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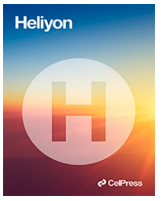

Research article

Chitosan mitigated the adverse effect of Cd by regulating antioxidant activities, hormones, and organic acids contents in pepper (*Capsicum annum* L.)

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ABSTRACT

Chitosan (CTS) is one of the natural healers' alternatives to chemical products within the scope of good agricultural practices. It can be used in the improvement of agriculture (prevention of toxic metal uptake by plants) due to its chelating feature of metal ions. This study aims to investigate the effectiveness of chitosan in eliminating the negative effects of cadmium (Cd) stress on pepper (*Capsicum annum* L.). The results showed that Cd stress significantly decreased plant growth, chlorophyll content, and leaf water relative content, followed by an increase in proline, antioxidant enzyme activities, and abscisic acid (ABA) content. According to the results, Cd treatment (200 mg kg-1) significantly increased the aspartate, glutamate, asparagine, histidine, and phenylalanine content, while it significantly decreased the content of endogenous hormones such as gibberellic acid (GA), indole-3-acetic acid (IAA), and salicylic acid (SA). However, CTS application decreased the uptake of Cd and caused a decrease in hydrogen peroxide (H_2O_2), abscisic acid (ABA), and melondialdehyde (MDA) content, as well as an increase in plant performance, and GA, IAA, and SA content in the plants grown under Cd pollution compared to the ones treated with Cd and without CTS. This study suggests that CTS application helps pepper seedlings tolerate Cd stress through a decrease in Cd uptake, and an increase in amino acids and hormone content.

1. Introduction

Chitosan (CTS), one of the most important derivatives of chitin, is a non-toxic, biodegradable, and biocompatible biopolymer from many natural sources such as the exoskeleton of mushrooms, crayfish, shrimp, and crabs [1]. It is used in many industrial fields such as food, cosmetics, medicine, and agriculture [2,3]. In addition to having antimicrobial properties, CTS also stands out with its elicitor

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and metal chelator properties in plant growth. It is seen these properties of CTS are the subject of many studies in the development of good agriculture and agricultural biotechnology. In agriculture, CTS is utilized for a range of purposes including disease control, nutrient chelation, and the prevention of pathogen entry into plant tissues. Additionally, CTS improves plants' natural defense mechanisms and enhances their ability to mitigate toxic metal uptake through chelation of harmful ions. The biopolymer's efficacy in inducing various biochemical and physiological responses varies with plant species and concentration, highlighting its adaptability in different agricultural contexts [4]. Economically, CTS provides substantial benefits by enhancing crop yield and quality, reducing reliance on synthetic fertilizers and pesticides, and increasing plant resilience to environmental stresses. Its biopesticide and biostimulant properties align well with organic farming practices, catering to the growing consumer demand for organic products and potentially commanding premium prices. Moreover, CTS contributes to extending the post-harvest shelf life of produce, thereby minimizing losses. By promoting soil health and reducing the regulatory burdens associated with chemical use, CTS represents a cost-effective and sustainable solution that supports farm profitability and environmental stewardship [5].

Both soil and water are precious natural resources that are essential to the long-term viability of agricultural production. But human-made activities have seriously harmed and polluted either soil or water [6]. Plants naturally have a propensity to absorb certain metals through their roots. A few micronutrients, such as copper, iron, cobalt, and molybdenum are essential to plant elements, while others, including mercuric, cadmium (Cd), nickel, and lead, may be harmful to plants [7,8]. Metals' toxicity is influenced by the type of ion, concentration of ion, plant genotypes, and development stage [9]. Cd is a prominent concern among the contaminants known as "heavy metals" because of its mobility in the plant-soil system. The additional uses of pesticides, phosphorus fertilizers, and sludges from the non - agriculture industries enhanced the concentration of this element in the soil. It is a poisonous and highly mobile element that may be quickly absorbed by plants in high amounts [10,11]. Cd stress has been found in previous research to decrease nutrient uptake, plant growth, and development. It affects photosynthesis and increases oxidative stress by boosting reactive oxygene species (ROS) production, which causes lipid peroxidation and modifies the antioxidative defense system [12].

CTS offers potential solutions to these issues through various molecular mechanisms. Its amino and hydroxyl groups facilitate interactions with other molecules, forming hydrogen bonds and electrostatic interactions. A key mechanism is metal chelation, where CTS binds to metal ions like Cd, thereby reducing their availability and toxicity to plants. Additionally, CTS exhibits antioxidant properties by scavenging free radicals and alleviating oxidative stress, which helps mitigate Cd-induced damage. It also stimulates the production of defense-related enzymes and compounds, enhancing plant resistance to heavy metal toxicity. Furthermore, CTS reinforces plant cell walls, improving structural integrity and enhancing the plant's ability to withstand environmental stresses [13,14].

To our knowledge, no prior research has specifically examined the impact of CTS on pepper plants' susceptibility to Cd stress. This study aims to explore the physiological and biochemical changes in pepper plants induced by CTS under Cd stress conditions. We hypothesize that CTS treatment will enhance the tolerance of pepper plants to Cd stress by improving their physiological and biochemical responses, thereby mitigating the adverse effects of cadmium toxicity. This research will contribute to developing strategies for sustainable plant production and addressing the risks associated with Cd contamination.

