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Efficacy of γ-Aminobutyric Acid (GABA) on Physio-biochemical Attributes of Lettuce *(Lactuca sativa* L.) Under Cadmium Toxicity

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Abstract

Cadmium can cause harmful effects in plants, even at low concentrations as it's easily enters the plant from the soil solution through root uptake. Current research was conducted to explore the potential of Gamma amino butyric acid in mitigation of Cadmium (Cd) toxicity in *Lactuca sativa*. Two varieties of *Lactuca sativa* (the iceberg and lettuce) were exposed to Cd concentrations i.e., (250 µM) and GABA (100 µM) to examined different morphological, physiological, biochemical parameter, antioxidant activity and mineral content of lettuce. Both varieties of Lettuce (*Lactuca sativa* L.) when treated with 250 µM Cd, it showed reduction in growth and physio-chemical parameters. Cd accumulates by roots and causes a noticeable reduction in length of root and it transfer to aerial parts and declines the photosynthesis process which ultimately, result in reduction of plant growth and yield. Cadmium exposed *L. sativa* seedlings showed elevation in malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content. On other hand, GABA supplementation increased growth of *L. sativa* plants beside incrementation in the activity of antioxidant enzymes (POD, CAT and POX), protein accumulation and proline content. GABA treatment induced tolerance in *L. sativa* plants treated with cadmium stress is credited to enhanced activity of antioxidant enzymes and reduced levels of oxidative stress markers (MDA, H_2O_2). Exposure to GABA also showed increased level of proline and total soluble proteins in *L. sativa* plants grown in normal and Cd-contaminated conditions.

Keywords Plants · Cadmium · Gamma aminobutyric acid · Lettuce

Introduction

Industrialization has become a major environmental concern in Pakistan and is poisoning the soil and water contaminated by heavy metals. Annually, about 20 thousand tonne Cadmium produced worldwide (Afzal et al. 2014). For the growth and development of plants, Cadmium is not an essential element (Hasan et al. 2015). Excessive assimilation of Cd in plants can cause injurious effects (Qi et al. 2020). Cadmium is a hazardous heavy metal found in agricultural soil that can damage crop development and yield (Zulfiqar et al. 2021). Toxicity of Cadmium also causes numerous nutrient imbalances by reducing the intake of essential mineral nutrients by plants (Rizwan et al. 2016).

In plants, Cd causes oxidative stress, disturb the activity of growth regulator and inhibit the growth and chromosomal aberrations (Pizzaia et al. 2019). Cadmium reduced the growth and productivity of many agronomic crops (Genchi et al. 2020). Cd is unfit for the proper health of plant because of its water soluble nature. Because of its

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toxicity and movement from soil to plant, this has gotten a lot of people's attention in recent years. Cadmium, even in trace amounts, is toxic to plants. cadmium high concentration effects the opening and closing of stomata due to disturbance of other essential ions uptake as a result of which plants start to secret wax on their surface to protect plants tissues (Rai et al. 2005).

Cadmium is extremely toxic for the living organism because of its unnecessary increase and due to its high solubility in biological systems. Phosphate fertilizers, sludge, and contributions from the mining and smelting industries all contribute to cadmium poisoning (Kaya et al. 2020). Cadmium poisoning disrupts hormonal and nutritional balance as well. Infertility, renal and bone problems, and cancer in humans are all possible side effects of Cd exposures (Soares et al. 2019). In the end, the toxic effect of cadmium damage the plant growth and metabolism in general (Qadir et al. 2014). The most prominent symptoms of Cd toxicity is stunting and chlorosis (Zhang & Shu 2006).

Human health is endangered by daily eating of Cd-contaminated products (Varun et al. 2017). Its great thread to human health because it is present in all kind of soil and easily become the part of food chain. Cd transporters also engage in the movement of important minerals like Zinc, iron, Manganese, or Copper complicates reducing Cd accumulation in vegetation (Huang et al. 2020). Consumption of heavy metal contaminated vegetables can cause to various long term illness including emphysema, bronchiolitis, and alveolitis as well as short term conditions like kidney, cardiovascular, nervous, and bone diseases. As a result of anthropogenic activities, Cd is unnecessary and highly harmful heavy metal in the environment. Because the Cd inhibits the plant from absorbing important nutrients, plant growth is reduced. (Shahzad et al. 2021).

Vegetables are important aspect of Pakistan's multiple agricultural system, according to the ministry of food, agriculture, and livestock's statistics on agriculture. Vegetable production is well-suited to the soil and climate conditions In Pakistan. Pakistan is incredibly diverse zoon and various types of veggies are cultivated (Asi et al. 2020). The industry of vegetables in Pakistan cope with the problem of stress by selecting the resistance varieties and also by growing the vegetables in greenhouse or controlled conditions (Nawaz et al. 2017). And the nature of plants is show specific and non-specific behaviour under stress condition (Haag 2013). Cadmium has excessive transportability and bioavailability. So, it enters the food chain via the ingestion of various vegetables, cereal grains obtained from Cd tainted soil (Awan et al. 2020).

Lactuca sativa is considered a highly nutritional and nutritional rich food (Senizza et al. 2020). Lettuce contains beneficial nutrients and compounds that boost health, such as fibre, iron, folate, fat and having low calories. lettuce has a high amount of antioxidants, such as tocopherols, which can minimize the generation of ROS and thus also prevent cellular components (Zhou et al. 2021). Lettuce (*Lactuca sativa* L.) is also having prominent Cd contents in their shoot, this naturally develop resistance protect the plant against the Cd toxicity. Lettuce is use in most of research due also bio-accumulator of Cd therefore it used mostly in research (Wang et al. 2020).

Plants use different copping techniques to decrease the Cd uptake from soil to understand the physiological response induced by Cd And to mitigate the Cd induced stress plant using biochemical and mechanical techniques (Lavres et al. 2019). Cadmium toxicity lowers food and water intake, enhance ROS, disturbs plant morphology, metabolic pathways and also negatively affect the plant physiology. Plants counteract these effects by activating enzymatic and non-enzymatic phytoconstituents (Haider et al. 2021). Under stress, this equilibrium is disturbed, leading to an elevation in ROS levels (Mansoor et al. 2022). It's important to look for an environmentally suitable way to get rid of heavy metal deposit in vegetables. For this purpose exogenous application of different plant protectants, hormones and organic acid are employed (Nayyar et al. 2014). like that GABA is also very important in ameliorating the heavy metal stress (Fromm 2020).

Gamma-Aminobutyric acid (GABA) level increase in plants under the stress of heavy metals. GABA's activity increases when plant supplemented with Cd for 24 h. that enhance the Cd tolerance in plant (Yang et al. 2020). In stress period, antioxidative enzymatic activity in plants also enhanced (Song et al. 2010). Furthermore, GABA increased the activities of antioxidative enzyme, that results in a considerable decline in H_2O_2 and MDA under stress (Seifikalhor et al. 2020a, b). GABA protects the plant from abiotic challenges by boosting leaf turgor, osmolytes, and antioxidant stimulation, as well as coping with oxidative damage.

The objective of present work was to study GABA treatments effect in improving tolerance against cadmium stress in *L. sativa* L. The objectives of the present study were to: Study the different growth parameters of lettuce varieties under Cd stress; Assess different physiological and biochemical attributes to identify GABA- controlled tolerance mechanisms against Cd; Explore the levels of GABA treatment effective in improving tolerance against Cd stress in *L. sativa*; examine the regulatory role of GABA in *L. sativa* tolerance to Cd stress.

Table 1 Three group of treatment were considered during experiment an one group for controlled

Sr. No	Treatment	Abbreviation	Concentration
1	Controlled	–	–
2	Gamma aminobutyric acid	GABA	(0 and 100 μM)
3	Cadmium chloride	CdCl2	(0 and 250 μM))
4	Combination of gamma aminobutyric acid and cadmium chloride	GABA + CdCl2	(100 μM + 250 μM)

Materials and Methods

Plant Material and Growth Conditions

Lettuce was planted in the University of Education's field area. All physico-chemical parameters were evaluated in the laboratories of Departments of Botany and zoology. *Lactuca sativa* L. and Iceberg (*Lactuca sativa* var. Capitata) are the Lettuce varieties used for the experiment. The first variety was taken from AL PAK nursery Township and second variety name Iceberg was taken from Model Town nursery in Lahore. All experiments were conducted in a complete randomized design with three replicate. Plastic pots were collected from the township market, Lahore. Using a red hot rod, a two-inch hole was drilled in the bottom of each pot, and cotton wool was placed over the top to avoid sand waste and easy drainage of excess water. Each pot had a length of twelve inches and a width of nine inches. Each variety have equal 12 pots in total (Table 1).

