. is widely used as a popular medicinal plant in homeopathy [65]. *Aconitum napellus* (ACON) is used for treating rheumatoid and joint pain and is generally safe when taken at doses dependent on its toxicity [66]. Aconitum species are reported to possess antifungal, anti-inflammatory, and immunostimulant properties [67].

This research was performed in light of the medicinal value and limited knowledge currently available regarding the heavy metal tolerance of *A. napellus*. However, the combined effect of chitosan and salicylic acid for the alleviation of chromium stress is unknown. Our study provides novel insights into the alleviation of chromium stress in *Aconitum napellus* growth improvement via the synergistic effect of chitosan and salicylic acid.

The goal of this study was to understand the changes in plant growth and physiological and biochemical attributes under Cr toxicity, as well as the role that chitosan and salicylic acid play in enhancing plant growth and physiological performance in chromium-rich soil. This research was performed in light of the medicinal value and limited knowledge currently available regarding the heavy metal tolerance of *A. napellus*.

Materials and methods

Propagules of *Aconitum napellus* (item no. CG931) were purchased/collected from a local nursery in Bahawalpur. Seedlings of *A. napellus* were established in plastic pots (20×30 cm) at the Botany Department, Islamia University of Bahawalpur. The newly transplanted seedlings were allowed to acclimate to greenhouse conditions for one week. Seedlings of a similar size (4–5 leaves) were selected for the experiments. The selection criteria for seedlings were root and shoot length, as well as leaf color, which was mostly green and dark green. This information has been written in the methodology section. All the seedlings were divided into three groups [Cr0-control, Cr1 (150 mg), and Cr2 (200 mg)] on the basis of the concentration of chromium spiked into the soil. Each group was further divided into three groups on the basis of the type of foliar treatment (three weeks after chromium spiking): (1) without spraying (control foliar treatment), (2) sprayed with 0.25 mmol/L salicylic acid, (3) sprayed with 0.4 g/L chitosan, and (4) sprayed with a combination of 0.25 mmol/L salicylic acid and 0.4 g/L chitosan (Table 1). Chitosan from Meron Biopolymers,

Table 1 C=control, Cr1=150 mg/kg chromium chloride, Cr2=200 mg/kg chromium chloride, chitosan=0.4 g/L, salicylic acid=0.25mmol/L

Treatment	Control (No Cr Stress)	Cr1 Stress 150 mg	Cr2 Stress (200 mg)
C	C	C+Cr1	C+Cr2
CTS 0.4 g L ⁻¹	CTS	CTS+Cr1	CTS+Cr2
SA 0.25 mmol L ⁻¹	SA	SA+Cr1	SA+Cr2
CTS+SA	CTS+SA	CTS+SA+Cr1	CTS+SA+Cr2

salicylic acid from Graham Chemicals, and Cr from the YILDIRIM Group of Companies were purchased and used in this research study.

Estimation of photosynthetic pigments

A foliage sample was collected for the spectrophotometric determination of photosynthetic pigments (a, b, a+b, carotenoids). The leaves (0.1 g) were crushed and homogenized in an ice-cooled mortar with 5 ml of 80% (v/v) acetone. Acetone was utilized as a blank following five minutes of centrifugation. To determine the absorbance at 663 nm, 645 nm, and 480 nm, we added cold acetone to the filtrate volume to adjust it to 5 ml. An EMCLAB German instrument spectrophotometer and acetone from INEOS were used in this study.

Determination of chlorophyll fluorescence parameters

The chlorophyll fluorescence parameters were measured via a portable syno device as previously described [68]. A fluorometer (model: PAM 2500-WALZ, Germany) was used to measure the amount of chlorophyll fluorescence in the final fifth of the leaves exposed to light. The following parameters were measured: chlorophyll (a, b), anthocyanin, and carotenoid contents.

The selection criterion for the seedlings was the use of roots and shoots to measure the amount of chlorophyll fluorescence in the final fifth of the leaves exposed to light. The following parameters were measured: chlorophyll (a, b), anthocyanin, and carotenoid contents.

Determination of antioxidants

Determination of catalase activity

The catalase (CAT) activity was determined following the method described by Chance et al. 1955 [69] with some modifications. The catalase reaction mixture (3 ml) was composed of 50 mM phosphate buffer, 5.9 mM H₂O₂, and 0.1 mL of enzyme extract. Upon the addition of the enzyme extract, the reaction commenced. Changes in the absorbance of the reaction mixture were monitored at 240 nm every 20 s, where a change of 0.01 units min⁻¹ in absorbance corresponded to 1 unit of catalase activity. Furthermore, absorbance changes at 470 nm were observed every 20 s. The catalase activities were expressed relative to the protein content, which was estimated via the method of [70].

Estimation of electrolyte leakage in leaves

This electrolyte leakage (EL) value indicates the percentage of ion leakage and reflects the extent of membrane damage. Three fully developed leaves were cut into 0.5 cm segments and immersed in 7 ml of sterilized water in a glass tube. The glass tube was placed over a roaster shaker (Model651: RYG) at 25 °C for one day, after which the initial electrical conductivity (Ec-i) was measured.

The second and final electrical conductivity (Ec-f) reading was recorded after autoclaving the sample for 30 min at 120 °C and cooling it to room temperature. Electrolyte leakage was calculated according to the formula of [71].

$$EL = (EC - i)/(EC - f) \times 100$$

Determination of flavonoids

The total flavonoid contents were determined via the aluminum chloride method outlined by [72]. Dried leaf powder (0.1 g) was extracted with 2 mL of 80% (v/v) methanol. Subsequently, 250 mL of the extract and 1 mL were mixed with 250 mL of 10% (w/v) potassium acetate and aluminum (Lianyungang Nuoxin Food Ingredient Co. Ltd.). The mixture was left at room temperature for 40 min before the absorbance was measured at 415 nm.

Determination of ascorbic acid peroxidase activity

The leaf samples (100–200 mg) were ground in 2 ml of 50 mM phosphate buffer (pH 7.8) containing 20 mg of polyclar AT and 1 mM ascorbate. For 15 min, the homogenate was centrifuged at 15,000 × g. The supernatant was used for protein measurements and enzyme tests. The Nakano et al., 1981 [73] technique was used to measure the oxidation of ascorbate at 290 nm to determine ascorbic acid (ASC) peroxidase activity. Protein content was determined according to a previously described method [70]. Bovine serum albumin was used as a standard curve, and the reaction mixture without protein was used as the control. Polyclars and ascorbate (Lianyungang Nuoxin Food Ingredient Co. Ltd.) were used.

Determination of secondary metabolites

Determination of total amino acids

The total amino acid content was estimated as previously described [74]. Approximately 0.5 g of fresh leaves were extracted with 0.2 M phosphate buffer at pH 7.0. Subsequently, 1 ml of the extract was added to individual testing tubes, together with 10% pyridine and aqueous 2% ninhydrin solutions. These test tubes, comprising the sample and reaction mixture, were heated in a boiling water bath for 30 min. Upon cooling, each test tube was adjusted to a volume of 50 mL with distilled water. The spectroscopic density of the resulting mixture was measured at 570 nm, with a standard curve based on various concentrations of leucine used to determine the total amino acid content.