2. Materials and methods

2.1. Plant material and design of experiment

In this study, we used the seeds of pepper (*Capsicum annum* L. cv. Yalova Carliston) as plant material and the experiment was conducted in a controlled greenhouse in pots. Pepper seeds were initially sown in peat, and when the seedlings had three true leaves, they were transplanted into 2.5 L pots as one seedling in each pot. The medium, which was made with a combination of garden soil, peat, and sand, was placed inside the pots (3: 1: 1, v: v: v), respectively. In the experiment, Cd concentrations of 0, 100, 150, and 200 mg kg^{-1} were added to the growth medium, watered to the field's capacity, and then incubated for three weeks, then pepper seedlings were planted in pots. CTS solutions were prepared in 0 mg L^{-1}, 0.5 mg L^{-1}, and 1.0 mg L^{-1} doses with 0.2 % Tween-20 and were sprayed to plant leaves, and the spraying was performed three times at one-week intervals. The experiment was set up randomized plot design with three replications and each replication consists of six plants. A total of 216 plants were used. The experiment was finished 50 days following the transplant.

2.2. Harvest and growth parameters

To assess the physiological and morphological parameters, the four plants were harvested per each sample. The plant material was maintained at 70 °C for 48 h to assess dry weight. Fresh leaves needed for additional examination were frozen in liquid nitrogen and kept at −80 °C. The assessments were carried out in quadruplicate.

2.3. Chlorophyll reading value, leaf area, leaf relative water content and Cd content

A chlorophyll meter was used to quantify the chlorophyll reading value (CRV). A leaf area meter was used to measure the leaf areas of the plants in each treatment. The leaf relative water content (LRWC) was determined according to the method of Araz et al. [15].

Determination of Cd content in the leaves and roots of pepper seedlings was done according to the method of Krishnamurti et al. [16].

2.4. Proline content

Shams et al. [17] method was used to determine proline content and the level of proline in the tissues was assessed using a standard curve made from pure proline.

2.5. Hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and antioxidant activities

Hydrogen peroxide and MDA content were determined according to Sardar et al. [18]. The H_2O_2 level was determined using a standard calibration curve (using different levels of H_2O_2). MDA was calculated from thiobarbituric acid-reactive compounds and its level was calculated from the absorbance curve using an extinction value of 155 mmol L^{-1} cm^{-1}.

The antioxidant enzyme activities (catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD)) were measured on fresh leaf samples using the techniques of Shams et al. [19] and Dadasoglu et al. [20].

2.6. Hormone analysis

For hormone analysis, extraction and purification steps were carried out exactly as specified by Koshita et al. [21]. The abscisic acid (ABA), gibberellic acid (GA), indole-3-acetic acid (IAA), and salicylic acid (SA) were analyzed by HPLC using a Zorbax Eclipse-AAA C-18 column (Agilent 1200 HPLC). The absorbance detection was fixed at either 220 nm or 265 nm depending on a given phytohormone class [22].

2.7. Amino acid analysis

To analyze amino acids, one g of fresh leaf sample was mixed with 0.1 N HCl, the homogenization was done with ultraturraks, and incubation was conducted at 4 °C for 12 h. The samples vortexed and spun at 1200 rpm for 50 min and then were filtered using a 0.22 m filter.

Detection of the amino acids was carried out by an Agilent 1200 HPLC (Zorbax Eclipse-AAA 4.6 × 150 mm, 3.5 μm column) and the following standard: O-phthaldialdehyde (OPA), fluorenylmethyl-chloroformate (FMOC), and 0.4 N borate. The absorbance of samples was recorded at 254 nm [23].

2.8. Statistical analysis

The statistical program SPSS was used to analyze the data, Two-way ANOVA were made and Duncan multiple range test (DMRT) tests were used to evaluate the differences among the means.

3. Results

The ability of CTS to diminish the hazard impact of heavy metal (Cd) stress on pepper (*Capsicum annum* L.) growth, physiology, and biochemical characteristics was examined in this study.

3.1. Plant growth, LRWC and CRV of pepper seedling

Cadmium (Cd) and chitosan (CTS) application significantly affected the growth, LRWC, and CRV of pepper seedling. As can be observed in Table 1, under Cd stress conditions, CTS treatment had a substantial impact on the fresh and dry weights of pepper leaves

Table 1
Effect of cadmium (Cd) and chitosan (CTS) applications on leaf and root weight of pepper seedling (±SD).

Cd (mg kg^{-1})	CTS (mg L^{-1})	Leaf fresh weight (g $plant^{-1}$)	Leaf dry weight (g $plant^{-1}$)	Root fresh weight (g $plant^{-1}$)	Root dry weight (g $plant^{-1}$)
0	0	23.23 ± 1.13 e	4.09 ± 0.03 f	13.17 ± 0.25 g	1.17 ± 0.01 e
	0.5	23.06 ± 0.30 e	4.19 ± 0.01 g	13.85 ± 0.29 h	1.15 ± 0.02 e
	1.0	21.83 ± 0.61 d	3.97 ± 0.05 e	13.49 ± 0.06 gh	1.17 ± 0.02 e
100	0	3.77 ± 0.06 ab	0.63 ± 0.01 b	3.25 ± 0.08 cd	0.24 ± 0.00 b
	0.5	5.20 ± 0.11 c	0.70 ± 0.01 c	4.19 ± 0.04 f	0.27 ± 0.00 c
	1.0	5.46 ± 0.02 c	0.83 ± 0.01 d	3.78 ± 0.05 e	0.31 ± 0.01 d
150	0	3.11 ± 0.04 ab	0.52 ± 0.01 a	2.86 ± 0.08 ab	0.20 ± 0.00 a
	0.5	3.66 ± 0.05 ab	0.73 ± 0.01 c	3.42 ± 0.05 de	0.31 ± 0.01 d
	1.0	4.34 ± 0.08 BCE	0.68 ± 0.01 BCE	3.40 ± 0.01 de	0.26 ± 0.01 c
200	0	3.00 ± 0.05 a	0.49 ± 0.01 a	2.57 ± 0.06 a	0.20 ± 0.00 a
	0.5	3.46 ± 0.07 ab	0.52 ± 0.01 a	2.96 ± 0.05 BCE	0.27 ± 0.01 c
	1.0	3.57 ± 0.04 ab	0.63 ± 0.01 b	3.02 ± 0.07 BCE	0.27 ± 0.01 c

There is no statistical difference between same letters given in each column (DMRT, $P < 0.05$).

and roots.

