scientific reports

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Mitigation of adverse effect of cadmium toxicity in lettuce (*Lactuca sativa* L.) through foliar application of chitosan and spermidine

Maham Rafique[1], Zahra Noreen[1]✉, Sheeraz Usman[1]✉, Anis Ali Shah[1]✉, Hafsa Taj[1], Mohamed A. El-Sheikh[2] & In Jung Lee[3]✉

Cadmium (Cd) stress is considered among the most harmful abiotic stresses because of its toxicity and ability to alter the ultrastructure of plants. Lettuce (*Lactuca sativa* L.) can readily accumulate Cd from the soil, but its elevated level posed negative effect on their development and nutritional quality. In this study, efficacy of chitosan and spermidine synergistic application was evaluated to improve Cd metal tolerance or its exclusion in lettuce. A pot experiment was conducted in a four-way completely randomized design (CRD) with 3 replicates, using two *L. sativa* varieties (VRIL-0205 and Green Check). Following treatments, Cd stress (10 ppm $CdCl_2$), chitosan (200 ppm) and spermidine (145 ppm) were applied along with their respective controls. The negative effects of Cd stress on the morphological, physiological, and biochemical attributes of both *L. sativa* varieties were evaluated along with counter effect of chitosan and spermidine alone and synergistic application. Cd stress resulted in significant accumulation of Cd^{2+} ions in the shoot of both varieties (0.038 mg kg^{-1} in VRIL-0205 and 0.041 mg kg^{-1} in Green Check). It also impaired growth, biomass, gas exchange, water relation, antioxidant activities and nutrient uptake in both varieties. Foliar application of both chitosan and spermidine improved growth, biomass, chlorophyll content, photosynthesis rate, stomatal conductance, water content, antioxidant activities and nutrient uptake in both control and stressed plants. Their combined treatment reduced stress indicators including relative membrane permeability (VRIL-0205; 19% and Green Check; 22%), H_2O_2 (VRIL-0205; 27% and Green Check; 26%) and malondialdehyde content (VRIL-0205; 6% and Green Check; 7%) in stressed plants, compared with stress only plants. These findings showed that chitosan and spermidine synergistic application effectively mitigated the Cd toxicity in both *L. sativa* varieties and improved their growth under stress condition. This study provides insight into the potential use of chitosan and spermidine foliar spray as sustainable tools for improving Cd resilience in crop plants.

Keywords Abiotic stress, Antioxidants, Heavy metals, Nutrient imbalance, Stress resilience, Sustainable tool

Currently, one of the foremost challenges faced by the modern industrial world is land and water pollution due to heavy metals. Owing to their toxic nature, HMs are significant contributors to the production of biosphere pollution, leading to increased serious environmental threats. This pollution has many harmful effects on living organisms, ranging from plants to animals[1]. Heavy metal (HM) stress is widely recognized as one of the most detrimental abiotic stresses, causing toxicity by targeting essential molecules and processes within plant cells[2]. Cadmium (Cd) ranks among the most hazardous metals to living organisms[3]. It has exceptional persistence in soil and is nonbiodegradable, posing a prolonged risk to both plants and humans[4]. Cadmium is a toxic heavy metal that is nonessential, highly soluble in water, and mobile. This facilitates its uptake by roots and transport to

[1]Department of Botany, Division of Science and Technology, University of Education, Lahore, Pakistan. [2]Botany and Microbiology Department, College of Science, King Saud University, Riyadh, Saudi Arabia. [3]College of Agriculture and Life Science, School of Applied Biosciences, Kyungpook National University, 80 Daehak-ro, Buk-gu, Daegu 41566, Korea. ✉email: zahranoreen@gmail.com; miansheerazusman@gmail.com; anisalibot@gmail.com; ijlee@knu.ac.kr

leaves through the xylem[5]. Cadmium poisoning leads to changes in the morphology, physiology, biochemistry, and ultrastructure of plants[6]. This results in inhibited growth of the root and shoot systems, as well as root tanning and leaf chlorosis[5]. Cadmium leads to the inhibition of plant growth, reduced productivity, compromised crop quality, and in severe cases, plant mortality[7]. Approximately 25,000 to 30,000 tons of cadmium are released into the Earth's ecosystem annually from different sources. Cadmium is a serious threat because of its mobility in soil and within plants. Therefore, comprehending plant cadmium intake levels and physiological responses to cadmium pollution is vital for conserving biodiversity and ensuring food safety[8].

The distribution and sources of Cd pollution vary significantly across different regions. In developed countries like Europe and North America, stringent regulations and effective pollution control measures have led to lower Cd concentrations in water bodies[9,10]. In contrast, developing regions such as Africa, Asia, and South America experience higher levels of Cd pollution due to less stringent environmental regulations and higher industrial and agricultural activities[9,10]. Water contaminated with Cd from industries can contaminate the soil, enter plants via their roots, and pose health risks. Vegetables grown in such areas irrigated with mixed industrial effluents take up Cd, which is harmful to both humans and plants.

Chitosan (CTS) is a polymer composed of β-1,4-linked 2-amino-2-deoxy-D-glucose units. It is derived from the degradation of chitin and is usually sourced from shellfish and crustacean waste. Chitosan is a natural biopolymer that deacetylates chitin and exhibits a variety of biological activities, such as antioxidant, antibacterial, and anticancer properties[11]. CTS effectively initiates plants' natural defense mechanisms, increases plant growth, and enhances the production of secondary metabolites[12]. CTS is extensively utilized in agricultural healthcare and medicine. It enhances cell growth, promotes plant cell health, enhances insect resistance, and initiates the production of enzymes that contribute to resistance against diseases[13]. Foliar treatment with CTS mitigated cadmium-induced toxicity in plants. Researchers have recently discovered that adding chitosan to soil treated with cadmium can modulate the distribution of cadmium within parts of plants while also increasing important plant characteristics[14].

Polyamines (PAs) are small aliphatic amines found in all plant cells and have been suggested as a new class of growth chemicals. They are linked with different biological processes in plants, such as the maturation of growth and ultimately the stress response[15]. Three primary PAs are naturally found in plants: spermidine (SPD), spermine (Spm), and their precursor putrescine (Put). Spermidine is one of the predominant natural forms of polyamine. Spermidine is a naturally occurring aliphatic amine molecule with an aliphatic nitrogen compound that functions as a hormone in plants[16]. Spermidine acts as a stress signaling regulator in mechanisms of stress tolerance and functions as a protective molecule during stressful conditions[17]. Spermidine, an endogenous chemical, modulates plant growth and supports resilience under drastic conditions[18]. Currently, investigations have revealed that the application of exogenous spermidine promotes plant resilience to stress induced by heavy metals across different plant species[19]. Additionally, researchers have shown that supplementation with exogenous spermidine might increase the accumulation of metals in plant tissues[20]. SPD can quickly chelate or deactivate metals within plants to stabilize and protect the membranes by preventing lipid peroxidation induced by metals[21]. The application of spermidine to plants under heavy metal stress can lead to increased metal deposition in plant tissues while also promoting plant tolerance[15].

Lettuce (*Lactuca sativa* L.) belongs to the Asteraceae family and is an herbaceous plant that can be either annual or biennial. This species originates from the Mediterranean areas of Europe[22]. Lettuce ranks as one of the most famous and economically significant leafy vegetable crops worldwide. Its leaves and stems possess various medicinal benefits, such as alleviating pain, reducing cholesterol, enhancing appetite, treating neurasthenia, and enhancing appetite and digestion[23]. Although lettuce is a low-calorie vegetable, it is rich in health-promoting substances, such as carotenoids, phenolic compounds, dietary fiber, essential minerals, and vitamins[24]. Lettuce has a significant tendency to absorb cadmium from the soil without showing visible signs of heavy metal toxicity, thereby leading to potential risks to human health[25]. As a result, various methods have been introduced to mitigate cadmium toxicity in lettuce plants.

Cadmium toxicity adversely impacts the development and nutritional quality of lettuce plants. Given the demonstrated efficacy of CTS and SPD in enhancing plant stress resilience it was hypothesized that their synergistic application would also alleviate Cd stress in *L. sativa*. Up to now, none of the previous studies have reported the synergistic application of chitosan and spermidine to improve lettuce resilience against Cd toxicity. Thus, this study presents a novel approach to use chitosan and spermidine as sustainable and ecofriendly tools for improving resilience against heavy metal stresses in crop plants. This study aimed to offer valuable insights into sustainable methods for increasing lettuce plant resilience against cadmium toxicity, with a focus on the synergistic effects of chitosan and spermidine. Therefore, the objectives of this study were to determine the effects of Cd toxicity on lettuce plants; to analyze the various growth, physiological, and biochemical characteristics of lettuce under the application of chitosan and spermidine; and to determine the effectiveness of chitosan and spermidine in the amelioration of Cd toxicity in lettuce plants.

Research methodology

Experimental design and plant material

This study was performed in a botanical garden at the Township Campus of the University of Education, Lahore, Pakistan. Seeds of two *L. sativa* varieties (VRIL-0205 and Green Check) were acquired from the Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. This study was conducted in a 4-way completely randomized design (CRD), with three replicates and 48 pots. The experiment was conducted in natural conditions with temperature range 24-33 °C during daytime and 9-22 °C overnight, 67% relative humidity and 22 mm rainfall during the whole experimental period. Initially, the sand was filtered through a perforated net. The sand was washed twice with distilled water. Pots (25 cm length and 20 cm diameter) were filled with prewashed sand. On 20 Oct. 2023, same sized healthy seeds (8–10) were sown at equal distance, in each pot. After germination, 5–6

seedlings were maintained in every pot with nearly equal distance from each other. Plants were watered every second day at start and then weekly at the end of experiment according to the changing weather conditions. To fulfil nutrients requirement, Hoagland's nutrient solution was used with irrigation water on regular intervals of one week. 21 days after germination seedling were subjected to different treatments. For Cd stress, $CdCl_2$ was mixed in Hoagland's solution at 10 ppm concentration (1 L for each pot), control plants were given Hoagland's solution only. This concentration was selected based on previous work done by Dawuda et al.[26] and Tang et al.[27]. On same day, after stress application, chitosan (200 ppm) and spermidine (145 ppm) solutions were prepared in 1% tween20 and foliar sprayed on plants using manual hand sprayer until plants get fully wet. Control plants were sprayed with the same amount of distilled water. These concentrations for chitosan and spermidine were partially selected based on previous work done by Ibrahim et al.[28] and Li et al.[29], respectively. These treatments were applied twice throughout the experiment with one weak interval. Two weeks after administration of prescribed treatments, growth, physio-biochemical attributes, nutrients uptake and Cd accumulation were assessed. Samples from every replicate were also stored at 4 °C for analyzing antioxidant activities and other biochemical parameters later.

Physiological attributes

Chlorophyll and carotenoid measurement

The chlorophyll and carotenoid content were calculated according to the protocol of Arnon[30] and Lichtenthaler[31]. Fresh, mature leaf samples from each replicate were weighed to 0.5 g and ground in 10 ml of 80% acetone. The samples were then filtered through Whatman filter paper and stored in a refrigerator at 4 °C for 24 h. The absorbance was measured at three specific wavelengths: 480 nm, 645 nm, and 663 nm using UV/VIS spectrophotometer (Shanghai Metash Instruments Co., Ltd.).

At LEAF chlorophyll measurement

A fully mature fresh lettuce leaf was placed in an atLEAF CHL BLUE (0131 – 58 Ver 1.3) chlorophyll meter, which displayed the chlorophyll value on the screen. Six readings were taken from each leaf (three on either side of the midrib) to calculate the average. On the measurement day, the weather was sunny, with a temperature of 10 °C and humidity of 96% in the morning. The at-LEAF meter uses a wavelength of 660 nm.

Chlorophyll fluorescence measurement

The variable fluorescence (Fv), maximal fluorescence (Fm), maximum quantum yield of PSII (Fv/Fm) and minimal fluorescence (Fo) were measured via an OS30p+ (Opti-sciences, Inc. | Hudson, NH 03051, USA) chlorophyll fluorometer. Fully matured leaf was placed between the device's clips to allow dark adaptation for 20 min before readings were taken. This procedure was conducted two weeks posttreatment, during the daytime in full sunlight.

Measurement of gas exchange parameters

The photosynthetic rate (P_n), stomatal conductance (g_s), transpiration rate (T_r), and intercellular CO_2 concentration (C_i) of the lettuce leaves were measured via an LCpro-SD (ADC Bio Scientific Ltd. Hoddesdons, UK) Infrared Gas Analyzer (IRGA). Ambient CO_2 concentration (C_{ref}) was 430 µmol mol^{-1}, the molar gas flow rate of the leaf chamber (U) was 200 µmol^{-1}, the leaf surface area was 6.25 cm^2, the temperature of the leaf chamber (T_{ch}) varied from 22 to 26 °C, the ambient pressure (P) was 997 kPa, and the photosynthetically active radiation (PAR/Q_{leaf}) at the leaf surface reached a maximum of 914 µmol m^{-2} s^{-1}.

