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Research Article

Exogenous selenium application enhances the photosynthetic pigment and antioxidant defense of mash bean (*Vigna mungo*) to confer tolerance to salt stress

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Abstract

Mash bean is an important crop enriched with proteins and highly praised in Pakistan due to its nutritional values. However, due to abiotic stresses like salinity, its production is decreased. This study was conducted to investigate the effect of selenium on mash bean to produce salt tolerance. Mash bean seeds were sown in plastic pots filled with sand. Three levels of NaCl (0 mM, 100 mM, 200 mM) and five levels of selenium (0 ppm, 20 ppm, 40 ppm, 80 ppm, 120 ppm) were applied with Hoagland solution. Salinity reduced plant height (28%), leaf area (33%), chlorophyll *a* (14%), chlorophyll *b* (9%), carotenoids (20%), potassium ions, calcium ions, superoxide dismutase, peroxidases, catalase, salt tolerance index and increased sodium ions (21%), H_2O_2 content as well as secondary metabolites. However, selenium application in low concentration enhanced plant height (31%), leaf area, chlorophyll *a* (17%), chlorophyll *b* (12%), carotenoids (40%), potassium, calcium, superoxide dismutase, peroxidases, catalase, salt tolerance index, proline, flavonoids, total phenol, while decreased sodium ions (25%) and hydrogen peroxide content under salt stress. Findings showed important function of selenium in improving physical characteristics, absorption of ions, photosynthetic pigments, and antioxidant defense in plants under salinity. Applying selenium at 40 ppm concentrations showed greatest efficacy in alleviating negative impacts of salt stress (100 mM) on plant growth and biochemical attributes. Maximum positive results of selenium application (40 ppm) were obtained at 0 mM of salinity.

Keywords: antioxidants; mash bean; photosynthesis; selenium; salinity; secondary metabolite

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Introduction

The world population level is rising up continuously making it possible to increase by more than 2.4 billion by 2050, resulting in demand to grow more food crops. However, drastic global climatic conditions are negatively affecting plant defense systems and crop production. FAO estimates that annually production of cereals must be increased by about 1 billion to provide food to projected population of 9.1 billion by 2050. For this, there is great need of maximizing food production (Iqbal *et al*., 2017) but climatic conditions are becoming extremely worse day by day globally, having negative impact on plants defense system and crops production (Abdelkhalek *et al*., 2020).

Mash bean (*Vigna mungo* L.) is associated to Fabaceae family which is third largest pulse crop of Pakistan, having great importance due to its nutritional values (Ali *et al*., 2022). It is rich in proteins, vitamins, carbohydrates, and fats that are necessary for good health. People use mash bean as an alternative source of meat as it contains sources of iron, calcium, magnesium, potassium, phosphorus, and vitamins (*Rahate et al., 2021*). Mash bean is a crop that can be cultivated in spring and autumn season and have crucial role in medicines and different disease treatments (Nasir *et al*., 2022). In Pakistan, it covers about 17,000 hectares area and Punjab is province that majorly produce mash bean (Qayyum *et al*., 2019). According to previous record (2011-2019) the average yield of mash bean was 676 kg ha^{-1} which was 12% less than demand. This reduction in yield may be due to severe environmental conditions like biotic and abiotic stresses (Ullah *et al*., 2020).

Toxicity of soil is a severe problem which causes loss of production. Soil toxicity is mainly due to overuse of salts and metals which have also effect on human health (Xie *et al*., 2018). Salinity is a global issue which have negative effect on production of plants by impairing physiological, biochemical, and morphological attributes. Salinity is the presence of excess number of salts like NaCl in soil. It may be osmotic stress or oxidative stress (Khan *et al*., 2024). Osmotically salinity causes closure of stomata which results in reduced photosynthesis by proteins denaturation and chlorophyll damage (Hameed *et al*., 2021). When plants are watered with saline water, the high concentration of soil salts directly affects the roots, leading to hindered growth and decreased agricultural output (Kumar *et al*., 2020). By the year 2050, it is believed that approximately 50% of agricultural land will be destroyed by salt (Naqve *et al*., 2021).

Salts higher accumulation affects legume crops in diverse ways. It retards the germination process of seed, root growth and shoot length. At first, salinity decreases leaf area, shoot length and leaf water content which ultimately cause stunted growth (Abdelgawad *et al*., 2019). Salt stress affects mash bean crops by reducing antioxidants defensive activities, leaf water status and photochemical efficiency. To cope with salinity stress, plants show several types of defensive responses. They produce antioxidants like SOD, POD and other enzymes which helps plants to detoxify salt produced substance like H_2O_2 and ROS (Nasrallah *et al*., 2022). However these detoxifying substances are reduced under high concentration of salts and then micronutrients, macronutrients and other growth promoting hormones are applied (Al-Zahrani *et al*., 2021).

Selenium is a vital micronutrient used for increasing antioxidants as well as photosynthetic activity of plants during salinity (*Ameen et al., 2024*). Selenium has a pivoting role in several plant activities like improving photosynthesis, growth, and tolerance of stress. Under salinity, selenium improves growth of plants, physiochemical characteristics and enhances level of carbohydrates in leaves of beans (Taha *et al*., 2021). Selenium facilitating stomatal opening, and also influence the cellular processes that indirectly enhances calcium signaling in plants. Selenium increases photosynthetic activity by improving chlorophyll formation and leaf area expansion rate (Niemi *et al*., 2020). Trace amount of selenium application is helpful for plant growth, but high dose can have negative effect on plants. However, its concentration varies from species to species, time duration and stages of growth. It has been observed that in *Vigna radiata,* selenium increased sucrose and starch level (Kamran *et al*., 2020).

Plants are provided with beneficial micronutrients like zinc, boron and silicon for their better growth and quality (Hmood *et al*., 2022). However, interaction of selenium with salt stress in legumes is still unclear and knowledge about high concentration of selenium in plants is still not clear. Therefore, this experiment was carried out to investigate the impact of selenium application on mash bean growth, photosynthetic pigment, and antioxidants defense system under salt stress. Main objectives of this research were to determine the role of selenium on mash bean morphology and uptake of sodium, potassium, calcium ions under salt stress as well as to investigate the effect of selenium on black gram (*Vigna mungo*) photosynthetic pigments and antioxidants under salt stress.

Materials and Methods

Experiment location and conditions

A pot experiment was carried out at the research area of University of Agriculture, PARS, during 2022 to examine the selenium effect on mash bean for enhancement of antioxidant defense activities and photosynthetic pigments under salinity stress. Mash bean seeds of variety ('Arooj-2011') were selected in present experiment. Plants were grown in sand filled plastic pots with three replications and pots were arranged in completely randomized design. Each pot was filled with approximately 6 kg of sand. The dimensions of each pot were approximately 25 cm in diameter and 30 cm in height. A total of 10 seeds were sown in each pot and after germination thinning was performed leaving 6 plants in each pot as well as Hoagland nutrient solution was applied. After 35 days of germination plants were treated with different levels of salt (0 mM NaCl, 100 mM NaCl, 200 mM NaCl) and Selenium (0 ppm, 20 ppm, 40 ppm, 80 ppm, 120 ppm). Root application method was used for salt and selenium application. After a few days of treatments plants were collected and different morphological, physiological, biochemical and antioxidants parameters were recorded according to the following protocols.

Morphological parameters

All growth parameters were measured by following Hunt (2012) protocols. One plant was collected from each replication. Then root, shoot length, and total plant height were measured by measuring scale as well as their mean values were calculated. Then the number of leaves and branches of that plant were recorded. After that their fresh weight was measured by using weighting balance. Then these roots and shoots were dried in sunlight for few hours to remove moisture. After that roots were fully dehydrated in oven at 100 °C and their dry weight was recorded.

Leaf area

To measure leaf area one leaf from each plant replication was plucked and area of leaf was determined by multiplying the leaf length with leaf width as well as correction factor according to this formula.

$$\text{Lea area} = \text{Leaf Length} \times \text{Leaf width} \times 0.75 \quad (1)$$

Whereas: 0.75 is the correction factor.

