Protective Role of Salicylic Acid against Cadmium Chloride-induced Stress in Punjab 2011 Wheat (*Triticum aestivum* L.) variety

Jamshid Ali [1], Nadia Mushtaq [2], Faizan Ullah [3], Muhammad Zubair [4*], Rida Nisar [5], Hadiqa Noor [6], Asia Arooj [7], Seemab Ali khan [8], Sidra Azam [9]

[1-4, 8] Department of Botany, University of Science and Technology Bannu, Khyber Pakhtunkhwa, Pakistan
[5] Department of Horticulture, Faculty of Agriculture Gomal University Dera Ismail Khan, Pakistan
[6, 9] Department of Botany, Islamia College University of Peshawar, Pakistan
[7] Department of Botany, University of Agriculture Faisalabad, Pakistan

* Corresponding Author: **Muhammad Zubair (zubirhasraat@gmail.com)**

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Abstract

Background: Heavy metal stress, particularly cadmium (Cd), poses a serious threat to crop productivity by impairing growth, physiology, and biochemical functions. For the management of Cd-stressed soils, intervention is at the utmost. Therefore, this study aimed to evaluate the effect of salicylic acid (SA) on biochemical, physiological and morphological growth attributes of wheat (*Triticum aestivum* L.) variety Punjab 2011 under cadmium chloride ($CdCl_2$) stress.

Method: The SA was applied at 10^{-4} mol/L as a seed soaking solution before sowing of seeds in the pots. The soil was contaminated with different concentrations of $CdCl_2$ (50 mg/kg, 100 mg/kg and 200 mg/kg soil).

Results: The $CdCl_2$ stress demonstrated adverse effects and decreased seed germination (%), root fresh weight, root dry weight, chlorophyll "*a*", and chlorophyll "*b*" content of wheat, significantly ($P<0.05$). The $CdCl_2$ stress resulted in the accumulation of total soluble phenolics and higher antioxidant activity in roots and leaves of maize. Both under unstressed as well as $CdCl_2$-induced stress conditions, SA application significantly ($P<0.05$) improved growth indices, including seed germination (%), index, index rate, shoot fresh and dry weight, root fresh and dry weight, and chlorophyll "*a*", and chlorophyll "*b*" content. It was also observed that SA exhibited stimulatory effects on the accumulation of phenolic compounds under $CdCl_2$ stress in both leaves and roots.

Conclusion: $CdCl_2$ had toxic effects and resulted in decreased different growth attributes, increase in carotenoid pigments and total soluble phenolics. The application of the SA significantly improved the overall health of *T. aestivum* crop under Cd-stressed soil.

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Keywords: Heavy Metal-Stress, Cadmium, Cadmium-Induced Stress, Plant Growth Regulators, Salicylic Acid, Wheat, Punjab 2011

1. Introduction

For this exceeding world population, wheat (*Triticum aestivum* L.) is a promising source of food and every year among the cultivated crops, and it is grown worldwide [1]. Meanwhile, in cereal crops, it holds a main position, and in Pakistan, about 66% of the food-cropped area is covered by *T. aestivum*, as it provides about 60% calories from the normal diet [2]. However, in recent years, its production has been low, which may be attributed to various reasons, such as drought, waterlogging, salinity, soil infertility, heavy metal stress and a lack of proper nutrients [3]. Additionally, heavy metals are elements with a density of more than 5 grams per cubic centimeter and are considered as one of the major environmental pollutants that adversely affect the

overall health of plants [4]. The accumulation of heavy metals in water, soil, and air has shown toxic effects, decreased agricultural yields and also caused contamination in the food chain, therefore, it has become a serious issue in recent years [5, 6]. Moreover, these heavy metals can act as stressors, causing physiological limitations and decreasing the growth of plants [7]. The different physiological and metabolic processes, such as cell elongation, plant-water relationships, photosynthesis, respiration, and mineral nutrition are inhibited by heavy metals [8]. In addition, these heavy metals in soil can cause different kinds of adverse effects in plants, such as limitations in enzyme activities, decreased chlorophyll content and photosynthesis, destroy biotic molecules, including lipids, proteins and nucleic acids, particularly DNA [9]. Meanwhile, industrialization, agricultural practices and urbanization are considered the main sources of heavy metals and adversely contaminated large areas of land [10].