Leaf osmotic potential

A fully expanded young leaf was taken from each replicate of the 48 pots and frozen at – 20 °C for 7 days. After freezing, the leaves were thawed and pressed with a glass rod to extract sap. This sap was directly used to quantify the leaf osmotic potential with an osmometer (Loser Messtechnik)[32].

Water potential measurement

A young leaf from each replicate was cut and immediately placed into the pressure chamber. Pressure was applied to the leaf until bubbles formed at the petiole, at this point the readings were recorded. The leaf water potential was measured via a pressure chamber instrument (PMS model 1000)[33].

Relative water content (RWC)

Leaves of similar size were collected from each replicate, weighed on a balance, and then quickly immersed in distilled water. The leaves were soaked at 25–26 °C for 3 h. After immersion, the turgid leaves were weighed. The samples were then dried in an oven at 75 °C for 24 h, after which their dry weights were recorded. The relative water content (RWC) of each sample was calculated via the provided formula[34].

$$RWC\,(\%) = [(\,FW - DW)/(TW - DW\,)] \times 100$$

Where, FW = fresh weight, DW = dry weight, and TW = turgid weight.

Biochemical attributes

Malondialdehyde (MDA)

The determination was performed following the mentioned protocol[35]. with some modifications. A 0.5 g fresh leaf sample from every replicate was weighed. The samples were then ground with 3 ml of 1.0% trichloroacetic acid with a mortar and pestle. The mixture was collected in conical flasks. The samples were subsequently

centrifuged at 20,000 rpm for 15 min at 4 °C in a centrifugation machine (Hermle Z 326 K). A total of 0.5 ml of the supernatant was separated in a test tube. Then, 0.5% thiobarbituric acid (TBA) was added to 20% trichloroacetic acid (TCA), and 3 ml of this mixture was added to 0.5 ml of the supernatant. The samples were then incubated in a water bath (Glas-Col model HSW-1/06) at 95 °C for 50 min. The reaction was terminated by cooling the samples in an ice water bath. The samples were again centrifuged at 10,000 rpm for 10 min, after which the absorbance at 532 nm and 600 nm was measured via a UV/VIS spectrophotometer. The MDA level (nmol) for each sample was determined via a given formula.

$$MDA\ level\ \ (nmol) = [(A532\ nm - A600\ nm)/1.56 \times 105] \times V/W \times 1,000,000$$

Hydrogen peroxide (H_2O_2) determination

This method was performed following the mentioned protocol[36]. Leaves (0.5 g) were freshly removed from each pot. Afterward, the leaves were ground with a prechilled mortar and pestle along with 0.1% trichloroacetic acid (5 ml). The sample extracts were subsequently transferred to falcon tubes. Centrifugation was performed at 12,000 rpm (15 min, 4 °C) using a centrifuge machine. The supernatant was separated and transferred to a test tube (0.5 ml) via a pipette. Then, 0.5 ml of potassium phosphate buffer (pH 7), potassium iodide (1 ml) and 0.5 ml of supernatant were mixed in the test tube. The mixture was then vortexed for a few seconds, and at 390 nm, the absorbance was measured via a UV/VIS spectrophotometer.

Relative membrane permeability (RMP)

First, equivalent-sized young leaves were collected from every replicate of 48 pots. The leaves were chopped with the help of a cutter and then added to test tubes containing distilled water (20 ml). After that, test tubes containing sample leaves were vortex for at least 5 s, and the electrical conductivity (EC_0) was determined via an EC meter (Milwaukee MW805 MAX). After that, the test tubes were kept in a refrigerator at 4 degrees Celsius for 24 h, and then, the EC_1 was noted. After measuring EC1, these samples were incubated in an autoclave at 121 °C for 20 min, after which EC_2 readings were taken. The percentage RMP was calculated via the formula provided by[37].

$$RMP\,(\%) = [(\,EC_1 - EC_0)/(EC_2 - EC_0\,)] \times 100$$

Total soluble protein

Fresh leaves were collected from each replicate, weighing 0.5 g each, and mixed in 10 ml of precooled 50 mM phosphate buffer (pH 7.8). The Falcon tubes containing the extracts were subsequently centrifuged at 6,000 rpm for 20 min at 4 °C. Upon centrifugation, the supernatant was carefully separated from the residue, which was then discarded. The supernatant was stored in a deep freezer for later use. Protein levels in the samples were determined following the provided protocol[38]. A Bradford mixture was prepared by dissolving 100 mg of Coomassie Brilliant Blue in 50 ml of 95% ethanol, after which this mixture was added to 100 ml of 85% phosphoric acid. These solutions were mixed and diluted with distilled water to achieve a total volume of one liter. For each sample, 5 ml of Bradford solution was taken, to which 0.1 ml of previously frozen leaf extract was added and thoroughly mixed via a vortex machine. The optical density was subsequently determined at a wavelength of 595 nm via a UV/VIS spectrophotometer. A standard curve for bovine serum was prepared by diluting a stock solution of bovine serum albumin to 100 mg, 200 mg, 300 mg, 400 mg or 500 mg from a stock solution of 7.5 mg/15 mL. Curves were used to identify proteins in unknown samples.

Catalase (CAT) activity

Catalase activity was assessed via the method outlined by[39] with some adjustments. To prepare the catalase reaction mixture, 3 mL was combined, consisting of 1 mL of 50 mM potassium phosphate buffer at pH 7, 1.9 mL of 5.9 mM H_2O_2, and 100 µL of enzyme extract previously prepared for total soluble proteins. The enzyme extract was introduced into the reaction mixture just before the cuvette was placed in the spectrophotometer. Absorbance readings at a wavelength of 240 nm were taken every 30 s within a 120-second timeframe.

Peroxidase (POD) activity

A 2 mL peroxidase reaction mixture was prepared by combining 700 µL of 50 mM potassium phosphate buffer adjusted to pH 5, 600 µL of guaiacol (20 mM), 600 µL of 40 mM H_2O_2, and 100 µL of enzyme extract. A spectrophotometer was used to measure the absorbance at 470 nm at 30-second intervals within a 150-second timeframe[40].

Ascorbate peroxidase (APX) activity

For APX, 3 ml of reaction mixture for each replicate was prepared, which included 50 mM phosphate buffer = 2.7 ml, 300 mM hydrogen peroxide (H_2O_2) = 0.1 ml, 7.5 mM ascorbic acid = 0.1 ml, and enzyme extract = 0.1 ml. The absorbance of the entire reaction mixture was measured at 290 nm for 30 s intervals within a 1-minute time frame via a UV/VIS spectrophotometer[41].

Superoxide dismutase (SOD) activity

For SOD, first, a reaction mixture comprising 0.3 ml of riboflavin (20 µL), 0.3 ml of methionine (130 mM), 0.3 ml of nitro blue tetrazolium (50 µM), and 0.3 ml of EDTA-Na2 (100 µM) was prepared. After that, 0.05 ml of the prepared enzyme mixture was added to all the mixtures. The mixture was then vortexed, and the samples were placed under 4000 lx light for 45 min. The color of the solution changed, after which the samples were

placed in the cuvette of a spectrophotometer one by one, and readings were taken with a 560 nm UV/VIS spectrophotometer[42,43].

Total phenolics

A 0.5 g dried leaf sample from every replicate was taken, ground and mixed in 80% acetone (10 ml) for 1 min. The samples were placed in conical flasks and centrifuged at 4000 rpm for 15 min at 4°C. Afterward, the supernatant was discarded, and the dried residue was retained for subsequent processing. This dried residue was then combined with 10 ml of methanol. The Folin–Ciocalteu protocol[44] was followed to determine the total phenolic content in these samples. Two millilitres from every replicate of these prepared samples were kept in test tubes. One milliliter of Folin-Ciocalteu reagent was combined with 0.8 ml of 7.5% sodium carbonate, mixed thoroughly, and allowed to stand for 30 min. The absorbance at 765 nm was measured via a UV/VIS spectrophotometer. The total phenolic content was calculated as gallic acid equivalents per gram of dry material.

Leaf proline

The proline content in the leaf samples was determined via the method outlined by[45]. Fresh leaf samples weighing 0.2 g each from 48 pots were ground in 5 mL of a 3% sulfo-salicylic acid solution. Whatman filter paper used to filter the mixture. To prepare acid ninhydrin, 2.5 g of ninhydrin was dissolved in a mixture of 40 mL of O-phosphoric acid and 60 mL of glacial acetic acid (6 M). Two milliliters of homogenate filtrate, 2 mL of glacial acetic acid, and 2 mL of acid ninhydrin were combined in a test tube. The test tubes in which the reaction mixture was present were kept in an incubator at 100 °C for 60 min. The reaction mixture in the test tubes was placed into an ice bath to cool, and the reaction was stopped after being incubated in an oven. After that, the reaction mixture was mixed with 4 mL of toluene and vortexed for one to two minutes. A chromophore containing toluene was separated from the aqueous phase. A spectrophotometer was used to measure the absorbance at 520 nm against a toluene blank.

Inorganic mineral ion measurement

First, 0.1 g of dried root and shoot samples were taken, ground with a mortar and pestle. The dried samples were transferred to test tubes, and 2 ml of H_2SO_4 was added for digestion via Wolf's method[46]. The samples were incubated for 24 h, after which they were heated on a hot plate, and H_2O_2 was added dropwise until the mixture was colorless. Next, distilled water was added to adjust the volume to 50 ml. The mixture was then filtered through Whatman filter paper. The readings for K^+ and Ca^{2+} were taken by using a flame photometer (Sherwood model 360).

Detection of Cd^{2+} and Fe^{2+}

The digested samples, which were prepared early for inorganic mineral ion measurement, were used. The concentrations of the heavy metals Cd^{2+} and Fe^{2+} in the prepared samples were determined via an atomic absorption spectrophotometer (Hitachi Polarized Zeeman AAS, Z-8200, Japan) following the conditions described in[47]. The Cd^{2+} conditions were as follows: wavelength, 228.8 nm; slit width, 1.3 nm; lamp current, 7.5 mA; oxidant gas pressure, 160 KPa; and fuel gas pressure, 6 KPa. The Fe^{2+} conditions were as follows: wavelength, 248.3 nm; slit width, 0.2 nm; lamp current, 10.0 mA; oxidant gas pressure, 160 KPa; and fuel gas pressure, 6 KPa.

Statistical analysis

ANOVA was applied to all the parameters via R software version 4.3.3. LSD mean comparison tests were conducted with a significance level of $p < 0.05$. Graphs were generated via MS Excel, data presented in the graphs is mean of three replicates ± standard errors. Bars sharing similar letters are not significantly different at $p < 0.05$. Pearson's correlation and principal component analysis were performed using R software at $p < 0.05$ confidence level.

Results

Effect of CTS and SPD foliar spray on growth attributes of Cd stressed *L. sativa*

Cd stress had negatively affected the growth of *L. sativa* plants. Shoot length was reduced by 21 and 20%, respectively, in VRIL-0205 and Green Check under 10 ppm Cd stress. Followed by 24, 22 and 26% reduction in root length, shoot fresh weight and shoot dry weight in VRIL-0205 and by 14, 22 and 23% in Green Check, respectively, compared to control plants. CTS and SPD treatments, alone or in combination, successfully retrieved the original status of the plants. These treatments improved growth attributes not only in control group but also in stressed plants. In stressed plants, maximum increase in growth attributes was observed with synergistic application compared to CTS and SPD alone treatment in both varieties (Supplementary Table S1 online).