Leaf specific area

Leaf specific area of one plant from each replication was measured by using given formula dividing leaf area by fresh weight of leaf.

$$\text{Leaf specific area} = \text{Fresh leaf area}/\text{Fresh leaf weight} \quad (2)$$

Similarly, the leaf area index of one plant from each replication was measured by formula given below.

$$\text{Leaf area index} = \text{Fresh leaf area} / \text{land area} \quad (3)$$

Physiological parameters

Chlorophyll content

Chlorophyll content of one plant from each replication was measured by using Davis (1976) and Arnon (1949) method. From each replication a fresh leaf was plucked, and 0.5 g of leaf was grinded into mortal and pestles. After this 5 ml of C_3H_6O (80%) was mixed with extract and kept for one night (10 °C temperature). On the next day the extract was centrifuged at 14,000 rpm value for 5 minutes. After that absorbance of extract was measured by using spectrophotometer at three wavelengths 480 nm, 645 nm, and 663 nm for carotenoids, chlorophyll *b,* chlorophyll *a* respectively by using formula given below.

$$\text{Carotenoids} = [4.16(OD_{480})\text{-}0.89(OD_{663})] \times V/1000 \times W \quad (4)$$
$$\text{Chl. a} = [12.7\ (OD_{663})\text{-}2.69(OD_{645})] \times V/1000 \times W \quad (5)$$
$$\text{Chl. b} = [22.9\ (OD_{645}\text{-}4.68(OD_{663})] \times V/1000 \times W \quad (6)$$

While OD describes optical density value (nm), V as volume (ml), W indicates weight (g).

Biochemical parameters

Shoot nutrient analysis

Mineral ions concentration of shoots was determined by digestion method proposed by Wolf (1982). 0.1 g of each plant dry shoot was added up in 2.5 ml of concentrated H_2SO_4 at 25 °C in flasks. After that 4 ml of H_2O_2 (35%) was added in flask and heated at 350 °C until solution turns colorless. After this solution was filtered and diluted by adding distilled water to 50 ml of volume. This filtrate was checked in flame photometer and ions concentration was determined. Similarly, Olson (1973) method was followed to measure the selenium ions concentration. Samples were prepared and digested with HNO_3 and $HCLO_4$. Then a blank was prepared, and titration was performed using NH_2OH-EDTA and eresol red. Then selenium ions content was measured at 378 nm wavelength by mixing freshly prepared 2,3-diaminonaphthalene (DAN) solution and cyclohexane.

Antioxidants and secondary metabolites

SOD, POD, CAT

Giannopolitis *et al*. (1977) method was followed to assess superoxide dismutase (SOD) activities. In this process, a mixture of phosphate buffer, H_2O, Triton, L-methionine, NBT, enzyme extract, and riboflavin was combined then introduced into cuvette. The cuvette was kept under a fluorescent lamp for 15 minutes and subsequently evaluated at a wavelength of 560 nm in order to determine the SOD activity levels.

In order to standardize the leaf sample, a quantity of 0.5 grams was combined with K_2HPO_4 buffer (50 mM). To determine the activity of Peroxidase (POD), a mixture was prepared consisting of K_3PO_4 buffer at 7.0 pH, guaiacol (20 mM), hydrogen peroxide (40 mM), as well as sample extract (0.1 ml). At a wavelength of (470 nm) absorbance was recorded continuously for a duration of 20 seconds. To determine catalase, a mixture comprising K_3PO_4 (pH 7.0) at a concentration of 50 mM, H_2O_2 at a concentration of 5.9 mM, and sample extract (0.1 ml) was prepared. The absorbance at 240 nm was recorded after every 20 seconds using the methodology outlined by Chance *et al*. (1955).

Proline, total phenol, flavonoids

To measure flavonoids content Skrypnik *et al*. (2019) method was used. 1 mL of sample extracts was mixed with 50 µL of $AlCl_3$ and 100 µL of 5% sodium nitrite. After ten minutes, at 510 nm absorbance was measured after adding 25 0µL of 1M NaOH. To measure proline content Bates *et al.* (1973) method was employed. For this 0.5 g of plant material was homogenized in 10 ml of 3% aqueous sulfosalicylic acid, and the filtrate (2 ml) was reacted with acid-ninhydrin and glacial acetic acid at 100 °C for 1 hour. The reaction was terminated in an ice bath, followed by extraction with 4 ml toluene. After mixing and separation, the chromophore containing toluene was measured at 520 nm using toluene as a blank. The Folin-Ciocalteu method (Singleton *et al*., 1965) was followed to measure the total phenol content. For this, 200 mg of leaf sample was ground up by adding 3 mL of 85% methanol and then filtered. After filtering, 1500 µL of 10% Folin reagent was mixed with 300 µL of the supernatant, and the mixture was allowed to sit at room temperature for five minutes. Then 1200 µL of 7% sodium carbonate was added and shaken for 2 hours. After shaking, absorbance was measured at 765 nm by using spectrophotometer.

H_2O_2 content

Similarly, to measure values for the hydrogen peroxide Alexieva *et al*. (2001) protocol was used. For this measurement enzyme extract was mixed with potassium iodide and absorbance of sample was recorded at 390 nm wavelength by using spectrophotometer.

Salt tolerance index (STI)

The salt tolerance index (STI) was calculated through a comparison between the total dry weight of plants grown from 100 seeds exposed to different salt concentrations and the dry weight of plants grown under normal conditions (control). The formula for computing the STI is as follows.

$$\text{Salt Tolerance Index} = (\text{Total Dry Weight at salinity} / \text{Total Dry Weight in control}) \times 100 \quad (7)$$

Statistical analysis

By following Steel *et al*. (1997) method data was analyzed by using COSTAT software and their means were compared by LSD test with 5% probability level.

Results

Morphological parameters

According to variance analysis salinity as well as selenium showed non-significant results for root length and significant results ($P \leq 0.001$) for fresh and dry weight of root. Salinity stress negatively affected root length, root fresh and dry weight of mash bean. Maximum reduction in root length and root fresh and dry biomass was recorded at 200 mM level. Selenium application under controlled conditions of salt stress increased root length and weight. It was also observed that 20 ppm and 40 ppm application of selenium mitigated salt stress and enhanced root length, root fresh weight and dry weight of mash bean by increasing antioxidants activity. Maximum root length and weight was observed at 40 ppm level of selenium. These results showed that selenium is a powerful essential nutrient to mitigate salt stress and increase root length, root fresh and dry weight in plants (Table 1 and Figure 1A, 1B, 1C).

A similar trend was followed by shoot length, shoot fresh and dry weight. According to variance analysis salinity and selenium showed significant ($P \leq 0.001$) results for shoot length, shoot fresh and shoot dry weight. Results indicated that salinity stress reduced shoot length, shoot fresh and dry weight of mash bean which were alleviated by selenium application. More reduction in shoot length and weight was noted at 200 mM as compared to 100 mM salt. Selenium application at 20 ppm and 40 ppm greatly enhanced shoot length, fresh and dry weight than other levels during salinity and controlled plants (Table 1 and Figure 1D, 1E, 1F).

Table 1. Analysis of variance for morphological parameters of mash bean under salinity stress with exogenous selenium application

Source	RL	RFW	RDW	SL	SFW	SDW	PH	NOL	NOB	LA	LSA	LAI
Salt	108.38***	0.09***	0.01***	169.8***	2.1***	0.6***	548.9***	6.75***	24.06***	3417237.6***	21215802***	1.23***
Selenium	12.38***	0.04***	0.02***	30.79***	0.93***	0.83***	80.3***	4.22***	18.8***	778226.96***	4327385.5***	0.28***
Salt * Selenium	0.92^{ns}	0.002*	0.0004**	7.35***	0.04***	0.03***	4.44*	0.08^{ns}	0.31^{ns}	151035.2***	152533.6*	0.05***
Error	0.54	8.84	1.35	1.07	5.24	0.001	1.7	0.17	0.51	4745.4	56696.9	0.001
LSD Salinity	0.47	0.022	0.008	0.77	0.01	0.02	0.97	0.53	0.31	51.3	177.5	0.03
LSD Selenium	0.61	0.028	0.011	0.99	0.02	0.038	1.25	0.68	0.4	66.3	229.2	0.03

***Significant at P≤0.001, ns=non-significant, SOV=Sum of variance, RL=Root length, RFW=Root fresh weight, RDW=Root dry weight, SL=Shoot length, SFW=Shoot fresh weight, SDW=Shoot dry weight, PH=Plant height, NOL=Number of leaves, NOB= Number of branches, LA=Leaf area, LSA=Leaf specific area, LAI=Leaf area index