Among the heavy metals, cadmium (Cd) is an extremely poisonous and non-essential heavy metal that mainly enter into the environment through phosphate fertilizers, industries, mining, sewage sludge, and irrigation with contaminated water [11, 12]. Numerous investigations have demonstrated that Cd adversely affects plant metabolism, including reduction in the uptake of nutrient elements [13, 14]. In addition, Cd reduces germination of seeds, shoot and root length and also decreases the proline and chlorophyll contents [15, 16]. Moreover, the biomass production is strongly reduced by the Cd stress in plants, which leads to several types of stresses like wilting, chlorosis, necrotic lesions, disturbance of carbohydrate metabolism and mineral nutrition [17]. The accumulation of Cd in *T. aestivum* grains is particularly alarming because it is one of the stable foods, therefore reducing its adverse effects on the growth and yield is utmost importance [18].

Given the widespread problems associated with Cd contamination, various strategies are used to alleviate the adverse effects. These strategies include phytoremediation, soil amendments, use of tolerant varieties, and application of plant growth regulators [19, 20]. Among these growth regulators, the application of signaling molecules, like salicylic acid (SA) is an effective signaling molecule of phenolic nature in plants which reduces the particular responses to abiotic as well as biotic stresses [21, 22]. Under Cd stress conditions, SA modulates osmolyte accumulation, antioxidant defense systems, and hormonal balance, thereby improving plant tolerance [23, 24]. Furthermore, SA also has beneficial effects in protecting against membrane damage generated by lead and mercury and alleviates toxicity of Cd in other crops, like rice, barley, and maize [25, 27].

The extensive research has highlighted the detrimental effects of Cd and the beneficial role of SA in plants, limited studies have focused specifically on the interaction of *T. aestivum* varieties and SA adapted to Asian agro-ecological conditions. Among varieties, Punjab 2011 is highly relevant to the local farming systems, yet its physiological, biochemical and morphological responses to SA under Cd stress remain inadequately understood. Therefore, addressing this gap can be helpful in optimizing agronomic practices and improving its productivity in Cd affected soils. Thus, this study was designed, to determine the SA effect on morphology, physiology and biochemical activities of *T. aestivum* under Cd stress.

2. Materials and Methods

2.1 Experimental design

The experiment was performed in the glasshouse following randomized design in the field, and each treatment was replicated three times and each replicate consisted of 10 plants.

2.2 Source of Seeds

Certified seeds of *T. aestivum* were procured from the agricultural research station, Sarai Naurang Lakki Marwat, Khyber Pakhtunkhwa (KPK), Pakistan. Seeds were surface sterilized using 0.2% mercuric chloride solution for 2-3 minutes, and then followed by rinsing thoroughly with distilled water. Sterilized seeds were sown in plastic pots (12 x 14cm^2) containing a mixture of clay and sand (1:1). These pots were then maintained in the glasshouse, and regular irrigation was applied to maintain the soil moisture.

2.3 Application of Cadmium

The soil was contaminated with Cd in the form of $CdCl_2$ at 50 mg/Kg, 100 mg/Kg, and 200 mg/Kg soil, respectively. The soil was filled in plastic pots 12 x 14cm^2. The pots were then arranged in a complete randomized design (CRD) in a glasshouse. The following treatments were made:

Control with SA and $CdCl_2$ exposure, SA group (10^{-4} mol/L), $CdCl_2$-1 group (50 mg/Kg soil), $CdCl_2$-2 group (100 mg/Kg soil), $CdCl_2$-3 group (200 mg/Kg soil), $CdCl_2$-1 + SA group (50 mg/Kg soil), $CdCl_2$-2 + SA group (100 mg/Kg soil), $CdCl_2$-3 + SA group (200 mg/Kg soil).

2.4 Application of Salicylic Acid

Ethanol (1 ml) was used to prepare the desired concentration of SA. The distilled water was used to increase the volume of the solution up to 10ml. The 10^{-4} mol/L solution was prepared by diluting the obtained stock solution further more. The volume of the solution became 100 Ml. Sterilization of seeds was done by soaking them for five hours. The seeds for control were soaked in distilled water.

For the determination of seed germination indication, the emerging seedlings were calculated after ten days.

2.5 Determination of Seed Germination (%), Index, and Rate Index

For the measurement of germination percentage of seed. The following formula was applied

$$\text{Seed germination \% age} = \frac{\text{No of seed germinated}}{\text{Total number of seed grown}} \times 100$$

While, the calculation of germination index was done by using the formula.

Germination Index= No of seeds germinated at 1st day+ No of seeds germinated at 2nd day+.....No of seeds germinated at 15th day /No of days.

To determine the Germination rate index, the following formula was used.

$$\text{Germination rate index} = \frac{\text{Germination index}}{\text{germination (\%)}}$$

The plants were harvested 30 days being sown and analyzed for the following morphological, physiological and biochemical growth parameters.

2.6 Determination of Morphological Characteristics

Shoot length of the seedling from every group and replica was measured separately in cm by using measuring tape. Physical balance was used to measure and record the fresh weight of shoot and root from every group and replica. Moreover, the shoot and root of plants were dried for 3 days in oven at 75˚C. By using the physical balance, the dry weight of shoot and root was determined and recorded separately from every group and replica.