Plantation and Thinning

In the fourth week of November seedlings were planted. The significant increase in growth after one week of plantation. Thinning was performed after 3 week of seedlings plantation. Six plants of uniform growth were selected per pot after careful observation of their growth conditions.

Treatments and Source of Treatments

After five weeks of plantation, Cd stress was applied to their respective plants according to the experimental layout. Then, according to the experimental plan, 12 pots were treated with Cadmium Chloride ($CdCl_2$). Supplementing 1 L Cadmium chloride in typical Hoagland's nutritional applied to each pot. After that 100 μM GABA was mixed well in 4 L distilled water and with help of plastic shower spray efficiently to each pot. Single plant was taken for study of growth parameters after 7 days of treatment application from each container and placed in labelled zipper bags. Roots were cleaned with regular tap water after all samples were collected. After that, a knife was used to separate the roots and shoots.

Growth parameters

Following sampling, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight was recorded using digital electronic weighing balance.

Determination of Photosynthetic Pigments

Chlorophyll and Carotenoid Contents

Arnon's (1949) method was used to determine the content of carotenoids and chlorophyll. Samples were taken by picking freshly grown lettuce leaves and weighing 0.05 g in a digital balance. Each sample was crushed in pestle and mortal in the solution of 10 mL Acetone (80%) and filter by using Whatman's filter paper No. 42. Using a spectrophotometer (Model T60UV) absorbance was note down at 480, 645, and 663 nm.

Following formula use for calculations;

$$\text{Chlorophyll a } = [12.7(A663) - 2.69(A645)] \times V1000 \times W$$

$$\text{Chlorophyll b} = 122.9(A645) - 4.68(A663)] \times V/1000 \times W$$

Carotenoid content was calculated using equation (Lichtenthaler, 1987) with a little variation in formula as follows;

$$\text{Carotenoids } = (1000x0D480nm) - (1.9 * \text{Ch1 a} \\ -63.14 * \text{Chl b})/214$$

Cell Membrane Injury (CMI)

Method of Yang et al., (1996) was use to determine the cell membrane injury. The uniform leaves sample from each pot were plucked and put in test tubes each containing 20 mL deionized distilled water. Before being tested for initial electrical conductivity (EC_0) the solution was vortexed for 5 s. After a day of keeping these tubes at 4 degrees Celsius, the EC_1 was measured. To estimate EC_2 autoclaved the same sample at 120 °C for 24 min.

Calculation of cell membrane injury by using following formula:

CMI (%) = [EC_1-EC_o/EC_2-EC_0] × 100.

Relative Water Content (RWC)

Leaves of same size from all replica were used to determine the water content in leaves. The leaves were weighed and then dipped into a Petri dish with double distilled deionized. Submerge the leaves in water for 4 h in the absence of light. First measured the turgor weight of the leaves and note it down. Then after drying the leaf for 24 h at 85 °C assessed the dry weight.

The relative water content of leaves was obtained by Jones and Turner's (1978) formula:

$$\text{Relative water content (\%)} = \left[(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})\right] \times 100,$$

where oven dry is indicating as DW, fresh weight in FW and turgid weights express as TW.

Determination of Hydrogen Peroxide (H_2O_2)

Following the Velikova et al.,(2000) method used for the estimation of hydrogen peroxide in the plant tissues. Fresh leaf tissue (0.5 g) was blended with 5 mL of 0.1 % (w/v) trichloroacetic acid in a pre-chilled pestle and mortar (TCA). Using the centrifuge machine (Model Z326k, HERMLE Company), the homogenate was centrifuge for 20 min at 12,000 rpm. Take the supernatant and mix it with 0.5 mL potassium phosphate buffer (pH 7.0) and 1 mL potassium iodide (KI). To read the absorbance of the mixture at 390 nm, UV–Visible spectrophotometer (Model T60UV) was used.

Determination of Malondialdehyde (MDA) Content

Cakmak and Horst (1991) protocol used for lipid peroxidation that measured in terms of thiobarbituric acid-reactive substances (TBARS) concentrations, with some slight modifications. Fresh leaf (0.5 g) was homogenized at 4 °C in 3 mL trichloroacetic acid (1.0 % w/v). 1.5 mL supernatant was added in a 20 percent TCA solution with 3 mL 0.5 % (v/v) thiobarbituric acid (TBA). After 50 min of incubation at 95 °C in a shaking water bath, the reaction was terminated by lowering the temperature of test tube. Then centrifuged at 10,000 rpm for 15 min, and measured the absorbance of supernatant at 532 nm with spectrophotometer. The value of non-specific absorption at 600 nm was subtracted from all reading taken. The absorption coefficient 155 mmol^{-1} cm.^{-1} used for the calculation of concentration of thiobarbituric acid-reactive substances (TBARS)

$$\text{MDA} = \Delta\,(\text{Abs 532nm} - \text{Abs 600nm})/1.56 \times 10^5$$

Note: Unit is (nmol) use for the level of MDA.

Determination of Total Phenolics

By following the Julkenen–Titto (1985) determined the total phenolic by using Folin–Ciocalteau reagent. Fresh leaf samples (50 mg) were crushed in an 80% acetone solution and centrifuged at 10,000 rpm for 10 min. After adding 5 mL of 20% sodium carbonate in Folic-phenolic Ciocalteau's reagent (1 mL), 2 mL water, and 0.1 mL supernatant vortex, the amount was increased to 10 mL with distilled water. A UV–Visible spectrophotometer was used to read the absorbance at 750 nm after the contents were vortexed. The results are expressed in mg/g of fresh leaf.

Estimation of Leaf Free Proline Determination

Bates et al., (1973) technique was used to estimate proline amount. Fresh leaf (0.5 g) was crushed with 10 mL of 3% sulfo-salicylic acid. A Whatman's filter paper was used for filter the extract. Then, in a separate test tube, 2.0 mL of acid ninhydrin (1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL of 6 M ortho-phosphoric acid) is added to the 2 ml of filtrate. The material was heated for 1 h at 100 degrees Celsius. The temperature of the mixture is then lowered. After that, 4 mL of toluene was added to the solution and stirred for 2–3 min with a continuous stream of air. A coloured toluene layer was extracted after adjusting the temperature of the solution to room temperature and reading the absorbance at 520 nm on a UV–Visible spectrophotometer (Model T60UV). As a blank, toluene was used. The concentration of proline was calculated using a standard curve as follows:- µmole proline g^{-1} fresh weight = (µg proline mL^{-1} × mL of toluene/115.5)/ (g of sample)

Determination of Total Soluble Proteins

At first step 0.1 g Coomassie brilliant blue, 50 mL ethanol (95%), 100 mL ortho-phosphoric acid (85%) were combined to 1 L distilled for making of one liter of Bradford reagent which is use in protein estimation. Fresh leaf (0.5 g) was crushed in 10 mL of cold phosphate buffer (50 mM) of (pH 7.8) on ice bath. After that, using a centrifuge machine centrifuges the mixture for 20 min at 6000 rpm at 4 °C. The extract was then subjected to more analysis. Protein content in leaf extracts was determined using Bradford's (1976) approach. In test tubes, we put 2 mL of Bradford reagent and 0.1 mL of leaf extracts. Each sample's absorbance was measured at 595 nm using a spectrophotometer (Model T60UV) after being held at room temperature for 5 min.

Determination of Antioxidant Enzymes Activities

Fresh leaves (0.5 g) were mashed in 10 ml of cold phosphate buffer (50 mM) with (7.8 pH) on an ice bath. The mixture was centrifuge at 10,000 rpm for 25 min at 4 degrees using centrifuge equipment. The antioxidant enzyme activities were determined using the supernatants.