Determination of proline

The proline (Pro) content was determined via the colorimetric method of [75]. A leaf sample (1 g) was mixed with a 3% (W/V) solution of sulfosalicylic acid in water. Afterward, a clear filtrate was acquired by passing the

homogenized mixture through two layers of filter paper. After the mixture of glacial acetic acid and ninhydrin was added to 1 ml of the filtrate, the reaction mixture was heated in a water bath at 100 °C for 1 h. The reaction was subsequently halted by placing the mixture in an ice bath. The absorbance was measured at a wavelength of 545 nm. The proline concentration was calculated via a standard curve.

Determination of primary metabolites

Determination of soluble sugar contents

The total soluble sugars were identified according to the protocol of [76]. Fresh leaf powder (0.5 g) was combined with ethanol (80%) and placed in a water bath at 100 °C for 10 min. After that, the material was centrifuged for 10 min at 5,000 rpm. After centrifugation, 1 ml of the supernatant was combined with 4 ml of anthrone reagent, and this mixture was then heated in a water bath at 100 °C for 10 min. To halt the reaction, the mixture was incubated in a water bath at room temperature (25 °C) for 5 min. The absorbance was subsequently recorded at a wavelength of 630 nm.

Statistical analysis

The data, which comprise the average values from three replicates, will be analyzed via one-way ANOVA and Duncan's multiple range test. To determine the significance of changes, P values were utilized, with values less than 0.05 being regarded as significant. The significance of differences between treatments will be shown by applying lowercase letters to the average values via Statistix software.

Results

Morphological characteristics

A significant difference was observed among the untreated and chromium-stressed plants, with a 3.22% decrease in shoot length in the chromium 1 treatment group and a 7.81% decrease in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved shoot length under the chromium 1 and chromium 2 treatments by 2.89% and 7.02%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with 181.9% in the chromium 1 treatment group and 272.8% in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved the root length under the chromium 1 and chromium 2 treatments by 27.75% and 3.36%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with 23.70% in the chromium 1 treatment group and 5.71% in the chromium 2 treatment group compared with the control plants. The synergistic

effects of chitosan with salicylic acid improved shoot fresh weight under the chromium 1 and chromium 2 treatments by 83.14% and 48.18%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with 64.38% in the chromium 1 treatment group and 5.09% in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved shoot dry weight under the chromium 1 and chromium 2 treatments by 25.93% and 72.56%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with 49.46% in the chromium 1 treatment group and 188.2% in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved the fresh weight of roots under the chromium 1 and chromium 2 treatments by 104.8% and 6.54%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with 27.39% in the chromium 1 treatment group and 479.5% in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved the root dry weight under the chromium 1 and chromium 2 treatments by 147.4% and 45.63%, respectively. Compared with those of the control plants, a significant difference was observed among the untreated and chromium-stressed plants, with 11.1% in the chromium 1 treatment group and 0% in the chromium 2 treatment group. All these morphological results are shown in Fig. 1. The synergistic effects of chitosan with salicylic acid improved floral bud development under the chromium 1 and chromium 2 treatments by 25.07% and 0%, respectively. These results are shown in Fig. 2.

Chlorophyll pigments and fluorescence parameters

A significant difference was observed among the untreated and chromium-stressed plants, with a decrease of 67.12% in chlorophyll *a*n in the chromium 1 treatment group and 0.4% in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved the chlorophyll content under the chromium 1 and chromium 2 treatments by 762.5% and 145.56%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with a 95.47% decrease in chlorophyll *b* in the chromium 1 treatment group and a 96.57% decrease in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved chlorophyll *b* content under the chromium 1 and chromium 2 treatments by 100% and 85.83%, respectively. These results are shown in Fig. 3. A significant difference was observed among the untreated and chromium-stressed plants, with a decrease of 89.13% in terms of carotenoid

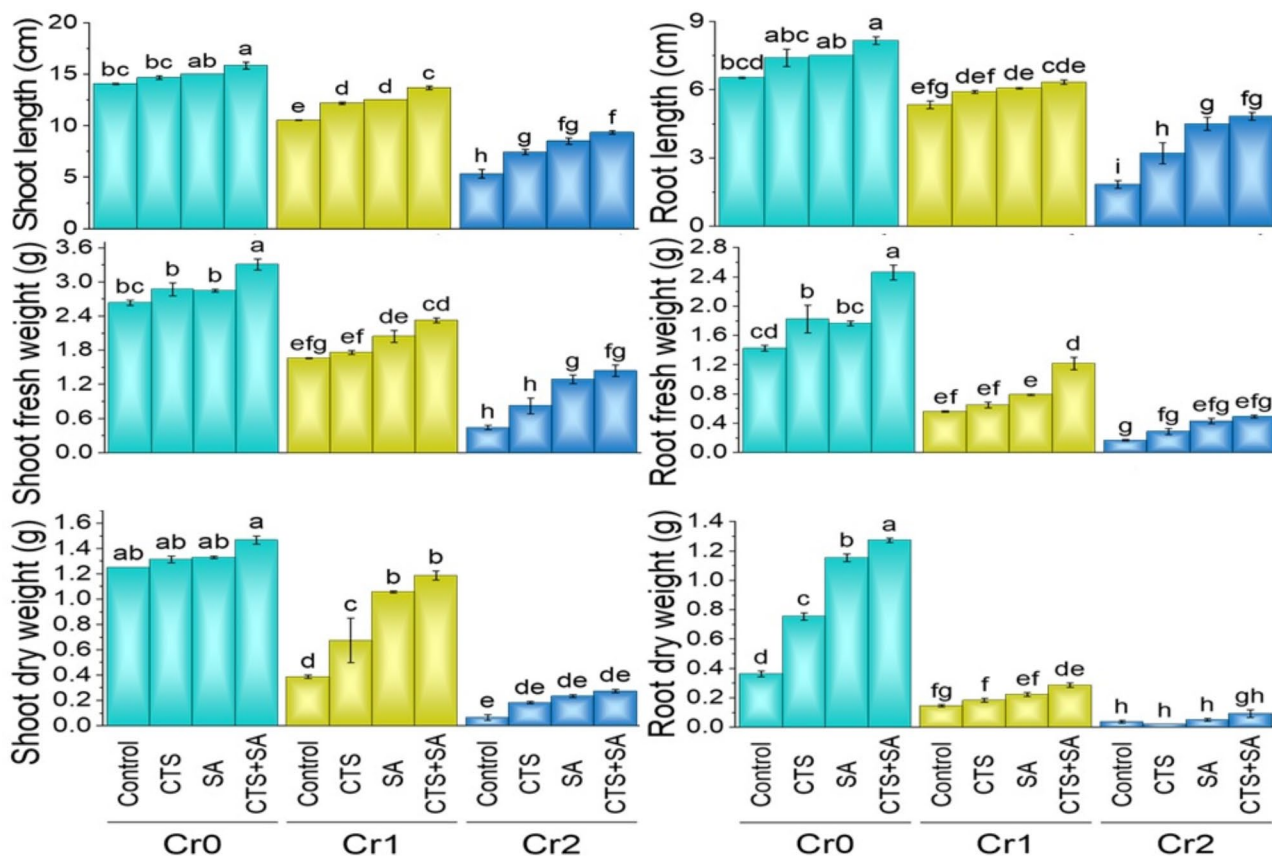


Fig. 1 Individual and combined effects of salicylic acid and chitosan foliar application on shoot length, root length, shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight under chromium stress in *Aconitum napellus*. Different letters indicate significant difference between the treatments. Data is Mean, \pm SE ($n=3$). C=control, Cr1 =150 mg/kg Chromium chloride, Cr2=200 mg/kg Chromium chloride, chitosan=0.4 g/L, salicylic acid=0.25mmol/L

