

Interactive effect of potassium and spermidine protects growth, photosynthesis and chlorophyll biosynthesis in *Vigna angularis* from salinity induced damage by up-regulating the tolerance mechanisms

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Abstract

Pot experiments were conducted to evaluate the role of potassium (100 mg KCl / kg soil) and the spermidine (100 μ M Spd) in regulation of growth, chlorophyll synthesis and photosynthesis in *Vigna angularis* under salinity stress (100 mM NaCl). Salinity declined chlorophyll synthesis by causing a significant decline in the synthesis of δ -amino levulinic acid (ALA), protoporphyrin IX (Proto IX) and Mg-protoporphyrin IX (Mg-Proto IX), however application of K and Spd alone as well as combinedly alleviated the decline to considerable extent. Further, K and Spd treated plants exhibited a significant decline in reactive oxygen species and the lipid peroxidation and such effects were also obvious under salinity stress. Photosynthetic rate, stomatal conductance, intercellular CO₂ concentration, Fv/Fm and photochemical quenching increased significantly due to K and Spd application, and salinity induced alleviation of the decline was maximal due to combined K and Spd treatment. Up-regulation of antioxidant enzymes activity, increased content of ascorbic acid and glutathione (GSH), and the accumulation of compatible osmolytes due to K and Spd application strengthened the tolerance against the salinity stress thereby lessening the oxidative effects considerably. Accumulation of phenols and flavonoids increased significantly due to application of K and Spd. Salinity caused significant increase in Na however K and Spd application induced a significant decline concomitant with increase in K content reflecting in decreased Na/K. Results suggest that K and Spd application protect the growth and photosynthesis from salinity induced oxidative damage by up-regulating the ion homeostasis, antioxidant system, osmolyte accumulation and secondary metabolite synthesis.

Keywords: antioxidants; osmolytes; salinity; secondary metabolites; photosynthesis; Na/K

Introduction

Salinity stress has been considered as one of the devastating environmental factors responsible for significant decline in yield productivity of crop plants (Sudhir and Murthy, 2004; Soliman *et al.*, 2020; El-Taher *et al.*, 2022). Reduction in plant growth due to salinity stress is a cumulative result of effect on processes including germination, root growth, mineral and water uptake, enzyme functioning and photosynthesis (Ahanger *et al.*, 2019a, b). Salinity stress induces ionic and osmotic stress thereby not only restricts the ion uptake but also imparts oxidative damage to macromolecules and the functioning of major cellular organelles

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(Khan et al., 2014). Excess accumulation of toxic reactive oxygen species (ROS) and hyper-accumulation of sodium ions within sensitive cellular spaces leads to intensification of damaging effects of salinity stress (Fatma et al., 2016; Ahanger et al., 2019a, b; Elkelish et al., 2019; Fariduddin et al., 2019). Plants up-regulate the tolerance mechanisms for averting the deleterious effects of salinity. These tolerance mechanisms include: antioxidant and osmolyte metabolism, salt exclusion and compartment (Ahmad et al., 2019; Zaid et al., 2020; Zhao et al., 2021). The functioning of these mechanisms determines the salinity tolerance potential of plants (Elkelish et al., 2019; Islam et al., 2021; El-Taher et al., 2022). Stresses like salinity trigger degradation of proteins, lipids etc thereby hampering the functional and structural stability of plant cells (Ahanger et al., 2017a; Hasanuzzaman et al., 2018). Therefore, up-regulating and strengthening the indigenously existing tolerance mechanisms can protect growth and metabolism from stress mediated damage.

Potassium is one the macro-elements required for normal growth and development, and is key to maintain the optimal plant growth and the productivity (White and Karley, 2010; Ahanger and Agarwal 2017a, b; Sardans and Penuelas, 2021). The importance of K in plant metabolism and growth regulation has been attributed to its key roles like enzyme activation, photosynthetic enhancement, stomatal functioning, uptake and metabolism of mineral elements like nitrogen, protein synthesis and sugar transport as well as yield attributes (Amanullah et al., 2016; Ahanger et al., 2017a, b; Hasanuzzaman et al., 2018; Xu et al., 2020). It has been reported that both root and foliar application of potassium impart beneficial effects on the plant growth, physiological and yield attributes (Amanullah et al., 2016; Bahrami-Rad and Hajiboland, 2017). Once taken by plants K exhibits a strong mobility within the plant system and regulates cellular osmotic pressure (Kaiser, 1982; Ahanger et al., 2017b). Besides potassium contributes to maintenance of turgor thereby protecting the key metabolic processes and the plant functioning through increasing water use efficiency (Bahrami-Rad and Hajiboland, 2017). Plant growth regulation by K depends on its concentration within soil as well as the applied K (Hu et al., 2016). Application of K protects wheat plants from the oxidative effects of water stress by enhancing the functioning of tolerance mechanisms. Transcriptomic studies have shown that K deficiency results in significant alteration in the expression of genes related to varied functions (Ma et al., 2012). K deficiency leads to growth reduction by reducing relative water content and chlorophyll synthesis (Liaqat et al., 2020).

Polyamines (PAs) include the widely distributed low molecular weight aliphatic compounds and exist as non-protein polycations at physiological pH, and having key biological roles (Pathak et al., 2014; Vuosku et al., 2018). PAs show strong binding capacity to negatively charged molecules like nucleic acids and have been shown to regulate key growth and developmental processes by affecting cell cycle functioning and signalling (Rakesh et al., 2021) as well as responses to environmental stresses (Ahanger et al., 2019b). Both free and conjugated forms of PAs have been reported in plants (Bano et al., 2020). PAs including spermine, spermidine and putresine regulate growth, stomatal functioning, enzyme activity and modulate responses to stresses (Gill and Tuteja, 2010; Liu et al., 2015; Ahanger et al., 2019b; 2020). PAs are believed to interact with key molecules including nitric oxide, abscisic acid, ROS and other signalling molecules for better elicitation of stress adaptation mechanism (Shi and Chan, 2014). Modulations of the PA biosynthesis under stresses have been observed to significantly affect the plants stress responses (Liu et al., 2015; Ahanger et al., 2019b).