Effect of CTS and SPD foliar spray on photosynthetic pigments and chlorophyll fluorescence attributes of Cd stressed *L. sativa*

Chlorophyll *a* content was significantly disintegrated under Cd stress, in both varieties. As depicted in Fig. 1A, it was reduced by 31% in VRIL-0205 and by 45% in Green Check, compared to control. In contrast, CTS and SPD alone or combined treatment elevated Chl *a* content in both control and stressed plants. Compared to non-treated stressed plants, foliar spray of CTS, SPD alone and their synergistic application improved Chl *a* content by 47, 33 and 55% in VRIL-0205 and by 44, 89 and 102% in Green Check, respectively. Chlorophyll *b* content was also reduced (VRIL-0205 by 29% and Green Check by 38%) in Cd stressed plants (Fig. 1B). In contrast, above mentioned treatments increased Chl *b* content in both control and stress conditions. The synergistic application of CTS and SPD had maximum effect, and it increased Chl *b* by 44 and 48% in VRIL-0205 and Green Check,

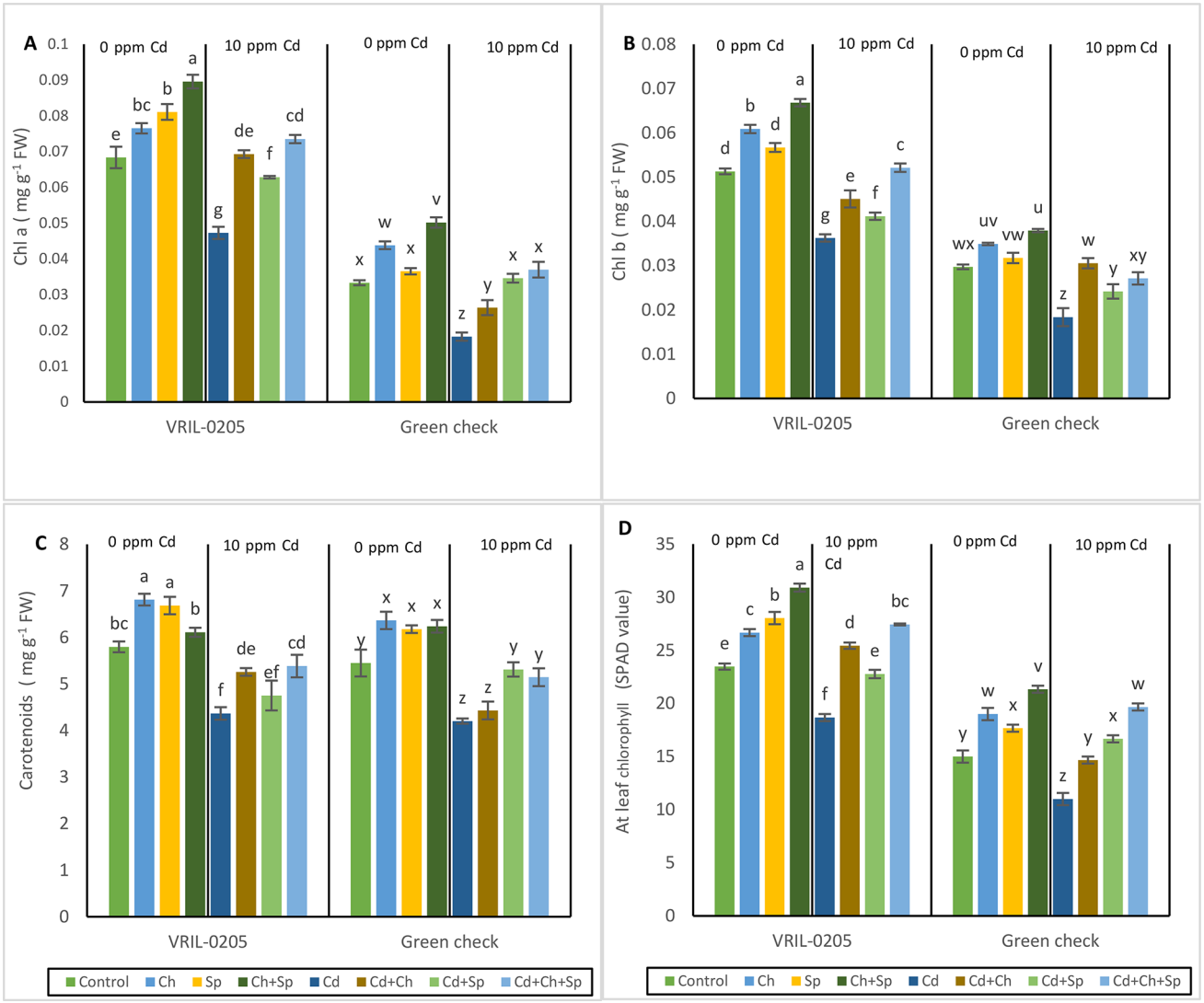

Fig. 1. Chlorophyll content of two *Lactuca sativa* varieties under Cd stress and foliar spray of chitosan and spermidine. (**A**) Chlorophyll *a*, (**B**) Chlorophyll *b*, (**C**) Carotenoids and (**D**) SPAD value. Data in (**A**-**D**) exhibits average value of three replicates. Error bars represent standard error (SE). Letters on bar graph (a, b, c, d and so on) showed statistically significant difference among treatments as determined by four-way ANOVA followed by LSD test ($p < 0.05$).

respectively, in Cd stressed plants. Similar trend was also observed in case of carotenoids content where Cd stress plants showed a significant reduction (VRIL-0205; 25% and Green Check; 23%) while, CTS and SPD treatments when applied alone or in combination retrieved this reduction in carotenoids content (Fig. 1C). These fluctuations in photosynthetic pigments in response to Cd toxicity and ameliorative role of CTS and SPD is also evident from SPAD values. Which also showed that Cd stress lowered SPAD value by 20 and 27% in variety VRIL-0205 and Green Check, respectively. On the other hand, CTS and SPD treatments either alone or combined reversed this negative effect of Cd toxicity in both varieties. Maximum increase (47% for VRIL-0205 and 79% for Green Check) in SPAD value was observed with synergistic application of CTS and SPD compared to non-treated plants under stress conditions (Fig. 1D).

Chlorophyll fluorescence parameters including minimal (F_O), maximal (F_M) and variable fluorescence (F_V) along with maximum quantum yield of photosystem II (F_V/F_M) were also analyzed in this study. The results showed that F_O was increased (VRIL-0205; 6% and Green Check; 35%) while F_M, F_V and F_V/F_M were lowered (by 16, 24 and 9% in VRIL-0205 and by 21, 28 and 9% in Green Check, respectively) under Cd stress. CTS and SPD treatments had a positive effect on chlorophyll fluorescence in both control and stress conditions. These treatments significantly increased F_M, F_V and F_V/F_M in control as well as stressed plants compared to non-treated plants. However, synergistic application of CTS and SPD showed maximum increase in F_M, F_V and F_V/F_M of both varieties (Fig. 2A-D).

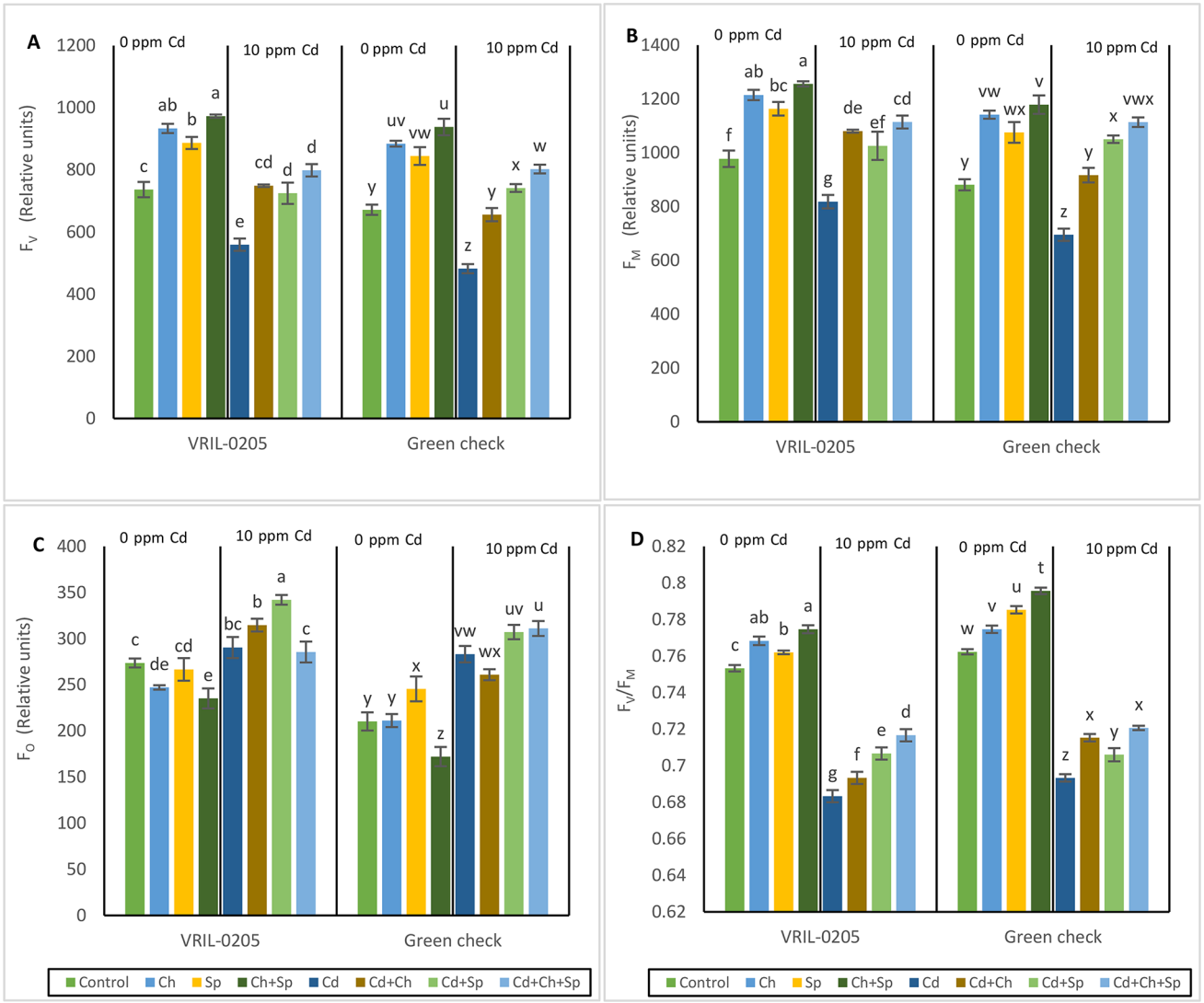

Fig. 2. Chlorophyll fluorescence parameters of two *Lactuca sativa* varieties under Cd stress and foliar spray of chitosan and spermidine. (**A**) Variable fluorescence (F_V) (**B**) Maximal fluorescence (F_M) (**C**) Minimal fluorescence (F_O) and (**D**) Maximum quantum yield of photosystem II (F_V/F_M). Data in (**A**-**D**) exhibits average value of three replicates. Error bars represent standard error (SE). Letters on bar graph (a, b, c, d and so on) showed statistically significant difference among treatments as determined by four-way ANOVA followed by LSD test ($p < 0.05$).

Effect of CTS and SPD foliar spray on gas exchange related attributes of Cd stressed *L. sativa*

Gas exchange attributes (P_n, T_r, g_s and C_i) were altered significantly in response to Cd toxicity. P_n was reduced by 66% in VRIL-0205 and 50% in Green Check, when plants were given 10 ppm Cd stress. Followed by 50, 55 and 22% reduction in T_r g_s and C_i for VRIL-0205, respectively. Similarly, in Green Check these attributes were reduced by 47, 42 and 18%, respectively. CTS and SPD had positive effect on gas exchange attributes either applied alone or in combination on both control and stress plants, compared with respective controls. But CTS and SPD synergistic treatment had more significant influence on gas exchange attributes, compared to their alone treatment in stress conditions. Their synergistic application over stressed plants enhanced P_n, T_r, g_s and C_i by 106, 50, 72 and 22% in VRIL-0205 and by 68, 63, 57 and 43% in Green Check, respectively (Fig. 3A-D).

Effect of CTS and SPD treatment on water content of Cd stressed *L. sativa*

Cadmium stressed *L. sativa* varieties (VRIL-0205 and Green Check) had significantly lowered water content, compared to control. Cd toxicity reduced osmotic potential, water potential and relative water content by 21, 26 and 20% in VRIL-0205 and by 24, 24 and 13% in Green Check, respectively. In contrast, CTS and SPD treatments, either alone or synergistic application not only retrieved this loss in water content of *L. sativa* plants but also increased it in both control and stressed plants, compared with their respective controls. For instance, CTS and SPD synergistic treatment over Cd stressed plants increased osmotic potential, water potential and RWC by 38, 31 and 30% in VRIL-0205 and by 33, 35 and 40% in Green Check, respectively (Fig. 4A-C).