Cd contamination had a deleterious impact on leaf fresh, leaf dry, root fresh, and root dry weights. However, CTS treatments mitigated the negative effects of Cd on the plant. These data show that, in the absence of Cd contamination, leaf fresh weight and dry weight dropped by 6 % and 2.9 %, respectively by applying 1 mg L^{-1} CTS, while root fresh weight and dry weight did not change when 1 mg L^{-1} CTS was sprayed, in comparison to the control (0 mg L^{-1} CTS). CTS treatment at a dosage of 1 mg L^{-1} enhanced fresh and dry weights of leaves by 19.01 % and 28.52 %, as well as fresh and dry weights of roots by 17.53 % and 35.22 %, respectively, in the plants grown under 200 mg kg^{-1} Cd contamination (Table 1).

Table 2 shows the effect of CTS treatments on the stem diameter, plant height, leaf area, CRV, and LRWC of the pepper grown under Cd treatment. In this respect, Cd treatment had a significant negative impact on the stem diameter, plant height, leaf area, CRV, and LRWC in the pepper, and the highest adverse effect was observed under 200 mg kg^{-1} Cd treatment. However, in the present of 200 mg kg^{-1} Cd, CTS spraying at a dosage of 1 mg L^{-1} raised the stem diameter, plant height, leaf area, CRV, and LRWC by 51.71 %, 20.52 %, 38.34 %, 47.69 %, and 54.61 % respectively, in comparison to 200 mg kg^{-1} Cd treatment (Table 2).

3.2. Cadmium content in roots and leaves of pepper seedlings

In this study, it was found that Cd content in leaves and roots of pepper seedlings was increased significantly under Cd pollution, but a decrease was realized with CTS application (Fig. 1a and b). The highest uptake of Cd was observed in the plants grown under 200 mg kg^{-1} of Cd relative to the control. In plants without CTS application, leaf Cd content increased approximately 7 thousand times and root Cd content increased approximately 5 thousand times with the highest dose Cd application (200 mg kg^{-1}) compared to the control (0 mg kg^{-1}). However, CTS (1 mg L^{-1}) application significantly decreased Cd uptake by 48.41 % and 54.30 % in leaves (Fig. 1a) and roots (Fig. 1b), respectively relative to the plants grown under 200 mg kg^{-1} of Cd.

3.3. Enzymes activities, MDA, H_2O_2, and proline contents of pepper seedlings

The change in enzyme activities after CTS (1 mg L^{-1}) applications to pepper grown under Cd (200 mg kg^{-1}) stress is shown in Table 3. As presented in Table 3, with rising Cd contamination, an improvement in CAT, POD, and SOD activity was seen in pepper seedlings. Under 200 mg kg^{-1} Cd stress, a 1 mg L^{-1} CTS treatment reduced CAT, SOD, and POD activity by 52 %, 53 %, and 37 %, respectively, relative to samples that did not receive CTS treatment. Whereas the maximum H_2O_2 and MDA content were gained under 200 mg kg^{-1} Cd stress, CTS application at a rate of 1 mg L^{-1} effectively ameliorated the detrimental impacts of Cd stress through reducing H_2O_2 and MDA content in the samples treated with 200 mg kg^{-1} Cd pollution by 41 % and 42 %, respectively.

As presented in Table 3, Cd pollution significantly increased proline content in the leaves of pepper seedlings. In this respect 100, 150 and 200 mg kg^{-1} of Cd caused an increase of 0.87, 3.00, and 4.35 fold in proline content in comparison to the untreated ones. However, CTS application decreased the proline content compared to the plants treated with Cd and without CTS. Additionally, solely application of CTS resulted in a drop in proline value relative to samples that did not receive Cd and CTS.

3.4. Hormones content of pepper seedling

According to the data obtained (Fig. 2), Cd pollution significantly decreased the GA (Fig. 2a, SA (Fig. 2b), and IAA (Fig. 2d) content in the pepper seedlings compared to the control, while it caused a raise in ABA (Fig. 2c). These decreases in GA, SA and IAA contents were 8 %, 22 % and 19 % at 100 mg kg^{-1}, 27 %, 41 % and 37 % at 150 mg kg^{-1}, and 38 %, 49 % and 37 % at 200 mg kg^{-1}, respectively. ABA content increased by 22 %, 46 % and 70 % in Cd applications, respectively. However, the CTS application increased the GA, SA, and IAA content, and decreased ABA content relative to the seedlings grown under Cd stress.

Nonetheless, an increase in the amount of GA (38 %), SA (37 %), and IAA (87 %) and a decrease in the amount of ABA by 34 % were

Table 2
Effect of cadmium (Cd) and chitosan (CTS) applications on stem diameter, plant height, leaf area, CRV and LRWC of pepper seedling (±SD).