Catalase Activity

Catalase activities in lettuce were calculated using the Chance and Maehly (1955) method. In test tubes, phosphate buffer (50 mM) and hydrogen peroxide (5.9 mM) with 7.0 pH were used to make a solution for catalase activity. The reaction was started in a Cuvette with 0.1 mL enzyme extract. The spectrophotometer (Model T60UV) was used to record the differences in absorbance measurements at each 20-s interval at 240 nm. An absorbance change of 0.01units/min equaled one unit of catalase activity.

Peroxidase activity

The 3 ml reaction mixture for peroxidase dismutase was made in test tubes using phosphate buffer (50 mM) with (5.0 pH), guaiacol (20 mM), and H_2O_2 (40 mM). Adding 0.1 mL enzyme extract to the Cuvette, the reaction began. After 20 s variations in the absorbance were noted at 470 nm wavelength on the spectrophotometer (Model T60UV). Absorbance change of 0.01 units per minute equaled one unit peroxidase activity. On the basis of soluble protein, enzyme activity was evaluated.

Ascorbate Peroxidase Activity

Nakano and Asada (1981) method was used to calculate the enzyme activity of ascorbate peroxidase. In test tubes, a reaction solution containing hydrogen peroxidase (300 mM), ascorbate (7.5 mM), and 100 percent sodium phosphate (50 mM) was prepared to determine APX. The activity of the enzyme was initiated in the Cuvette by a 0.1 mm extract of the enzyme in the reaction solution. By noticing the drop in absorbance level at 290 nm, the H_2O_2 dependent oxidation of ascorbate was determined. Distil water use as a blank ($E = 2.8\ mM^{-1}\ cm^{-1}$).

Determination of Mineral Content

For the measurement of specific minerals, the Chapman and Pratt (1978) method was employed to digest dry lettuce shoots and roots. In a 250 mL digestion flask, 1 g of ground plant material (oven dried at 80 °C) was washed with acid 2 mL sulphuric acid. All samples were placed on a heated plate at 80 °C and H_2O_2 was added drop by drop until orange vapours formed and then turned into a clear white liquid. The samples were then allowed to cool for a few minutes before being diluted with water. Whatman's No 42 was used to filter all of the samples. After that, the filtrates were transferred into volumetric flasks, with the volume of distilled water in the flasks being increased to 50 ml. To evaluate the levels of various ions in plants, all solution samples of lettuce shoot and root digestion were saved.

By using the flame photometer (Sheerwood model 360) the Na^+, K^+, and Ca^+ content in the shoot and root digestion were estimated. Titration was used to determine the value of a solute in a solution by Mohr's method (1938). For the calculation of unknown chloride ions, silver nitrate was utilized as a reagent of known volume 1. The molar ratio of silver ions to chloride ions is 1:1.

In a beaker, 5 m of chloride solution was poured, followed by a few drops of sodium dichromate (0.1 N) as an indicator and adjustment beneath the burette, which was tilled with silver nitrate solution (I M). The colour of the solution changed between the initial and final readings until it ceased changing colour. The solution had reached the equivalence point, when the number of silver ions equaled the amount of chloride ions, as indicated by the reddish colour.

Statistical Analysis

The analysis of variance (ANOVA) of all lettuce (*Lactuca sativa* L.) parameters was performed using the widely used statistical tool CoStat (Cohort software, 1988).

Results

Effect of γ-Aminobutyric Acid on Growth Attributes

ANOVA indicated that under Cd stress, shoot fresh weight decrease 38% in iceberg and 31% in lettuce as compare to control plant but with exogenous GABA application significant increase in shoot fresh weight up to 70% and 89% in both iceberg and lettuce, respectively, as compare to the non-treated plants. GABA increased shoot fresh weight up to 94% in iceberg and 90% in *L. sativa* plants exposed to Cd stress, in comparison of Cd-only treated plants. As far as varieties concern shoot fresh weight of lettuce under GABA application was highest as compare to the iceberg. So collective result was the Cd caused decrease in shoot fresh weight and GABA enhanced shoot fresh weight to many extents. The interaction of three factors (varieties*GABA*cadmium) was also significantly.

Cadmium stress decreases the shoot dry weight up to 56% in iceberg and 46% in lettuce as compare to control plant. In comparison to non-treated plants, GABA spray improves the dry weight of the shoot by 97% and 69% in iceberg and lettuce, respectively. Supplementation of GABA increased shoot dry weight in *L. sativa* cultivars exposed to Cd stress.

250 μM of Cd cause significant decrease in shoot dry weights of two lettuce varieties and GABA restore this loss.

As compare the non-treated plant, under Cd treatment the considerable reduction in root fresh weight of 45% in iceberg lettuce and 32% in lettuce. And under GABA application alone plant show highly significant increase in root fresh weight of both iceberg and lettuce 80% and 66%, respectively as compare to non-treated plant. In contrast to control plants, GABA and Cd combined treatment increases fresh weight of root by 15% and 2% in both iceberg and lettuce, respectively. In interaction of whole three factors (Variety*Cadmium*GABA) was significant.

Under cadmium stress variety lettuce exhibited marked reduction in dry weight of root that was 52% but in iceberg only 37% in lettuce in comparison of control plants. But GABA supplement plant show highly significant increase in root dry weight as compare to without treatment plant that was 85% and 79% in iceberg and lettuce, respectively. And dry biomass of both varieties with combine treatment of GABA and Cd also show highly significant increase specially 226% in iceberg and 93% in lettuce that was different in concern of varieties. The interaction of complete three factors (Variety*Cadmium*GABA) was highly significant.

The result explain that the shoot length decrease under Cd stress up to 27% in iceberg and 39% in lettuce as compare to control plant. But with foliar application of GABA the 65% and 50% increase in iceberg and lettuce, respectively. Combine treatment of GABA and Cd cause significant increase in shoot length as compare to cadmium treated plant such as 188% in ice berg and 176% lettuce. As for varieties concern iceberg show better percentage of shoot length as compare to control by the spray of GABA in both normal and stress condition. Interaction of three factors (Variety*Cadmium*GABA) was non-significant.

Under Cd stress the root length of iceberg decrease 36% and 20% decrease in *L. sativa*. But the exogenous spray of GABA increase the root length up to 52% in iceberg and 46% in lettuce in comparison of control plant. Combine treatment of GABA and Cd increase the root length up to 166% in iceberg and same 110% in lettuce in comparison of Cd stressed plant. So results show that the Cd reduced the root length of plant and exogenous application of GABA was overcome the adverse effect of Cd stress.

The numbers of leaves in iceberg and lettuce declined under Cd stress. In iceberg 30% decrease in no. of leaf under cadmium stress while in lettuce 18% as compare to the control plant. But when plants treated with GABA increased in carotenoids content up to 40% in iceberg and 59% in lettuce in comparison of control plants. The plant in which GABA and cadmium was collectively applied show significant increase of 57% and 38% in both iceberg and lettuce, respectively, as compare to Cd stressed plants. The interactions of three factors was (Variety*Cadmium*GABA) significant (Fig. 1).

Effect of γ-Aminobutyric Acid on Photosynthetic Pigments of *Lactuca Sativa*

Result revealed that the Chl a content of two varieties of lettuce under cadmium stress is decreases up to 29% and 33% in both iceberg and lettuce, respectively, in comparison of controlled plant. And under GABA treatment alone increase in Chl content was highly significant that was 76% in iceberg and 89% in lettuce. But under combine treatment of GABA and Cd iceberg show 65% and lettuce show 84% boost in Chl a content as compare to the Cd treated plants. The interaction of three factors (Variety*Cadmium*GABA) was significant.

The estimation of Chl contents revealed that the Chl a content of two varieties of lettuce under Cd stress is decreases up to 22% and 24% in both iceberg and lettuce, respectively, in comparison of controlled plant. And under GABA treatment alone increase in Chl content was highly significant that was 116% in iceberg and 127% in lettuce. But under combine treatment of GABA and Cd iceberg show minor increase of only 110% and lettuce show 133% increase in chlorophyll content as compare to the plants treaded with Cd. The interaction of three factors (Variety*Cadmium*GABA) was significant (Fig. 2).