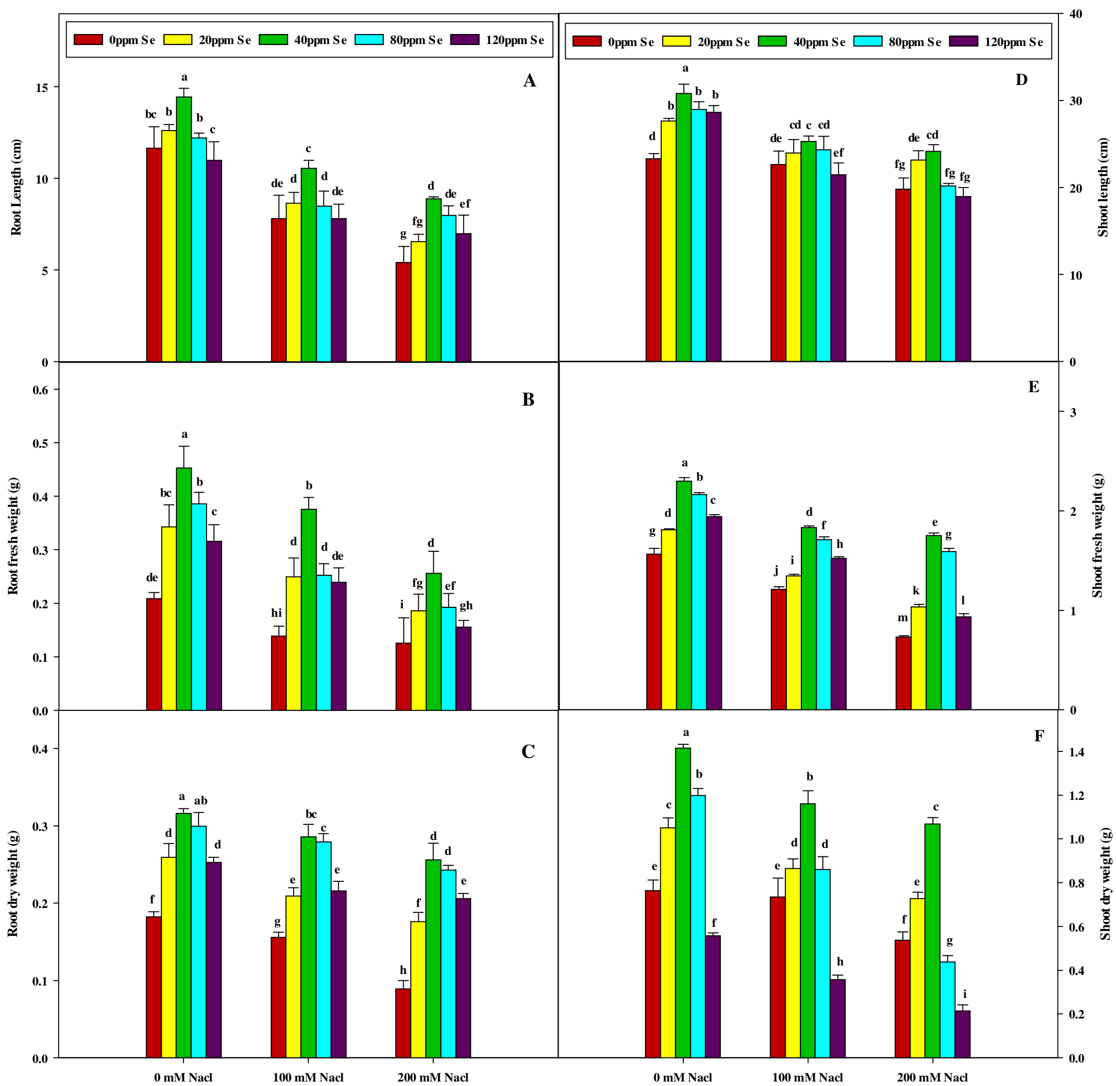

Figure 1. Effect of exogenous selenium application on root length (A), root fresh weight (B), root dry weight (C), S=shoot length (D), shoot fresh weight (E) and, shoot dry weight (F) of mash bean under salinity stress

Salinity and selenium showed significant (P≤0.001) results for plant height while non-significant results were recorded for the number of leaves and number of branches. According to observations, results indicated that plant height, number of leaves and branches of mash bean were decreased by salinity stress. Results obtained from data showed that selenium at lower dose (20 ppm and 40 ppm) helped in increasing plant height, no. of leaves and branches under normal and salt stressed conditions. However, selenium application at maximum level (120 ppm) under normal conditions and stressed conditions reduced these parameters (Table 1 and Figure 2A, 2B, 2C).

Similarly, significant (P≤0.001) results for leaf area and leaf specific area as well as leaf area index. Salinity stress adversely affected leaf area parameters of mash bean. According to the results, it was noted that during salinity, surface area of leaf, specific area of leaf and area index were decreased. However, selenium application at 20 ppm and 40 ppm increased leaf area, and leaf area index in salt treated and non-treated plants while selenium induced toxicity at higher level (80 ppm, 120 ppm) leading to reduce leaf area parameters as shown in (Table 1 and Figure 2D, 2E, 2F).

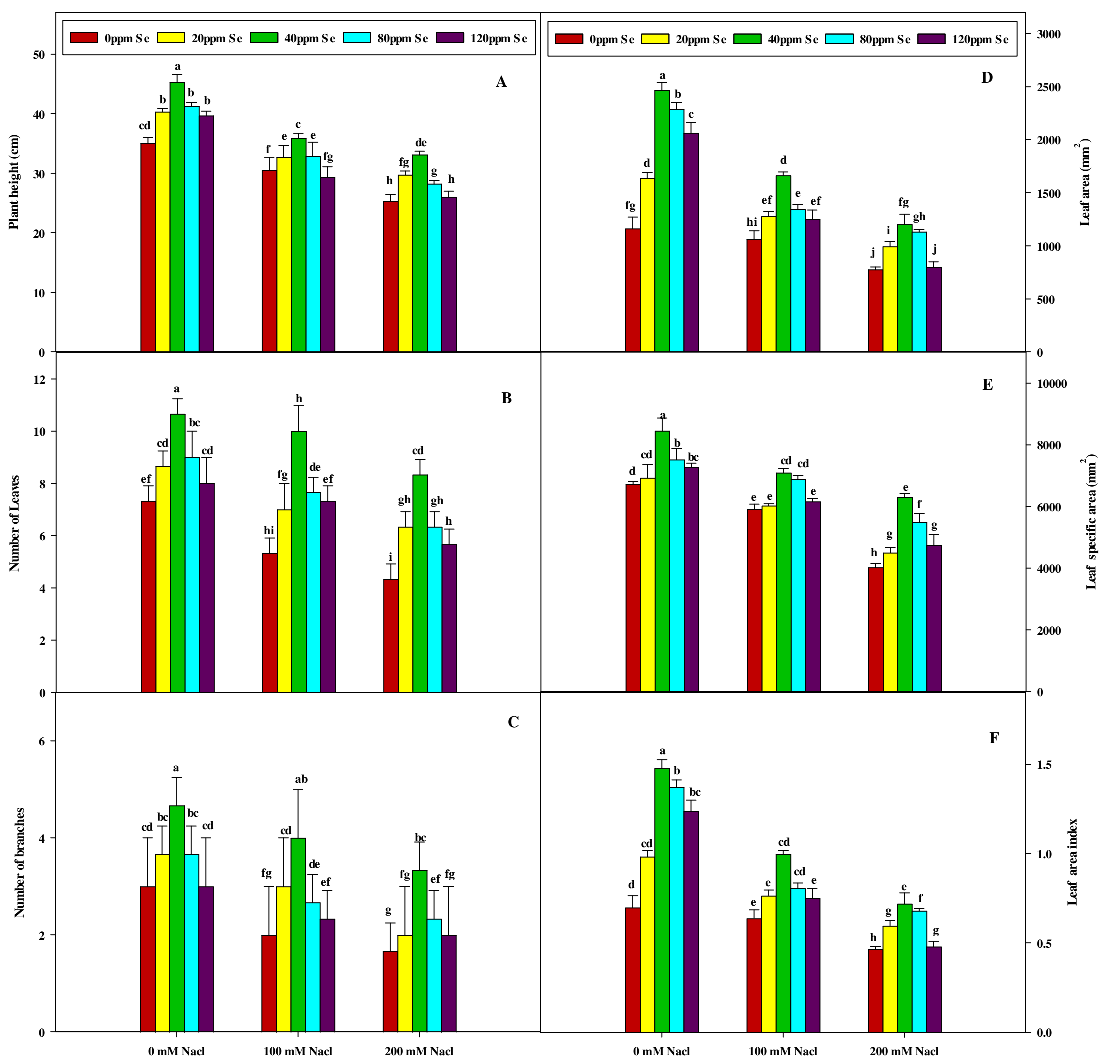

Figure 2. Effect of exogenous selenium application on plant height (A), number of leaves (B), number of branches (C), leaf area (D), Leaf specific area (E) and leaf area index (F) of mash bean under salinity stress