Cd (mg kg^{-1})	CTS (mg L^{-1})	Stem diameter (mm)	Plant height (cm)	Leaf area (cm^2 $plant^{-1}$)	CRV (SPAD)	LRWC (%)
0	0	5.71 ± 0.08 h	29.92 ± 0.51 f	30.42 ± 0.49 f	56.40 ± 0.78 d	70.81 ± 1.41 g
	0.5	5.83 ± 0.08 h	31.42 ± 0.51 g	30.83 ± 2.09 f	54.77 ± 1.11 d	71.81 ± 1.76 g
	1.0	5.89 ± 0.08 h	31.00 ± 0.17 fg	31.50 ± 0.10 f	53.00 ± 0.72 d	71.33 ± 1.66 g
100	0	3.53 ± 0.03 b	15.94 ± 0.17 BCE	16.83 ± 0.24 BCE	44.02 ± 0.49 c	53.08 ± 1.27 c
	0.5	3.76 ± 0.02 c	18.22 ± 0.15 e	19.63 ± 0.38 de	46.88 ± 1.04 c	62.50 ± 1.05 ef
	1.0	4.57 ± 0.03 ef	18.29 ± 0.29 e	20.37 ± 0.64 e	45.87 ± 1.93 c	65.87 ± 0.64 f
150	0	3.43 ± 0.05 ab	15.01 ± 0.13 ab	14.97 ± 0.26 ab	33.80 ± 0.35 b	46.27 ± 1.11 b
	0.5	4.50 ± 0.04 de	17.74 ± 0.33 de	16.83 ± 0.43 BCE	44.90 ± 1.03 c	59.65 ± 1.70 de
	1.0	4.75 ± 0.06 f	18.14 ± 0.22 e	17.53 ± 0.64 cd	46.83 ± 2.44 c	61.00 ± 0.62 e
200	0	3.29 ± 0.03 a	14.32 ± 0.12 a	13.47 ± 0.24 a	29.87 ± 1.58 a	36.87 ± 0.95 a
	0.5	4.32 ± 0.16 d	16.57 ± 0.22 cd	17.17 ± 0.12 BCE	45.17 ± 0.38 c	54.80 ± 1.07 c
	1.0	4.97 ± 0.11 g	17.19 ± 0.23 cde	18.63 ± 0.54cde	44.20 ± 1.39 c	56.93 ± 1.41 cd

There is no statistical difference between same letters given in each column (DMRT, $P < 0.05$).

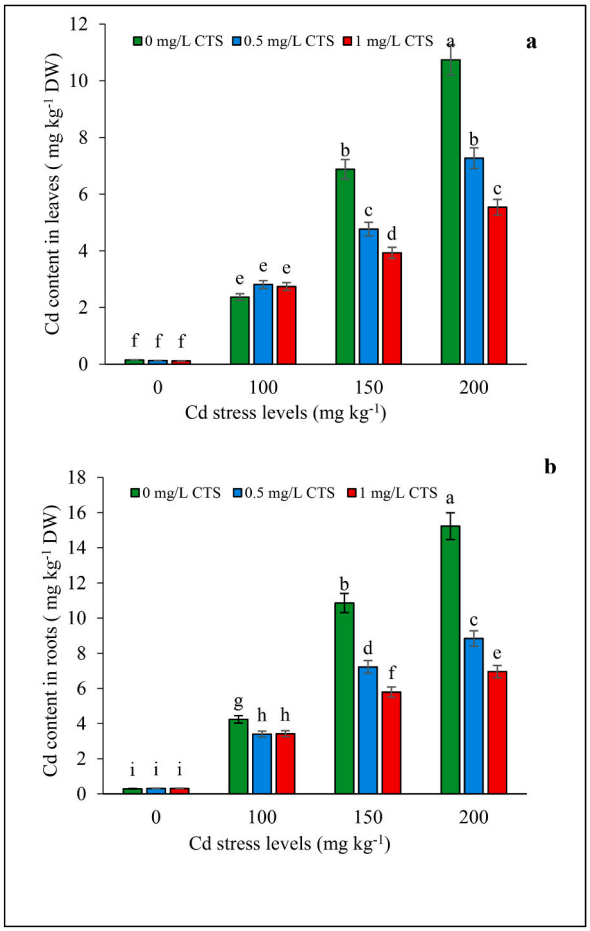

Fig. 1. The effect of chitosan (CTS) and cadmium (Cd) treatment on Cd content in leaves (a) and roots (b) of pepper seedlings. Different letters on top bars indicate differences (DMRT, $p < 0.05$ at each treatment); Vertical bars indicate the mean ± standard deviation for three biological replicates.

determined by the application of 1 mg kg^{-1} of CTS in the plants grown without Cd pollution (Fig. 2a, b, 2c and 2d).

3.5. Amino acids profile of pepper seedling

In this study, it was determined that Cd pollution decreased the accumulation (20–30 %) for all amino acids tested in pepper leaves, but the accumulation could be improved (max. 128 % increase) with chitosan application (Table 4). In this respect, Cd treatment (200 mg kg^{-1}) significantly increased the aspartate, glutamate, asparagine, histidine, and phenylalanine content in the leaves of pepper seedlings in comparison to the control. These increases occurred as 26 %, 18 %, 30 %, 27 % and 19 % at the highest Cd level (200 mg kg^{-1}), respectively. In addition, the CTS application increased their content again in the pepper plants grown under Cd stress (200 mg kg^{-1}). Under normal conditions, CTS application also increased threonine, arginine, alanine, tyrosine, cystine, valine, methionine, aspartate, asparagine, serine, glutamine, histidine, glycine, tryptophan, phenylalanine, isoleucine, leucine, lysine, hydroxyproline and sarcosin contents.

4. Discussion

Heavy metal pools have formed recently as a result of the excessive application of untreated wastewater and industrial effluents to agricultural lands. Cd stress has been proven to affect nutrient absorption, plant growth and development, and photosynthesis, as well as to increase oxidative stress by increasing ROS formation, which causes lipid peroxidation and modifies the antioxidative defense system [24,18]. In this experiment, we looked into how using CTS as a metal ions chelator may affect the physio-biochemical characteristics, oxidative stress, and plant performance as well as the uptake of mineral elements and Cd in various organs of the pepper.

Table 3
Effect of cadmium (Cd) and chitosan (CTS) applications on antioxidant enzyme activities and H_2O_2, MDA and proline content of pepper seedling (±SD).