2.7 Determination of Physiological and Biochemical Characteristics

Leaf photosynthetic pigments: The Arnon (28) method was used to measure the leaf photosynthetic pigments. The fresh leaf material (0.1gm) was extracted with (5 mL) (80%) acetone and placed at 4˚C overnight in dark. The supernatant was carefully collected and used for chlorophyll determination. Absorbance of solutions was read at 645 nm for chlorophyll "*a*" and at 663nm for chlorophyll *"b"* and 480 nm (carotenoid) on spectrophotometer (Hitachi's U-5100 Japan) against a blank (acetone).

Antioxidant potential: Methanolic extract was used for the determination of free radicals by using the method of Blois (29) (2, 2 di Phenyl-1 picrayl-hydrazyle). The absorbance was recorded at 517 nm. Triplicate value test was taken. The percentage inhibition was determined by formula.

Percent (%) inhibition = [(A blank/A sample)/A blank] x 100.

A blank = Absorbance of control reaction.

A sample = Absorbance of extract.

Phenolic compounds of root and shoot: The total phenolic were measured on the basis of folin-ciocalteau method [30]. The shoot or root tissue (0.1 mg) were extracted in 5 mL of acetone. The extract (130 µl) was mixed with freshly prepared folin ciocalteu reagent and then released for five minutes. The mixture was allowed to stand for 5 minutes. The 2.5 mL solution of sodium carbonate (7.5%) and distilled water (0.5 mL) was added to the mixture and then placed for 90 minutes at room temperature. After that using a spectrophotometer optical density of all the samples was determined at 765 nm.

2.8 Statistical Analysis

All obtained data were subjected to analysis of variance (one-way ANOVA) for data analysis using SPSS-16 statistical software. Mean comparison for treatments was performed using least significant difference (LSD) test at a significance level of 0.05 [31].

3. Results

3.1 Effect of Salicylic Acid on the Seed Germination (%), Index, And Index Rate

The application of SA has no significant effect on seed germination (%) of *T. aestivum* L. in comparison with control (Figure 1A). The $CdCl_2$ at 100 mg/Kg soil and 200 mg/Kg soil significantly ($P<0.05$) reduced seed germination (%). Meanwhile, the effect was more significant ($P<0.05$) at the concentration of 200 mg/Kg soil compared to control. Overall, application of SA minimized the adverse effect of $CdCl_2$ and resulted in high seed germination (%).

Likewise, the SA under control conditions has no significant ($P>0.05$) effect on germination index than control. All the concentration of $CdCl_2$ significantly ($P<0.05$) decreased seed germination index than control. However, application of SA to $CdCl_2$ treatments (SA+50 mg/Kg soil and 100 mg/kg soil) significantly ($P<0.05$) reduced the toxicity of $CdCl_2$ on seed germination index (Figure 1B).

Similarly, on the seed germination seed index, SA demonstrated no significant ($P>0.05$) effect than control. However, higher amount of $CdCl_2$ at the rate of 200 mg/Kg soil significantly ($P<0.05$) reduced the germination rate index. The effect of SA along with $CdCl_2$ significantly ($P<0.05$) increased seed germination rate index by reducing the adverse effect of $CdCl_2$ stress (Figure 1C).