Physiological parameters

According to analysis of variance salinity and selenium showed non-significant results for chlorophyll *a,* and significant results ($P \leq 0.001$) were noted for chlorophyll *b* and carotenoids. Salinity negatively affected photosynthetic pigments of mash bean. it was observed that salinity minimized chlorophyll *a, b* and carotenoids content and this negative effect was alleviated by selenium treatment. The minimum content of photosynthetic pigments was noted at 200 mM salt level. It was noted that selenium treated mash bean plants showed higher chlorophyll *a, b,* and carotenoids content than control (without salt application). Additionally, Se at 20 ppm and 40 ppm increased photosynthetic pigments values under salt stress condition however selenium at higher concentration 80 ppm and 120 ppm become toxic and minimized chlorophyll content (Table 2 and Figure 3A, 3B, 3C).

Table 2. Analysis of variance for physiological and biochemical parameters of mash bean under salinity stress with exogenous selenium application

Source	Chl. *a*	Chl. *b*	Car	Total Chl.	Chl. ratio	Na^+	K^+	Ca^{2+}	Selenium	STI
Salt	0.00003***	0.00009***	0.0002***	0.00008***	0.032***	382.4***	84.86***	42.46***	0.21***	8912.1***
Selenium	0.00008***	0.0001***	0.0002***	0.00002***	0.03***	208.5***	86.96***	76.9***	0.24***	11190***
Salt * Selenium	0.0000014^{ns}	0.000004*	0.000007**	0.0000006^{ns}	0.001***	8.18^{ns}	8.95***	1.07^{ns}	0.01***	380.8***
Error	1.22	1.64	2.46	6.8	2.71	4.33	1	1.13	1.99	18.78
LSD Salinity	8.24	9.56	0.001	6.18	0.01	1.55	0.57	0.54	0.01	3.23
LSD Selenium	0.001	0.001	0.001	7.99	0.01	2.004	0.74	0.7	0.01	4.17

***Significant at $P \leq 0.001$, ns=non-significant, SOV=Sum of variance, Chl. a=Chlorophyl a, Chl. b=Chlorophyl b, Car=Carotenoids, Total Chl.=Total chlorophyll, Chl. ratio= Chlorophyll ratio, Na^+=Sodium ions, K^+= Potassium Ions, Ca^{2+}= Calcium ions, STI= Salt tolerance index.

Moreover, non-significant results for total chlorophyll and significant results ($P \leq 0.001$) for chlorophyll ratio were obtained under salinity and selenium treatment. Salinity at 200 mM level showed maximum reduction in total chlorophyll content and chlorophyll ratio of plants without selenium application. However, selenium at 40 ppm showed maximum total chlorophyll content and chlorophyll ratio under salinity and on further increasing selenium value more decrease in total chl. and chlorophyll ratio was observed (Table 2 and Figure 3D, 3E).

Biochemical parameters

According to analysis of variance salinity and selenium showed non-significant results for sodium and calcium ions while significant results ($P \leq 0.001$) for potassium ions. It was clearly observed that sodium ions concentration increased by increasing salt stress level. Maximum sodium ions were observed at 200 ppm salinity. Sodium ions content was decreased by application of selenium. Selenium at 20 ppm and 40 ppm reduced salinity by decreasing sodium ions non-significantly. However, selenium at 80 ppm and 120 ppm increased sodium ions concentration under salinity stress. Meanwhile, potassium and calcium ions showed opposite results under salinity and selenium application. Potassium and calcium ions concentration was reduced by salinity stress and maximum reduction of potassium and calcium ions was observed at 200 mM level. Selenium application resulted in increasing potassium and calcium ions under saline and controlled plants. Selenium (40 ppm) showed maximum potassium and calcium ions concentration. Above 40 ppm selenium treatment with salinity K^+ and Ca^{2+} ions level was observed decreasing. Results showed that selenium concentration was significantly increased with application of selenium under salinity stress. However, at higher concentrations of salinity a little reduction was recorded. Maximum selenium concentration was observed at 80 ppm and 120 ppm of selenium application (Table 2 and Figure 4A, 4B, 4C, 4D).

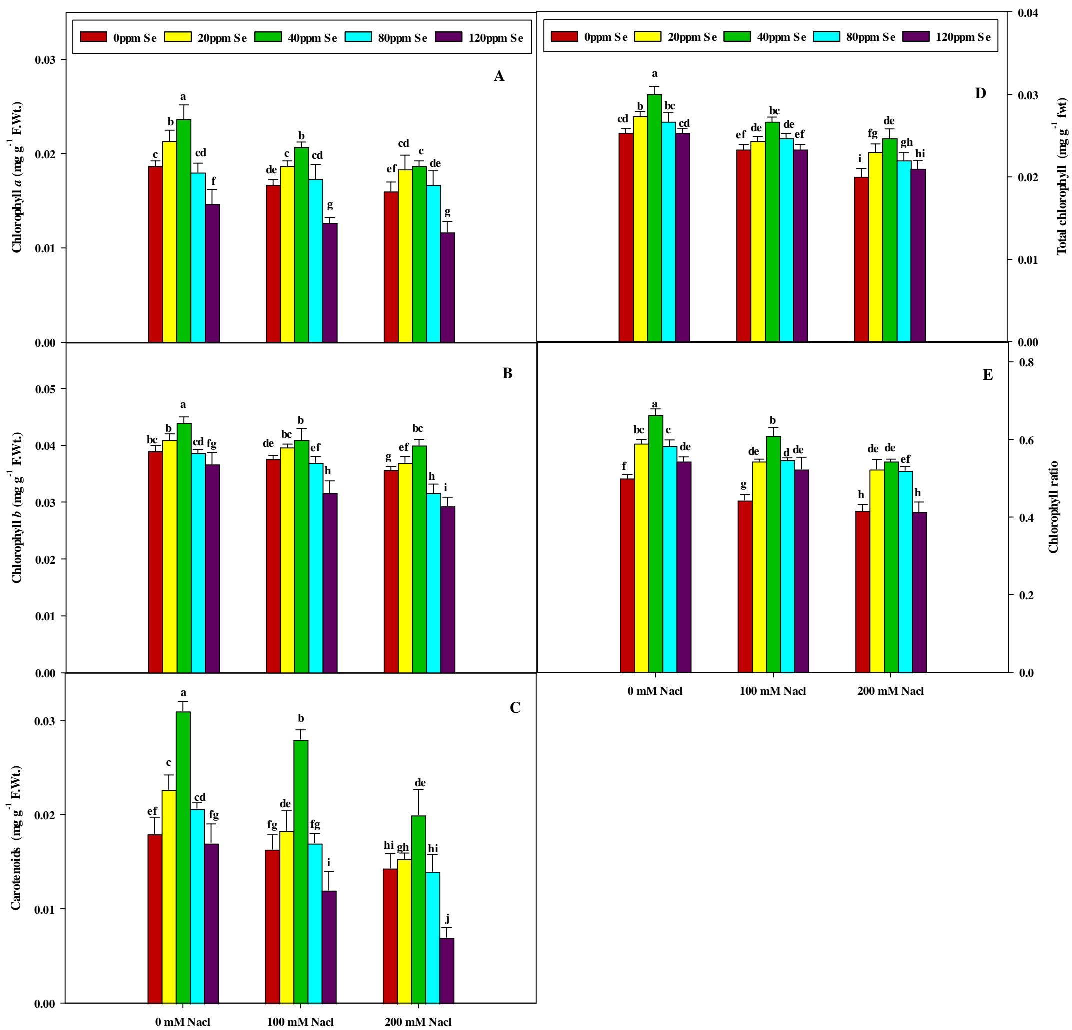

Figure 3. Effect of exogenous selenium application on chlorophyll *a* (A), chlorophyll *b* (B) and carotenoids (C), total chlorophyll (D), and chlorophyll ratio (E) of mash bean under salinity stress