Cd (mg kg^{-1})	CTS (mg L^{-1})	H_2O_2 (mmol kg^{-1})	MDA (nmol g^{-1} DW)	CAT (EU g $leaf^{-1}$)	POD (EU g $leaf^{-1}$)	SOD (EU g $leaf^{-1}$)	Prolin (µg g^{-1} FW)
0	0	12.97 ± 0.27 c	6.90 ± 0.44 a	138.69 ± 2.02 b	6161.67 ± 282.32 ab	525.69 ± 22.79 c	83.49 ± 9.92 cde
	0.5	10.37 ± 0.34 b	5.92 ± 0.22 a	128.19 ± 1.75 b	5110.67 ± 56.88 a	436.22 ± 10.19 b	64.43 ± 4.90 abc
	1.0	8.45 ± 0.24 a	5.44 ± 0.28 a	105.19 ± 2.81 a	4901.67 ± 59.68 a	365.64 ± 5.72 a	47.86 ± 3.95 ab
100	0	18.54 ± 0.47 d	9.20 ± 0.26 a	182.55 ± 8.51 c	9803.00 ± 64.78 ab	796.44 ± 7.03 e	156.66 ± 5.82 g
	0.5	14.07 ± 0.25 c	6.72 ± 0.31 a	131.49 ± 2.32 b	8652.33 ± 20611.68 ab	550.27 ± 11.75 c	92.73 ± 5.09 de
	1.0	11.24 ± 0.27 b	5.80 ± 0.32 a	94.21 ± 2.34 a	5182.67 ± 105.10 a	433.84 ± 32.02 b	45.98 ± 2.34 a
150	0	20.97 ± 0.78 g	12.23 ± 1.16 a	258.29 ± 14.21 g	45857.00 ± 35442.12 b	862.85 ± 8.84 f	334.65 ± 17.55 i
	0.5	16.34 ± 0.28 d	11.36 ± 0.26 a	188.70 ± 2.37 c	8951.00 ± 253.80 ab	693.07 ± 25.07 d	123.51 ± 3.33 f
	1.0	13.49 ± 0.48 c	6.73 ± 0.09 a	132.91 ± 4.35 b	6222.67 ± 112.41 ab	425.65 ± 3.08 b	72.69 ± 11.23 bcd
200	0	24.81 ± 1.04 g	14.70 ± 0.54 a	291.31 ± 3.19 f	12144.00 ± 569.78 ab	997.46 ± 34.42 g	446.80 ± 8.13 j
	0.5	20.45 ± 0.60 g	43.93 ± 425.04 b	214.42 ± 4.74 d	9228.33 ± 76.78 ab	753.82 ± 12.53 e	212.51 ± 3.42 h
	1.0	14.46 ± 0.69 e	8.42 ± 0.27 a	138.75 ± 1.93 b	7547.67 ± 453.37 ab	468.83 ± 9.38 b	104.94 ± 7.87 ef

There is no statistical difference between same letters given in each column (DMRT, $P < 0.05$).

4.1. CTS effect on the growth, LRWC, and CRV of pepper grown under Cd stress conditions

As presented in Tables 1 and 2, the fresh and dry weight of the plant and LRWC and CRV values significantly decreased by 200 mg kg^{-1} of Cd. It can be related to the toxicity effects of Cd accumulation on cell division, and oxidative stress, all of which may impede plant growth. However, we identified that CTS application (1 mg L^{-1}) greatly decreased the hazard effect of Cd on plant growth, LRWC, and CRV in pepper. This can be related to the CTS's role in reducing the Cd uptake from the soil and transporting it to the shoots. These findings are consistent with those of Moenne and González [25], who found that CTS treatment minimized heavy abundance and distribution in plants by complexing heavy metals with surface functional groups. Another possible mechanism of CTS can be related to the respective functions of CTS such as the induction of various protective genes in plants, including glucanase and chitinase [26].

4.2. Antioxidants, proline, MDA, and H_2O_2

SOD converts oxygen radicals to hydrogen peroxide as the first line of defense against oxidative stress. However, the byproduct of SOD activity (hydrogen peroxide) remains hazardous and must be removed in subsequent reactions by CAT and POD. The balance between the activities of SOD and CAT is required for determining the stable level of hydrogen peroxide and oxygen radicals [27]. In this experiment, Cd stress raised the SOD, POD, and CAT activities, and proline content in leaves of pepper significantly, which were accompanied by a raise in hydrogen peroxide and MDA content. However, the CTS application decreased proline content and the activities of SOD, POD, and CAT. This can be related to the direct role of CTS on heavy metal uptake. In this study, CTS treatment by decreasing Cd uptake from the growth medium and inhibited the overaccumulation of Cd in leaves, as well as mitigated the adverse effect of Cd stress in plant cells. In contrast to our results, It was shown that Cd stress decreased the activities of SOD, POD, and CAT in Cucurbita pepo but the application of CTS raised their activities [12]. As a result, responses to Cd and CTS treatment may differ between species and genotypes.

4.3. Hormones and amino acids

The role of plant phytohormones in the responses to trace metal elements in various plant genotypes has been investigated. In this respect, Guo et al. [28] reported an enhancement in ABA, GA and IAA in wheat cultivars under Cd treatment (3 mg kg^{-1}). Besides, Jan et al. [29] found an increase in ABA content in the epidermis and exodermis in the roots of *Kandelia obovate* during Cd treatment (110 µM Cd^{2+}). Contrastingly, Hu et al. [30] repoted a reduction in IAA concentration in model and grown species in reaction to Cd-induced stress. In barley root tips, short-term cadmium exposure disturbed IAA homeostasis [31]. In this experiment, Cd stress significantly increased ABA content and decreased the GA, IAA, and SA content in the leaves of pepper (Fig. 2). However, the application of CTS mitigated the adverse effect of Cd on plant growth and raised the content of GA, IAA, and SA, as well as decreased the ABA content significantly.