Vigna angularis known as adzuki bean, is an important food legume crop grown worldwide for food. In this backdrop it was hypothesised that combined treatment of K and spermidine (Spd) can protect photosynthesis and growth of *Vigna angularis* by up-regulating the key tolerance mechanisms like osmolytes, antioxidant system and secondary metabolite accumulation as well as the components of chlorophyll biosynthesis pathways.

Materials and Methods

Experimental design and treatment

Healthy seeds of *Vigna angularis* were disinfected by washing in 0.001% HgCl₂ for five min and followed by repeated washing with distilled water (DW) for five times. Sterilised seeds were sown in pots filled with soil and sand in 3:1 ratio. During pot filling soil was thoroughly mixed with 0 and 100 mg KCl/kg soil. Indigenous concentrations of N, P, K and Na in soil were 59.11, 25.33, 67.20 and 1.02 mg/kg soil respectively. Ten days after germination four plants per pot were maintained and salinity stress was induced by applying 100mM NaCl on alternate days. Spermidine (100 µM Spd; Sigma Aldrich) application was carried onto the foliage using a sprayer and was given twice a week. Therefore, the detailed experimental treatments included: (a) control (0 mg KCl), (b) K (100 mg KCl / kg soil), (c) 100 µM Spd, (d) 100 mM NaCl (NaCl + 0 mg KCl + 0 Spd), (e) 100 mM NaCl + K, (c) 100 mM NaCl + 100 µM Spd and (f) 100 mM NaCl + 100 µM Spd + K. After twenty-five days of growth plants were carefully up-rooted, washed and analysed for oxidative stress parameters, chlorophyll synthesis, osmolytes and antioxidant components. Standard methods were adopted for different estimations. Pots were arranged in complete randomized block design and maintained in green house. Height was measured using tape while plant dry weight was determined after drying whole plant in oven for 48 h at 70 °C.

Estimation of δ-Amino levulinic acid (ALA), Prototoporphyrin IX (Proto IX) and Mg-prototoporphyrin IX (Mg-Proto IX)

For estimation of δ-ALA content method of Harel and Klein (1972) as described by Dalal and Tripathy (2012) was followed. Two separate sets (200 mg each) of leaf samples were prepared in which one set was incubated for 4hrs in 60 mM levulinic acid (LA) under light while another set was immediately extracted in 1M sodium acetate buffer (pH 4.6) under cold conditions. After centrifuging the homogenate at 15000g for 10 min, 1 mL of supernatant was made to 4 mL using DW followed by addition of acetyl-acetone and contents were thoroughly mixed. Thereafter, samples were kept in boiling water bath for 10 min and after cooling to room T, Ehrlich's reagent was added and samples were vortexed. After 10 min samples were read at 555 nm and ALA content was calculated by subtracting 10h ALA content from 4h ALA content. Content of Proto IX and Mg-Proto IX were estimated following the method of Hodgins and Van Huystee (1986). Briefly, 0.3 g fresh leaf tissue sample was extracted in 5mL alkaline acetone (80%) and volume was made up to 25 mL by 80% alkaline acetone. Extract was allowed to bleach by incubating it under dark. Thereafter, homogenate was subjected to centrifugation at for 10 min at 15000 g and absorbance of supernatant was measured at 575 nm, 590 nm and 628 nm.

Estimation of photosynthetic pigments and gas exchange parameters, chlorophyll fluorescence

Total chlorophylls and carotenoids were extracted by homogenising fresh 100 mg leaf tissue in 80% acetone using pestle and mortar (Arnon, 1949). After centrifuging the homogenate at 3000 g for 20 min volume of supernatant was made to 10 mL by 80% acetone. Thereafter optical density was recorded at 480, 645 and 663 nm. For measurement of photosynthetic efficiency, intercellular CO₂ concentration and stomatal conductance, infrared gas analyzer (CID-340, Photosynthesis System, Bio-Science, Washington, USA) was used and measurements were recorded on fully expanded leaf. Chlorophyll fluorescence parameters including maximum photochemical efficiency, photochemical quenching and non-photochemical quenching were measured using Chlorophyll Fluorometer (PAM 2500; Walz, Germany) and leaves were dark adapted for 30 min.

Estimation of relative water content, proline, glycine betaine and soluble sugar content

Relative water content (RWC) was determined by the method of Smart and Bingham, (1974) and following formula was used for calculation:

$$RWC = \frac{FW - DW}{TW - DW} \times 100$$

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

Proline was estimated by macerating dry powder in sulphosalicylic acid and reacting the extract with ninhydrin reagent. Optical density was taken at 520nm (Bates *et al.*, 1973). Content of glycine betaine (GB) was estimated by extracting the dry powdered sample in DW. Concentration was determined by dissolving the periodide crystals in 1,2-dichloroethane and recording the optical density at 365nm (Grieve and Grattan, 1983). Soluble sugars were extracted in 80% ethanol and anthrone method was used for determination and absorbance was taken at 585 nm (Shields and Burnet, 1960). Standard curves of proline, GB and glucose were used for calculation.