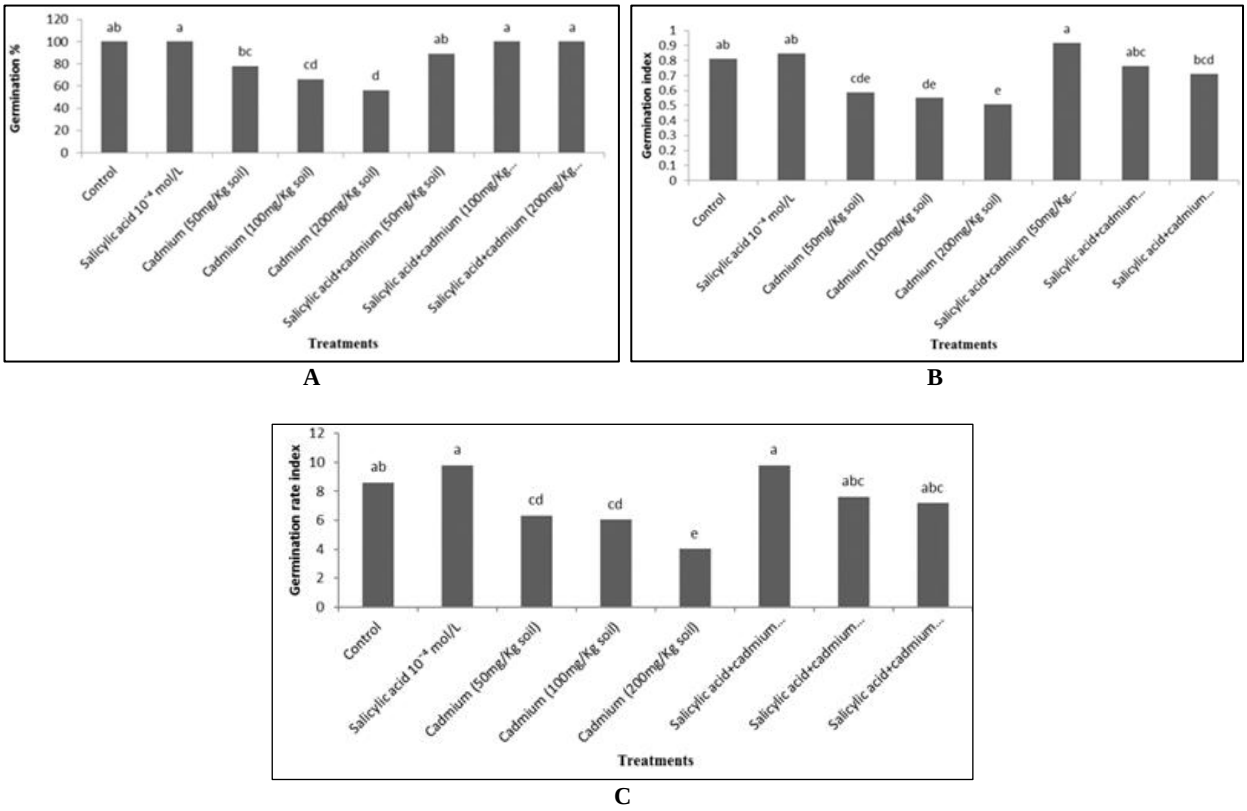


Fig 1: Effect of SA and $CdCl_2$ on wheat A. seed germination (%) (LSD: 17.468). B. Seed germination index (LSD: 0.1964). C. Seed germination rate index (LSD: 3.347). All means which have same English letters are statistically similar.

3.2 Effect of Salicylic acid on the Shoot Fresh Weight, Shoot Dry Weight

The shoot fresh weight of wheat seedling was significantly ($P<0.05$) increased by the application of SA than control (Figure 2A). The $CdCl_2$ at higher concentration (200 mg/Kg soil) highly reduced the fresh weight of shoot than the control treatment. Application of SA increased the fresh weight of seedling thereby reducing the adverse effect of $CdCl_2$ stress (Figure 2A).

Similarly, SA also significantly ($P<0.05$) increased shoot dry weight in comparison with control. The effect of $CdCl_2$ was non-significant ($P>0.05$) on dry weight of shoot compared with control. The $CdCl_2$ stress on dry weight of shoot was vastly minimized by the exogenous application of SA (Figure 2B). The application of SA under $CdCl_2$ resulted in a significantly ($P<0.05$) higher shoot dry weight as compared to untreated control.

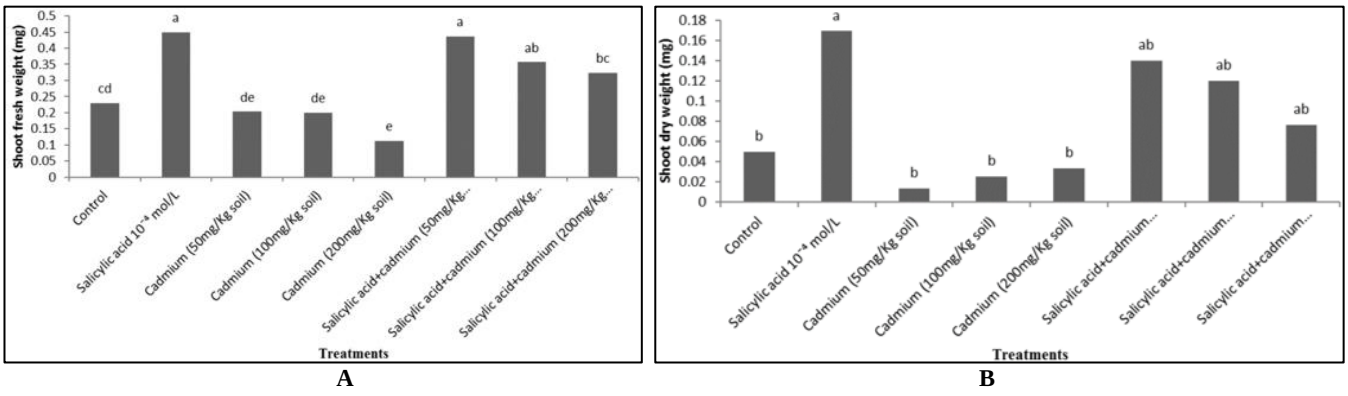


Fig 2: A. Effect of salicylic acid and $CdCl_2$ on wheat. A. Shoot fresh weight (LSD: 0.1128). B. Shoot dry weight (LSD: 0.3264). All means which have same English letters are statistically similar.