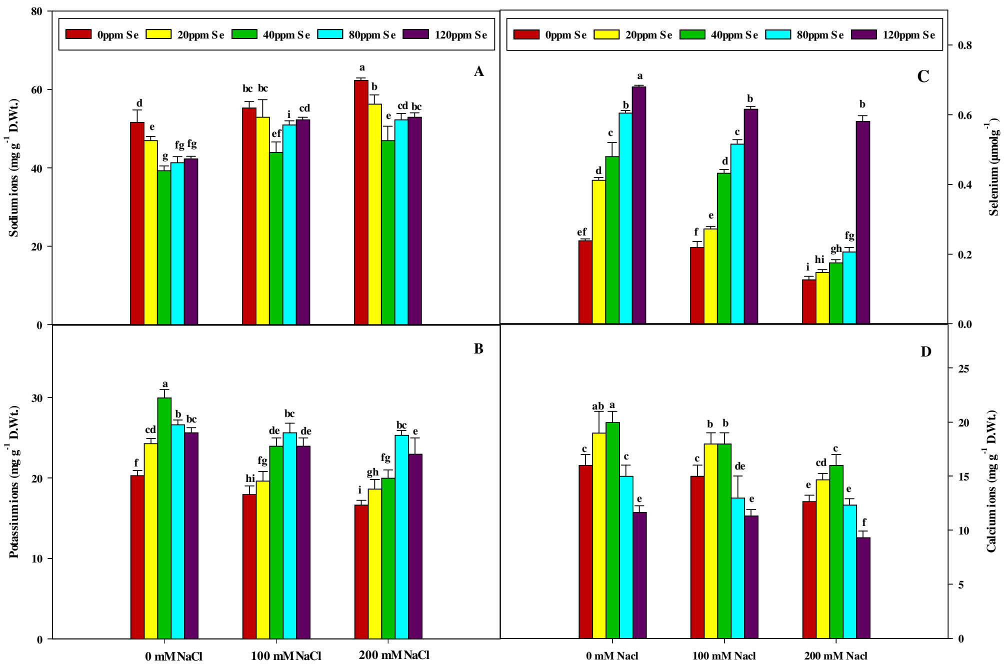

Figure 4. Effect of exogenous selenium application on shoot sodium ions (A), potassium ions B), selenium (C) and calcium ions (D) of mash bean under salinity stress

Antioxidants

Significant results ($P \leq 0.001$) for SOD, POD and CAT content were recorded under salinity and selenium treatment. Salinity significantly reduced SOD, POD, and CAT activities in mash bean. According to the results, it was observed in mash bean that SOD, POD, and CAT were reduced during salt stress at 100 mM while selenium application at 20 ppm and 40 ppm improved antioxidants enzymes activities. However, high dose (80 ppm, 120 ppm) of selenium resulted in reduction of SOD, POD, and CAT levels in mash bean during salinity. Maximum enzymatic activities were observed at 40 ppm of selenium application under salinity as shown in (Table 3 and Figure 5A, 5B, 5C).

Table 3. Analysis of variance for antioxidants and secondary metabolites of mash bean under salinity stress with exogenous selenium application

Source	SOD	POD	CAT	H_2O_2	TPC	Proline	Flavonoids
Salt	1239.9***	1233***	129.8***	76.6***	35.45***	55.86***	1.10***
Selenium	106.5***	99.3***	135.3***	15.1***	3.50***	49.16***	0.50***
Salt * Selenium	4.49**	3.92*	8.22***	1.16***	0.99***	2.01^{ns}	0.01^{ns}
Error	1.22	1.298	0.14	0.20	0.04	0.97	0.007
LSD Salinity	0.82	0.84	0.28	0.33	0.16	0.73	0.06
LSD Selenium	1.06	1.09	0.36	0.43	0.20	0.95	0.08

***Significant at $P \leq 0.001$, ns=non-significant, SOV=Sum of variance, SOD= Superoxide dismutase, POD= Peroxidase, CAT= Catalase, H_2O_2= Hydrogen peroxide, TPC=Total phenol content.

H_2O_2 content

According to analysis of variance salinity and selenium showed non-significant results for H_2O_2. From results, it was clearly observed that H_2O_2 concentration increased by increasing salt stress level. Maximum H_2O_2 was observed at 200 ppm salinity. H_2O_2 content was decreased by application of selenium. Selenium at 20 ppm and 40 ppm reduced salinity by decreasing H_2O_2. However, selenium at 80 ppm and 120 ppm increased H_2O_2 concentration under salinity stress as shown in (Table 3 and Figure 5D).

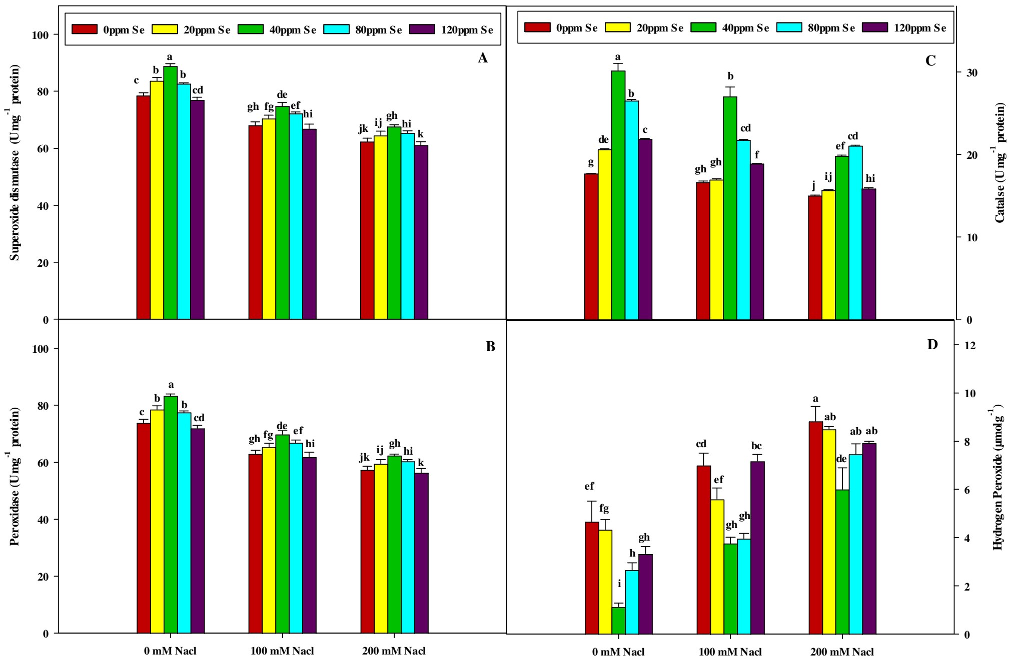

Figure 5. Effect of exogenous selenium application on SOD (A), POD (B) and CAT (C), and H_2O_2 (D) of mash bean under salinity stress

Secondary metabolites

It was observed that lower-level selenium application enhanced secondary metabolites concentration in mash bean under salinity. However, at higher concentration of selenium (120 ppm) and salinity (200 mM) significant reduction ($P \leq 0.001$) was recorded in mash bean plants. Proline content of mash bean plants was observed maximum at 100 mM salt stress and lower levels of selenium application leading to produce salinity tolerance. However, with more at higher concentration of salinity and selenium it was noted that proline content tends to reduce. Similar trend was followed by flavonoids and total phenol content. Maximum improvement in proline, total phenol content and flavonoids was noted at 40 ppm selenium application under 100 mM salinity (Table 3 and Figure 6A, 6B, 6C).

Salt tolerance index (STI)

It was revealed from the results that salinity has significantly ($P \leq 0.001$) affected salt tolerance index (STI) of mash bean. Salt stress (100 mM, 200 mM) resulted in reduction of STI. Under controlled conditions an increase in STI was recorded with selenium application. Maximum STI was observed on (40 ppm Se) application under control conditions. However, it was observed that selenium under higher concentrations (80 ppm, 120 ppm) tends to decrease STI in mash bean plants (Table 2 and Figure 7).