Determination of hydrogen peroxide, superoxide and lipid peroxidation

Hydrogen peroxide was estimated by macerating fresh 500 mg sample in 0.1% TCA using pestle and mortar. After centrifuging the extract at 12,000 g for 15 min, 500 μ L supernatant was mixed with equal amount of potassium phosphate buffer (pH 7.0) followed by addition of 1 mL potassium iodide solution. Absorbance was taken at 390 nm and standard curve of hydrogen peroxide was used for calculation (Velikova *et al.*, 2000). For measuring the concentration of superoxide fresh 100 mg tissue was extracted in potassium phosphate buffer (65 mM, pH7.8). After centrifuging the homogenate at 5000 g for 10 min, supernatant was reacted with 10 mM hydroxylamine hydrochloride and allowed to stand for 20 min. Thereafter sulfanilamide and naphthylamine were added and mixture was incubated for 20 min at 25 °C. Optical density was taken at 530 nm and computations were done using a standard graph of NaNO₂ (Yang *et al.*, 2011). Lipid peroxidation was estimated by measuring the malonaldehyde (MDA) content. Briefly, 100 mg fresh leaf tissue was homogenised in 1%TCA and extract was centrifuged at 10,000 g for 5 min. To 1.0mL supernatant 4 mL of 0.5% thiobarbituric acid was added and mixture was heated at 95 °C for 30 min. Samples were cooled on ice bath and again centrifuged at 5000 g for 5 min. Absorbance was measured at 532 and 600 nm (Heath and Packer, 1968).

Determination of nitrate reductase

The activity of nitrate reductase (E.C. 1.6.6.1.) was assayed by cutting the 500 mg fresh leaf tissue into pieces in polythene vials containing 2.5 mL of phosphate buffer (pH 7.5) containing 20mM potassium nitrate and 5% isopropanol. After 2 hrs of incubation in dark aliquot was reacted with sulphanylamine (1%) and naphthylethylene diamine hydrochloride (0.02%) and optical density was taken at 540 nm using a spectrophotometer (Jaworski, 1971).

Estimation of activities of antioxidant enzymes

Fresh 1.0 gm of leaf tissue was homogenised in liquid nitrogen using pestle and mortar. Thereafter powdered tissue was transferred to centrifuge tubes and 50mM sodium phosphate buffer (pH 7.0) supplemented with 1% polyvinyl pyrrolidone and 1 mM EDTA was added to each tube. After centrifuging the extract for 20 min at 15,000 g supernatant was collected and used as enzyme source. Activity of superoxide dismutase (SOD, EC 1.15.1.1) was assayed following Bayer and Fridovich (1987) and the ability of enzyme to inhibit photochemical reduction of nitroblue tetrazolium chloride (NBT) was spectrophotometrically recorded at 560 nm. For assaying activity of ascorbate peroxidase (APX, EC 1.1.1.11) method of Nakano and Asada (1981) was followed and change in absorbance at 290 nm was recorded for 3 min. For calculation extinction coefficient of 2.8 mM⁻¹ cm⁻¹ was used. Method of Carlberg and Mannervik (1985) was adopted for assaying glutathione reductase (GR; EC 1.6.4.2) activity and glutathione dependent oxidation of NADPH was recorded as change in absorbance at 340 nm for 2min. For calculation extinction coefficient of 6.2 mM⁻¹ cm⁻¹ was used. Activities of antioxidant enzymes are expressed as EU mg⁻¹ protein and protein content was estimated according to Lowry *et al.* (1951).

Determination of ascorbate and reduced glutathione

Ascorbate (AsA) was extracted by homogenising 1gm fresh plant sample in 5mL of TCA (6%). Homogenate was centrifuged at 1000g for 10 min and supernatant was mixed with 2% dinitrophenylhydrazine (prepared in 9M H₂SO₄) and thiourea (10%). After incubating the samples in water bath for 15 min, ice cooled 80% H₂SO₄ (5 mL) was added and absorbance was taken at 530 nm (Mukherjee and Choudhuri, 1983). Method of Ellman (1959) was followed for estimation of reduced glutathione (GSH). Briefly 100 mg fresh leaf tissue was extracted in phosphate buffer (pH 8.0) and centrifuged at 3000 g for 15 min. Thereafter, 500 µL supernatant was mixed with 5, 5-dithiobis-2-nitrobenzoic acid and after 10 min optical density was taken at 412 nm and concentration of GSH was calculated using standard curve of GSH.

Determination of total phenols and total flavonoids

Content of total phenol in dry leaf samples was extracted in methanol following Singleton and Rossi, (1965). Phenols were estimated in supernatant by reacting a known volume of aliquot with Folin-Ciocalteu reagent and absorbance was measured at 765nm. For estimation of flavonoids method of Zhishen *et al.* (1999) was followed and calculation was done using quercetin as standard.

Estimation of sodium and potassium

Dry samples were digested in acid (H₂SO₄ and HClO₄ in the ratio of 3:1). Thereafter K and Na were estimated in digested samples using flame photometer.

Statistical analysis

Data presented is mean of four (\pm SE) replicates. Data was statistically analysed using ANOVA and least significant difference was calculated using Duncan's Multiple Range Test at $p < 0.05$.

Results

Salinity stress resulted in significant decline in plant height and dry weight however application of K and Spd individually as well as combinedly enhanced plant height and dry weight (Table 1). Relative to control, decline in plant height and dry weight due to NaCl stress was 34.26% and 39.02% respectively. Supplementation of K and foliar application of Spd improved height and dry weight significantly with maximal increase of 49.03% and 41.46% in plants treated with both K and Spd. Relative to control, decline in plant height and dry weight was 12.83% and 8.78% in NaCl + Spd + K treated plants (Table 1).