3.3 Effect of Salicylic Acid on the Root Fresh and Dry Weight

The root fresh weight was expressively increased in comparison with control when treated with SA. The stress of $CdCl_2$ on root fresh weight was significantly ($P<0.05$) less than control (Figure 3A). Therefore, the treatment of SA along with $CdCl_2$ extremely improved the root fresh weight.

Under unstressed conditions, SA highly improved the dry weight of root in comparison with control ($P<0.05$). The $CdCl_2$ stress was not much effective on the root dry weight in comparison with control. At lower concentration (50mg/Kg soil) SA reduced the adverse effect of $CdCl_2$ by increasing the root dry weight (Figure 3B). The effect of treatments SA+$CdCl_2$ (200mg/Kg soil) was non-significant on the dry weight of root in comparison with control.

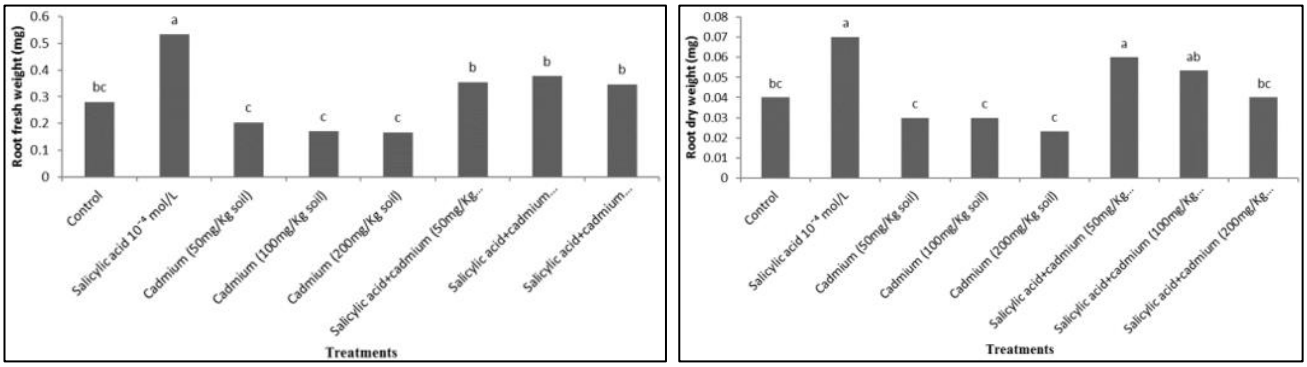


Fig 3: A. Root fresh weight of wheat as influenced by salicylic acid and $CdCl_2$ (LSD: 0.1332). B. Root dry weight of wheat as influenced by salicylic acid and $CdCl_2$ (LSD: 0.0200). All means which have same English letters are statistically similar.

3.4 Effect of Salicylic Acid on Chlorophyll "*A*" And "*B*" Content

The chlorophyll "*a*" content of wheat seedling was significantly increased with the application of SA compared to control. While, the $CdCl_2$ stress at higher concentrations of 100 and 200mg/Kg soil $CdCl_2$ reduced the chlorophyll "*a*" content than control. Application of SA in $CdCl_2$ stress significantly ($P<0.05$) increased the chlorophyll "*a*" content in all the treatments by minimizing the adverse effect of $CdCl_2$ stress (Figure 4A).

Figure 4B demonstrated that chlorophyll "*b*" content of wheat seedling was significantly ($P<0.05$) increased by SA application than control. While, non-significant ($P>0.05$) difference was observed in chlorophyll "*b*" content in all the concentrations of $CdCl_2$ than control. Meanwhile, application of SA significantly ($P<0.05$) increased the chlorophyll "*b*" content of wheat leaves under $CdCl_2$ stress at 50 mg/Kg soil and 100mg/Kg soil (Figure 4B).