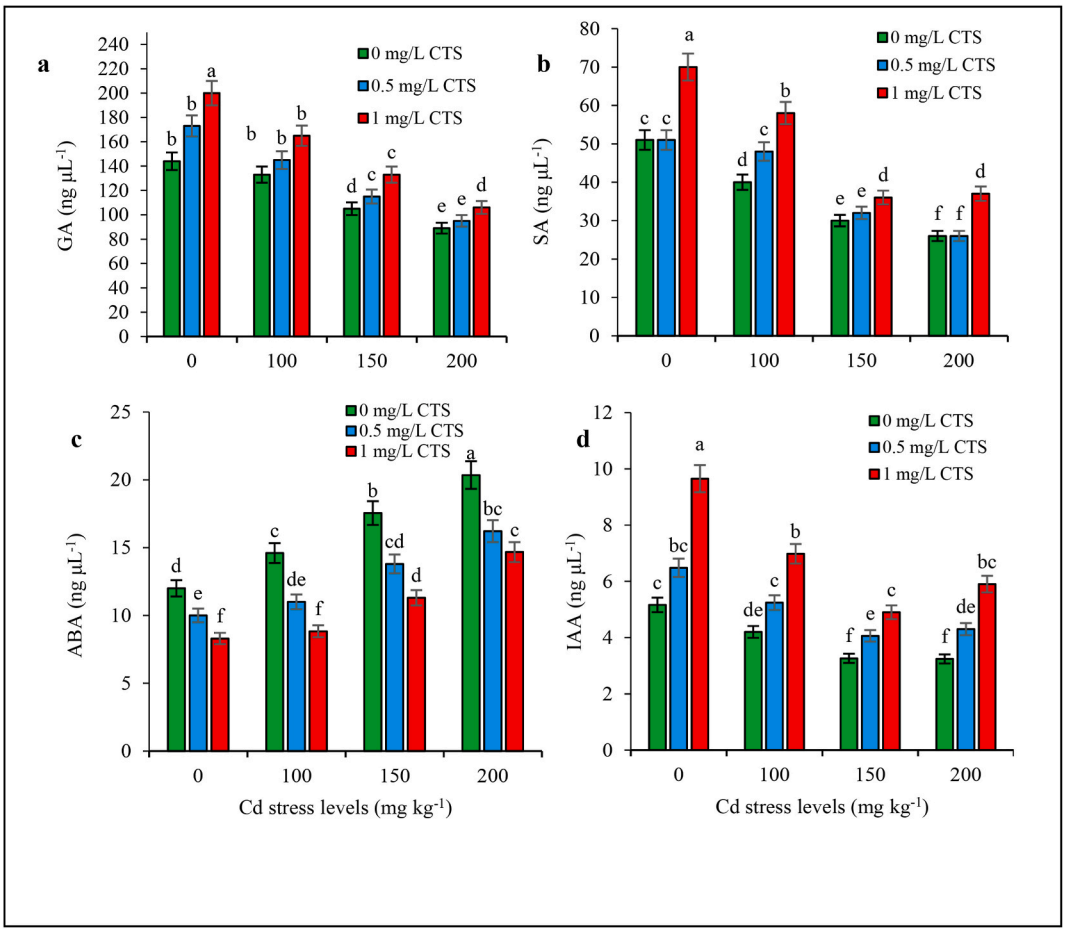

Fig. 2. The effect of chitosan (CTS) and cadmium (Cd) treatment on hormones content in leaves of pepper seedlings. Different letters on top bars indicate differences (DMRT, $p < 0.05$ at each treatment); Vertical bars indicate the mean ± standard deviation for three biological replicates.

During Cd stress, raising ABA concentration is a useful method for plants to continue photosynthesis and acclimatize to adverse situations. Under Cd pollution, plants can limit water loss by raising ABA concentration and shutting leaf stomata, hence minimizing cadmium stress harm to the plants [13,32]. Also, it was demonstrated that IAA and SA raised Fv/Fm and Pn, and GA boosted the activity of important photosynthetic enzymes [12,23]. According the results of this study, it can be concluded that CTS application improved pepper seedlings' tolerance to cadmium stress by regulating endogenous hormones such as SA, GA, IAA, and ABA content (Fig. 2). Therefore, it can be concluded that chitosan (CTS) modulates the biosynthesis and degradation pathways of key plant hormones, thereby influencing their levels to promote growth and stress tolerance. Specifically, CTS may upregulate the expression of genes involved in GA biosynthesis while downregulating genes responsible for its degradation, resulting in elevated GA levels. Similarly, CTS enhances SA biosynthesis by activating key enzymes such as phenylalanine ammonia-lyase (PAL) [33].

Cadmium pollution can disturb amino acid metabolism in plants, and variations in amino acid levels may have also a substantial impact on how plants respond to Cd stress [10,34]. Besides, the homeostasis of amino acids is crucial for plants to grow, develop, and protect themselves from environmental stress. De novo biosynthesis, absorption and translocation, and protein synthesis and degradation all play a role in maintaining homeostasis [11]. Furthermore, it was discovered that Cd can cause an increase in asparagine, methionine, and lysine levels in lettuce [35]. It was demonstrated that asparagine, glutamine, and branched-chain amino acids (valine, isoleucine, phenylalanine, and tryptophane) were considerably increased in the roots of tomatoes under Cd pollution [36]. In this study, Cd treatment (200 mg kg^{-1}) significantly increased the aspartate, glutamate, asparagine, histidine, and phenylalanine content in the leaves of pepper seedlings compared to the control. In addition, the CTS application boosted their content again in the pepper plants grown under Cd stress (200 mg kg^{-1}). This indicates that pepper plants responded to Cd stress by an increase in the aforementioned amino acids, and CTS application also played a great role in boosting their content. The main role of CTS in mitigating the adverse effect of Cd stress can be related to its impact on the glutamate, asparagine, and histidine contents, because they play a major function in nitrogen transport in plants. Additionally, these amino acids are utilized to store nitrogen at times when it is available for usage later on in growth, defense, and reproductive functions [37]. In contrast with our findings, it was identified that a higher amount of these amino acids was found in *A. halleri* and *N. caerulescens* for higher stress adaptation under Cd pollution [38,39]. As a

Table
4Effect of cadmium and CTS applications on amino acid content (pmol μL^{-1}) of pepper seedling (±SD).