Plants treated with Spd and K exhibited significant increase in the content of proline, glycine betaine and sugars under normal and NaCl treated conditions. Relative to control, proline, glycine betaine and sugars increased by 22.11%, 8.97% and 22.39% due to Spd, by 45.70%, 21.88% and 87.71% by K and by 59.06%, 34.07% and 158.80% by Spd + K (Figure 8). Salinity resulted in increase of 83.14%, 64.65% and 56.75% in proline, glycine betaine and sugars respectively over control. Maximal accumulation was observed in NaCl + Spd + K treated plants (Table 1).

Activity of nitrate reductase (44.39%) declined due to NaCl treatment over control. Application of Spd and K individually and combinedly enhanced the activities of nitrate reductase over control with maximal increase of 80.57% due to their combined application (Table 1). Application of Spd and K ameliorated the decline in nitrate reductase significantly with a percent amelioration of 69.73% in NaCl + Spd + K treated plants over the NaCl counterparts (Table 1).

Salinity stress reduced the content of K by 47.18% over control while as application of Spd (39.62%), K (61.74%) and Spd + K (86.74%) resulted in increased K uptake (Table 1). Reduction in K uptake was mitigated by application of Spd and K with maximal amelioration of 70.74% observed in NaCl + Spd + K treated plants over NaCl counterparts (Table 1). Content of Na increase by 221.76% in NaCl treated plants and a reduction

of 13.18% in NaCl + Spd, 21.58% in NaCl + K and 36.68% in NaCl + Spd + K was observed over NaCl treated plants. Under normal conditions Na declined by 36.94%, 44.78% and 56.74% respectively in Spd, K and Spd + K treated plants (Table 1).

Table 1. Effect of salinity (100 mM NaCl) stress with and without potassium (100 mg KCl/kg soil) supplementation and spermidine (100 μ M Spd) application on plant height, plant dry weight, proline, glycine betaine, sugars, relative water content, nitrate reductase activity, sodium, potassium and Na/K in *Vigna angularis*

	Control	NaCl	Spd	K	Spd + K	NaCl + Spd	NaCl + K	NaCl + Spd + K
Plant height (cm / plant)	25.33 $\pm 1.95d$	16.65 $\pm 1.62h$	29.58 $\pm 1.01c$	33.95 $\pm 1.99b$	37.75 $\pm 2.13a$	18.03 $\pm 0.94g$	19.83 $\pm 1.46f$	22.08 $\pm 1.92e$
Plant dry weight (g/ plant)	2.05 $\pm 0.26d$	1.25 $\pm 0.12h$	2.33 $\pm 0.14c$	2.55 $\pm 0.13b$	2.90 $\pm 0.14a$	1.44 $\pm 0.11g$	1.58 $\pm 0.14f$	1.87 $\pm 0.12e$
Proline (mol /g DW)	20.35 $\pm 1.2h$	37.27 $\pm 2.0d$	24.85 $\pm 1.5g$	29.65 $\pm 1.6f$	32.37 $\pm 2.4e$	44.87 $\pm 2.5c$	53.47 $\pm 3.1b$	70.67 $\pm 4.4a$
Glycine betaine (g/g DW)	1.80 $\pm 0.21h$	2.97 $\pm 0.21d$	1.96 $\pm 0.11g$	2.20 $\pm 0.19f$	2.420 $\pm 0.10e$	3.21 $\pm 0.22c$	3.42 $\pm 0.21b$	3.86 $\pm 0.18a$
Carbohydrates (mg/ g DW)	5.28 $\pm 0.81h$	8.28 $\pm 0.93f$	6.46 $\pm 0.59g$	9.91 $\pm 1.09e$	13.63 $\pm 1.6d$	10.74 $\pm 1.4c$	14.26 $\pm 2.0b$	18.40 $\pm 2.12a$
RWC (Percent)	84.65 $\pm 4.8b$	63.15 $\pm 4.0e$	84.65 $\pm 4.8b$	89.07 $\pm 4.8a$	88.30 $\pm 3.7a$	67.32 $\pm 3.7d$	70.12 $\pm 3.0c$	73.87 $\pm 3.7c$
Nitrate Reductase (nmol NO ₂ ⁻ released /g FW/ hr)	200.5 $\pm 9.79d$	111.5 $\pm 7.5h$	278.7 $\pm 8.22c$	338.3 $\pm 12.2b$	362.1 $\pm 10.0a$	151.0 $\pm 7.4g$	166.7 $\pm 7.4f$	189.2 $\pm 8.3e$
Na (mg /g DW)	2.15 $\pm 0.17c$	6.91 $\pm 0.63a$	1.36 $\pm 0.14f$	1.19 $\pm 0.22g$	0.933 $\pm 0.09h$	6.00 $\pm 0.30b$	5.42 $\pm 0.42c$	4.38 $\pm 0.23d$
K (mg /g DW)	14.56 $\pm 1.7d$	7.69 $\pm 0.46h$	20.33 $\pm 1.9c$	23.55 $\pm 2.7b$	27.19 $\pm 1.8a$	10.30 $\pm 1.1g$	10.73 $\pm 0.91f$	13.13 $\pm 1.18e$
Na/K	0.146 $\pm 0.014e$	0.895 $\pm 0.050a$	0.067 $\pm 0.012c$	0.048 $\pm 0.005f$	0.034 $\pm 0.004g$	0.586 $\pm 0.062b$	0.507 $\pm 0.062c$	0.336 $\pm 0.042d$

Values presented are mean (\pm SE) of four replicates and different letters denote significant difference at $P < 0.05$.