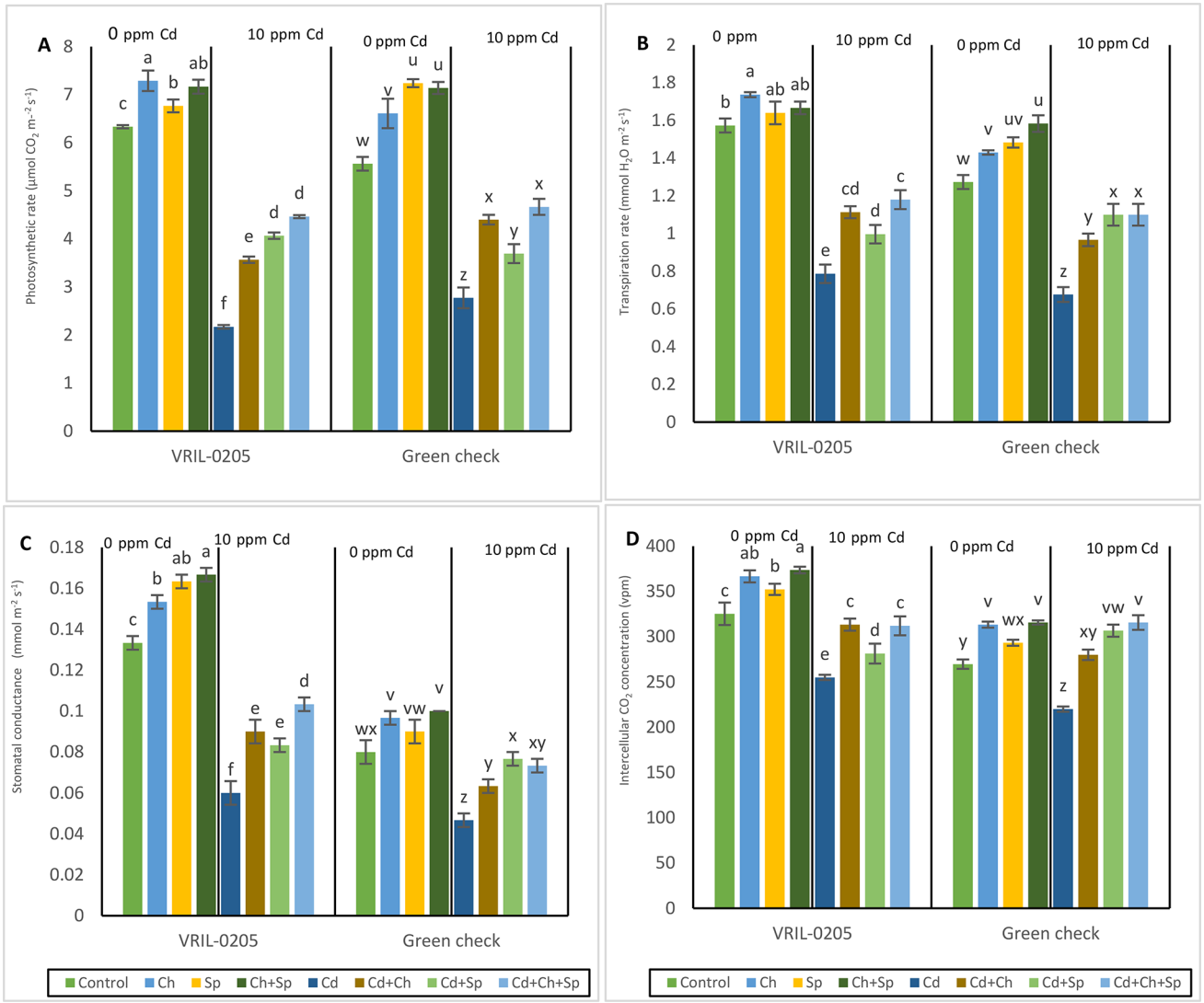

Fig. 3. Gas exchange parameters of two *Lactuca sativa* varieties under Cd stress and foliar spray of chitosan and spermidine. (**A**) Rate of photosynthesis (**A**), (**B**) Rate of transpiration (**E**), (**C**) Stomatal conductance (g_s) and (**D**) Intercellular CO_2 concentration (C_i). Data in (**A**-**D**) exhibits average value of three replicates. Error bars represent standard error (SE). Letters on bar graph (a, b, c, d and so on) showed statistically significant difference among treatments as determined by four-way ANOVA followed by LSD test ($p < 0.05$).

Effect of CTS and SPD foliar spray on stress markers and total soluble proteins in Cd stressed *L. sativa*

As depicted in Fig. 5A-C, Cd stress caused oxidative damage to *L. sativa* plants in the form of lipid peroxidation. This is indicated by the increase in MDA content (56% in VRIL-0205 and 49% in Green Check) of Cd stressed plants when compared to control. Cd toxicity also caused ROS generation and posed oxidative damages. As depicted from Fig. 5B, Cd stressed plants had elevated levels of H_2O_2 in both varieties (VRIL-0205; 29%, Green Check; 35%). These effects also disrupted membrane permeability which is evident from 37% increase in RMP of both varieties. CTS and SPD treatments alone or in combination successfully lowered MDA content, detoxified ROS and maintained RMP of both *L. sativa* varieties exposed to Cd toxicity. Under stress conditions, maximum reduction in MDA content was observed with SPD alone treatment in both varieties, compared with stress only plants. Similarly, for H_2O_2 and RMP this effect was observed with CTS and SPD synergistic application in both varieties (Fig. 5A-C).

Total soluble proteins were significantly uplifted (49% in VRIL-0205 and 66% in Green Check) in Cd stressed plants, compared with control group. On the other hand, CTS and SPD treatments, in all combinations further increased TSP in both *L. sativa* varieties in control as well as stress conditions, as equated to their respective controls. For stressed plants maximum increase in TSP was observed with synergistic treatment of CTS and SPD for both varieties, which is 26% in VRIL-0205 and 22% in Green Check (Fig. 5D).

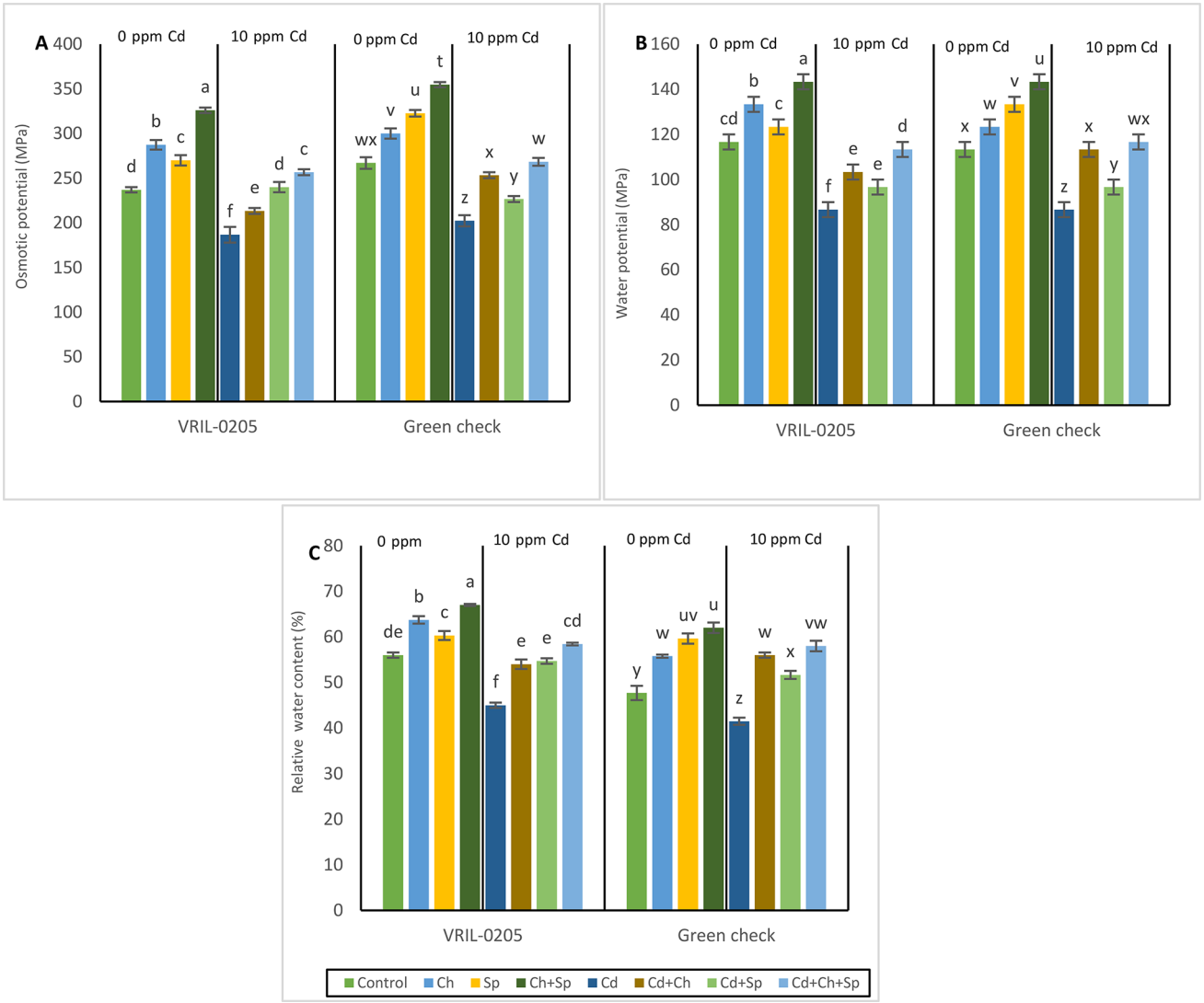

Fig. 4. (**A**) Osmotic potential, (**B**) Water potential and (**C**) Relative water content (RWC) of two Lactuca sativa varieties under Cd stress and foliar spray of chitosan and spermidine. Data in (**A**-**C**) exhibits average value of three replicates. Error bars represent standard error (SE). Letters on bar graph (a, b, c, d and so on) showed statistically significant difference among treatments as determined by four-way ANOVA followed by LSD test ($p < 0.05$).

Effect of CTS and SPD foliar spray on enzymatic antioxidants of Cd stressed *L. sativa*

Catalase (CAT) enzyme activity was increased (VRIL-0205; 112%, Green Check; 43%) in Cd treated plants compared to control. CTS and SPD treatment improved CAT activity in both control and stress conditions. For instance, in Cd treated plants, the synergistic application of CTS and SPD increased CAT activity by 19 and 21% in VRIL-0205 and Green Check, respectively, compared with stress only plants. In response to Cd stress, *L. sativa* plants significantly enhanced peroxidase (POD) activity. Activity of this enzymes in Cd treated plants was 175 and 87% higher than control plants for variety VRIL-0205 and Green Check, respectively. Cd stressed plants along with synergistic treatment of CTS and SPD had further improved POD activity by 17% in variety VRIL-025 but it had no significant effect in Green Check. Activities of APX and SOD were increased greatly (by 61 & 108% in VRIL-0205 and by 94 & 57% in Green Check, respectively) under toxic Cd stress. CTS and SPD treatments alone or synergistic further increased the activities of these enzymes. Maximum activities of these enzymes were confined in Cd stressed plant with synergistic effect of CTS and SPD (Fig. 6A-D).