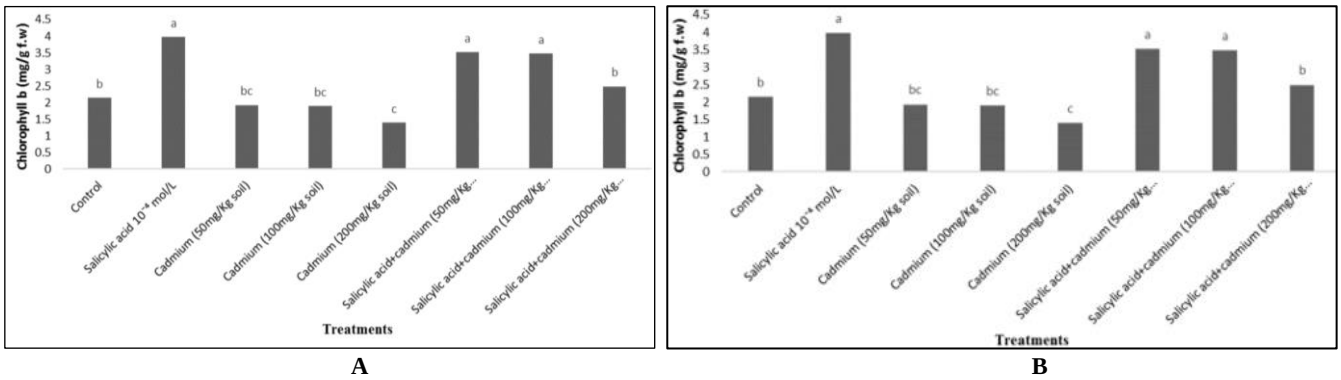


Fig 4: Effect of salicylic acid and $CdCl_2$ on the wheat A. Chlorophyll "*a*" content (LSD1.4683). B. Chlorophyll "*b*" content (LSD: 0.7353). All means which have same English letters are statistically similar.

3.5 Effect of Salicylic Acid on the Leaf Carotenoid Content

Application of SA significantly ($P<0.05$) increased the carotenoid content of wheat leaves as compared to control. $CdCl_2$ stress did not affect the carotenoid content of wheat leaves, while the application of SA under $CdCl_2$ induced stress significantly ($P<0.05$) increasing leaf carotenoid content over untreated control (Figure 5).

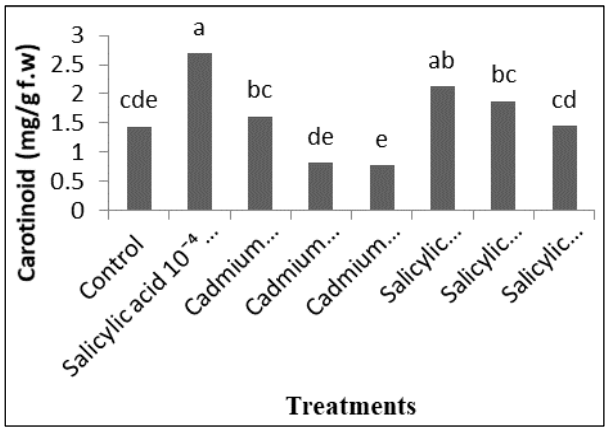


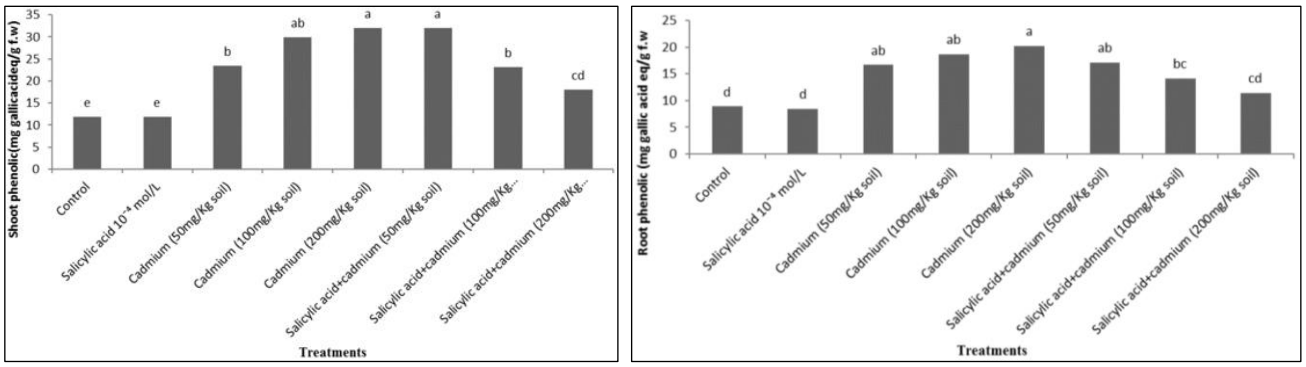
Fig 5: Leaf carotenoids of wheat as influenced by salicylic acid and $CdCl_2$ (LSD: 0.6674). All means which have same English letters are statistically similar

3.6 Effect of Salicylic Acid on Leaf and Root Total Soluble Phenolic Content

Salicylic acid had no effect on leaf phenolic content in comparison with control treatment. While, the $CdCl_2$ stress significantly (P<0.05) increased phenolic contents at 100 mg/Kg soil and 200 mg/Kg soil in comparison with control. The application of SA in $CdCl_2$ stress significantly (P<0.05) increased the phenolic content of leaf. It was found

exogenous application of SA under $CdCl_2$ stress augmented the deposition of total soluble phenolic (Figure 6A).