Cd (mg kg^{-1})	CTS (mg L^{-1})	Aspartate	Glutamate	Asparagine	Serine	Glutamine	Histidine	Glycine
0	0	475.43 ± 11.44 a	466.63 ± 31.11 ab	766.87 ± 13.86 a	1097.40 ± 32.42 a	805.61 ± 34.22 a	254.13 ± 10.69 a	234.25 ± 9.71 a
	0.5	551.60 ± 8.64 BCE	397.98 ± 11.08 a	1331.29 ± 52.24 c	1490.95 ± 99.80 BCE	767.75 ± 35.25 a	325.41 ± 12.44 b	378.93 ± 15.73 b
	1.0	688.97 ± 8.42 f	539.45 ± 25.23 bcd	1719.81 ± 55.13 e	1991.94 ± 94.86 e	1050.76 ± 59.06 b	329.71 ± 10.36 b	585.11 ± 24.16 c
100	0	497.39 ± 11.97 a	471.01 ± 31.40 abc	797.26 ± 14.41 a	1091.61 ± 32.25 a	812.73 ± 34.53 a	261.85 ± 11.02 a	236.32 ± 9.79 a
	0.5	577.09 ± 9.03 cd	401.71 ± 11.18 a	1384.05 ± 54.31 cd	1483.09 ± 99.28 BCE	774.54 ± 35.56 a	335.29 ± 12.81 b	382.28 ± 15.87 b
	1.0	720.80 ± 8.81 f	544.51 ± 25.46 bcd	1787.97 ± 57.31 ef	1981.44 ± 94.36 e	1060.05 ± 59.58 b	339.71 ± 10.67 b	590.29 ± 24.37 c
150	0	531.67 ± 12.79 b	497.17 ± 33.15 bcd	859.28 ± 15.53 ab	1125.57 ± 33.25 a	837.72 ± 35.59 a	279.70 ± 11.77 a	243.58 ± 10.10 a
	0.5	616.86 ± 9.66 e	502.55 ± 13.99 bcd	1517.58 ± 54.51 d	1529.22 ± 102.37 c	798.35 ± 36.66 a	358.14 ± 13.69 b	394.04 ± 16.36 b
	1.0	770.48 ± 9.42 g	574.75 ± 26.88 d	1927.06 ± 61.77 f	2043.07 ± 97.30 de	1092.64 ± 61.41 b	362.87 ± 11.40 b	608.43 ± 25.12 cd
200	0	598.45 ± 14.40 de	548.99 ± 36.60 cd	1000.20 ± 18.08 b	1252.70 ± 37.01 ab	909.25 ± 38.63 a	322.66 ± 13.58 b	264.38 ± 10.96 a
	0.5	694.33 ± 10.87 f	554.93± 15.45 d	1766.45 ± 63.45 ef	1701.95 ± 113.93 c	866.52 ± 39.79 a	413.15 ± 15.79 c	427.68 ± 17.75 b
	1.0	867.24 ± 10.60 h	670.59 ± 16.89 e	2285.65 ± 113.93 g	2273.84 ± 108.29 e	1185.93 ± 66.65 b	418.61 ± 13.15 c	660.39 ± 27.27 d

Cd (mg kg^{-1})	CTS (mg L^{-1})	Threonine	Arginine	Alanine	Tyrosine	Cysteine	Valine	Methionine
0	0	634.68 ± 23.71 a	856.50 ± 44.68 a	704.57 ± 36.04 a	116.05 ± 4.31 a	83.36 ± 2.04 a	46.22 ± 0.55 d	174.39 ± 7.83 a
	0.5	765.15 ± 63.42 ab	1247.87 ± 79.06 BCE	804.65 ± 17.77 ab	144.29 ± 5.32 b	136.61 ± 5.30 b	100.57 ± 7.83 BCE	252.57 ± 11.41 BCE
	1.0	909.98 ± 85.20 BCE	1445.86 ± 89.76 cd	1064.64 ± 73.16 c	180.58 ± 6.73 de	239.06 ± 7.61 d	125.89 ± 10.63 a	278.08 ± 19.90 cde
100	0	631.34 ± 23.58 a	882.49 ± 46.03 a	710.80 ± 36.36 a	115.44 ± 4.29 a	82.92 ± 2.03 a	45.97 ± 0.54 cd	180.82 ± 8.12 a
	0.5	761.12 ± 63.08 ab	1285.74 ± 81.46 BCE	811.77 ± 17.93 BCE	143.53 ± 5.30 b	135.89 ± 5.27 b	100.04 ± 7.79 b	261.89 ± 11.83 bcd
	1.0	905.18 ± 84.75 BCE	1489.74 ± 92.49 cd	1074.05 ± 73.80 cd	179.63 ± 6.69 de	237.80 ± 7.57 d	125.23 ± 10.58 a	288.33 ± 20.63 cde
150	0	650.97 ± 24.31 a	942.65 ± 49.17 a	732.65 ± 37.47 a	119.03 ± 4.42 a	85.50 ± 2.09 a	47.40 ± 0.56 bcd	191.56 ± 8.60 a
	0.5	784.79 ± 65.05 ab	1373.39 ± 87.01 cd	836.72 ± 18.48 ab	148.00 ± 5.46 BCE	140.11 ± 5.44 BCE	103.16 ± 8.03 b	277.43 ± 12.54 cde
	1.0	933.34 ± 87.39 BCE	1591.30 ± 98.79 d	1107.07 ± 76.07 c	185.22 ± 6.90 e	245.20 ± 7.80 d	129.12 ± 10.91 a	305.45 ± 21.86 de
200	0	724.50 ± 27.06 ab	1087.44 ± 56.72 ab	795.21 ± 40.67 ab	132.47 ± 4.92 ab	95.16 ± 2.33 a	52.76 ± 0.62 bcd	221.64 ± 6.74 ab
	0.5	873.44 ± 72.39 BCE	1584.34 ± 100.38 d	908.17 ± 20.06 b	164.71 ± 6.08 cd	155.94 ± 6.05 c	114.81 ± 8.93 b	321.02 ± 10.09 ef
	1.0	1038.76 ± 97.26 c	1835.72 ± 113.97 e	1201.60 ± 82.57 c	206.14 ± 7.68 f	272.90 ± 8.68 e	143.71 ± 12.14 a	360.52 ± 25.80 f