Salinity stress resulted in significant decline in content of ALA, Proto-IX, Mg-Proto-IX and total chlorophylls while as K and Spd application significantly increased the content over control and ameliorated the salinity mediated decline. Relative to control, Spd, K and Spd + K treated plants exhibited an enhancement of 12.57%, 27.54% and 38.27% for ALA, 14.29%, 28.02% and 49.07% for Proto-IX, 10.80, 21.88 and 27.85% for Mg-Proto-IX and 29.52%, 65.54% and 85.79% for total chlorophylls. Application of Spd and K individually as well as combinedly mitigated the NaCl induced decline. Relative to NaCl treated seedlings, decline in ALA, Proto-IX, Mg-Proto-IX and total chlorophylls was mitigated by 53.06, 48.11, 61.56 and 65.76% respectively in NaCl + Spd + K treated plants (Figure 1A-D). In addition, the content of carotenoids was decreased by 33.87% due to NaCl and increased by 10.26%, 24.10% and 36.69% in Spd, K and Spd + K treated plants (Figure 1E). Relative to NaCl stressed plants, the maximal amelioration of 36.05% was observed in NaCl + Spd + K treated plants (Figure 1E).

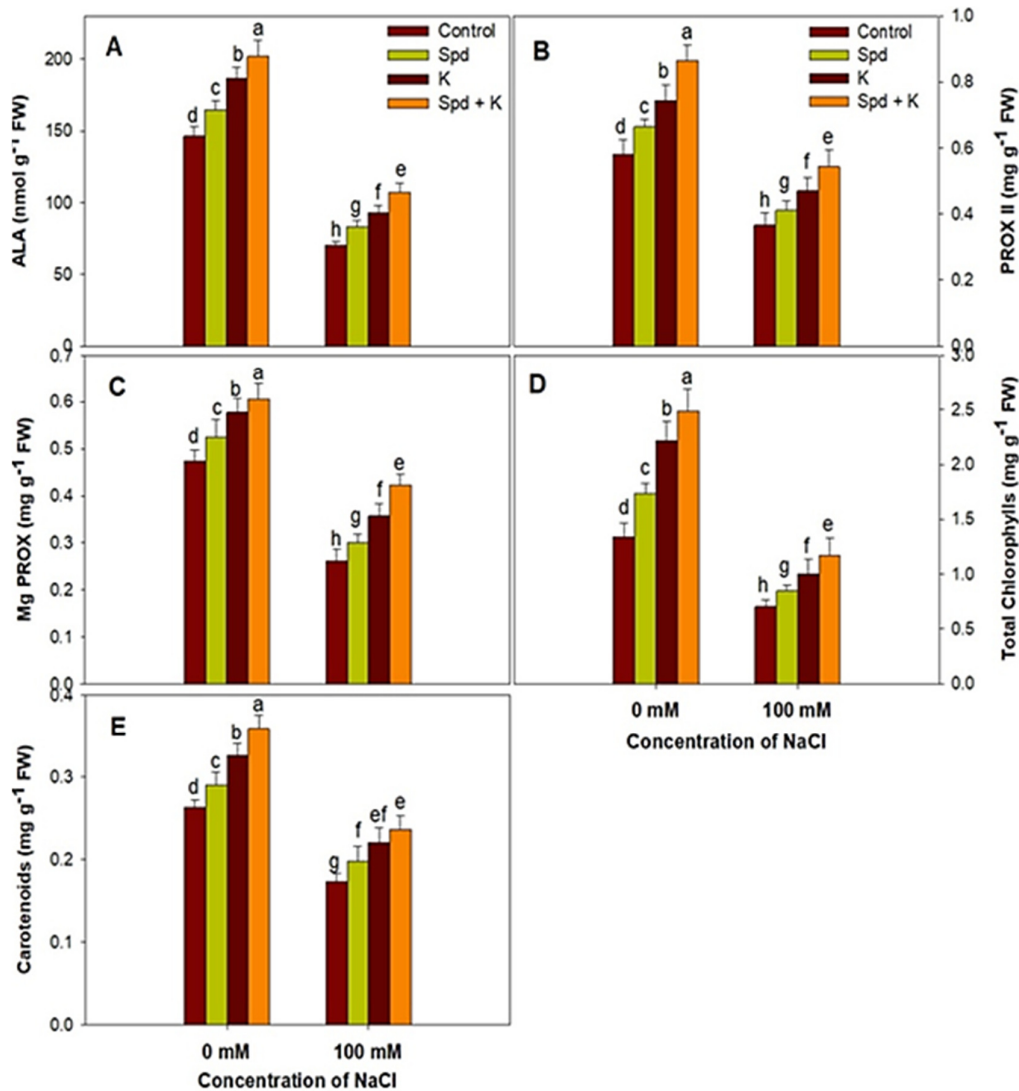


Figure 1. Effect of salinity (100 mM NaCl) stress with and without potassium (100 mg KCl / kg soil) supplementation and spermidine (100 μ M Spd) application on (A) δ -Amino levulinic acid (ALA), (B) protoporphyrin IX (Proto IX), (C) Mg-protoporphyrin IX (Mg-Proto IX), (D) total chlorophylls and (E) carotenoids in *Vigna angularis*. Values presented are mean (\pm SE) of four replicates and different letters denote significant difference at $P < 0.05$.

Plants treated with Spd or K or Spd + K exhibited significant enhancement in the photosynthetic parameters including Pn, gs and Ci. However, NaCl treatment declined Pn, gs and Ci by 41.47%, 27.64% and 39.37% respectively over the control. Under normal growth conditions the increase in Pn, gs and Ci was maximal of 93.72%, 43.99% and 55.15% in plants treated with both Spd and K. Application of Spd and K resulted in amelioration of the NaCl mediated decline and NaCl + Spd + K treated plants exhibited a maximal amelioration of 52.47%, 31.85% and 57.03% over NaCl treated plants (Figure 2A-C). Application of NaCl imparted a significant decline in the maximal PSII activity (Fv/Fm, 25.98%) and photochemical quenching (qP, 29.25%) while as increased non-photochemical quenching (NPQ, 31.96%) over control. Plants treated with Spd, K and Spd + K exhibited an enhancement of 9.45%, 19.22% and 26.43% in Fv/Fm and 5.71%, 13.66% and 21.37% in qP while as NPQ was observed to reduce by 8.12%, 10.71% and 17.46% respectively.