content in the chromium 1 treatment group and 69.56% in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved the carotenoid content under the chromium 1 and chromium 2 treatments by 17.03% and 28.57%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with 8.584% lower anthocyanin content in the chromium 1 treatment group and 317.24% lower anthocyanin content in the chromium 2 treatment group than in the control plants. The synergistic effects of chitosan with salicylic acid improved the anthocyanin content under the chromium 1 and chromium 2 treatments by 112.01% and 47.96%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with values of 7.34% in the chromium 1 treatment group and 2.67% in the chromium 2 treatment group compared with those of the control plants. These results are shown in Fig. 3. The synergistic effects of chitosan with salicylic acid improved the F_s under the chromium 1 and chromium 2 treatments by 22.78% and 59.41%, respectively. Compared with those of the control

plants, a significant difference was observed among the untreated and chromium-stressed plants, with 2.08% in the chromium 1 treatment and 12.52% in the chromium 2 treatment. The synergistic effects of chitosan with salicylic acid improved ϕ_2 under the chromium 1 and chromium 2 treatments by 17.42% and 16.33%, respectively. Compared with those of the control plants, a significant difference was observed among the untreated and chromium-stressed plants, with 15.32% in the chromium 1 treatment group and 37.97% in the chromium 2 treatment group. The synergistic effects of chitosan with salicylic acid improved the ϕ NPQ under the chromium 1 and chromium 2 treatments by 32.26% and 21.65%, respectively. Compared with those of the control plants, a significant difference was observed among the untreated and chromium-stressed plants, with 19.98% in the chromium 1 treatment group and 19.09% in the chromium 2 treatment group. The synergistic effects of chitosan with salicylic acid improved the qL under the chromium 1 and chromium 2 treatments by 1.22% and 2.59%, respectively. Compared with those of the control plants, a significant difference was observed among the untreated

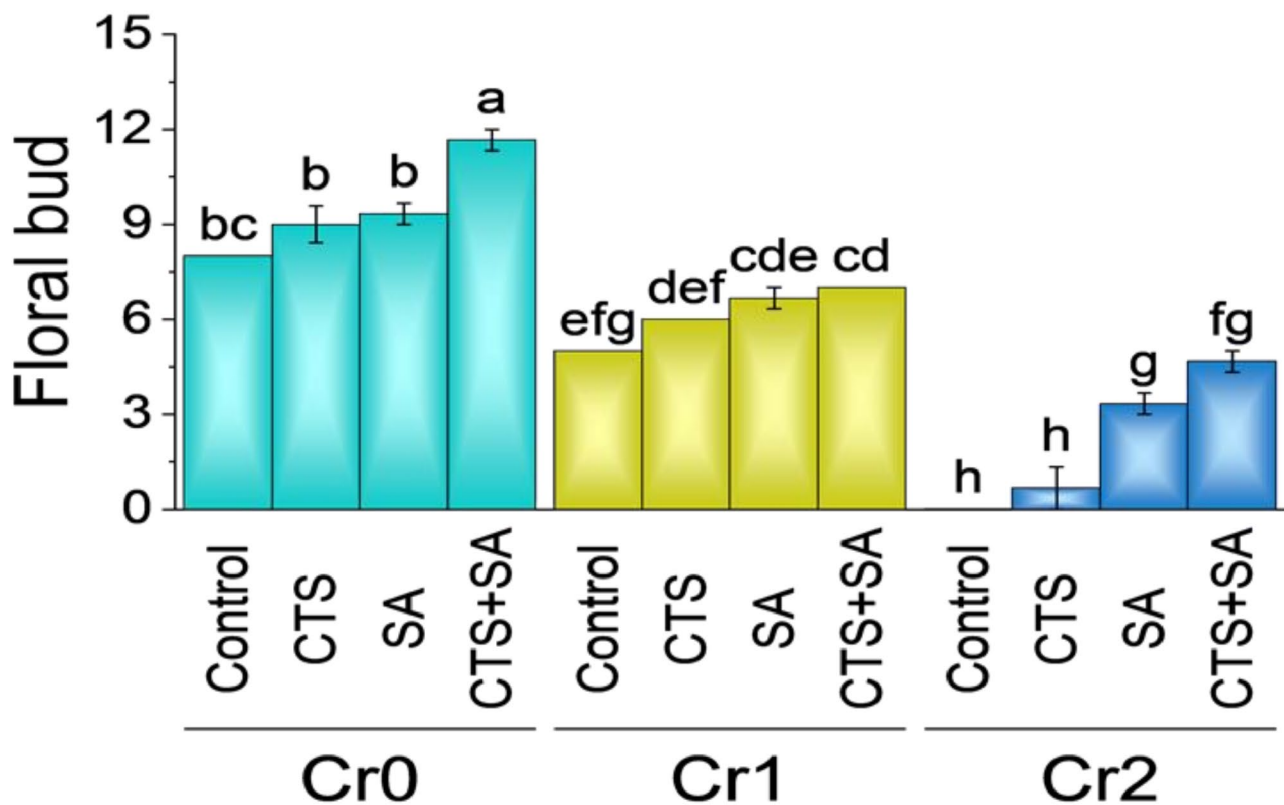


Fig. 2 Individual and combined effects of salicylic acid and chitosan foliar application on floral bud under chromium stress in *Aconitum napellus*. Different letters indicate significant difference between the treatments. Data is Mean, \pm SE ($n=3$). C= control, Cr1=150 mg/kg Chromium chloride, Cr2=200 mg/kg Chromium chloride, chitosan=0.4 g/L, salicylic acid=0.25mmol/L