Under cadmium stress total Chl contents of plant decreased in both varieties about 40% in lettuce and 22% in iceberg as compare to non-treated plants. The foliar application of GABA increases the total Chl content up to 63% in iceberg and 92% in lettuce in comparison of non-treated plant. Combine treatment of GABA and Cd also increase the catalase activity in iceberg and lettuce that was 113% and 101% in both iceberg and lettuce in comparison of Cd treated plants. The results show that cadmium GABA and their combine treatment increase the total carotenoid content. Interaction of factors (varieties*GABA*cadmium) was significant.

The results revealed that carotenoids contents in iceberg and lettuce both varieties show decreased under Cd stress. In iceberg 42% decrease in carotenoids under cadmium stress while in lettuce 35% as compare to the control plant. But when plants treated with GABA increased in carotenoids content up to 38% in iceberg and 85% in *L. sativa* in comparison of control plants. The plant in which GABA and cadmium was collectively applied show significant increase of 118% and 185% in both iceberg and lettuce, respectively, as compare to Cd stressed plants. So results are indicated that the Cd reduced the carotenoids and GABA increase its level in both normal and stress condition. The interactions of three factors was (Variety*Cadmium*GABA) show significant result.

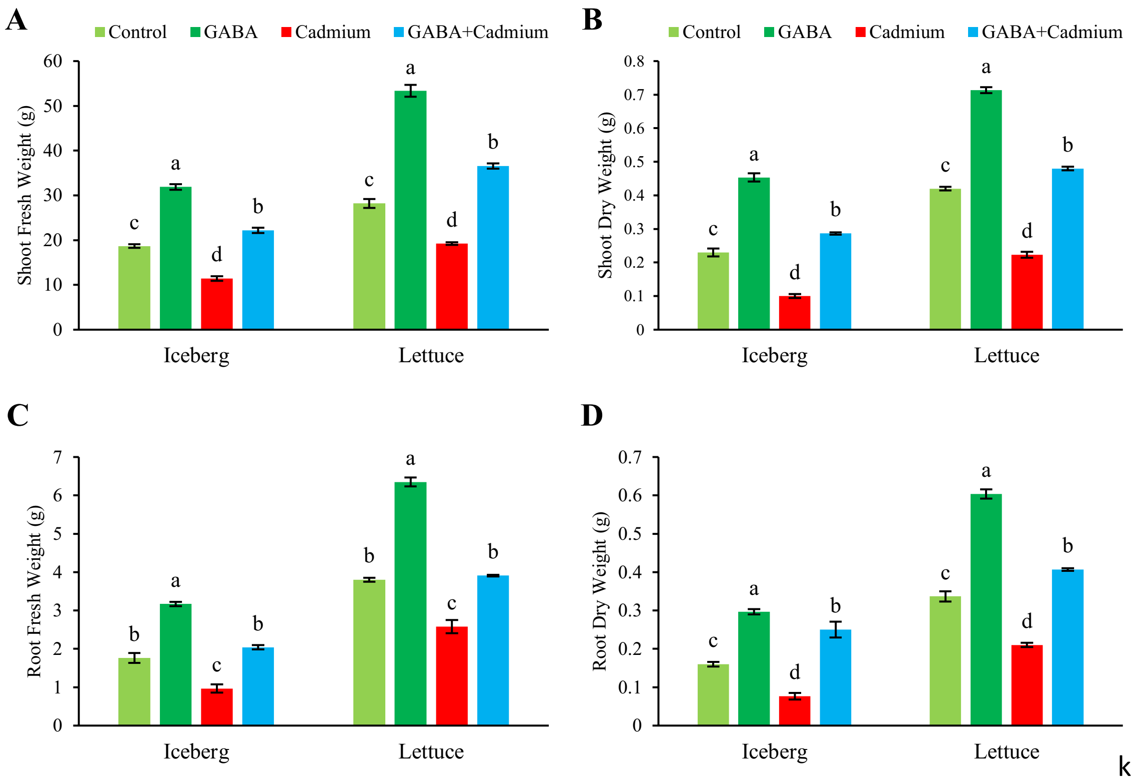


Fig. 1 Effect of foliar application of 100 µM GABA on shoot fresh weight, shoot dry weight, root fresh weight and root dry weight of six weeks old iceberg and lettuce (*Lactuca sativa* L.) plants under 250 µM Cd stress

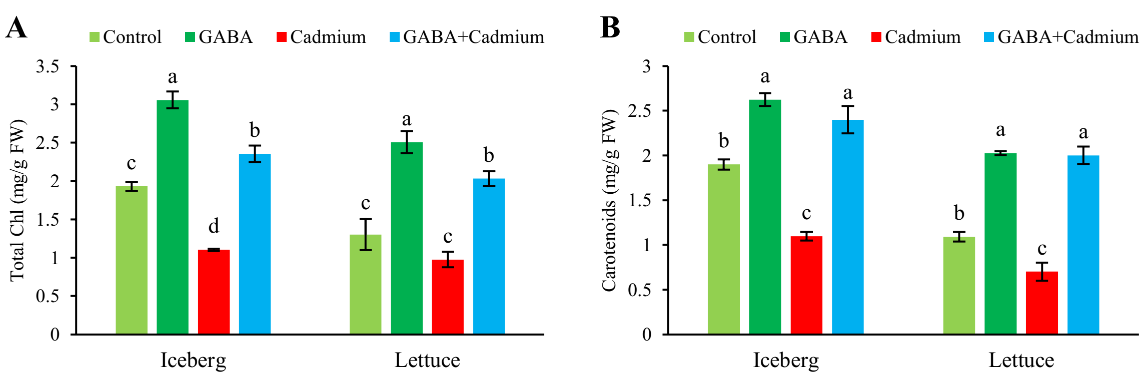


Fig. 2 Effect of foliar application of 100 µM GABA on total chlorophyll and carotenoids of six weeks old iceberg and lettuce (*Lactuca sativa* L.) plants under 250 µM Cd stress

Effect of γ-Aminobutyric Acid on Relative Water content of *Lactuca Sativa*

Results explained that both varieties of lettuce (*Lactuca sativa* L.) show almost same kind of behaviour toward the Cd stress i.e. 26% and 29% declined in relative water content in iceberg and lettuce, respectively, as compare to the non-treated plant. And also show non-significant results in interaction of varieties and GABA because only 26% increase in RWC in iceberg and 20% in lettuce under GABA spray alone. But 45% increase in RWC in iceberg and 50% in lettuce under combine treatment of Cd and exogenous GABA application in contrast of Cd stress plants (Fig. 3).