Under salinity stress the application of Spd or K significantly ameliorated the decline in Fv/Fm and qP with maximal amelioration of 23.09% in Fv/Fm and 35.25% in qP due combined application of Spd + K (Figure 3A and B). Plants treated with NaCl + Spd + K exhibit a decline of 23.91% in NPQ over the NaCl treated plants (Figure 3C).

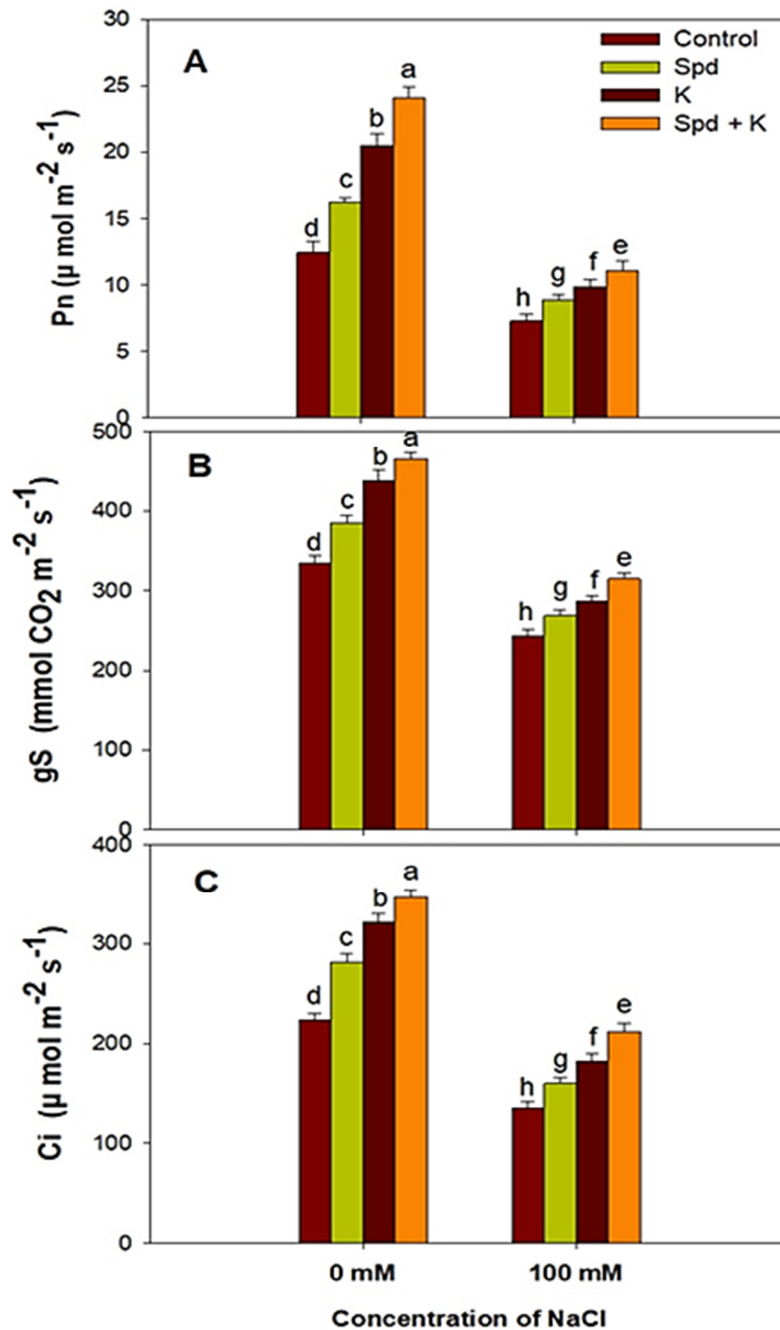


Figure 2. Effect of salinity (100 mM NaCl) stress with and without potassium (100 mg KCl / kg soil) supplementation and spermidine (100 μM Spd) application on (A) photosynthesis, (B) stomatal conductance and (C) intercellular CO₂ concentrations in *Vigna angularis*. Values presented are mean (\pm SE) of four replicates and different letters denote significant difference at P<0.05.