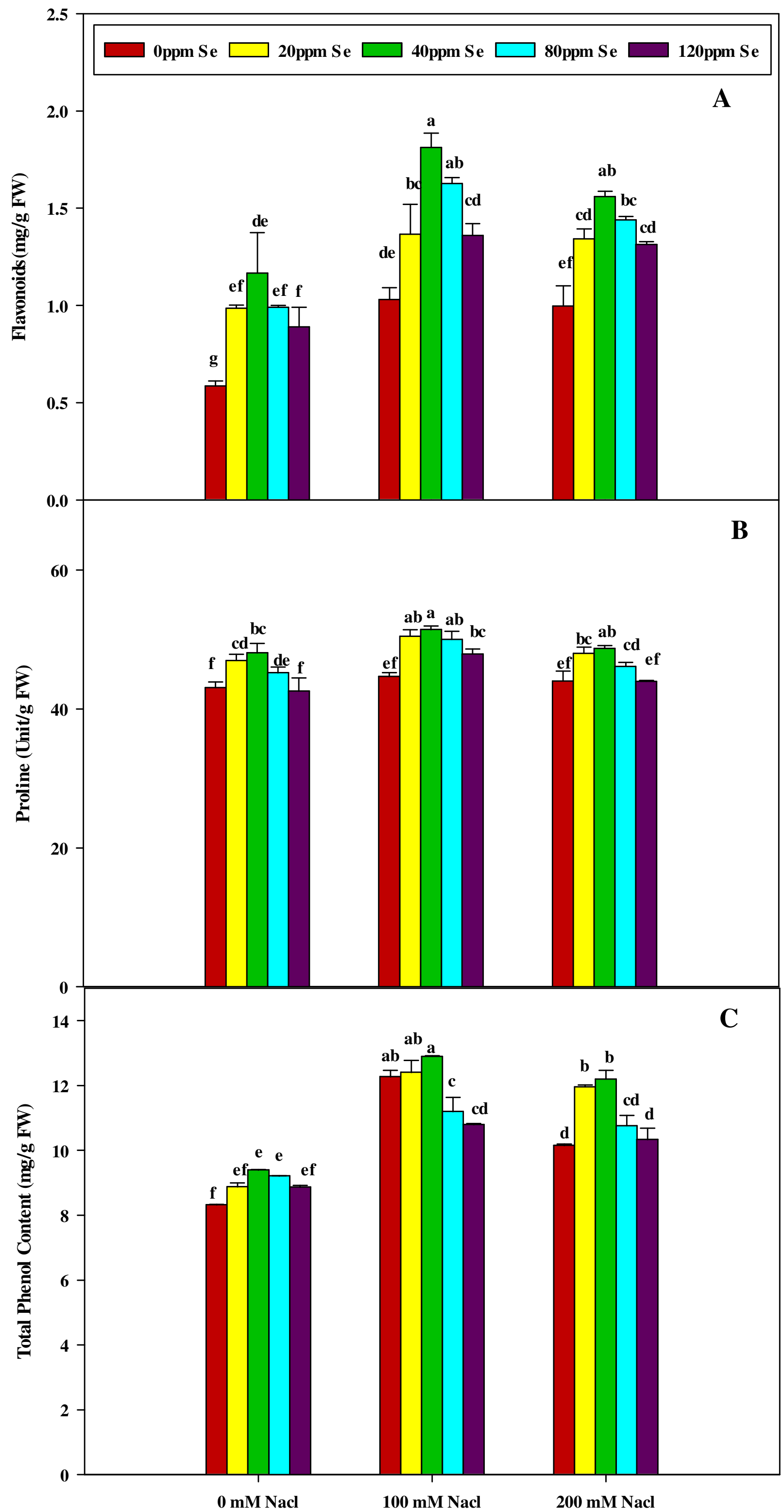

Figure 6. Effect of exogenous selenium application on Flavonoids (A), Proline (B) and Total Phenol Content (C) of mash bean under salinity stress

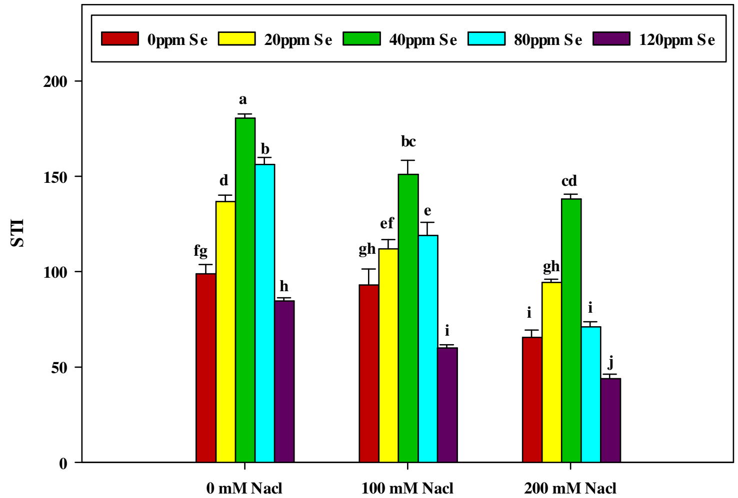

Figure 7. Effect of exogenous selenium application on Salt tolerance index (STI) of mash bean under salinity stress

Correlation analysis

Pearson's correlation

Morphological attributes at different salinity levels were correlated: at 0mM NaCl i.e., SDW, RDW, RFW, SFW, SL, RL, LA, NOB, NOL and LSA strongly positively correlated with physiological attributes i.e., K^+, Ca^{2+}, Ch*a*, Ch*b* and CAR, SOD, POD, CAT, and negatively correlated with Na^+, K^+, Ca^{2+}, H_2O_2. While at 100 mM NaCl there is a negative correlation between physiological attributes and positive correlation between morphological attributes. But at 200 mM NaCl there exists less positive correlation between Chla, Chlb, CAR, K+ with morphological attributes. K^+ didn't show any correlation with other morpho-physiological attributes (Figure 8).

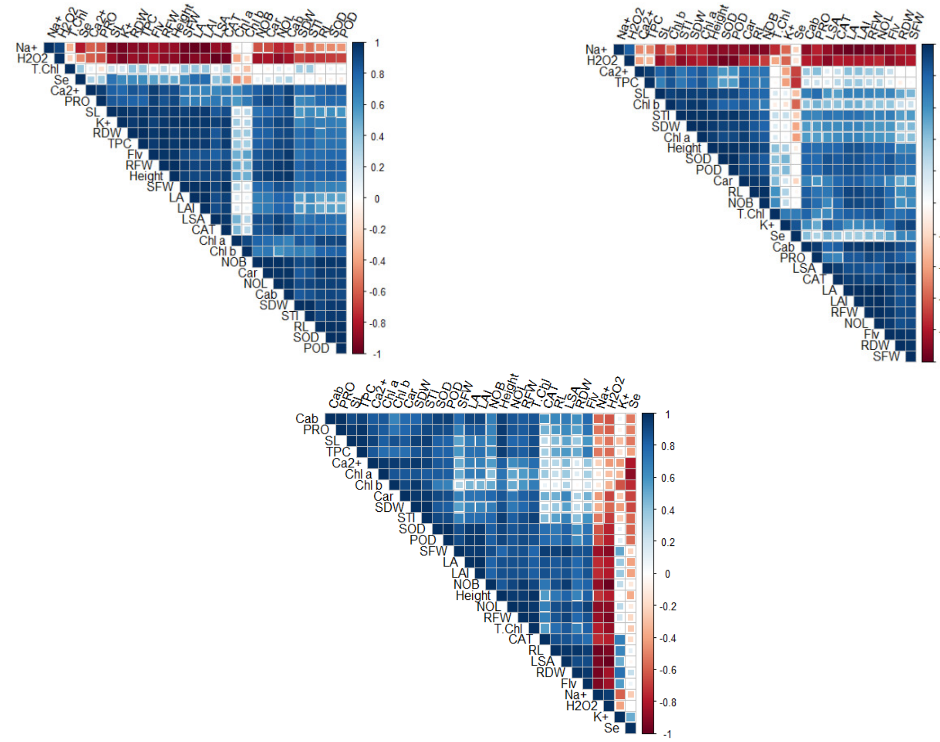

Figure 8. Pearson correlations of different morpho-physiological attributes of mash bean