Effect of CTS and SPD foliar spray on total phenolics and leaf proline content of Cd stressed *L. sativa*

Total phenolics and leaf proline contents were increased in Cd stress plants by 38 & 84% in VRIL-0205 and by 70 & 76% in Green Check, respectively, compared to control. CTS and SPD treatments, alone or in combination, further enhanced these attributes. In Cd stressed plants, synergistic application of CTS and SPD was more effective in elevation of these antioxidants compared to their alone treatments. In Cd treated plants combined

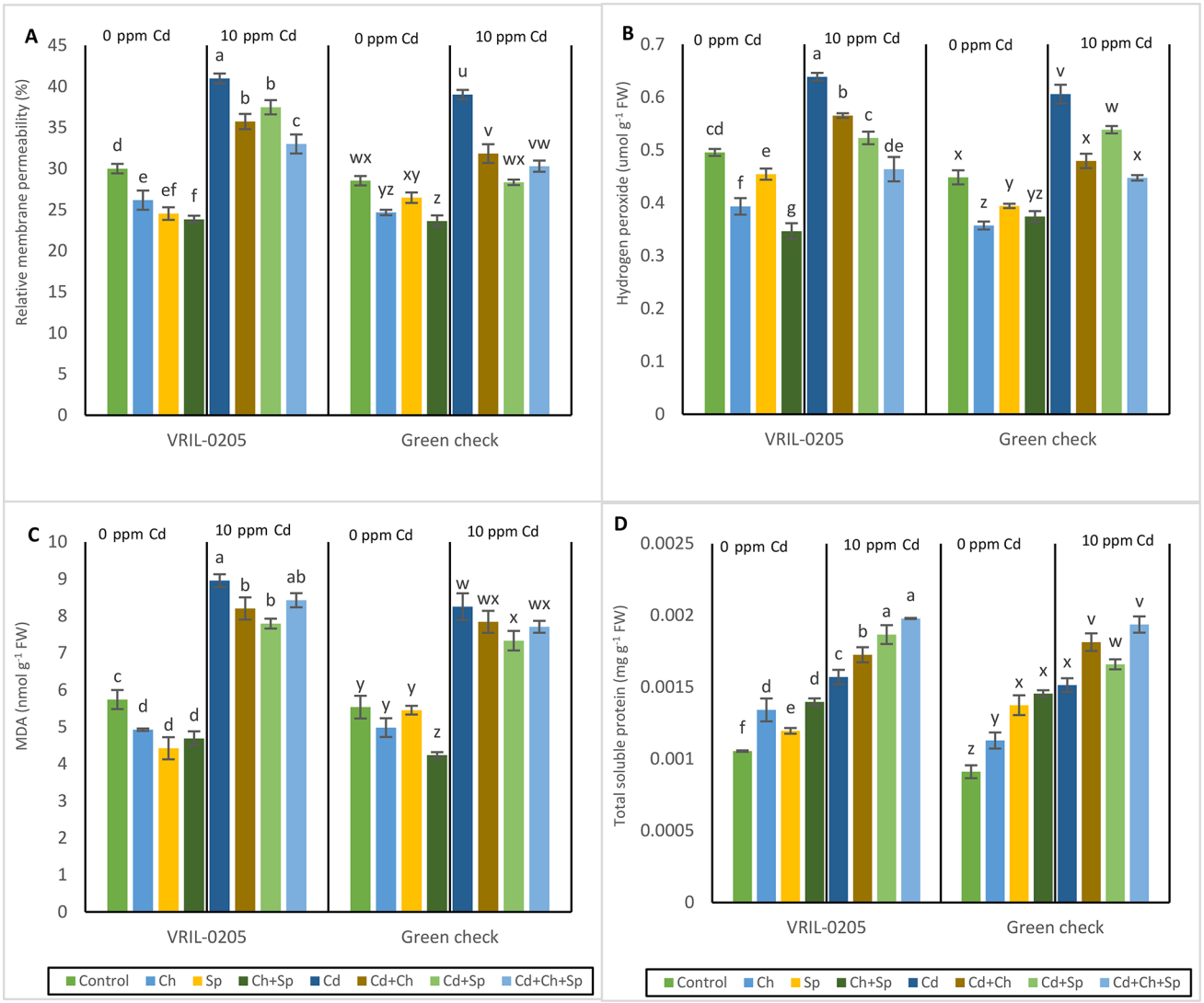

Fig. 5. (**A**) Relative membrane permeability, (**B**) Hydrogen peroxide, (**C**) Malondialdehyde (MDA) and (**D**) Total soluble proteins in two *Lactuca sativa* varieties under Cd stress and foliar spray of chitosan and spermidine. Data in (**A**-**D**) exhibits average value of three replicates. Error bars represent standard error (SE). Letters on bar graph (a, b, c, d and so on) showed statistically significant difference among treatments as determined by four-way ANOVA followed by LSD test ($p < 0.05$).

application of CTS and SPD increased total phenolics by 15 and 27%, respectively, in VRIL-0205 and Green Check. Similarly, leaf proline was increased by 36 and 10%, respectively, in VRIL-0205 and Green Check, compared with Cd-stressed plants (Fig. 7A & B).

Effect of CTS and SPD foliar spray on K^+ and Ca^{2+} in shoot and root of Cd stressed *L. sativa*

Cd stress significantly lowered K^+ and Ca^{2+} concentrations in both root and shoot of *L. sativa* plants, compared with control group. For instance, in VRIL-0205, K^+ ions were reduced by 26 and 29% in shoot and root, respectively. Similarly, in Green Check it was reduced by 22 and 21%, respectively, in shoot and root as equated to control. The foliar spray of CTS and SPD, either alone or combined, improved K^+ and Ca^{2+} concentration in root and shoot of both *L. sativa* varieties. Under stress conditions, synergistic treatment of CTS and SPD increased shoot K^+ by 61 and 16% and root K^+ by 73 & 68%, respectively, in VRIL-0205 and Green Check. Similar effects of Cd stress and counter effects of CTS and SPD alone and synergistic treatments were observed for shoot and root Ca^{2+} ions in both *L. sativa* varieties. The synergistic application of CTS and SPD was more efficient in improving shoot Ca^{2+}, while their alone treatments were more effective in improving root Ca^{2+} in both *L. sativa* varieties (Fig. 8A-D).

Effect of CTS and SPD foliar spray on Fe^{2+} and Cd^{2+} content in shoot of Cd stressed *L. sativa*

Under Cd stress 0.038 and 0.041 mg Kg^{-1} of Cd^{2+} ions were detected in shoot of *L. sativa* varieties VRIL-0205 and Green Check, respectively. CTS and SPD alone and their combined treatment decreased Cd metal ions in

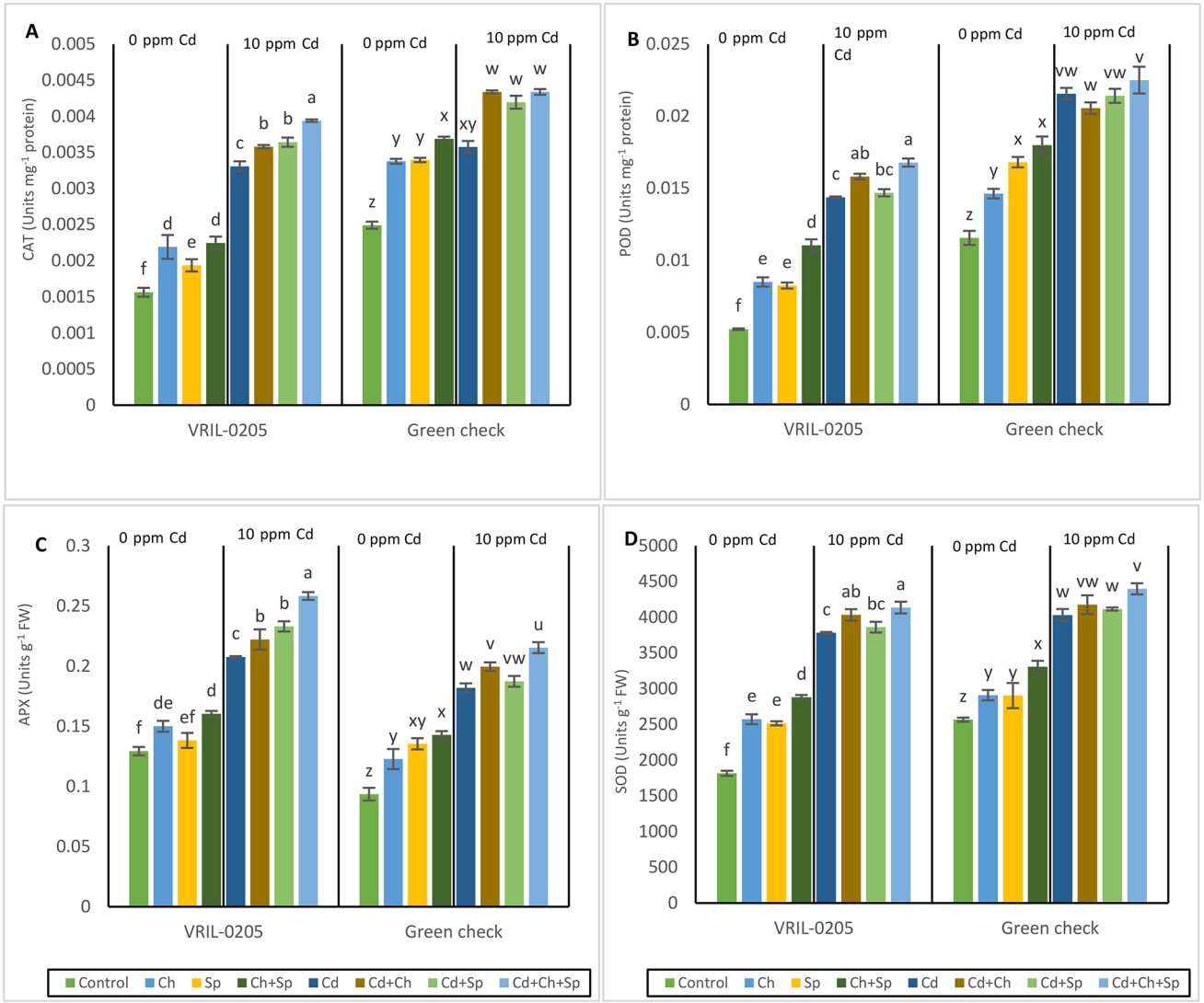

Fig. 6. Antioxidant enzymes activities of two *Lactuca sativa* varieties under Cd stress and foliar spray of chitosan and spermidine. (**A**) Catalase (CAT), (**B**) Peroxidase (POD), (**C**) Ascorbate peroxidase (APX) and (**D**) Superoxide dismutase (SOD). Data in (**A**-**D**) exhibits average value of three replicates. Error bars represent standard error (SE). Letters on bar graph (a, b, c, d and so on) showed statistically significant difference among treatments as determined by four-way ANOVA followed by LSD test ($p < 0.05$).

the shoot of both *L. sativa* varieties. Synergistic application of CTS and SPD decreased shoot Cd by 62 and 58% in Cd stressed VRIL-0205 and Green Check plants, respectively, compared to stress only plants (Fig. 9A). Similarly, shoot Fe^{2+} in both *L. sativa* varieties, VRIL-0205 and Green Check, was reduced by 25 and 13%, respectively, in Cd stressed plants compared to control. CTS and SPD foliar sprays effectively reversed this reduction of shoot Fe^{2+} in both varieties. In stressed plants, maximum shoot Fe^{2+} content was observed under synergistic foliar spray of CTS and SPD (VRIL-0205; 35% and Green Check plants 19%) compared to stress only plants (Fig. 9B).

Pearson's correlation and principal component analysis

Pearson's correlation results for both varieties (upper triangle; VRIL-0205 & lower triangle; Green Check) of *L. sativa* are shown in Fig. 10. Both varieties had comparatively similar results, where Cd contents in shoot is in negative correlation with growth and physiological attributes. This revealed that Cd stress had negative impacts on growth and development of plants. Furthermore, a positive correlation between Cd contents in shoot and stress markers (antioxidants, lipid peroxidation, and relative membrane permeability) also confirm adverse effects of Cd stress applied in this study. Principal component analysis shown in the Figs. 11 and 12 for variety VRIL-0205 and Green Check, respectively, also support the results of Pearson's correlation. PCA results showed that first two components (PC1 and PC2) had the maximum contribution 92.62% for variety VRIL-0205 and 92.13% for Green Check. Arrows length from origin depicts the contribution of respective parameters. The numeric 1, 2, 3…, 8 showed that individual effect of each treatment applied in this study. For both varieties, as

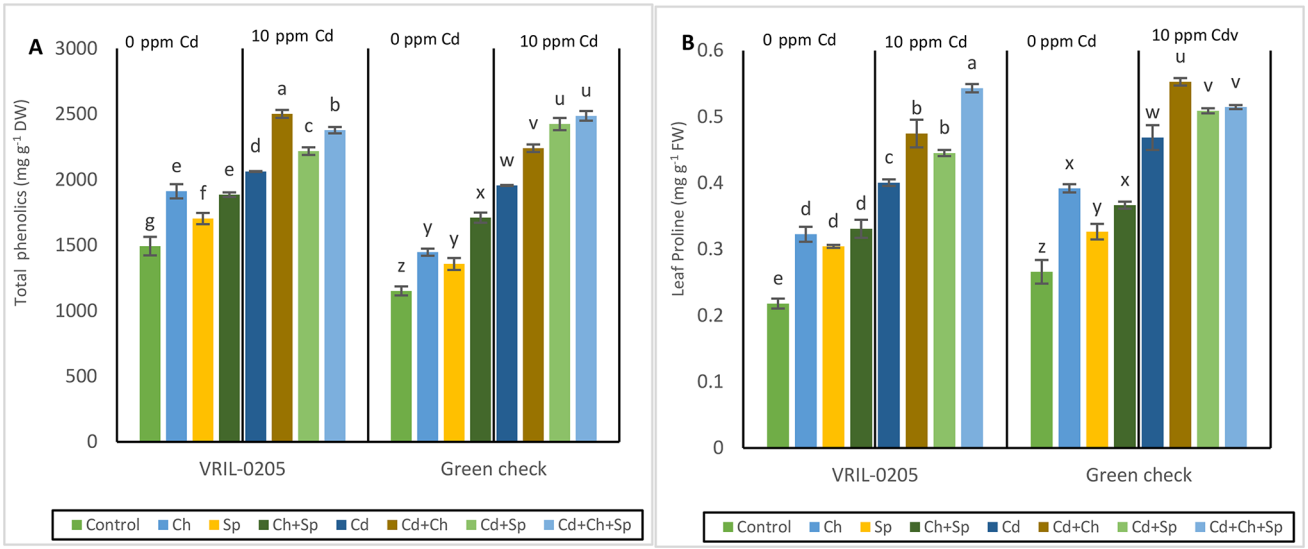

Fig. 7. (**A**) Total phenolics and (**B**) Leaf proline of two *Lactuca sativa* varieties under Cd stress and foliar spray of chitosan and spermidine. Data in (A and B) exhibits average value of three replicates. Error bars represent standard error (SE). Letters on bar graph (a, b, c, d and so on) showed statistically significant difference among treatments as determined by four-way ANOVA followed by LSD test ($p < 0.05$).

shown in Figs. 11 and 12 Cd stress (5) treatment is separated well from all other treatments. This revealed that Cd stress significantly influenced all studied parameters for both varieties.