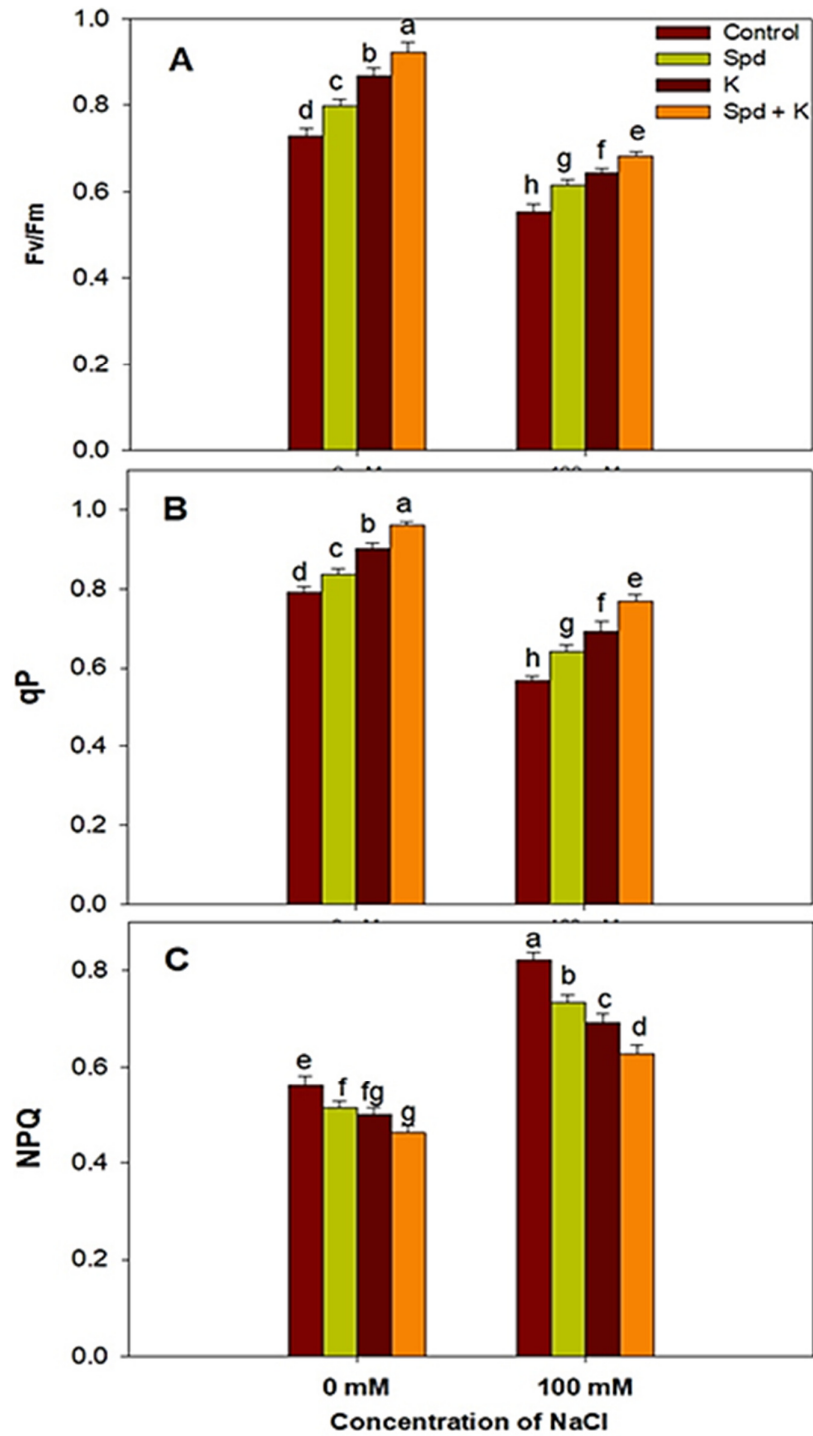


Figure 3: Effect of salinity (100 mM NaCl) stress with and without potassium (100 mg KCl / kg soil) supplementation and spermidine (100 μ M Spd) application on (A) Fv/Fm, (B) photochemical quenching (qP) and (C) non-photochemical quenching (NPQ) in *Vigna angularis*. Values presented are mean (\pm SE) of four replicates and different letters denote significant difference at P<0.05.

NaCl stress resulted in increased accumulation of reactive oxygen species like H_2O_2 and O_2^- over the control and Spd or K or Spd + K application decreased their accumulation declining the lipid peroxidation significantly. Relative to control an increase of 88.52%, 75.01% and 149.29% in H_2O_2 , O_2^- and lipid peroxidation was observed in NaCl treated plants. Application of Spd or K or Spd + K to NaCl stressed plants resulted in decline of 17.23%, 27.20% and 32.61% in H_2O_2 , 21.61%, 30.39% and 35.96% in O_2^- and 15.59%, 22.86% and 42.08% in lipid peroxidation over the NaCl stressed plants (Figure 4A-C). Maximal decline in H_2O_2 (39.13%), O_2^- (51.52%) and lipid peroxidation (43.95%) was observed in plants treated with Spd + K under normal conditions (Figure 4A-C).

Salinity stress increased total phenols and flavonoids by 23.09% and 10.94% over the control (Figure 5). Supplementation of Spd or K individually as well as combinedly increased the accumulation of phenols and flavonoids attaining maximal increase of 41.79% and 41.41% due to Spd + K treatment. Application of Spd and K to NaCl treated plants imparted further increase in phenol and flavonoids. Relative to control, content of total phenols and flavonoids exhibited an increase of 83.87% and 50.37% in NaCl + Spd + K treated plants (Figure 5A and B).

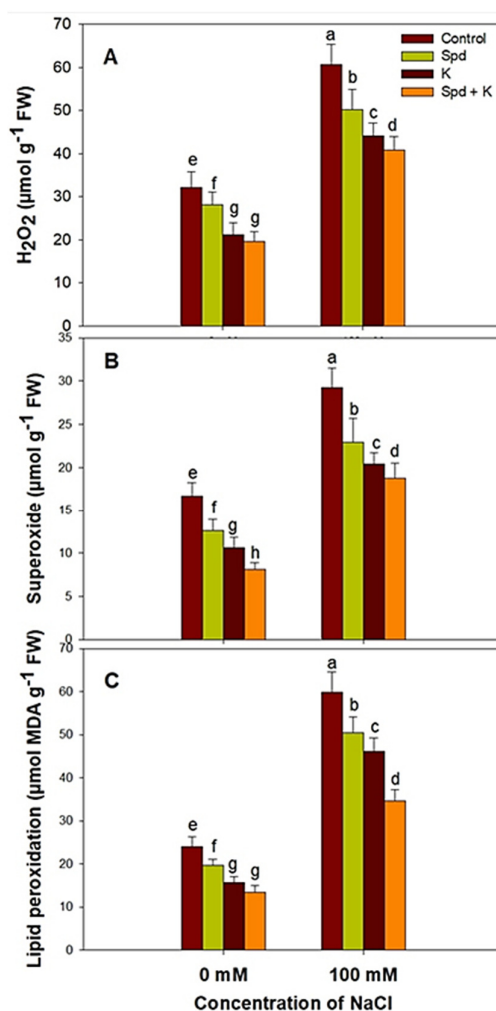


Figure 4. Effect of salinity (100 mM NaCl) stress with and without potassium (100 mg KCl / kg soil) supplementation and spermidine (100 μM Spd) application on (A) hydrogen peroxide, (B) superoxide and (C) lipid peroxidation in *Vigna angularis*

Values presented are mean (\pm SE) of four replicates and different letters denote significant difference at $P < 0.05$.