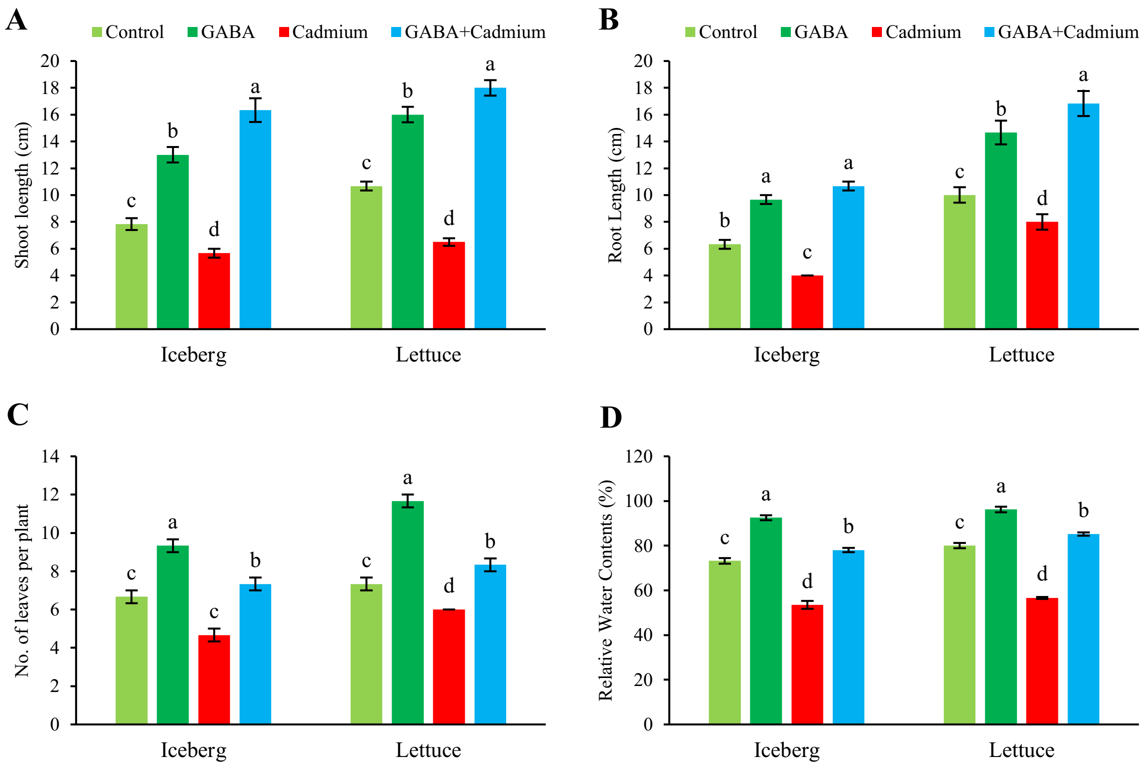


Fig. 3 Effect of foliar application of 100 µM GABA on shoot length, root length, number of leaves and relative water contents of six weeks old iceberg and lettuce (*Lactuca sativa* L.) plants under 250 µM Cd stress

Effect of γ-Aminobutyric Acid on Cell Membrane Injury of *Lactuca Sativa*

The application of Cd stress notably increase the cell membrane injury of the plant that was 47% in iceberg and 42% in lettuce as compare to control plants. GABA shows the less cell membrane injury in plants in comparison of control plant that was 61% and 49% in iceberg and lettuce respectively. GABA show marked decrease in cell membrane injury which was 67% in iceberg and in lettuce plants grown in Cd-toxic conditions, as compared to Cd stressed plants. The interaction of whole three factors (Variety*Cadmium*GABA) was non-significant.

Effect of γ-Aminobutyric Acid on Malondialdehyde Content of *Lactuca Sativa*

Results depicted significant increases in MDA in both iceberg and lettuce 100% and 23%, respectively, under Cd stress as compare to non-treated plant. But GABA application was reduce the MDA content up to 26% in iceberg and 21% in lettuce in comparison of plants without GABA spray. In case of combine treatment of GABA and cadmium the MDA reduce up to 43% in iceberg and 41% in lettuce in contrast of plant with Cd stress alone. So results show that Cd stress increase the MDA contents in plants but GABA supplementation reduced MDA content in *L. sativa* plants.

Effect of γ-Aminobutyric Acid on Hydrogen Peroxide Content of *Lactuca Sativa*

The result revealed that the significant increases in H_2O_2 in both iceberg and lettuce 120% and 97%, respectively, under Cd stress as compare to non-treated plant. But GABA application cause decrease in H_2O_2 content up to 19% in iceberg and 13% in lettuce in comparison of plants control plants. In case of combine treatment of GABA and cadmium the H_2O_2 reduce up to 40% in iceberg and 41% in lettuce in contrast of plant with Cd stress. So results show that the Cd stress increased the H_2O_2 contents in plants but GABA show reduction in H_2O_2 contents. The interaction of three factors (Variety*Cadmium*GABA) was non-significant (Fig. 4).

Effect of γ-Aminobutyric Acid on Free Proline Content of *Lactuca Sativa*

Figure 5 indicating the effect of cadmium on two varieties of lettuce. Under Cd stress proline content increases in both

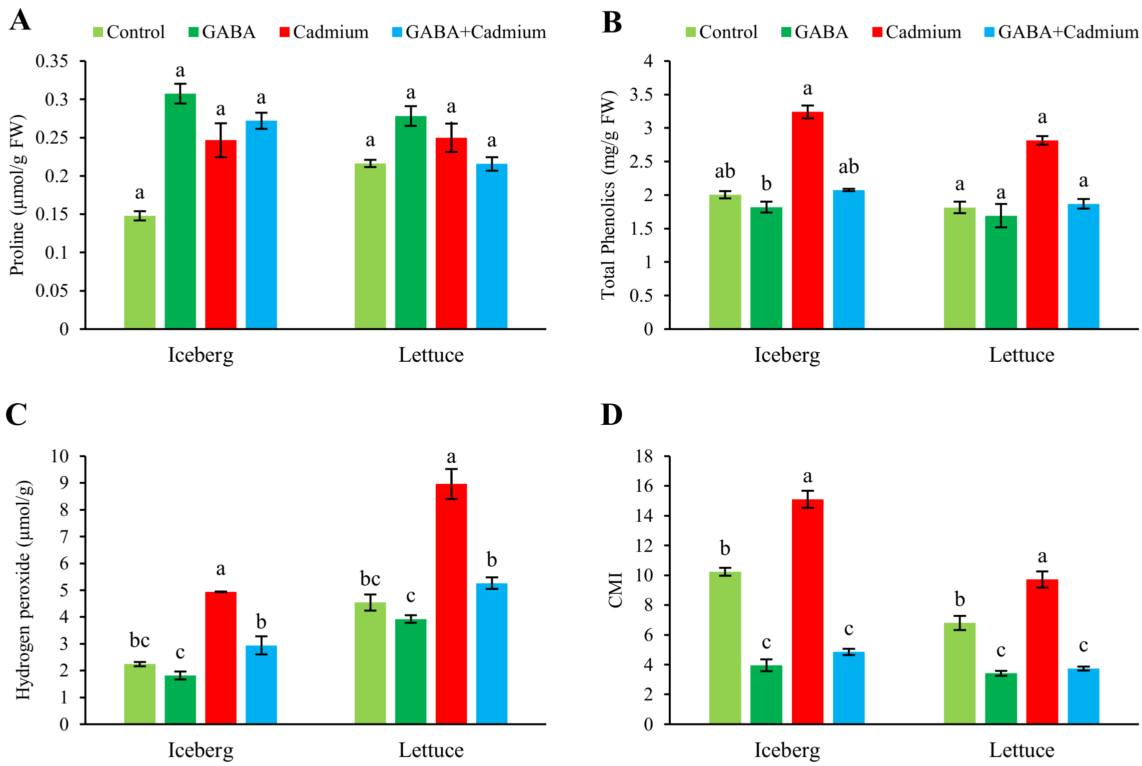


Fig. 4 Effect of foliar application of 100 µM GABA on proline, total phenolics, hydrogen peroxide and CMI of six weeks old iceberg and lettuce (*Lactuca sativa* L.) plants under 250 µM Cd stress

varieties about 48% in lettuce and 33% in iceberg as compare to non-treated plants. The foliar application of GABA increases the proline up to 24% in iceberg and 20% in lettuce in comparison of control plants. Combine treatment of GABA and Cd also increase the proline content in iceberg and lettuce that was 23% and 25%, respectively, in contrast of control plants. The results show that the cadmium GABA and their combine treatment increase the proline content. Interaction of factors (varieties*GABA*cadmium) was significant.

Effect of γ-Aminobutyric Acid on Total Phenolic Content of *Lactuca Sativa*

Under Cd stressed conditions, total phenolic was increase in both varieties such as 61% increase in total phenolic of iceberg and 55% increase was observed in lettuce as compare to control plant. And GABA show down regulation in phenolic content such as 9% decreased was observed in iceberg and 6% in lettuce as compare to non-treated plants. But 35% and 33% decrease was noticed in iceberg and lettuce, respectively, under combine treatment of Cd and GABA as compare to cadmium stressed plant. The interaction of three factor (varieties*GABA*cadmium) was non-significant.