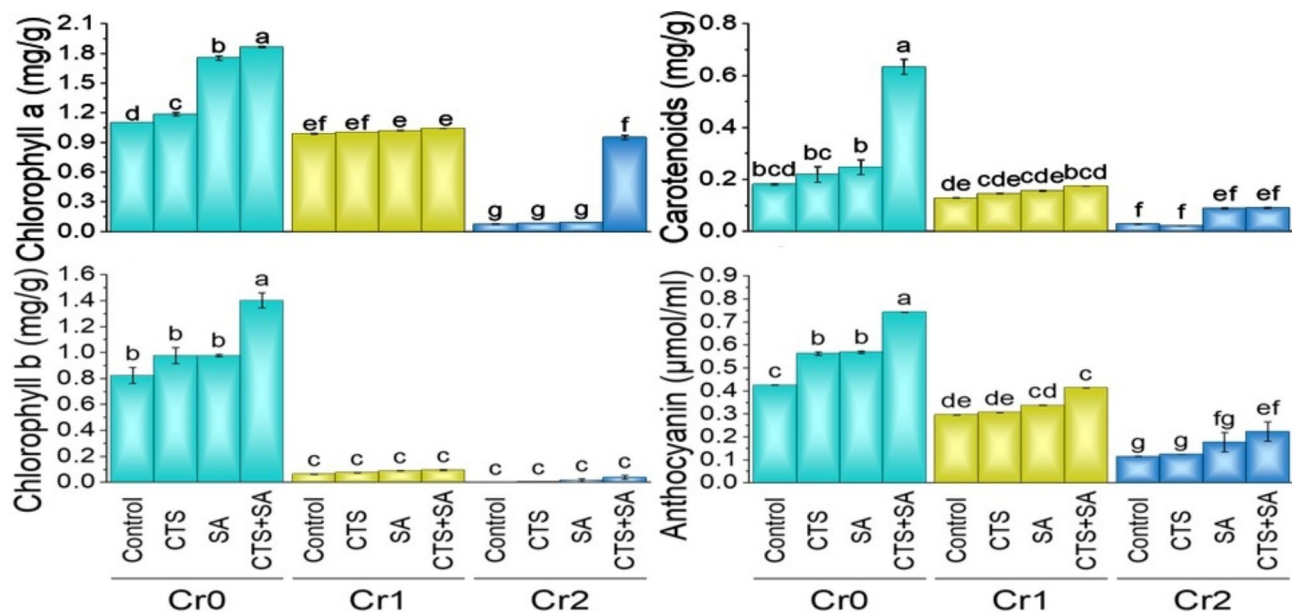


Fig. 3 Individual and combined effects of salicylic acid and chitosan foliar application on chlorophyll a, chlorophyll b, carotenoids, and anthocyanin under chromium stress in *Aconitum napellus*. Different letters indicate significant difference between the treatments. Data is Mean, \pm SE ($n=3$). C= control, Cr1=150 mg/kg Chromium chloride, Cr2=200 mg/kg Chromium chloride, chitosan=0.4 g/L, salicylic acid=0.25mmol/L

and chromium-stressed plants, with 46.39% in the chromium 1 treatment group and 91.81% in the chromium 2 treatment group. The synergistic effects of chitosan with salicylic acid improved NPQt under the chromium 1 and chromium 2 treatments by 44.07% and 28.24%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with 8.96% in the chromium 1 treatment group and 16.93% in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved the F_v/F_m' under the chromium 1 and chromium 2 treatments by 14.51% and 12.15%, respectively. These results are shown in Fig. 4.

Antioxidant parameters

A significant difference was observed among the untreated and chromium-stressed plants, with 27.59% in the chromium 1 treatment group and 43.79% in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved catalase activity under the chromium 1 and chromium 2 treatments by 55.84% and 45.97%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with 35.19% in the chromium 1 treatment group and 85.32% in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved electrolyte leakage under the chromium 1 and chromium 2 treatments by 9.88% and 25.59%, respectively. A significant difference was observed among the untreated and chromium-stressed

plants, with 78.56% in the chromium 1 treatment group and 31.73% in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved the flavonoid content under the chromium 1 and chromium 2 treatments by 107.72% and 27.11%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with a decrease of 131.79% in the ascorbic acid content in the chromium 1 treatment and 5.769% in the chromium 2 treatment compared with the control plants. The synergistic effects of chitosan with salicylic acid improved the ascorbic acid content under the chromium 1 and chromium 2 treatments by 85.76% and 37.69%, respectively. These results are shown in Fig. 5.

Osmoprotectant parameters

A significant difference was observed among the untreated and chromium-stressed plants, with a decrease of 56.52% in the chromium 1 treatment and 4.34% in the chromium 2 treatment compared with the control plants. The synergistic effects of chitosan with salicylic acid improved the proline content under the chromium 1 and chromium 2 treatments by 41.50% and 18.18%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with 72.62% in the chromium 1 treatment group and 68.43% in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved the total amino acid content under the chromium 1 and chromium 2 treatments by 592% and

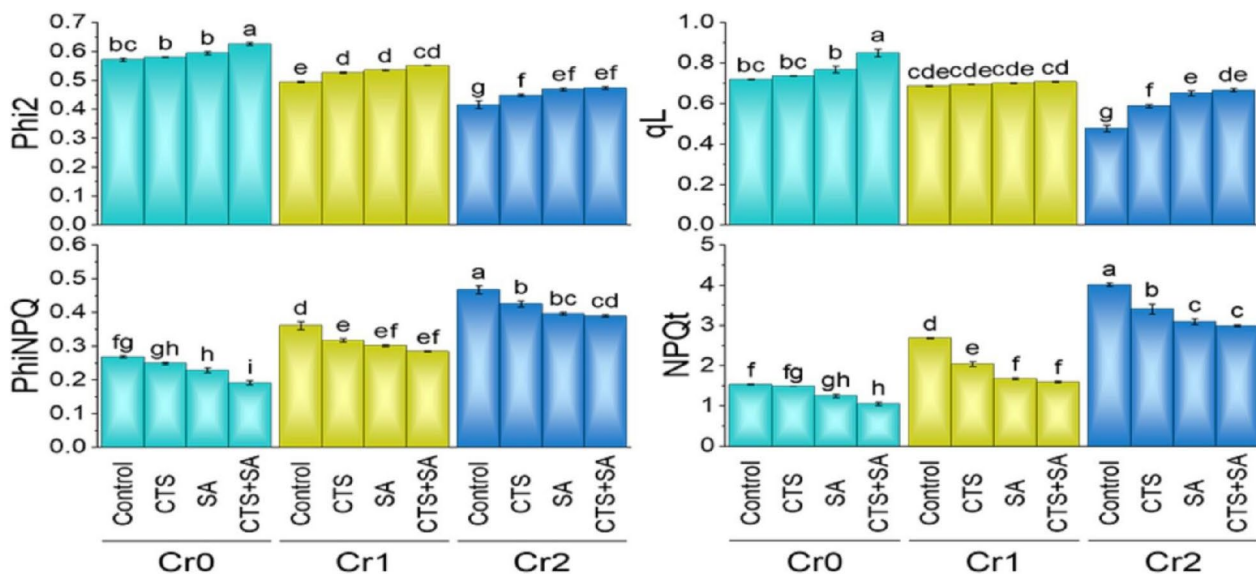


Fig. 4 Individual and combined effects of salicylic acid and chitosan foliar application on Phi2, PhiNPQ, qL, and NPQt under chromium stress in *Aconitum napellus*. Different letters indicate significant difference between the treatments. Data is Mean, ±SE (n=3). C = control, Cr1 = 150 mg/kg Chromium chloride, Cr2 = 200 mg/kg Chromium chloride, chitosan = 0.4 g/L, salicylic acid = 0.25mmol/L