The $CdCl_2$ stress significantly (P<0.05) increased root phenolic contents as compared to control (Figure 6B). All the concentrations of $CdCl_2$ significantly (P<0.05) increased the phenolic contents, while the higher concentration (200mg/Kg soil) showed more profound effect. The application of SA in Cd Cl_2 stress condition also increased the phenolic contents of roots. However, higher content of total soluble phenolic was recorded for treatments SA+$CdCl_2$ (50 mg/Kg soil) and SA+$CdCl_2$ (100 mg/Kg soil), as described in Figure 6B.



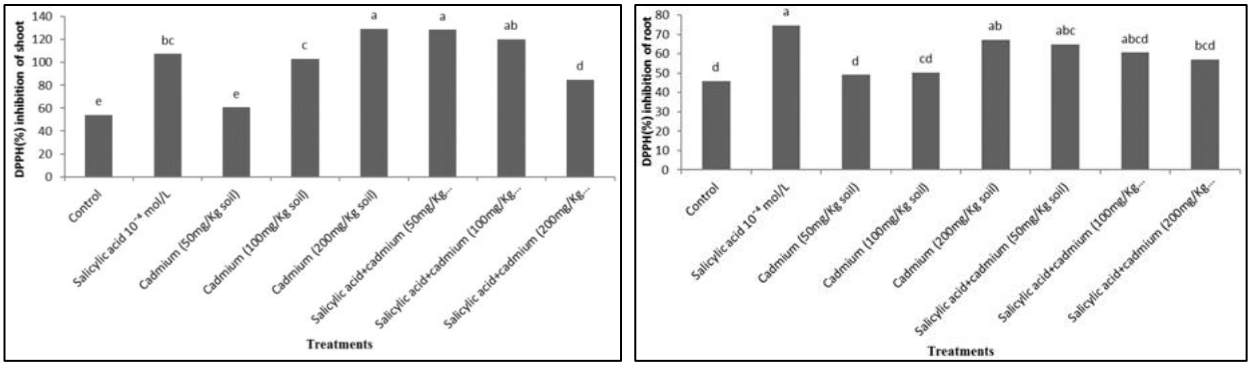
A B

Fig 6: Effect of salicylic acid and $CdCl_2$ on wheat. A. Leaf phenolic (LSD: 4.5276). B. Root phenolic (LSD: 4.8128). All means which have same English letters are statistically similar.

3.7 Effect of Salicylic Acid on Antioxidant Activity in Leaf And Roots

The application of SA significantly (P<0.05) increased the antioxidant activity of wheat seedling as compared to control (Figure 7A). $CdCl_2$ stress also increased the antioxidant activity and it was highly significant (P<0.05) in higher concentrations of 100 mg/Kg soil and 200 mg/Kg soil (Figure 7A). Thus, SA has stimulatory effect on antioxidant activity both under stress and non-stress conditions.

Similarly, root antioxidants were also significantly (P<0.05) increased with the application of SA as compared to control (Figure 7B). Meanwhile, $CdCl_2$ stress also increased antioxidant activity, and the high concentration was recorded in high treatment of $CdCl_2$ (200 mg/Kg soil), as described in Figure 7B.



A B

Fig 7: Effect of salicylic acid and $CdCl_2$ on wheat. A. Shoot antioxidant activity (LSD: 17.404). B. Root antioxidant activity (LSD: 14.839). All means which have same English letters are statistically similar.

4. Discussion

The contamination of agricultural soils with heavy metals is rapidly increasing due to advancement in industrial sectors and ever-increasing population of human beings [32]. These heavy metals have detrimental effects on growth of crop plants. The present investigation showed that $CdCl_2$ inhibited seed germination indices such as seed germination (%) and germination index of wheat. This may be due to the oxidation stress and lipid peroxidation of cell membranes, which is usually developed by heavy metals, including Cd. As a result, seed fails to germinate because embryo is destroyed [33].