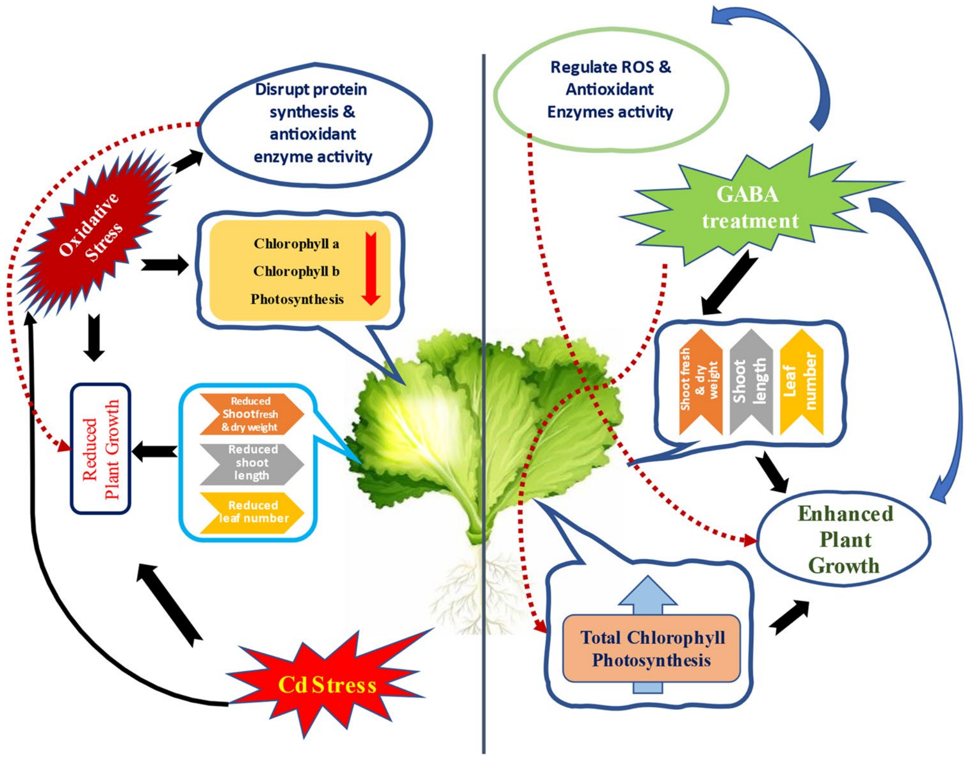
Effect of γ-Aminobutyric Acid on Total Soluble Proteins of *Lactuca Sativa*

Under cadmium stress total soluble protein decreased in both varieties such as 41% in iceberg and 48% in lettuce in comparison of control plants. Application of GABA increased the total soluble protein 96% in iceberg and 60% in lettuce in contrast of control plants. Combine treatment of GABA and Cd stress show the increased in the total soluble proteins such as 92% and 119% in iceberg and lettuce, respectively, as compare to Cd stressed plants. According to results the Cd stress cause significant decreased in total soluble protein. The interaction of three factors (Variety*Cadmium*GABA) was non-significant.

Effect of γ-Aminobutyric Acid on Activity of Antioxidative Enzymes of *Lactuca Sativa*

Foliar application of GABA increased the activity of POD in iceberg and lettuce by 4% and 3%, respectively, as compared to control treatment. Cadmium toxicity increased POD activity in iceberg and lettuce by 27% and 63%, respectively, as compared to control treatment. Combine treatment of GABA and Cd also cause significantly increase POD activity in iceberg and lettuce 7% and 17%, respectively, as compare to

Fig. 5 Schematic diagram showing the role of γ-aminobutyric acid on biochemical attributes of *Lactuca sativa* exposed to Cd stress



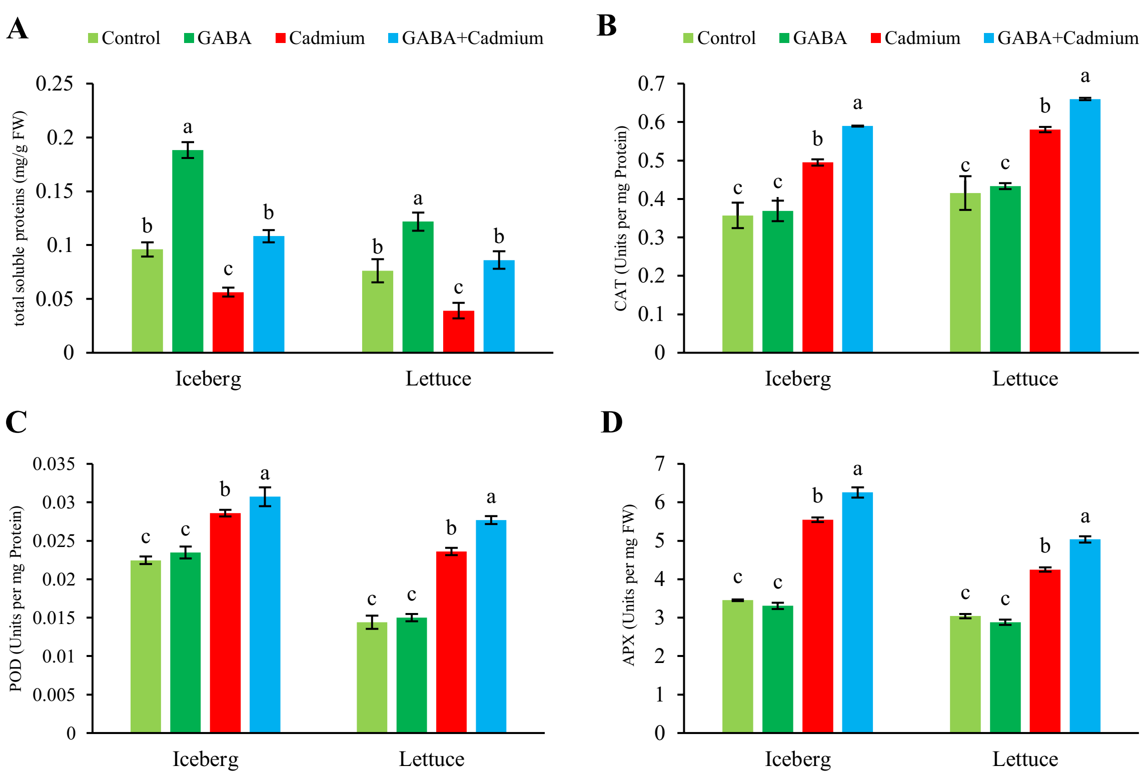


Fig. 6 Effect of foliar application of 100 µM GABA on total soluble protein, CAT, POD and APX of six weeks old iceberg and lettuce (*Lactuca sativa* L.) plants under 250 µM Cd stress

Table 2 Sodium content in root of six weeks old two varieties of lettuce (*Lactuca sativa* L) under cadmium stress and foliar application of GABA

Source of variation	DF	Mean square of sodium in roots
Variety	1	0.04335***
Cadmium	1	0.01401**
GABA	1	0.02161**
Variety*cadmium	1	0.00135 ns
Variety*GABA	1	0.00026 ns
Cadmium*GABA	1	0.00426 ns
Variety*Cadmium*GABA	1	0.00026 ns

* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.01$

ns means $p \geq 0.05$ (or non-significant)

Table 3 Potassium content of shoot of six weeks old two varieties of lettuce (*Lactuca sativa* L.) under cadmium stress and levels of GABA

Source of variation	DF	Mean square of potassium in shoot
Variety	1	0.0057042 ns
Cadmium	1	3.248704***
GABA	1	5.0142042***
Variety*cadmium	1	0.0000375 ns
Variety*GABA	1	0.0022042 ns
Cadmium*GABA	1	0.549037***
Variety*Cadmium*GABA	1	0.16170421*

* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.01$

ns means $p \geq 0.05$ (or non-significant)

Table 4 Potassium content in root of six weeks old two varieties of lettuce(*Lactuca sativa* L) under cadmium stress and foliar application of GABA

Source of variation	DF	Mean square of potassium in roots
Variety	1	0.0022042 ns
Cadmium	1	0.825142***
GABA	1	0.595042***
Variety*cadmium	1	0.095042**
Variety*GABA	1	0.000935 ns
Cadmium*GABA	1	0.0532041*
Variety*Cadmium*GABA	1	0.010837 ns

* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.01$

ns means $p \geq 0.05$ (or non-significant)

control treatment. Cadmium stress also enhanced the activity of CAT enzymes in both varieties of *L. sativa*. In case of *L. sativa* plants grown in Cd-contaminated soil, GABA application increased CAT activity in iceberg and lettuce varieties by 19% and 13% as compared to Cd-only treatment. Cadmium stress also increased APX activity in both iceberg and lettuce 60% and 39%, respectively, in contrast of control plants. GABA application alone don't show any prominent response only 4% and 5% decreased in APX activity as compare to non-treated plant. Application of GABA enhanced the APX activity up to 12% in iceberg and 18% in *L. sativa* grown in Cd-contaminated soil as compare to Cd-treatment without GABA (Fig 6).