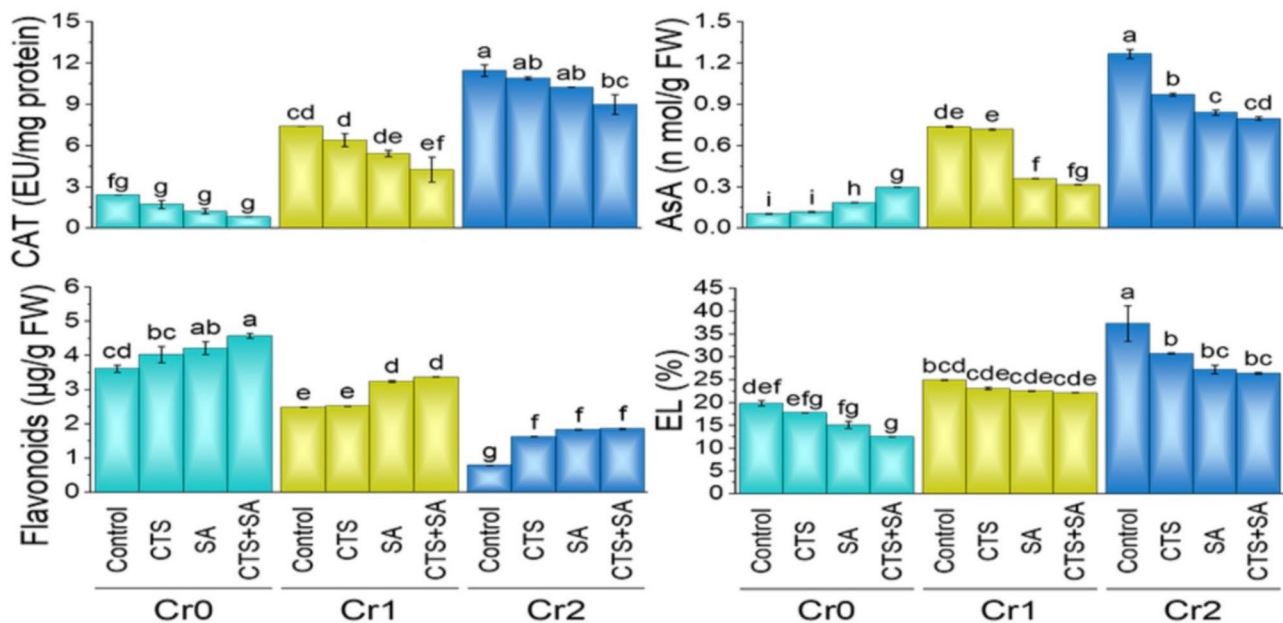


Fig. 5 Individual and combined effects of salicylic acid and chitosan foliar application on catalase (CAT), electrolyte leakage (EL), flavonoids and ascorbic acid (AsA) under chromium stress in *Aconitum napellus*. Different letters indicate significant difference between the treatments. Data is Mean, \pm SE ($n=3$). C = control, Cr1 = 150 mg/kg Chromium chloride, Cr2 = 200 mg/kg Chromium chloride, chitosan = 0.4 g/L, salicylic acid = 0.25 mmol/L

15%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with 50.29% in the chromium 1 treatment group and 14.42% in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved the total protein content under the chromium 1 and chromium 2 treatments by 54.11% and 20.07%, respectively. A significant difference was observed among the untreated and chromium-stressed plants, with 28.32% in the chromium 1 treatment group and 60.94% in the chromium 2 treatment group compared with the control plants. The synergistic effects of chitosan with salicylic acid improved the total soluble sugar content under the chromium 1 and chromium 2 treatments by 78.17% and 49.82%, respectively. These results are shown in Fig. 6.

Pearson correlation analysis

The Pearson correlation analysis results present a matrix of correlation coefficients that quantify the relationships between various variables in the dataset. First, there was a strong positive correlation between several pairs of variables, such as shoot fresh weight (g) and shoot length (cm), with a correlation coefficient of 0.97902. This suggests that as the shoot fresh weight increases, the shoot length tends to increase proportionally. Similar strong positive correlations were found between other pairs of variables, including shoot dry weight (g) and shoot length (cm) (0.95326), root fresh weight (g), shoot fresh weight (g) (0.95000), root dry weight (g) and root fresh weight

(g) (1.00), among others. These highly positive correlations imply that these variables tend to move in the same direction, which is essential for understanding biological and experimental contexts. Conversely, notable negative correlations were detected, particularly between the antioxidant variables ASA (n mol/g FW) and proline (μ g/g) (-0.9469) and between ASA (n mol/g FW) and CAT (EU/mg protein) (-0.96981). These negative correlations suggest that as the levels of one antioxidant increase, the levels of the other antioxidants tend to decrease. It is worth exploring the implications of these negative associations in the context of antioxidant responses. Furthermore, there were strong positive correlations among variables related to photosynthetic pigments, such as chlorophyll a (mg/g) and chlorophyll b (mg/g) (0.76859), as well as chlorophyll b (mg/g) and carotenoids (mg/g) (0.82853). These correlations highlight the interdependencies of these variables in the context of plant pigments. Figure 7 shows all the attributes studied via Pearson correlation analysis.

Convex hull and hierarchical cluster analysis

The results of the convex hull analysis offer valuable insights into the distribution and relationships of data points within a two-dimensional space defined by PC 1 and PC 2, which represent 91.81% and 3.39% of the total variation, respectively. This suggests that PC 1 is the primary source of variation and likely carries more meaningful information for distinguishing treatments. The data are categorized into four treatments: control, CTS,

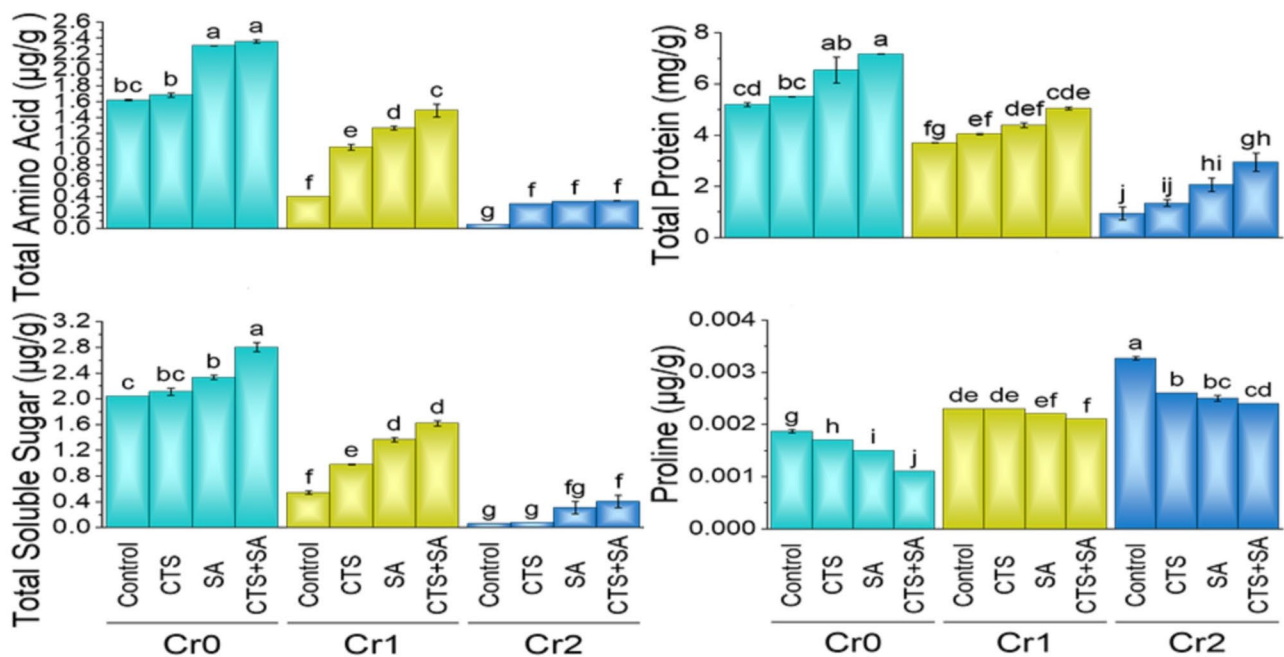


Fig. 6 Individual and combined effects of salicylic acid and chitosan foliar application on total amino acid under, total soluble sugar, total protein and proline content under chromium stress in *Aconitum napellus*. Different letters indicate significant difference between the treatments. Data is Mean, ±SE (n=3). C = control, Cr1 = 150 mg/kg Chromium chloride, Cr2=200 mg/kg Chromium chloride, chitosan=0.4 g/L, salicylic acid=0.25mmol/L

SA, and CTS+SA. Analyzing the convex hulls of these treatments reveals distinctive patterns. The control group was widespread, indicating significant variation within the treatment group, which may be a sign of heterogeneity or a lack of clear clustering. In contrast, the CTS and SA groups were more tightly clustered, suggesting that these treatments may have more consistent effects. The CTS+SA group exhibited a broader spread, suggesting a wider range of responses to this combined treatment (Fig. 8A).