Discussion

Environmental pollution has become an alarming and global issue. More specifically, heavy metal contamination of the soil has severely affected plant growth and crop production. This is caused due to various anthropogenic activities. Cadmium is highly noxious due to its toxicity at low level and mobility in the soil. Results of this study as illustrated in Supplementary Table S1 online, showed that Cd stress had highly negative effect on growth, fresh and dry biomass, leaf area and leaf count of both studied *L. sativa* varieties. Comparable reductions in growth characteristics resulting from Cd exposure have been noted in peas[48], maize[49], tomatoes[50] and wheat[19]. Cd stress impedes cell division and elongation rates, resulting in reduced biomass production in seedlings[51]. Cd induces several changes in cell structure and stimulates programmed cell death, which remarkably contributes to decreases in root growth, shoot length and biomass[52]. The reduced growth of plants exposed to Cd may also be related to lowered nutrient uptake.

In the present study, foliar spray of CTS and SPD mitigated the adverse effects of Cd and enhanced growth in both *L. sativa* varieties. This is because CTS has ability to form complexes with metals, reducing its availability or turning into less harmful form. Plants with lower proximity to metal ions show increased uptake of essential elements, including K, P, and Ca[53]. CTS can chelate various nutrients and minerals, increasing their solubility and making them more accessible for plant uptake[13,54]. Results of this experiment are consistent with those of earlier studies demonstrating the ability of CTS to ameliorate the adverse effects of Cd on rapeseed (*Brassica napus*) growth[55]. Along with this SPD application also increased the growth of both *L. sativa* varieties by suppressing damaging effects of Cd stress. It is observed that SPD might act as a signaling molecule in stress response mechanisms, influencing the expression of genes involved in cell division and differentiation in plants[56]. Polyamines are suggested to work as metal chelators, enabling plants to potentially utilize increased polyamine levels directly to chelate additional metal ions. This could help to increase the ability of plants to accumulate metals[57]. Our findings are consistent with previous study, where SPD application increased biomass, promoted the levels of Fe, Cu, Zn, soluble proteins, and certain antioxidants in *S. matsudana* subjected to Cd stress[58].

Our findings reveal that Cd stress significantly impacts photosynthetic pigments, chlorophyll fluorescence, and gas exchange attributes, ultimately contributing to reduced growth in both varieties of *Lactuca sativa*. Cadmium stress was found to decrease total chlorophyll content and SPAD values, indicating disruption in chlorophyll biosynthesis. This disruption may be attributed to Cd's ability to compete with Mg^{2+} for binding sites in chlorophyll molecules[59], thereby impairing pigment synthesis. These observations align with previous studies, such as those by Dias et al.[60] and Zhou et al.[61] which reported reductions in chlorophyll content in lettuce plants exposed to Cd stress. Chlorophyll fluorescence efficiency, as measured by parameters such as F_V, F_O, F_M, F_V/F_M, and F_V/F_O, was also adversely affected by Cd stress in both lettuce varieties. These parameters serve as indicators of PSII efficiency in primary photochemical reactions and provide insights into the health of the photosynthetic apparatus. Cd toxicity caused significant damage to the photosynthetic machinery[62], leading to reduced fluorescence efficiency. These findings corroborate earlier reports, for example in canola stress conditions negatively impacted chlorophyll fluorescence[63].

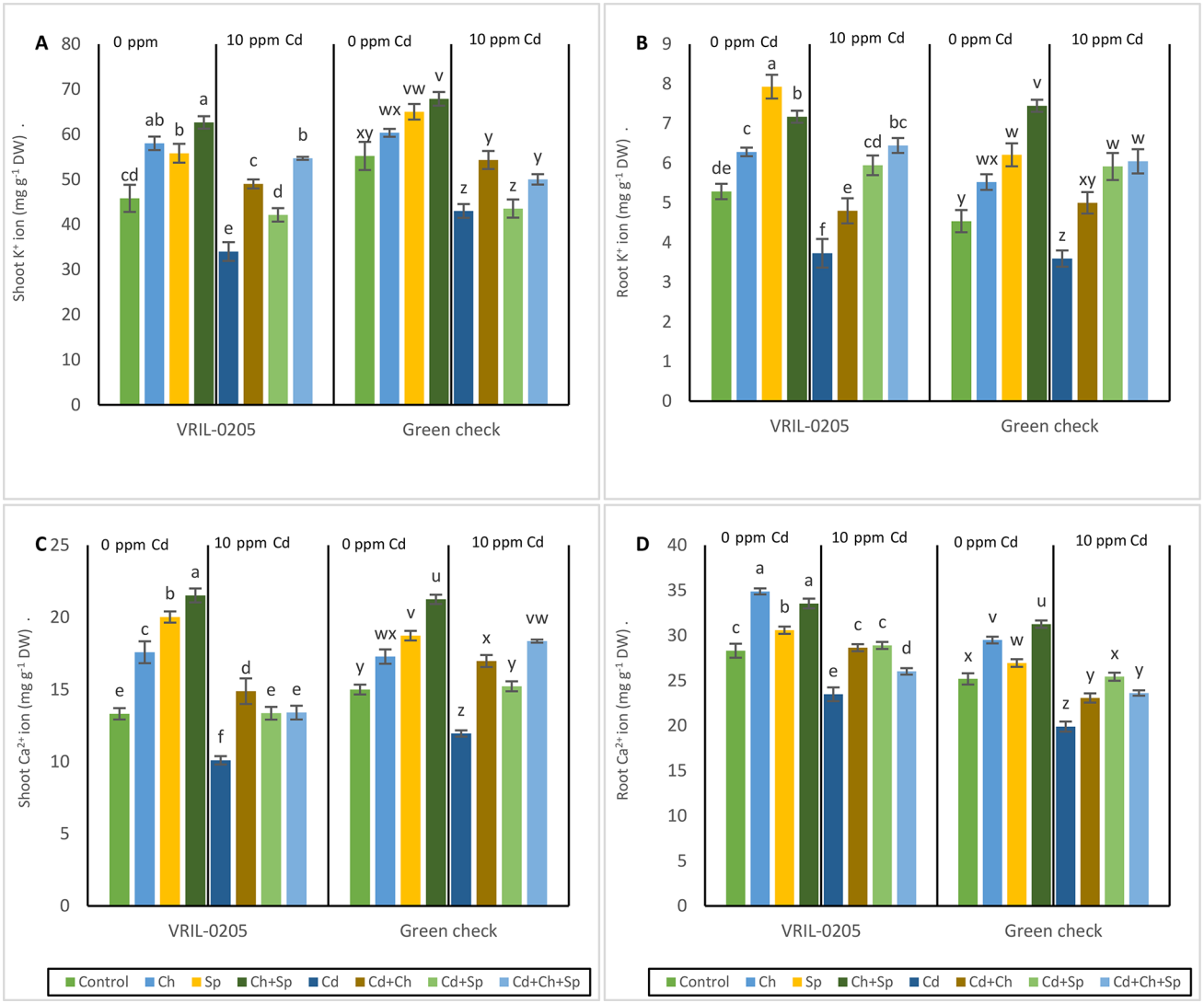

Fig. 8. Inorganic ions of two *Lactuca sativa* varieties under Cd stress and foliar spray of chitosan and spermidine. (**A**) Shoot K^+ ion, (**B**) Root K^+ ion, (**C**) Shoot Ca^{2+} ion and (**D**) Root Ca^{2+} ion. Data in (**A**-**D**) exhibits average value of three replicates. Error bars represent standard error (SE). Letters on bar graph (a, b, c, d and so on) showed statistically significant difference among treatments as determined by four-way ANOVA followed by LSD test ($p < 0.05$).

The detrimental effects of Cd stress extended to gas exchange attributes, with decreases observed in the photosynthetic rate (Pn), transpiration rate (Tr), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i). These declines suggest that Cd disrupts multiple facets of the photosynthetic process, including electron transport on thylakoid membranes, stomatal conductance, and the carbon cycle[60]. Since photosynthesis efficiency depends on factors such as leaf surface area, stomatal density, cell size, and conductance, Cd-induced disruptions in these processes directly contribute to reduced photosynthesis. Previous investigations of lettuce plants have similarly demonstrated the negative impact of Cd stress on photosynthetic efficiency and plant growth. The interplay between the reduced synthesis of photosynthetic pigments, impaired PSII efficiency, and disrupted gas exchange ultimately culminates in a significant decline in the growth of *L. sativa* plants under Cd stress.

Our research demonstrated that Cd stress significantly reduced water content (WC) in both studied varieties of *Lactuca sativa*, as evidenced by decreases in osmotic potential, water potential, and relative water content (RWC). This reduction in WC can be attributed to the lowered transpiration rate under Cd stress, which impairs water absorption through the roots, leading to insufficient water availability within the plant. Similar findings were reported by Dawuda et al.[26] who observed that Cd stress at 100 µM drastically reduced RWC in Cd-sensitive lettuce plants. The decline in WC under Cd stress has a direct impact on plant growth and photosynthesis. Reduced water availability disrupts cell turgor, which is essential for cell expansion and overall plant development. Additionally, water stress interferes with stomatal conductance, limiting CO_2 uptake and thereby reducing the photosynthetic rate. The impaired photosynthetic process, combined with decreased

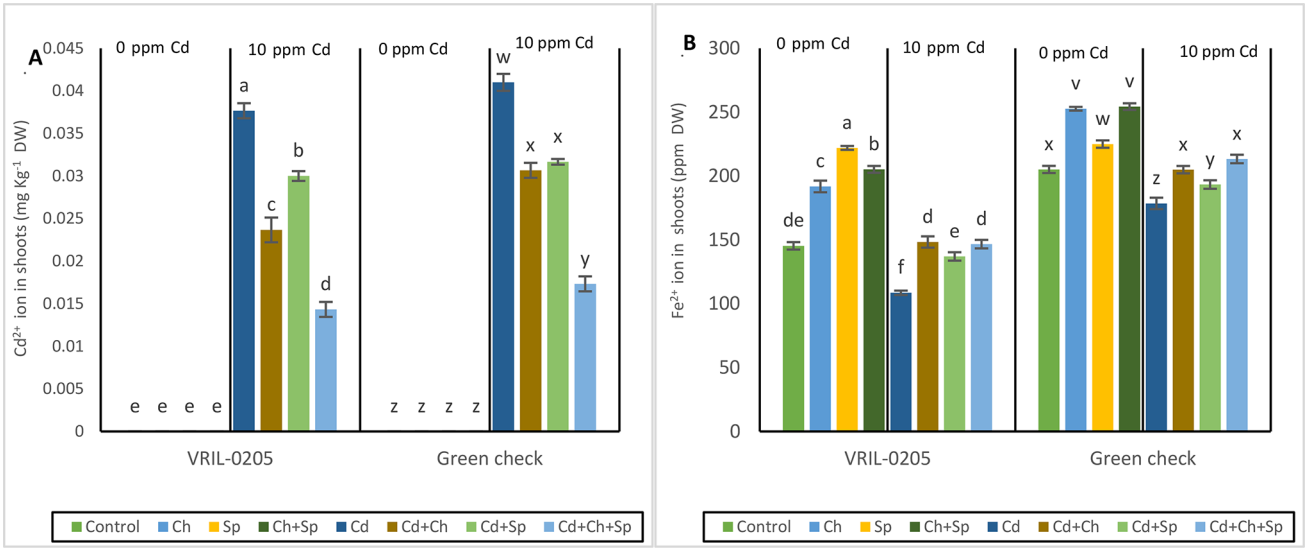

Fig. 9. (**A**) Cd^{2+} and (**B**) Fe^{2+} ion in shoot of two *Lactuca sativa* varieties under Cd stress and foliar spray of chitosan and spermidine. Data in (**A** and **B**) exhibits average value of three replicates. Error bars represent standard error (SE). Letters on bar graph (a, b, c, d and so on) showed statistically significant difference among treatments as determined by four-way ANOVA followed by LSD test ($p < 0.05$).

water potential and RWC, hinders the plant's ability to maintain energy production and metabolic functions, ultimately resulting in stunted growth. These interconnected effects highlight the critical role of water content in sustaining photosynthesis and overall plant vitality under Cd stress.