Morphological attributes: Shoot dry weight (SDW), Root dry weight (RDW), Shoot fresh weight (SFW), Root fresh weight (RFW), Shoot length (SL), Root length (RL), Plant height, Leaf area index (LAI), Specific leaf area (SLA), Number of branches (NOB), NOL(Number of leaves), Leaf area (LA), Chlorophyll *a* (Cha) , Chlorophyll *b* (Chb), Carotenoids (CAR), Total chlorophyll (T.Chl), SOD (Superoxide dismutase), CAT (Catalase), POD (Peroxidase), Na^+ (Sodium), K^+ (Potassium), Calcium (Ca^{2+}), H_2O_2 (Hydrogen peroxide), Salt tolerance index (STI), Selenium (Se), Proline (PRO), Total phenolic contents (TPC), Flavonoids (FLV)

<u>PCA-Biplot</u>

There are greater variations among different morpho-physiological attributes at different salinity levels. PC $_1$ shows 75.9% variations while PC_2 shows 15.8% variations at 0mM NaCl. The biplot showed morpho-physiological attributes are affected by $S_0NP_{20,}$ S_0NP_{40} and $S_0NP_{80\ i.e.}$, LA, LAI, CAT, K^+, TCP, Se. S_0NP_{120} didn't contribute to affect any morph-physiological attribute. The second biplot represented growth and physiological parameters at 100 mM NaCl showing positive engine values at NP_{20} and NP_{40}. But various parameters RDW, SFW, CAT showed -2 engine values at S0NP80. At 200 mM NaCl, the major contributor in affecting growth and physiological parameters was NP_{20}, NP_{40} and NP_{80}, while Na and H_2O_2 were varied according to different salinity level (Figure 9).

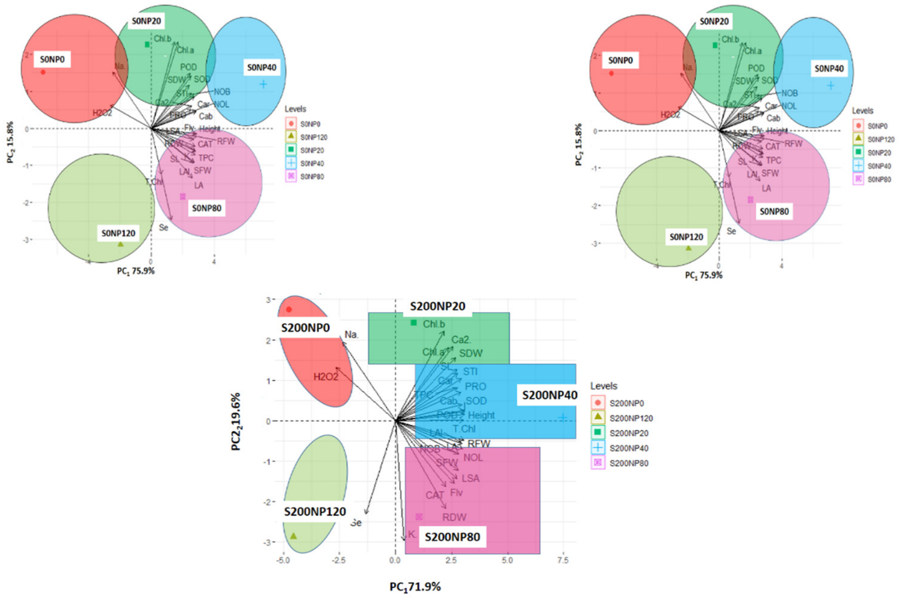

Figure 9. PCA-Biplot of different morpho-physiological attributes of mash bean with different treatments of salt and nanoparticles S_0NP_0, S_0NP_{20}, S_0NP_{40}, S_0NP_{80}, S_0NP_{120}, $S_{100}NP_0$, $S_{100}NP_{20}$, $S_{100}NP_{40}$, $S_{100}NP_{80}$, $S_{100}NP_{120}$, $S_{200}NP_0$, $S_{200}NP_{20}$, $S_{200}NP_{40}$, $S_{200}NP_{80}$, $S_{200}NP_{120}$.

Morphological attributes: Shoot dry weight (SDW), Root dry weight (RDW), Shoot fresh weight (SFW), Root fresh weight (RFW), Shoot length (SL), Root length (RL), Plant height, Leaf area index (LAI), Specific leaf area (SLA), Number of branches (NOB), NOL (Number of leaves), Leaf area (LA), Chlorophyll *a* (Cha) , Chlorophyll *b* (Chb), Carotenoids (CAR), Total chlorophyll (T.Chl), SOD (Superoxide dismutase), CAT (Catalase), POD (Peroxidase), Na^+ (Sodium), K^+ (Potassium), Calcium (Ca^{2+}), H_2O_2 (Hydrogen peroxide), Salt tolerance index (STI), Selenium (Se), Proline (PRO), Total phenolic contents (TPC), Flavonoids (FLV).

Clustered heat map

Clustered heat map showing two sub clustering, where morphological attributes grouped with physiological attributes. In cluster 1 morphological attributes RFW, LA, SL and Height showed strong positive association at 0 mM NaCl and NP_{40}. All parameters showed positive association at 0 mM NaCl. As salinity increases up to 200 mM NaCl the growth hampered as depicted in clustering except Na^+ and H_2O_2 which shows counter association (Figure 10).

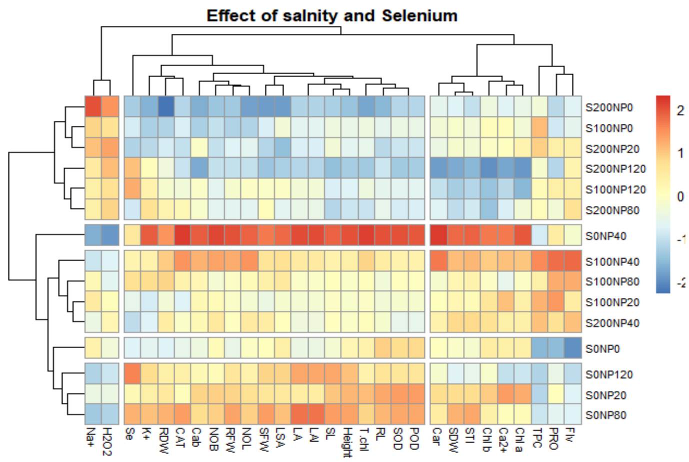

Figure 10. Clustered Heatmap of different morpho-physiological attributes of mash bean with different treatments of salt and nanoparticles S_0NP_0, S_0NP_{20}, S_0NP_{40}, S_0NP_{80}, S_0NP_{120}, $S_{100}NP_0$, $S_{100}NP_{20}$, $S_{100}NP_{40}$, $S_{100}NP_{80}$, $S_{100}NP_{120}$, $S_{200}NP_0$, $S_{200}NP_{20}$, $S_{200}NP_{40}$, $S_{200}NP_{80}$, $S_{200}NP_{120}$.
Morphological attributes: Shoot dry weight (SDW), Root dry weight (RDW), Shoot fresh weight (SFW), Root fresh weight (RFW), Shoot length (SL), Root length (RL), Plant height, Leaf area index (LAI), Specific leaf area (SLA), Number of branches (NOB), NOL (Number of leaves), Leaf area (LA), Chlorophyll *a* (Cha) , Chlorophyll *b* (Chb), Carotenoids (CAR), Total chlorophyll (T. Chl), SOD (Superoxide dismutase), CAT (Catalase), POD (Peroxidase), Na^+ (Sodium), K^+ (Potassium), Calcium (Ca^{2+}), H_2O_2 (Hydrogen peroxide), Salt tolerance index (STI), Selenium (Se), Proline (PRO), Total phenolic contents (TPC), Flavonoids (FLV).

Discussion

Plants face several types of environmental factors like salinity during their growth phase and reproduction. These factors affect plant growth negatively and result in reduction of morphological parameters like plant height, biomass, number of leaves and branches (Chattha *et al*., 2022). Salt induce significant decrease in shoot length, root length and plant height due to nutrient imbalance and osmotic potential (Safdar *et al*., 2019). Present study showed that length of root and shoot and total height was reduced during salt condition. However, selenium application at lower level (20 ppm and 40 ppm) resulted in increasing plant height, root, and shoot length. It is also observed in other studies that lower dose of selenium enhances plant height and morphological attributes of plants during salinity by adjusting osmotic and ionic balance (Ashraf *et al*., 2018; Sattar *et al*., 2017). Higher concentration of selenium become hazardous and negatively affect plant growth. Present findings revealed similar results that higher concentration of selenium (80 ppm and 120 ppm) inhibited height, root length, and shoot length of mash bean in salt stress.