The convex hull analysis results offer valuable insights into the distribution and relationships of data points in a two-dimensional space defined by PC 1 and PC 2, accounting for 91.81% and 3.39% of the total variation, respectively. The data points are categorized into three distinct Cr groups: Cr0, Cr1, and Cr2. Analyzing the convex hulls for these groups reveals interesting patterns and implications. The dominance of PC 1 in capturing most of the variation underscores its importance in distinguishing treatments.

The convex hulls visually represent the outermost points for each Cr category, providing insights into the spread and clustering of data within this space. In this context, the Cr0 group displays a relatively tight cluster in the PC 1 and PC 2 spaces, suggesting a relatively homogeneous response within this treatment category. Conversely, the Cr1 group shows a broader spread, implying that this category may encompass a more diverse set of responses, potentially due to various underlying factors.

The Cr2 group appears to exhibit a similar pattern to that of Cr1, with a relatively broad spread, indicating a potentially wide range of responses within this category. These convex Hull patterns suggest that Cr0 may have a more consistent and focused response, whereas Cr1 and Cr2 may involve a broader range of effects or could be influenced by different variables (Fig. 8B).

The hierarchical cluster analysis results illustrate a hierarchical grouping of variables on the basis of their similarity, with corresponding labels denoting the variables being compared. Several noteworthy patterns and relationships emerge from this analysis. First, variables such as shoot length (cm) and Fv/Fm' presented a moderate similarity of 0.54694, suggesting some shared characteristics. In contrast, shoot fresh weight (g) and fresh biomass (g) were highly similar at 0.85717, indicating a strong correlation between these two variables. Similarly, total amino acid (µg/g) and total soluble sugar (µg/g) are highly similar, with values of 0.94648, implying a shared response or association. Variables such as root length (cm) and EL (%) have a substantial similarity of 1.19116, indicating a close connection between root length and electrical conductivity. Interestingly, anthocyanin (µmol/ml) and Fs are significantly similar, with a value of 1.20042, suggesting a potential relationship between these variables. Furthermore, down the hierarchy, variables such as root dry weight (g) and chlorophyll b (mg/g) presented a high similarity of 3.79227, indicating a strong correlation in their behavior. In contrast, variables such

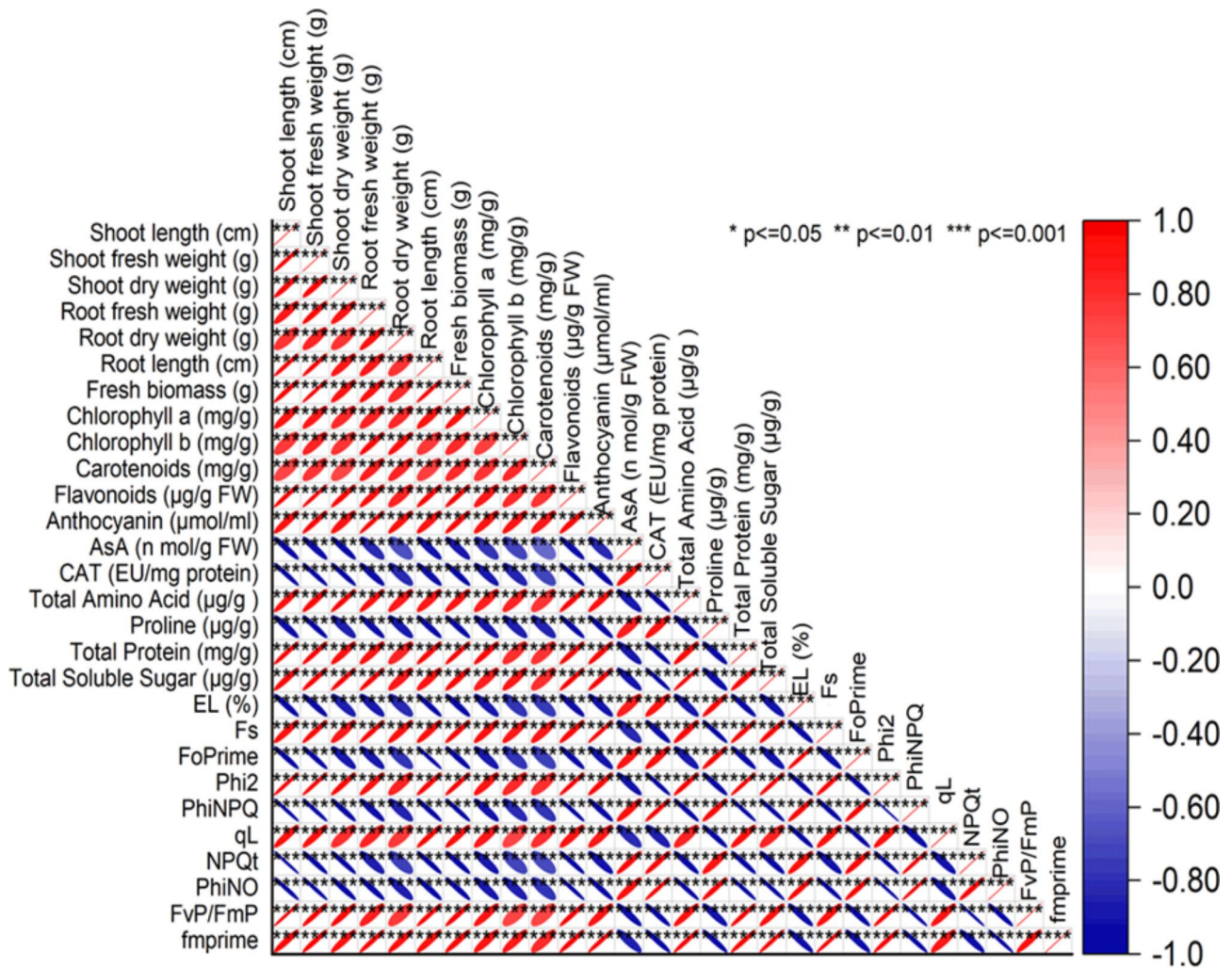


Fig. 7 Pearson correlation for studied attributes

as carotenoids (mg/g) and AsA (n mol/g FW) had an exceptionally high similarity of 10.17629, indicating a very close connection between the carotenoid and ascorbic acid contents (Fig. 8C).