The application of chitosan (CTS) and spermidine (SPD) demonstrated a remarkable capacity to alleviate Cd-induced stress in *Lactuca sativa* by enhancing photosynthetic efficiency and mitigating the adverse effects of Cd toxicity on key physiological processes. The observed increase in chlorophyll content with CTS and SPD treatment coincided with the restoration of photosynthetic parameters, suggesting that the improvement in photosynthesis occurred partly through increased chlorophyll synthesis[64]. This enhancement was likely driven by CTS-induced expression of chloroplast-related genes, enlargement of chloroplasts, and improved accessibility of amino compounds essential for chlorophyll synthesis[65]. These findings align with previous studies, such as Rasheed et al.[66] who reported increased chlorophyll and carotenoid levels in *Pisum sativum*, and Ibrahim et al.[28] who noted similar results in lettuce.

CTS and SPD also improved chlorophyll fluorescence and efficiency of photosystem II which contribute to enhanced photosynthetic activities in both *L. sativa* varieties under Cd stress. This protective effect can be attributed to CTS's role in stabilizing cellular structures, counteracting Cd-induced oxidative stress, and promoting the activity of antioxidant systems. SPD also alleviated Cd-induced photosynthesis inhibition by enhancing chlorophyll content and preserving the structural and functional integrity of the photosynthetic apparatus[67,68]. Its acid-neutralizing and antioxidant properties, along with its ability to stabilize membranes and bind with proteins, helped maintain the integrity of the thylakoid membrane and protect photosynthetic functions[69]. In addition to improving photosynthesis, CTS and SPD also contributed to the maintenance of water relations under Cd stress. CTS enhanced RWC by expanding cell layers and boosting antioxidant activity, as demonstrated in tomato plants Hassnain et al.[70]. SPD, being a nitrogenous compound, also increased leaf RWC, particularly under stress conditions[71].

In this study, Cd stress induced oxidative stress in plants by triggering excessive production of reactive oxygen species (ROS), particularly H_2O_2. This leads to oxidative damage to the photosynthetic machinery and other physiological processes, including disrupted membrane integrity caused by lipid peroxidation[26]. Consequently, elevated levels of MDA and RMP were observed, consistent with findings in wheat under Cd stress[72]. These changes highlight the detrimental effects of ROS on cellular structures, resulting in membrane leakage. To combat ROS, plants activate enzymatic and non-enzymatic antioxidant mechanisms. In our study, Cd stress increased the activities of antioxidant enzymes, including CAT, SOD, POD, and APX in both *Lactuca sativa* varieties. These enzymes play a critical role in ROS scavenging, with SOD converting superoxide radicals into H_2O_2 and POD and CAT detoxifying H_2O_2[73]. Similar trends have been reported in other species, such as *Hibiscus cannabinus*[74] and *Pistia stratiotes*[75]. Cd stress also enhanced total soluble proteins in both studied varieties. This could be attributed to the fact that most of the enzymes are proteins that play a key role in stress responses in plants. Besides this synthesis of stress responsive proteins such as heat shock proteins is also triggered under stress conditions[76]. Non-enzymatic antioxidants, such as proline and phenolics, also contribute to oxidative stress mitigation. Proline, an osmo-protectant, stabilizes enzymes and cellular structures, alleviating heavy metal stress. Phenolic compounds, synthesized via the phenylpropanoid pathway, act as antioxidants by reducing lipid peroxidation and quenching ROS[77]. In our study, Cd stress increased both proline and phenolic contents in lettuce, consistent with reports from other species[78].

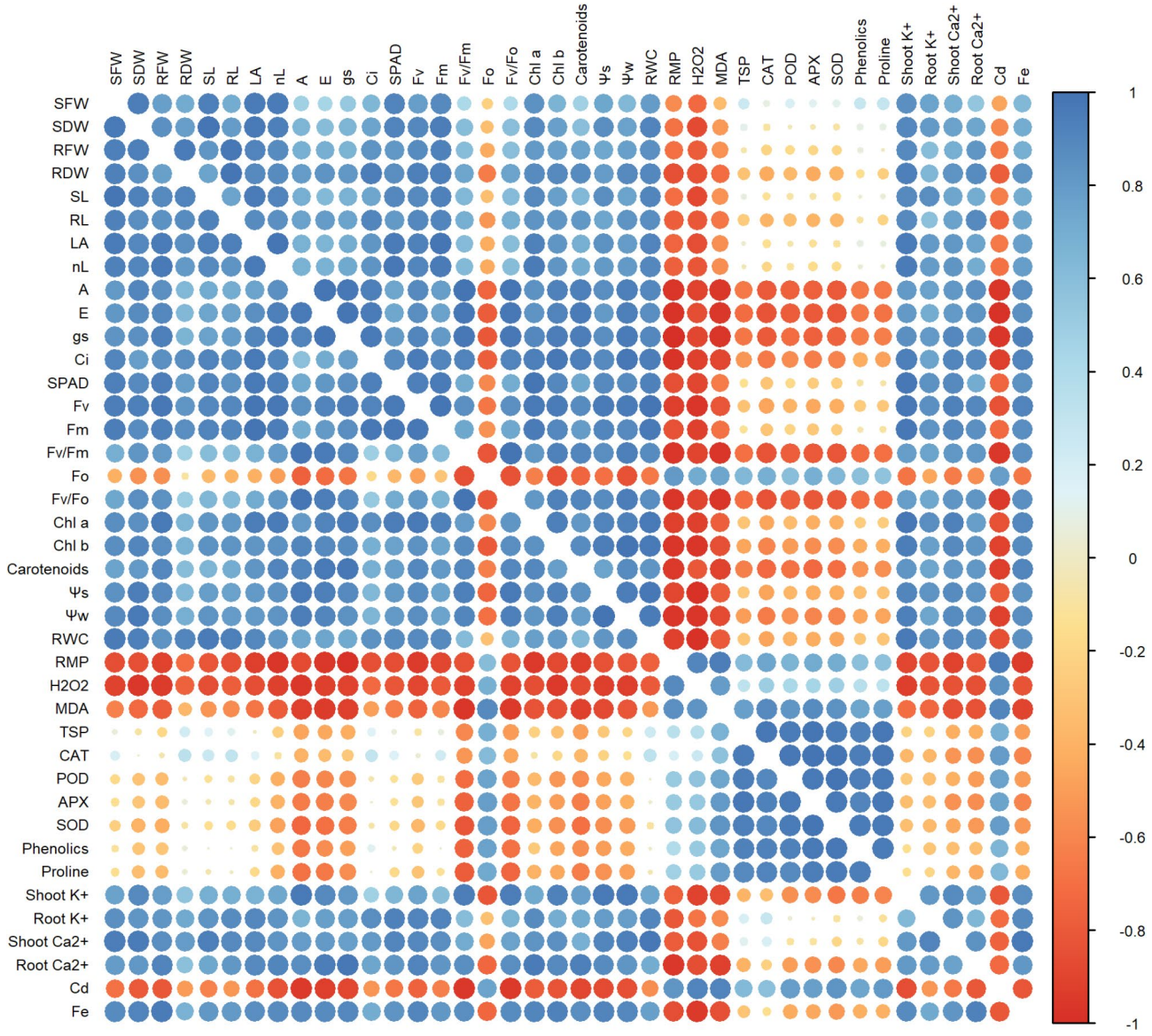

Fig. 10. Pearson's correlation for all studied attributes of *L. sativa* plants under the effect of Cd toxicity with or without chitosan and spermidine foliar spray. Upper triangle is for variety VRIL-0205 and lower triangle is for Green Check. (Various abbreviations used are as follows; SFW; shoot fresh weight, SDW; shoot dry weight, RFW; root fresh weight, RDW; root dry weight, SL; shoot length, RL; root length, LA; leaf area, nL; number of leaves, A; photosynthesis rate, E; transpiration rate, gs: stomatal conductance, Ci: intercellular CO_2, SPAD; chlorophyll content, Fv; variable fluorescence, Fm; maximal fluorescence, Fv/Fm; maximum quantum efficiency of PSII, Fo; minimal fluorescence, Fv/Fo: Maximum Efficiency of the Water-Splitting Complex on the Donor Side of PSII, Chl; chlorophyll, Ψs; osmotic potential, Ψw; water potential, RWC; relative water content, RMP; relative membrane permeability, H_2O_2; hydrogen peroxide, MDA; malondialdehyde, TSP; total soluble proteins, CAT; catalase, POD; peroxidase, APX; ascorbate peroxidase and SOD; superoxide dismutase).

On the other side, CTS and SPD treatments significantly decreased H_2O_2 and MDA content, while stabilizing RMP in both varieties, reflecting reduced lipid peroxidation and improved membrane integrity. CTS's ability to scavenge ROS, as demonstrated by Prashanth et al.[79] and Mehmood et al.[80] is attributed to its antioxidant properties, which protect cellular components. Similarly, SPD reduces MDA and H_2O_2 levels by interacting with negatively charged membrane components, maintaining structural stability, and directly scavenging ROS, as reported by Gong et al.[81].

Both CTS and SPD enhanced total soluble proteins (TSP), likely through increased synthesis of stress-related structural and signaling proteins[82]. CTS promotes the production of osmotin-like proteins and influences cell wall remodeling, while SPD acts as a signaling molecule, regulating stress-responsive gene expression and stabilizing proteins against degradation. These mechanisms contribute to the improved stress tolerance observed in plants treated with CTS and SPD.

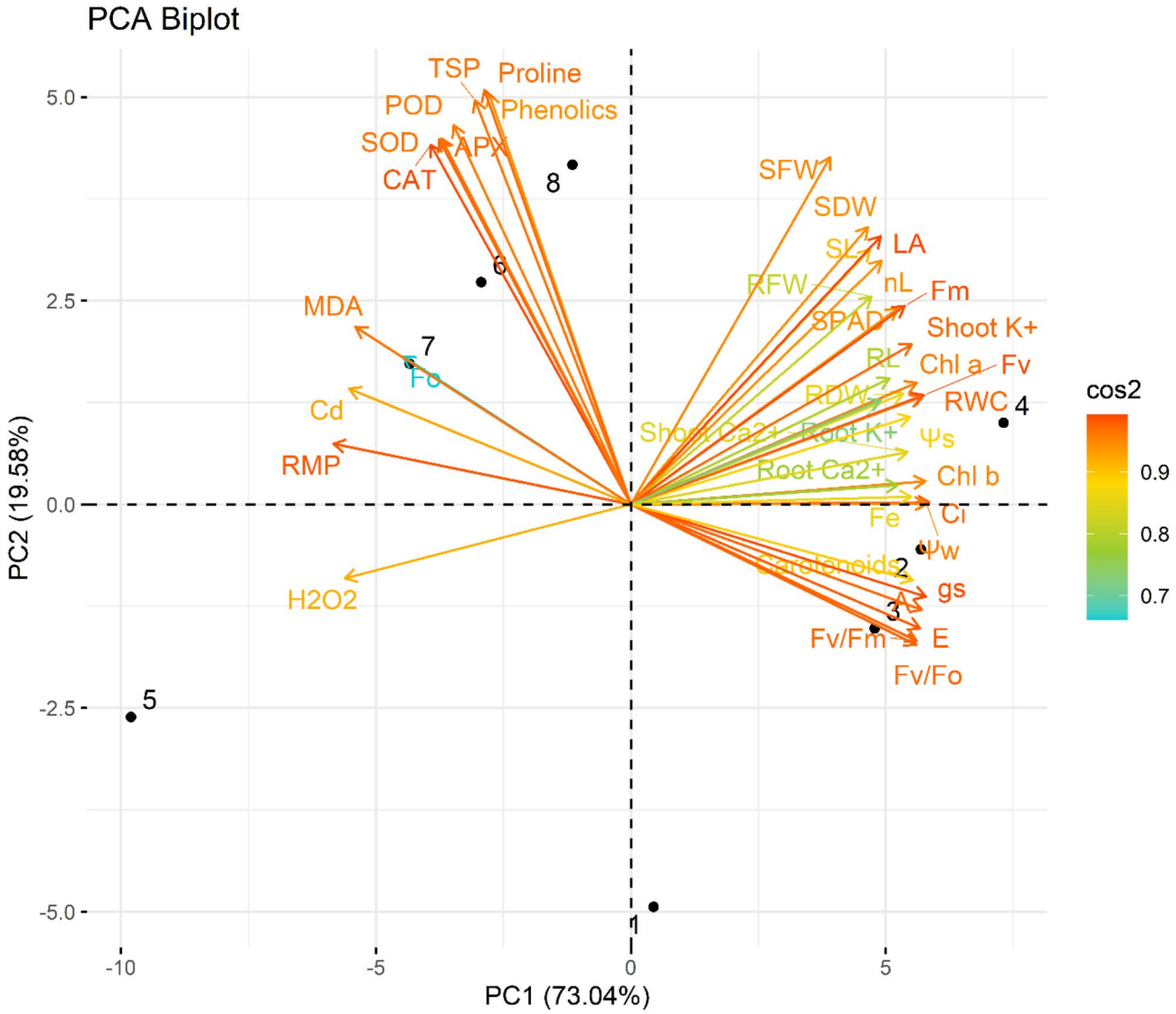

Fig. 11. Principal components analysis of all studied attributes of *L. sativa* variety VRIL-0205 under the effect of Cd toxicity with or without chitosan and spermidine foliar spray. The arrow length showed the contribution of each attribute. Numbers 1, 2, 3…, 8 represents different treatments chitosan and spermidine applied in control (1-4) and Cd stress (5-8) conditions. (Various abbreviations used are same as given in Fig. 10).