Effect of γ-Aminobutyric Acid on Mineral Content of *Lactuca Sativa*

Cadmium stress increased sodium content in iceberg and lettuce varieties by 26% and 56% as compared to control conditions. Foliar application of GABA reduced sodium content in *L. sativa* varieties in comparison with control treatment. In case of *L. sativa* plants exposed to Cd stress, GABA reduced sodium content in iceberg and lettuce varieties by 20% and 12%, respectively, as compared to control plants.

Cadmium stress increased sodium content in *L. sativa* varieties. And the foliar application of GABA reduce only 13% and 9% in root sodium content in iceberg and lettuce, respectively, as compare to the control plants. When GABA and Cd was supplied collectively then the 22% and 24% reduction in sodium content was observed as compare to Cd stressed plant. The interaction of all three factors (varieties*GABA*cadmium) was non-significant (Table 2).

ANOVA exposed that the significant increases in potassium of shoot in both iceberg and lettuce 38% and 57%, respectively, with foliar application of GABA as compare to non-treated plant. But under Cd stress potassium content was reduce up to 26% in iceberg and 21% in lettuce in comparison of plants without treatment plants. In case of combine treatment of GABA and Cd the potassium content enhanced up to 43% in iceberg and 41% in lettuce in contrast of plant with Cd stress alone (Table 3).

The ANOVA show that the highly significant result of two varieties of lettuce (*Lactuca sativa* L.) in concern potassium content. Under Cd stress variety lettuce exhibited noticeable reduction in potassium content of root that was 38% but in iceberg only 22% in lettuce in comparison of control plants. But GABA supplement plant show highly significant increase in potassium content in root as compare to without treatment plant that was 47% and 44% in iceberg and lettuce, respectively. And K^+ content of both varieties with combine treatment of GABA and Cd also show highly significant increase specially 29% in iceberg and 42% in lettuce that was different in concern of varieties. So results show that the Cd stresses reduce the potassium contents in plants but GABA show reveres behavior. The interaction of three factors (Variety*Cadmium*GABA) was highly significant (Table 4).

Table 5 Calcium content of six weeks old shoot of two varieties of lettuce (*Lactuca sativa* L.) under cadmium stress and foliar application of GABA

Source of variation	DF	Mean square of Calcium in shoot
Variety	1	0.0084375*
Cadmium	1	0.023437***
GABA	1	0.0126041**
Variety*cadmium	1	0.0026042 ns
Variety*GABA	1	0.0001041 ns
Cadmium*GABA	1	0.0026042 ns
Variety*Cadmium*GABA	1	0.0001047 ns

* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.01$

ns means $p \geq 0.05$ (or non-significant)

Under Cd stress, calcium content reduced in both varieties such as 41% in iceberg and 38% in lettuce as compare to control plant. And GABA upregulate in calcium contents such as 16% in iceberg and 9% in lettuce as compare to non-treated plants. But 57% and 15% decrease was noticed in iceberg and lettuce, respectively, under combine treatment of Cd and GABA as compare to Cd stressed plant. The interaction of three factor (varieties*GABA*cadmium) was non-significant (Table 5).

The results exposed that calcium contents in iceberg and lettuce both varieties show decreased under Cd stress. In iceberg 36% decrease in calcium under cadmium stress while in lettuce 35% as compare to the control plant. But when plants treated with GABA increased in calcium content up to 45% in iceberg and 47% in lettuce in comparison of control plants. The plant in which GABA and cadmium was collectively applied show significant increase of 85% and 63% in both iceberg and lettuce, respectively as compare to Cd stressed plants. So results are indicated that the cadmium reduced the calcium content and GABA increase its level in both normal and stress condition. The interactions of three factors (Variety*Cadmium*GABA) was show non-significant (Table 6).

The chloride content enhanced due to the Cd stress in the shoot of iceberg and lettuce increased up to 27% and 25%, respectively, as compare to plants without treatment. And the foliar application of GABA reduce 11% and 9% in chloride content of shoot in iceberg and lettuce, respectively, as compare to the control plants. When GABA and Cd was supplied collectively then the 27% and 30% reduction in chloride content was observed as compare to Cd stressed plant. The interaction of all three factors (varieties*GABA*cadmium) was non-significant (Table 7).

The result of cell membrane injury of two varieties of lettuce. The application of Cd stress notably increased the chloride contents of the plant that was 101% in iceberg and 75% in lettuce as compare to control plants. GABA shows

Table 6 Calcium contents of six weeks old root of two varieties of lettuce (*Lactuca sativa* L.) under cadmium stress and foliar application of GABA

Source of variation	DF	Mean square of Calcium in shoot
Variety	1	0.0104167**
Cadmium	1	0.0337523***
GABA	1	0.015123***
Variety*cadmium	1	0.0000605 ns
Variety*GABA	1	0.0004106 ns
Cadmium*GABA	1	0.0004116 ns
Variety*Cadmium*GABA	1	0.0016667 ns

* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.01$

ns means $p \geq 0.05$ (or non-significant)

Table 7 Chloride contents of six weeks old shoot two varieties of lettuce (*Lactuca sativa* L.) under cadmium stress and foliar application of GABA

Source of variation	DF	Mean square of Chloride in shoot
Variety	1	0.2426187*
Cadmium	1	0.707877***
GABA	1	2.124291***
Variety*cadmium	1	0.0268171 ns
Variety*GABA	1	0.0008154 ns
Cadmium*GABA	1	0.6504472***
Variety*Cadmium*GABA	1	0.0025896 ns

* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.01$

ns means $p \geq 0.05$ (or non-significant)

the reduction in chloride content of root in lettuce in comparison of control plant that was 17% and 4% in iceberg and lettuce, respectively. Combined treatment of cadmium and GABA show marked decrease in percentage of cell membrane injury which was 56% in iceberg and in lettuce was 46% as compare to cadmium stressed plants. The interaction of whole three factors (Variety*Cadmium*GABA) was non-significant (Table 8).

Discussion

The aim of this research is to learn more about Cd toxicity and its impact on physio-biochemical attributes of lettuce, as well as the effects of GABA which acts as a growth regulator on plants under stress, which has been well demonstrated in previous literature reviews. Cd is an abiotic stress that has a deleterious impact on plant growth and development. In many researches the effects of GABA on different plants under several biotic and abiotic stress conditions have been

Table 8 Chloride contents of six weeks old root two varieties of lettuce (*Lactuca sativa* L.) under cadmium stress and foliar application of GABA

Source of variation	DF	Mean square of Chloride in root
Variety	1	6.602280***
Cadmium	1	20.96124***
GABA	1	29.93891***
Variety*cadmium	1	0.0862503 ns
Variety*GABA	1	0.327419 ns
Cadmium*GABA	1	19.64257***
Variety*Cadmium*GABA	1	0.000349 ns

* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.01$

ns means $p \geq 0.05$ (or non-significant)

investigated; however the GABA's effect on lettuce under Cd exposure has yet to be investigated.

The application of a high dose of Cd stress on two varieties of lettuce (*Lactuca sativa* L.) cause reduction in growth and has a depressing impact on the plants physio-chemical parameters. The aim of this study was to find the regulatory role of GABA against Cd stress in lettuce. The endogenous signalling molecule & metabolite GABA have very promising effect in plant cells, which can improve tolerance to biotic and abiotic stress (Rezaei–Chiyaneh et al. 2018). However, the effect of foliar application of GABA on lettuce growth is not well known. GABA concentration in plant tissue is variable in normal condition but rapid increases when plants face the environmental stress (Ramesh et al. 2017).