Salinity affect plant weight and it is observed in different studies that salinity reduce weight by ionic imbalance and more sodium ions concentration (Tanveer *et al*., 2020; Zahra *et al*., 2020). In this experiment reduction in fresh and dry weight of root and shoot was recorded under salinity stress condition which were improved by application of selenium (20 ppm and 40 ppm). Selenium application under minute level enhance fresh and dry weight of root and shoot in plants (Mozafariyan *et al*., 2016) while higher level of selenium negatively affect plant growth and cause decrease in fresh and dry weight (Lehotai *et al*., 2012). This experiment revealed that higher concentration of selenium (80 ppm, 120 ppm) decreased weight because maximum concentration of selenium caused more absorption of sodium ions.

According to Sadak *et al*. (2019) salinity reduce no. of branches and leaves in plants. Similarly, adverse impacts of salt stress on morphology like no. leaves and branches have also been observed by different authors (El Nahhas *et al*., 2021; Ul Haq *et al*., 2023). In the present study salt stress reduced no. of leaves and branches.

Application of selenium at low concentration showed improvement in increasing number of branches and leaves in present study. Selenium has been found as a growth promoter in other plants under salinity stress conditions (Ali *et al*., 2021).

Plant photosynthesis is facilitated by chlorophyll that is responsible for this process. Salinity interferes with chlorophyll synthesis in plant cells and causes reduction in photosynthetic pigments by reducing leaf area. It is observed from several studies that salt stress reduces photosynthetic pigments (Chen *et al*., 2022; Harizanova *et al*., 2019; Hmaeid *et al*., 2019; Shin *et al*., 2020). Our study showed that salinity stress reduced *Vigna mungo* chlorophyll which is enhanced by selenium application. Same results in previous studies have been reported for selenium application on other plants during salt condition (Kamali-Andani *et al*., 2022; Silva *et al*., 2020).

Salinity causes reduction in leaf area and leaf specific area of plants. Plants face difficulty in absorbing water and keeping a good water balance in their tissues when they are exposed to excessive salt concentrations (Toshtemirovna *et al*., 2019). According to Rodríguez Coca *et al*. (2023) salinity enhanced leaf area in rice. Similarly, in another study on rice revealed that due to increase sodium ions during salinity stress, leaf area indices were decreased (Haque *et al.*, 2021). In our findings it was clearly shown that salinity inhibited leaf area, leaf specific area and leaf area index. According to different research selenium use boosts photosynthetic efficiency and activity, which favorably affects expansion of leaf area (Samynathan *et al*., 2023; Semida *et al.*, 2021). Present study also showed enhancement in leaf area and specific area on selenium application under low concentrations. However, higher concentration of selenium resulted in negative results.

Absorption and accumulation of sodium ions in plants due to salinity stress represent a notable adverse consequence which causes toxicity (Hanif *et al*., 2024). The presence of ionic toxicity in plant tissues due to salinity stress induces various physio-biochemical disturbances, generates free radicals, and disrupts nutrients balance (Azeem *et al*., 2023a). The following experiment revealed that salt enhanced sodium ions accumulation and reduced potassium as well as calcium ions. However, application of selenium enhanced potassium and calcium ions following to decrease in sodium ions concentration. In addition to this selenium ions concentration in mash bean plants was recorded maximum on increasing selenium application and similar results were observed in previous studies (Jawad Hassan *et al*., 2020). It is reported that application of Se and its nanoparticles mitigates the impacts salinity and enhances synthesis of antioxidants in lemon verbena plants by regulating ion homeostasis, reducing sodium ions accumulation and increasing potassium ions and calcium ions (Ghanbari *et al*., 2023). Additionally, it is revealed that in snap bean seedlings, the application of 5μM Se improved potassium and calcium ions while decreasing sodium ions demonstrating a positive impact on plant responses to salinity stress. However, higher selenium concentrations further intensified the decrease in sodium ions levels (Farag *et al*., 2022).

Antioxidants serve as a plant defense system during salinity. SOD acts as powerful armer against salinity stress by conversion of superoxide radicles into H_2O_2 and O_2 (Dumanović *et al*., 2021). However, SOD, POD, and CAT activities are affected by higher concentration of salt stress. During salt stress it is observed that activities of antioxidants are reduced in different plants due to nutrient imbalance and ROS accumulation (Azeem *et al*., 2023b). In the present study salt stress reduced activity level of SOD, POD, and CAT. Low selenium concentration enhances enzymatic activities in plants during salinity and other abiotic stresses (Lanza *et al*., 2021; Moussa *et al*., 2018). This study results showed agreement with previous literature findings as in this study selenium at lower concentration also improved SOD, POD, and CAT activities while higher doses of selenium became toxic and caused reduction in enzymatic activities under salt stress. Similarly, application of salinity enhanced hydrogen peroxides content which was reduced by selenium application at lower level. However, higher levels of selenium resulted in maximizing H_2O_2 content. Moreover, in current experiment proline content of mash bean plants was recorded maximum under minimum salt stress and selenium application. It is reported that proline content is associated with stress tolerance in plants acting as an osmo-protectant. Similar results for proline and H_2O_2 were observed in previous studies (Rasool *et al*., 2023).

The synthesis and production of secondary metabolites in plants are influenced by abiotic stresses. The biochemical pathways that lead to the synthesis of secondary metabolites are significantly shaped by these external factors (Rasheed *et al*., 2022). In our present study salinity stress and selenium application at lower levels enhanced flavonoids and total phenol content in mash bean plants and led to enhance stress tolerance. However, it was noted that higher concentration of selenium and salinity caused a small reduction in secondary metabolites. Similar results were shown in previous studies (Assaf *et al*., 2022; Ghanbari *et al*., 2023).

In the context of the salt tolerance index, prior research has indicated a decline in salinity tolerance as salt stress increases (Awasthi *et al*., 2016). Our investigation also observed a decrease in the salinity tolerance index among mash bean plants exposed to saline conditions. However, selenium application at lower concentrations enhanced salt tolerance index in mash bean.

In our present study the correlation analysis data also showed that morphological and physiological attributes have strong positive correlations at 0 mM NaCl. However, a negative correlation at 100 mM NaCl and a weaker positive correlation at 200 mM NaCl. K^+ doesn't correlate with any other morpho-physiological characteristics. Significant differences in morpho-physiological characteristics are shown by the PCA-Biplot at various salinity levels, with NP_{20}, NP_{40}, and NP_{80} having an impact on these attributes. These results are supported by the clustered heat map, which shows sub-clusters where morphological and physiological attributes grouped together revealing the effect of salinity on these parameters.

Conclusions

Selenium significantly enhanced the morphological parameters, ion uptake, photosynthetic pigments, and biochemical attributes in mash bean during salinity. Selenium application at lower concentrations (20 ppm and 40 ppm) proved to be powerful in reducing the detrimental impacts of salt and improving secondary metabolites. Results highlight selenium potential as a valuable micronutrient for increasing yield and stress tolerance in mash bean, particularly in saline environments. Further study is required to explore mechanisms of selenium-mediated stress tolerance and optimize its application methods to ensure its efficient utilization in agricultural practices. Salinity stress and selenium interaction regarding secondary metabolites synthesis also need more exploration at molecular level. Moreover, selenium mode of application from root to foliar application on mash bean is needed to study. By harnessing the beneficial effects of selenium, we can contribute to sustainable food production and address the challenges posed by increasing population growth and climate change. Furthermore, selenium application at 20 ppm and 40 ppm might be helpful for other crop plants under saline conditions.

Authors' Contributions

ZUA, SB; Conducted investigations and drafted paper, SB, AM; supervised research, AM NE; Conception and design, analyses and interpretation of the data, ZE, ÇCT, AM, SS, MI and AES; analyses and interpretation of the data Drafting of paper; Application of Statistics Analyses and Software, IAA; Provide Resources and Revising it critically for intellectual content, AM, SB, MI, KG, AES, IAA, NE; revising it critically for intellectual content; and the final approval of the version to be published. All authors agree to be accountable for all aspects of the work. All authors reviewed the manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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