In the present study, the application of SA minimized the adverse effect of $CdCl_2$ by increasing seed germination (%) and germination index. Similar outcomes were observed in another study and concluded that the seed priming with SA increased seed germination (%) and germination index of wheat under abiotic stress [34]. Furthermore, the current study presented that $CdCl_2$ reduced the shoot fresh weight and shoot dry weight of wheat seedling. Our results are similar to the previous findings of Rahnama, Torabi (35) that Cd inhibited shoot fresh and dry weight of sunflower. Application of SA minimized adverse effect of $CdCl_2$ on

seedling fresh and dry weight. While, in normal conditions, it has been found that SA application increased fresh and dry weight of *T. aestivum* [36]. The minimization of adverse effect of $CdCl_2$ on seedling weight by SA may be due to less consumption or uptake of heavy metal by roots. As a result, the shoot fresh and dry weight is increased in SA treatments. It has been found that SA improves movements of photosynthesis to the storage organs which might have contributed to the increased weight of seedling [37]. Similarly, another study also found improved plant growth, gas exchange attributes, relative water content, and chlorophyll content in Cd-stressed Anaj-17 and Akbar-19 *T. aestivum* varieties, when treated with foliar application of SA [38]. The present research also showed that Cd reduced the root fresh and dry weight of wheat seedling. This result supports the result of Raza and Fahad (39) that Cd decreased the dry and fresh weight of root in radish (*Raphanus sativus*). Likewise, the application of SA under Cd stress also increased the dry and fresh weight of root. Similar findings were observed in the study performed by Metwally, Finkemeier (27) that SA improved the root dry and fresh weight of barley under Cd stress.

The present study showed that chlorophyll "*a*" chlorophyll "*b*" and carotenoid content of wheat seedlings was reduced by the Cd stress and with the application of SA in Cd stress increased the chlorophyll "*a*", chlorophyll "*b*" and carotenoid content in all the treatments by minimizing the adverse effect of Cd stress. Our results are similar to the previously published study and concluded that the chlorophyll "a", chlorophyll "*b*" and carotenoid content of wheat was decreased by the high concentration of heavy metal [40]. Another study also observed significantly high chlorophyll "*a*" and "*b*" and carotenoid content and demonstrated that application of SA at the concentration of 0.5 mM and 0.1 mM of $CdCl_2$, yielded a high growth in the wheat crop [41]. Application of SA in Cd stress increased the chlorophyll "*a*", chlorophyll "*b*" and carotenoid content in all the treatments by minimizing the adverse effect of Cd stress. The treatment of SA in soybean and maize plants increased the chlorophyll content and photosynthesis [42, 43]. Application of SA increased the photosynthetic pigments by ameliorating the adverse effect of Cd stress. The possible mechanism may be that SA increased uptake of nutrients; thus, they have positive effect on growth of *T. aestivum* [44].

The present investigation showed that $CdCl_2$ stress markedly increased phenolic contents in roots and leaves of *T. aestivum* and when these $CdCl_2$ stressed plants were treated with SA, it increased the phenolics content of shoot. These findings are supported by Cervilla, Blasco (45) that tomato leaves under heavy metal stress increases total soluble phenolic compounds. Similarly, when treated with SA, it declined the negative effect of Cd on *T. aestivum* plants and contributed to increases the total soluble phenolic [46]. Previous studies demonstrated that heavy stress tolerance of plants is correlated with higher endogenous phenolic content [32]. Moreover, present investigation revealed that application of SA increased the antioxidant activity in leaves and roots under $CdCl_2$ stress, *T. aestivum* seedling and roots also increase their antioxidant activities. Further the application of SA along with Cd also increased antioxidant activity. Thus, SA has stimulatory effect on antioxidant activity both under stress and non-stress conditions. Earlier studies suggested that the antioxidant content in *T. aestivum* was increased by the application of SA under salt stress [47].

The findings of this study highlight the significant protective role of SA in mitigating the adverse effects of Cd stress on *T. aestivum* by improving growth, physiological performance, biochemical and antioxidant mechanism. This study also endorses the application of SA, which can be cost-effective and practically enabled strategy to enhance crop tolerance under stress, thereby contributing to sustainable crop production in contaminated soils. However, future studies should focus on optimizing the concentration and application methods of SA under filed conditions, exploring its synergistic effect with other growth regulators and investigating its molecular mechanism of action.

5. Conclusion

This study demonstrated that Cd had toxic effects and resulted in decrease in different growth attributes such as seed germination indices, shoot and root weight, and photosynthetic pigments of wheat. In addition, Cd in soil also increases carotenoid pigments and total soluble phenolics in leaves of *T. aestivum*. Meanwhile, application of SA successfully reduces the adverse effects of Cd on *T. aestivum*. While, the SA at 10^{-4} mol/L might be a cost-effective concentration of SA for application in farmer's field. However, filed studies are required for the validation of these findings.

6. Conflict of Interest

All authors declare that there was no conflict of interest.

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