Discussion

In the present study, plants exposed to CrCl₂ presented a noteworthy increase in shoot length when subjected to low concentrations of CrCl₂ (150 mg), and these results are similar to previous findings on *Myriophyllum spicatum* branch length at lower Cr concentrations (0.05 mg/L) [77]. However, considerable reductions in shoot length and shoot weight were observed under conditions of high chromium content (1 mg/L) [78]. The effects of Cr exposure on the shoot length and biomass of many plant species have been independently verified, as comparable results were reported by [79], who confirmed that exposure to Cr had a noticeable effect on shoot length and biomass. Roots are highly important in plants because of their ability to absorb water and nutrients and

appear to be major sites of Cr toxicity. In our study, CrCl₂ (200 mg/L) increased the number of roots of *A. napellus*; however, relatively high concentrations of CrCl₂ inhibited root development. Similar outcomes were previously reported for *Pistia stratiotes* [80] (O₃) and *C. sinensis* [81]. In addition, *Pisum sativum* roots are thin and delicate when exposed to high Cr concentrations (>1000 mg/L [82]). In our study, CrCl₂ stress reduced the levels of photosynthetic pigments, as reported by [83]. In *A. napellus*, excess CrCl₂ had no effect on the characteristics of chlorophyll fluorescence, indicating a stable PS II under Cr stress, as also reported by [84]. The light-harvesting complex of photosystem II in *T. aestivum* was substantially affected after treatment with Cr (≥0.10 mM) [84, 85]. Additionally, the PhiNO and NPQ values also markedly increased under Cr stress, indicating that an antenna pigment was unable to successfully convert light energy into chemical energy and instead dissipated as heat [86]. According to the findings of our investigation, catalase and ascorbic acid activities were increased by Cr

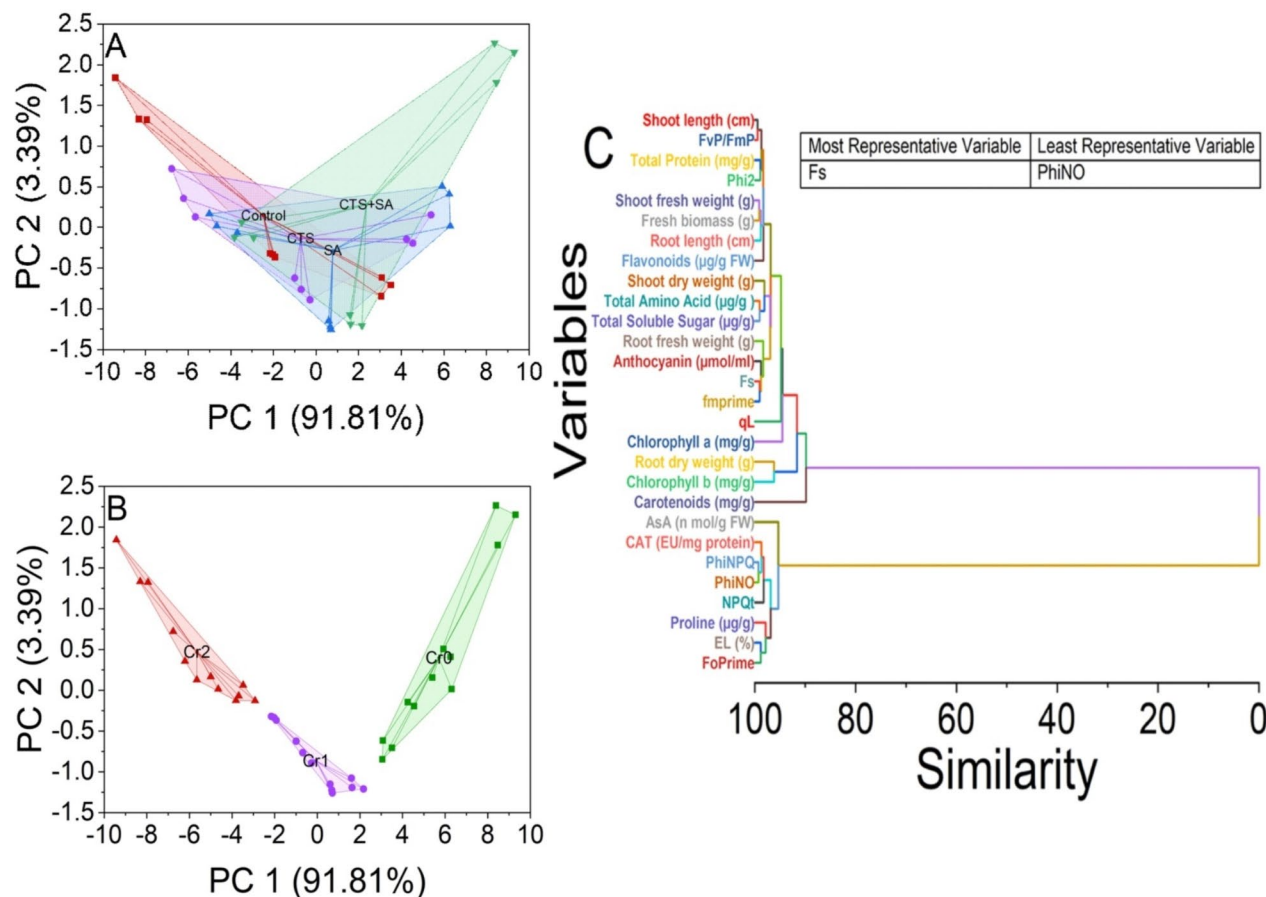


Fig. 8 Cluster plot convex hull for treatments (A) for studied attributes, (B) for Cr stress, and Hierarchical cluster analysis (C) for studied attributes

toxicity. Moreover, two antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), are activated in response to cadmium-induced stress, and similar findings were reported by [86]. These findings demonstrated that CAT was critical for protecting *Aconitum napellus* against Cr stress. Our findings revealed an increase in proline levels under Cr stress, as previously reported in different studies [87, 88]. Proline inhibits the formation of reactive oxygen species, functions as an osmoprotectant, and stabilizes cell structures [14, 89]. Our results revealed that the total protein content and total soluble sugar content decreased under CrCl₂ stress in *Aconitum napellus*.

The growth and development of *A. napellus* improved, and the negative effects of Cr were mitigated by the foliar application of CTS. These findings are consistent with studies demonstrating that CTS reduces the negative effects of Cd on the growth of flamingo anthurium [90], rapeseed (*Brassica napus*), and other plants [91, 92]. The findings of the present study revealed that foliar application of 0.4 g/L CTS had the greatest mitigating effect. It has also been reported that CTS, which has a relatively low Mw, has great potential as a plant growth promoter

[92]. In plants, a CTS spray frequently decreased the accumulation of heavy metals [92], but shoot Cr levels dramatically increased when 0.4 g/L CTS was applied to the leaves of *A. napellus* under Cr stress. In our experiments, we found that Cr toxicity was suppressed in the leaves of *A. napellus*. In contrast, CTS treatment markedly increased the amount of photosynthetic pigments in *A. napellus* under Cr stress, suggesting that 0.4 g/L CTS might prevent stomatal closure due to Cr stress and increase photosynthesis. However, this outcome was incongruent with other studies in which CTS caused stomatal closure in pepper [92, 93] tomato and Asiatic dayflower (*Commelina communis* L.) [94], as well as diminished the effects of drought stress on rice [95]. In addition, the increase in chlorophyll content was accompanied by the recovery of the photosynthetic machinery, similar to the ability of CTS to minimize the negative effects of Cr on the Fv/Fm, Fv/Fo, and NPQ. After the plants were sprayed with 1 kDa CTS, the NPQ value drastically decreased, indicating that CTS improved the light utilization efficiency. The accumulation of proline (a potential indicator of stress tolerance) increased in *A. napellus* under Cr stress after (0.4 g/L) CTS treatment,

which could help in protecting osmoregulation, the chelation and detoxification of metals, enzyme protection, cytosolic acidity regulation, the stabilization of protein synthesis machinery, and the trapping of reactive oxygen species [96]. In our study, the application of CTS (0.4 g/L) considerably increased the ASC content in plants subjected to chromium stress. More significantly, the level of ascorbic acid (ASC) increased to a greater extent in non-stressed plants that received CTS (0.4 g/L) than in plants that were not treated with CTS. These findings indicate that CTS could increase ASC synthesis and sustain protection against Cr stress. In this study, CTS increased the CAT and ASA activities, which may have reduced the generation of H₂O₂.