The development of plant organs bears detrimental effect of heavy metals such as Cd and Pb which reduce biomass of several plant species (Shahzad, et al*.,* 2021). In present study shoot and root fresh weight was decreased in both iceberg and lettuce under Cd stress as study earlier in wild lettuce variety (Zafar-ul-Hye et al. 2020) and in parsley (Ulusu et al. 2017). Foliar treatment of GABA increased the fresh weight of shoot and root also observed earlier in banana because of improvement in the photosynthetic and enzymatic activity under stress (Wang et al. 2014).

Roots are the inital target of Cd, which affects root morphology and inhibits root growth (Rizwan et al. 2016). under Cd stress, root fresh and dry weight decreased in both varieties of (*Lactuca sativa* L.) iceberg and lettuce, in comparison of plants without treatment. A similar outcome was found in two different types of (*Brassica juncea* L.) (Irfan et al. 2014). In this way GABA enhance the root fresh and dry weight in iceberg and lettuce like in maize plant (Seifikalhor et al. 2020a, b). in Cd toxic plants, GABA also mitigate the Cd stress effect and recovered the plant growth as observed in tea plant (Ren et al. 2021).

Cadmium accumulates by roots causes a remearkable reduction in length of root and it transfer to aerial parts and declines the photosynthesis process which ultimately, result in reduction of plant growth and yield (Awan et al. 2020). Cd can be considerably accumulated in edible shoot parts as leaves or grains. (Rizwan, et al., 2016).In comparison to the control or non-treated plant, the shoot and root length and no. of leaf under Cd stress as observed earlier in wheat (Perveen et al. 2020) and also in lettuce (Tang et al. 2020). GABA application alone have some better growth effect on root and shoot length similarly in same (Bor et al. 2009) but by the exogenous spray of GABA not only recovered the growth but also increased the length of root and shoot and leaf numbers was also increased as discussed in *Brassica juncea* L. (Mahmud et al. 2017a, 2017b).

Furthermore, Cd suppresses photosynthesis process by inhibiting the enzymes actrivity in the C3 cycle and by damaging the light harvesting system (Qi et al. 2020). Combine effect of GABA and Cd stress also enhance the photosynthetic pigments as compare to cadmium stress plant like in maize plant that effected by cadmium stress their photosynthetic activity enhanced by the application of GABA and remarkable increased in chlorophyll and carotenoids content was observed so it proved that the GABA boost up the photosynthetic activity (Seifikalhor et al. 2020a, b).

Cadmium is not crucial element but absorbed abruptly in plants by divalent metal carrier and ion channels specificity. As Cd accumulates, its damage normal metabolism which leads to morpho-physiological and cellular changes (Hasan et al. 2015). Large amount of Cd increase the level of oxidative species and damage the plant. Moreover, Cd leads to the excessive generation of ROS responsible for oxidative injury, which adverserly affects the plant antioxidant defense system (Awan et al 2020).

Increased in phenolic and H_2O_2 contents of both lettuce varieties the iceberg and lettuce due to the toxicity of Cd was observed under current study and in *Biologia Plantarum* same results was found earlier (Sakihama & Yamasaki 2002) and GABA normalized the oxidative stress in both varieties of lettuce under Cd stress and alone also same result was found in tobacco plants under Cd stress (He et al. 2021) improve resistance against stress GABA was utilized in reduction of phenolic and H_2O_2 in *Brassica napus* L. (Lei et al. 2016) In any situation either plant stressed with Cd or without stress, GABA always play positive role to reduce the phenolic content and H_2O_2 and protect the plant from oxidative damage (Sheteiwy et al. 2019).

For Cd toxicity, Oxidative stress is responsible and widely recognized as the most crucial molecular mechanism (Waheed et al. 2022).Level of MDA increased in current study under Cd stress as compared to non-stressed plant due to increase in of oxidative species and same result was recorded in Ujala wheat variety under Cd stress (Thind et al. 2021) also in bean species(Gutiérrez-Martínez et al. 2020). GABA not only reduce the level of MDA and also restore plant to normal activity by increasing the enzymatic activities as same in maize (Li et al. 2016) and also in tomato seedling (Malekzadeh et al. 2014).

Level of free proline in lettuce plant under Cd stress is increased in present study as compare to plants without treatment as similar results observed in pea (Zdunek-Zastocka et al. 2021) and also noticed in wheat (Perveen et al. 2020) and on the other hand proline level improvement noticed in tobacco under salt stress and with the application of GABA (Lei et al. 2016) as same increased in proline content was observed in *Sedum alfredii* (Sun et al. 2007).It is earlier reported that Cd toxicity in plants generates higher levels of proline in species of Brassicaceae family (Waheed et al. 2022).

Enzymatic activity of both lettuce (*Lactuca sativa* L.) varieties increased under stress for defense of plant against ROS was observed in earlier researches like in wheat ROS start accumulate when they was exposed to a high dose of Cd (Perveen et al. 2020). Activity of antioxidant enzyme APX, CAT, POD increased under Cd stress in lettuce varieties and also as previously observed in (*Hibiscus cannabinus* L.) under Cd stress (Li et al. 2013a, b). in *P. stratiotes* activity of CAT and POD enhanced by the treatment of 100 µM Cd (Yong et al. 2013b) GABA application eliminate the growth inhibitor, reduce ROS and oxidative damage cause by stress due to increase in enzymes APX, catalase, and POX and in barley relative finding was observed (Ma et al. 2019) and also in mustard (Mahmud et al. 2017a, b).

Cell membrane injury is also increased by the application of Cd in present study and as same in *Triticum aestivum* (Xuebin et al. 2020). Cadmium caused cell membrane injury because of osmolytes and ROS production. But GABA restored the cell membrane injury under Cd stress and also protected the cell from further damages by foliar application as in maize (Seifikalhor et al. 2020a, b). Relative water content of lettuce is decreased due to Cd high dose as similar in *Brassica oleracea*. its decreased because turgor pressure is lost in Cd stress (Shah et al. 2020). GABA eliminate this stress effect and enhance the relative water content of lettuce leaf and same results were found in tea plants (Ren et al. 2021). Under combine application of GABA and Cd on lettuce varieties, GABA mitigate the stress effect as noticed in *Brassica napus* L. under heavy metal stress (Wang et al. 2022).

Cadmium higher concentration in soil minimize nutrients uptake in plants such as P, Zn, Fe and Mn (Shahzad et al. 2021). In lettuce varieties, Cd cause decrease in potassium and calcium ions in current study under Cd stress and also same results were observed in wheat seedlings (Veselov et al. 2003) on the other hand Upregulation of sodium and chloride content in two varieties of lettuce was observed due to stress of cadmium as in Fava Bean (*Vicia faba* L.) (Piršelová & Ondrušková, 2021). And in cause of mineral ions GABA reduce the content of Na^+ in root and shoots of plants also studied in Tomato (Wu et al. 2020) because GABA enhance all cell mechanism of plants and improve the protein level (Sheteiwy et al. 2019). GABA treatment also considerably improved the contents of calcium and potassium in plant with and without stress similarly in barley (Gut et al. 2009).

Conclusion

The present research proved that cadmium (250 µM) exposure reduced the growth and development of both varieties of *Lactuca sativa*. Cadmium toxicity can also lower the chlorophyll and carotenoid content in *L. sativa* plants and its toxicity was suppressed by supplementation of GABA. On the other hand, (100 µM) GABA treatment improved growth, photosynthetic pigmentation and activity of antioxidative enzymes (APX, CAT and POD) in *Lactuca sativa* varieties that grown in normal and Cd-toxic conditions. This non-protein amino acid (GABA) also reduced MDA, H_2O_2 content besides reduction in sodium and chloride contents. Moreover, calcium and potassium ions boosted up in plants that supplemented with GABA treatment and with cadmium as compared to cadmium toxic plants.

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Author Contributions ZW: performed experiments; ZN: supervised, conceptualization, research design; AAS: review and drafting; SU: statistical analysis and experimentation; ANS: validation and writing; MR: statistical analysis; RC: writing, drafting and funding; HOL: writing, validation and funding.

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Declarations

Conflict of interest All the authors declare no conflict of interest.

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