Cd (mg kg^{-1})	CTS (mg L^{-1})	Tryptophan	Phenylalanine	Isoleucine	Leucine	Lysine	Hydroxyproline	Sarcosine
0	0	108.46 ± 2.98 a	270.10 ± 7.74 ab	150.70 ± 3.94 a	193.04 ± 5.97 a	200.11 ± 5.54 a	102.12 ± 6.30 a	337.73 ± 10.97 a
	0.5	170.16 ± 5.22 b	263.34 ± 16.43 a	193.39 ± 10.80 BCE	362.59 ± 19.87 b	482.08 ± 19.24 b	329.44 ± 16.29 c	607.24 ± 34.78 b
	1.0	249.51 ± 9.34 d	401.12 ± 18.19 c	237.49 ± 12.93 d	373.20 ± 22.18 b	667.46 ± 26.13 d	194.63 ± 11.58 b	766.55 ± 41.06 c
100	0	109.47 ± 3.01 a	270.77 ± 7.75 ab	149.90 ± 3.92 a	200.16 ± 6.19 a	200.61 ± 5.56 a	103.02 ± 6.36 a	335.95 ± 10.91 a

(*continued on next page*)

Table (*continued*)

Cd (mg kg^{-1})	CTS (mg L^{-1})	Tryptophan	Phenylalanine	Isoleucine	Leucine	Lysine	Hydroxyproline	Sarcosine
	0.5	171.76 ± 5.27 b	263.99 ± 16.47 a	192.37 ± 10.74 BCE	375.96 ± 20.60 b	483.28 ± 19.29 b	332.36 ± 16.43 c	604.04 ± 34.59 b
	1.0	251.85 ± 9.43 d	402.13 ± 18.24 c	236.23 ± 12.86 d	386.95 ± 22.99 b	669.13 ± 26.19 d	196.35 ± 11.68 b	762.50 ± 40.85 c
150	0	115.55 ± 3.17 a	281.42 ± 8.06 ab	154.56 ± 4.05 a	212.04 ± 6.56 a	208.50 ± 5.78 a	106.19 ± 6.55 a	346.40 ± 11.25 a
	0.5	181.30 ± 5.57 b	274.37 ± 17.12 ab	198.36 ± 11.08 BCE	398.28 ± 21.82 b	502.28 ± 20.05 b	342.57 ± 16.94 c	622.83 ± 35.67 b
	1.0	265.84 ± 9.95 d	417.93 ± 18.96 c	243.58 ± 13.26 d	409.93 ± 24.36 BCE	695.43 ± 27.22 d	202.38 ± 12.04 b	786.22 ± 42.12 c
200	0	130.36 ± 4.61 a	320.88 ± 11.52 b	175.75 ± 6.16 ab	245.43 ± 3.65 a	237.57 ± 5.57 a	119.81 ± 8.49 a	393.89 ± 15.77 a
	0.5	204.46 ± 6.91 c	312.44 ± 17.26 ab	225.64 ± 14.36 cd	460.70 ± 18.46 cd	572.76 ± 26.47 c	386.67 ± 24.32 d	708.74 ± 47.45 BCE
	1.0	302.90 ± 11.34 e	479.95 ± 21.77 d	279.73 ± 15.23 e	483.84 ± 28.75 d	798.64 ± 31.26 e	232.42 ± 13.83 b	902.91 ± 48.37 d

There is no statistical difference between same letters given in each column (DMRT, $P < 0.05$).

consequence, increasing the amino acid contents in pepper seedlings by CTS application has a great role in increasing the pepper plant tolerance under Cd contamination.

5. Conclusions

According to the results of this study, CTS boosted plant growth, chlorophyll value, enzyme activity, and dry matter accumulation of pepper via boosting the amino acid content in leaves of pepper, which is favorable for dry matter accumulation and transport. Under Cd pollution, CTS alleviated the Cd toxicity by reducing Cd uptake and regulating endogenous hormone content, as well as, amino acid contents. In order to boost pepper seedling susceptibility to Cd stress and increase the sustainability of pepper output in cadmium-polluted environments, we suggest that a particular dosage of CTS may be used as a high-efficiency stimulator. For an effective amendment, the effects of the applications on the physical and chemical properties of the soil and the Cd behavior in different environmental conditions should also be determined. In addition, long-term field trials are necessary to detail the benefits and risks of the applied amendment.

Data availability statement

Not applicable.

CRediT authorship contribution statement

Melek Ekinci: Investigation. **Mostafakamal Shams:** Formal analysis, Data curation. **Metin Turan:** Resources, Methodology. **Sumeyra Ucar:** Writing – review & editing, Software. **Esra Yaprak:** Software, Methodology. **Esra Arslan Yuksel:** Writing – original draft, Validation, Methodology. **Murat Aydin:** Writing – review & editing, Software. **Emre Ilhan:** Writing – review & editing, Software. **Guleray Agar:** Software, Methodology. **Sezai Ercisli:** Writing – review & editing. **Ertan Yildirim:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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