In the present study, the application of salicylic acid (SA) resulted in significant changes in nutrient uptake, total chlorophyll content, and the antioxidant response in *A. napellus* plants, irrespective of Cr stress. SA is assumed to be a principal controller of a range of biological processes, encompassing the shaping of plant form and structure, development, flowering, and closure of stomata [97]. The better growth in the presence of SA is in agreement with previous reports on corn soybean [98] and maize [99]. SA plays a role in increasing proline levels under HM toxicity [100, 101]. This effect could be attributed to the increased activity of enzymes involved in proline synthesis [102]. Our research revealed that foliar application of SA (0.25 mmol/L) considerably increased the amount of photosynthetic pigments in *A. napellus* under CrCl₂, as reported for wheat by Sharma [103]. The contribution of SA in plants is to decrease the level of reactive oxygen species by boosting the antioxidant defense system and improving membrane stability under HM stress [104, 105]. As a prominent regulator of photosynthesis, SA influences various aspects of plants, including the chlorophyll content, stomatal conductivity, and activities of photosynthesis-related enzymes [106]. Under heavy metal (HM) stress, SA enhances photosynthetic efficiency and improves the functionality of the photosynthetic apparatus [107]. A recent investigation examined the mechanisms by which SA increased pigment levels and increased photosynthetic efficiency [28]. The findings of this study demonstrated that pretreatment with SA increased the levels of carotenoids and PS II either in the presence of low levels or in the absence of Cd. These findings imply that salicylic acid positively protects photosynthetic pigments and the photosynthetic machinery. The application of SA has been shown to increase the total chlorophyll content in plants subjected to stress conditions [108, 109]. Our study revealed an increase in chlorophyll content and chlorophyll fluorescence in *A. napellus* following the foliar application of SA (0.25 mMol/L) under Cr stress. In several plant species, salicylic acid (SA) treatment confers defense against

various metals, including lead (Pb), mercury (Hg), and cadmium (Cd) [41, 110]. Recent research findings indicated that the addition of salicylic acid (SA) to *Nymphaea tetragona* and *Lemna minor* plants under Cd stress conditions led to the stimulation of antioxidant enzymes (SOD, APX, POD) [111].

The addition of Cr to the soil significantly decreased plant growth and biomass in spinach (*Spinacia oleracea* L.) [112]. Cr and Cd stress in plants significantly reduces root length, shoot length, dry weight, and fresh weight [113]. Higher levels of chromium stress (200 μM) significantly reduce sweet potato plant growth, biomass, photosynthetic attributes, antioxidants, and enzyme activities [114]. Similarly, in the present study, the root length, shoot length, fresh weight, dry weight, chlorophyll content, catalase activity, and ascorbic acid activity strongly decreased with increasing concentrations of Cr. Foliar salicylic acid and zinc treatments increase proline, carotenoid, and chlorophyll contents in *Galanthus elwesii*, with higher doses enhancing antioxidant enzyme activity [115]. Salicylic acid application to *Salvia coccinea* plants can improve growth, photosynthetic pigments, and antioxidant activity under salinity stress, making it a promising source of antioxidants [116]. Foliar application of salicylic acid reduces oxidative damage, increases photosynthetic efficiency, and improves tomato production under environmental stress [117]. Similarly, in this study, the synergistic effects of salicylic acid strongly improved the biomass, chlorophyll a content, chlorophyll b content, carotenoid content, anthocyanin content, catalase content, electrolyte leakage, and flavonoid and ascorbic acid contents of *Aconitum napellus*.

Chitosan alleviates boron toxicity in cucumber plants and strengthens antioxidant defenses while reducing the boron concentration in tissues [118]. In our present research, chitosan reduced the effects of Cr by increasing catalase, ascorbic acid, and electrolyte leakage. Foliar application of chitosan dissolved in ascorbic or citric acid can improve tomato plant growth and antioxidant activity under salinity stress conditions [119]. In our study, the synergistic effects of chitosan with salicylic acid alleviated Cr stress and improved ascorbic acid, proline, and amino acid contents and photosynthetic efficiency. Biochar and chitosan effectively alleviate the negative impacts of drought stress on barley plants, increasing growth traits and improving anatomical and yield characteristics [120]. In our research, chitosan with salicylic acid mitigated Cr stress and increased growth traits, improving the physiological characteristics and Cr stress tolerance of *Aconitum napellus*.

In our study, exposure to CrCl₂ resulted in the suppression of antioxidant enzymes, such as catalase. However, the coapplication of salicylic acid (0.25 mmol/L) and chitosan (0.4 g/L) effectively resulted in superior efficiency

in comparison with their concentrations and had a more positive effect on the treatment. However, further investigations are needed for the alleviation of stress caused by other heavy metals and other plant species due to the combined effects of chitosan and salicylic acid. The combined effects of chitosan and salicylic acid can be used as an environmentally friendly approach in the agricultural sector for the mitigation of abiotic stress.

Conclusion

In summary, Cr stress is highly toxic to *Aconitum napellus*. Foliar applications of CTS (0.4 g/L) or SA (0.25 mmol/L) led to significant improvements in the growth, chlorophyll content, fluorescence, and photosynthetic traits of *Aconitum napellus* plants under Cr stress. The most notable effects were observed with the combined application of CTS and SA, resulting in enhancements in various morphological parameters. Additionally, several physiological parameters, such as chlorophyll (a, b), carotenoids, and anthocyanins, were notably improved. Moreover, the combined treatment (CTS+SA) improved the fluorescence parameters while decreasing the levels of enzymatic antioxidants such as catalase and electrolyte leakage by 5.19% and 85.32%, respectively, under the chromium 1 and 2 treatments. The application also notably increased osmoprotectant parameters, such as total protein content and total soluble sugar content, in the leaves of *A. napellus* in the presence of Cr. This combined effect of chitosan and salicylic acid represents an innovative approach that can be successfully used in *Aconitum napellus* plants to alleviate Cr stress. To further improve plant health and tolerance to chromium stress, future research can explore the synergistic effects of chitosan and salicylic acid with additional growth regulators, antioxidants, or nanoparticles.

Abbreviations

CTS	Chitosan
SA	Salicylic Acid
Cr	Chromium
TF	Translocation Factor
ROS	Reactive Oxygen Species
HM	Heavy Metals
ASC	Ascorbic Acid
SOD	Superoxide Dismutase
CAT	Catalase

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Author contributions

[MR] conceived the study and wrote the manuscript. [TJ] added valuable comments and improved the paper. Ariba Hassan [AH] contributed various inputs during the manuscript preparation. [MZA] finalized the manuscript. Statistical analysis, validation, and funding contributed by [HA], [AAS], and [MAES]. [MI] performed the data analysis and revised and arranged the figures and manuscript. [AAS], [MAES] and [VR] were involved in drafting, revision the article and final approval of the version to be published. All the authors read and approved the finalized manuscript for publication.

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Data availability

All data is presented in this manuscript. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors permit the publisher to publish this research.

Conflict of interest

All authors declare that they have no conflicts of interest.

Competing interests

The authors declare no competing interests.

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