CTS also facilitates nitrogen metabolism, potentially elevating proline content, a key osmo-protectant[83], while SPD enhances proline and phenolic compounds through its role in enzyme regulation and synthesis pathways. These effects were consistent with findings by Tabassum et al.[84] and Xie et al.[85] demonstrating enhanced antioxidant activity and secondary metabolite synthesis under stress. Together, CTS and SPD alleviate oxidative damage, improve membrane stability, and enhance the antioxidant defense system, reversing the detrimental impacts of Cd stress on lettuce plants.

Cd, a divalent cation, competes with the transport of other nutrients across the membrane. Despite the uptake of essential nutrients, Ca channels and Fe transporters start absorbing Cd. The reduction in K^+ Ca^{2+} and Fe^{2+} uptake mediated by Cd accounts for the growth suppression as observed in this study[86]. Similar results were previously reported for pea plants[66]. Ca is a multifunctional essential element for plants. Ca dependent proteins e.g. calmodulin proteins are involved in defense and various other physiological responses in plants. Thus, the uptake of Cd in place of Ca would replace Ca everywhere it is needed and disrupt the functioning of Ca dependent ions channels and other proteins. This ultimately led to reduced growth and development of plants. Similarly, iron (Fe) is a mineral nutrient required for healthy growth of plants. It is a crucial component of proteins involved in photosynthesis process especially energy transfer reaction. It is component of cytochrome proteins required for electron transfer in cellular respiration. As a cofactor of several enzymes, it is also involved in various physiological and metabolic processes in plants[87,88]. Fe is also involved in synthesis of chlorophyll. Fe deficiency can cause chlorosis, reduced photosynthesis and stunted growth in plants[89]. In this study, elevated level of Cd imitated this divalent ion and caused its deficiency thus disrupting key physiological processes. *L.*

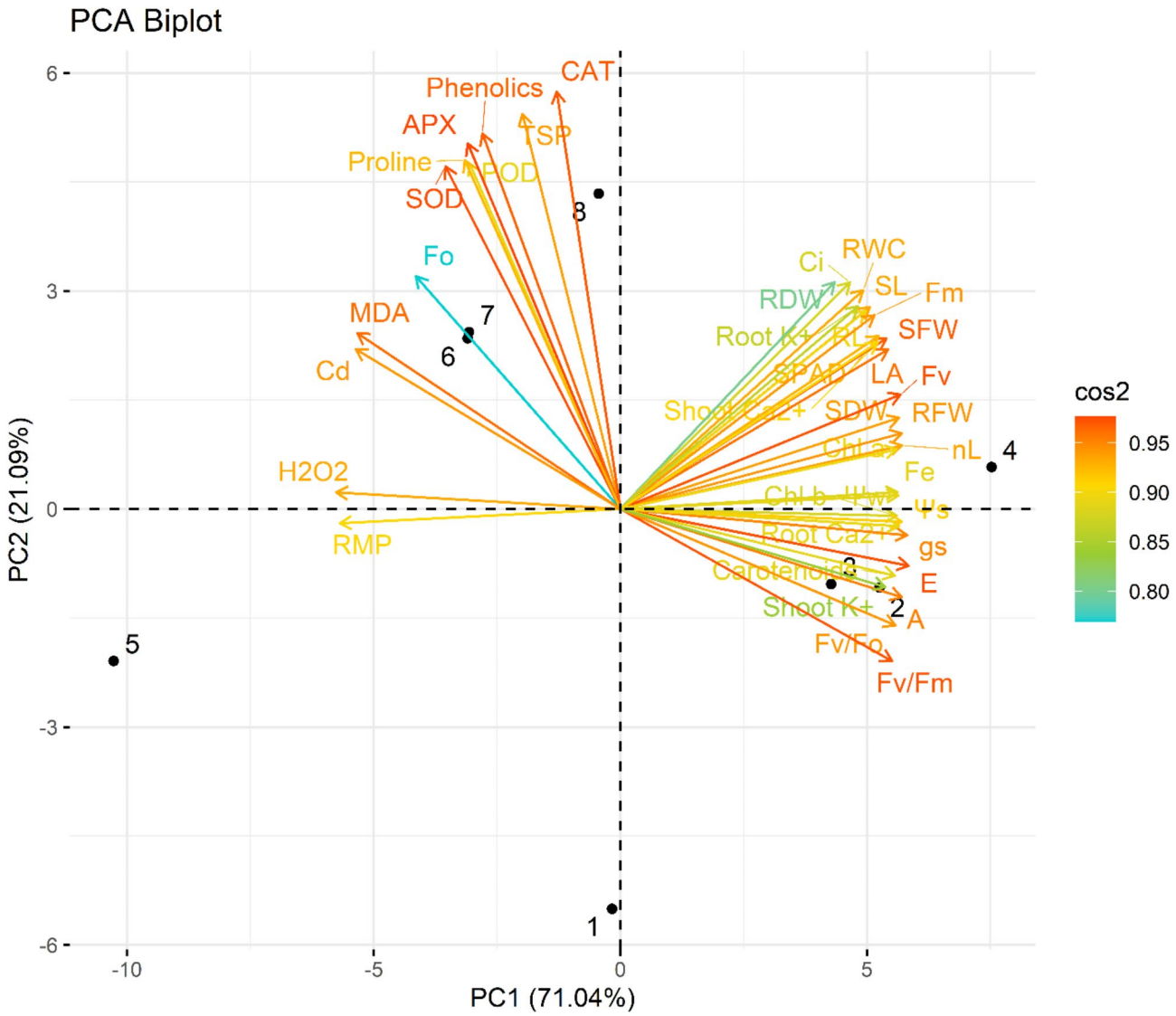

Fig. 12. Principal components analysis of all studied attributes of *L. sativa* variety Green Check under the effect of Cd toxicity with or without chitosan and spermidine foliar spray. The arrow length showed the contribution of each attribute. Numbers 1, 2, 3…, 8 represents different treatments chitosan and spermidine applied in control (1-4) and Cd stress (5-8) conditions. (Various abbreviations used are same as given in Fig. 10).

sativa is also a source of Fe in the human diet which could also be compromised in this way. Foliar application of CTS and SPD increased plant growth by regulating nutrients level and reducing ionic disbalance[90]. Foliar spray of CTS also increased the K^+ content and decreased the Na^{2+} level in the shoots and roots of stressed *P. sativum*[84]. Another study revealed that application of SPD restricted Na^{2+} influx in exposed roots and maintained the K^+/Na^{2+} balance in barley seedlings by preventing K^+ depletion from stems[91]. These results also aligned with prior observations documented in oats[92].

While comparing the both varieties in term of Cd tolerance, both varieties were adversely affected by Cd stress, Green Check appeared more sensitive. Both varieties showed some level of tolerance to Cd stress. The application of CTS and SPD, especially their synergistic application, helped to mitigate the negative effects of Cd stress in both varieties. This suggests that both varieties possess some inherent tolerance mechanisms, which can be further enhanced by exogenous applications of CTS and SPD. The study did not explicitly investigate the exclusion mechanism of Cd by CTS and SPD. However, it is likely that these compounds interact with Cd ions, reducing their bioavailability and uptake by the plant. This could involve chelation, complexation, or precipitation reactions. The increased growth and reduced Cd accumulation in treated plants support the idea that CTS and SPD can effectively mitigate Cd toxicity.

The mechanism behind CTS and SPD synergistic application in alleviating cadmium (Cd) stress in *Lactuca sativa* could involve complex biochemical and physiological processes. Chitosan effectively chelates Cd, reducing its bioavailability and limiting uptake by plant tissues. Furthermore, it fortifies cell walls, creating a barrier against

Cd intrusion and enhancing structural integrity[93]. Conversely, spermidine stabilizes cellular membranes and maintains ion homeostasis, often disrupted by Cd stress[94]. The synergistic action of CTS and SPD modulate the antioxidant defense system by upregulating key enzymes, thereby mitigating oxidative damage from ROS under Cd toxicity[95]. In this study their combined treatment also enhanced photosynthetic efficiency by preserving chlorophyll content, ensuring energy production despite Cd stress. These findings reinforce the effectiveness of CTS and SPD combined treatment in eliminating Cd stress. Foliar application of CTS and SPD can be a cost-effective and environmentally friendly approach to mitigate Cd toxicity in lettuce and other crops. This strategy could be helpful in improving plant growth, yield, and quality, even in Cd-contaminated soils. Further research is needed to optimize the application rates and timing of these treatments. For practical implication of this study, it is essential to conduct field trials under various environmental conditions. These trials should assess the long-term effects of CTS and SPD on plant growth, yield, and fruit quality. While the study provides valuable insights into the short-term effects of CTS and SPD, it is crucial to investigate their long-term impacts on plant growth, yield, and soil health.

Conclusion

This study revealed that Cd stress posed negative impact on the morphological, physiological, and biochemical attributes of both *L. sativa* varieties. While chitosan and spermidine application either alone or synergistic efficiently mitigated the toxic effects of Cd stress. Cd stress resulted in significant accumulation of Cd^{2+} ions in the shoot of both varieties. It also significantly impaired growth, biomass, gas exchange, water relation, antioxidant activities and nutrient uptake in both varieties. Foliar application of both chitosan and spermidine significantly improved growth, biomass, chlorophyll content, photosynthesis rate, stomatal conductance, water content, antioxidant activities and nutrient uptake in both control and stressed plants. These treatments significantly reduced stress indicators including relative membrane permeability, H_2O_2 and malondialdehyde content in stressed plants, compared with respective controls. These findings showed that chitosan and spermidine foliar spray effectively mitigated the Cd toxicity in both *L. sativa* varieties and improved their growth under stress condition. This study provides insight into the potential use of chitosan and spermidine foliar spray as sustainable tools for improving Cd resilience in crop plants. While the study provides valuable insights into the short-term effects of CTS and SPD, it is crucial to investigate their long-term impacts on plant growth, yield, and soil health.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Received: 12 September 2024; Accepted: 10 March 2025
Published online: 17 March 2025

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Acknowledgements

The authors would like to extend their sincere appreciation to the Researchers Supporting Project Number (RSP2025R182) King Saud University, Riyadh, Saudi Arabia.

Author contributions

MR; Experimentation and Methodology, ZN; Supervision and Validation, SU; writing-original draft preparation and Statistical analysis, AAS; Conceptualization and Review, drafting, HT; Formal analysis, MAES; Conceptualization, data curation and review, IJL; writing-revised draft preparation. All authors read and approved the final

manuscript.

Funding
Researchers Supporting Project Number (RSP2025R182) King Saud University, Riyadh, Saudi Arabia.

Declarations

Competing interests
The authors declare no competing interests.

Ethical approval and consent to participate
We declare that the manuscript reporting studies do not involve any human participants, human data or human tissues. So, it is not applicable. Our experiment follows with the relevant institutional, national, and international guidelines and legislation.

Consent for publication
Not applicable.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-93672-4.

Correspondence and requests for materials should be addressed to Z.N., S.U., A.A.S. or I.J.L.

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