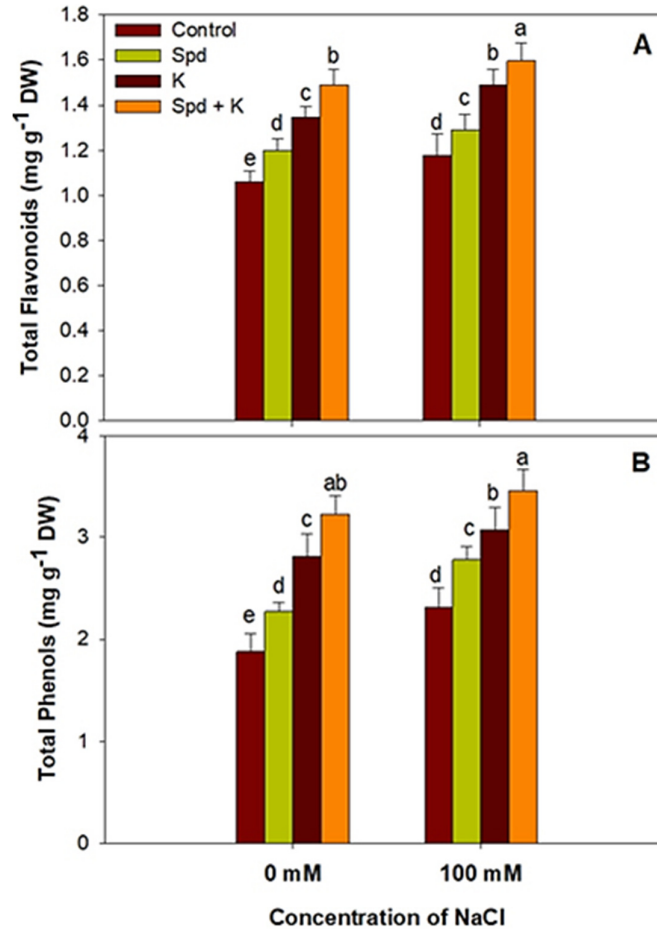


Figure 5. Effect of salinity (100 mM NaCl) stress with and without potassium (100 mg KCl / kg soil) supplementation and spermidine (100 μ M Spd) application on (A) total flavonoids and (B) total phenols in *Vigna angularis*

Values presented are mean (\pm SE) of four replicates and different letters denote significant difference at $P < 0.05$.

Application of Spd and K resulted in increase in the activity of antioxidant enzymes and the content of non-enzymatic antioxidants (Table 2). Relative to control the activity of SOD (35.56%), APX (53.20%), MDHAR (45.37%) and GR (26.91%) and the content of AsA (20.76%) and GSH (18.49%) increased maximally in Spd + K treated plants under normal conditions (Table 2). Plants stressed with NaCl exhibited an increase of 81.81% for SOD, 76.53% for APX, 56.21% for MDHAR, 26.91% for GR and 23.29% for GSH while as reduced AsA by 6.58% over control. Application of Spd or K or Spd + K increased the activity of all assayed enzymes over the NaCl counterparts. Relative to NaCl treated plants the activity of SOD, APX, MDHAR and GR was further increased by 70.17%, 36.03%, 31.41% and 75.30% respectively due to NaCl + Spd + K treatment (Table 2). Content of GSH increased maximally by 46.58% in NaCl + Spd + K treated seedlings over control (Table 2). Reduction in AsA was ameliorated due to treatment of Spd and K with maximal amelioration of 12.81% in NaCl + Spd + K treated plants over the NaCl treated counterparts (Table 2).

Table 2. Effect of salinity (100 mM NaCl) stress with and without potassium (100 mg KCl / kg soil) supplementation and spermidine (100 μ M Spd) application on activity of SOD, APX, MDHAR and GR, and the content of ascorbate and reduced glutathione in *Vigna angularis*

	Control	NaCl	Spd	K	Spd + K	NaCl + Spd	NaCl + K	NaCl + Spd + K
SOD (U /mg protein)	6.27 $\pm 0.58h$	11.4 $\pm 0.92d$	6.97 $\pm 0.37g$	7.75 $\pm 0.63f$	8.50 $\pm 0.69e$	14.30 $\pm 1.15c$	17.1 $\pm 1.3b$	19.4 $\pm 1.32a$
APX (U /mg protein)	0.9732 $\pm 0.10h$	1.718 $\pm 0.106d$	1.261 $\pm 0.179g$	1.330 $\pm 0.151f$	1.491 $\pm 0.140e$	1.884 $\pm 0.040c$	2.053 $\pm 0.208b$	2.337 $\pm 0.072a$
MDHAR (U /mg protein)	45.82 $\pm 3.89h$	71.58 $\pm 4.68d$	49.15 $\pm 3.43g$	58.05 $\pm 4.09f$	66.61 $\pm 5.08e$	78.51 $\pm 4.18c$	84.95 $\pm 6.04b$	94.07 $\pm 4.51a$
GR (U /mg protein)	0.4065 $\pm 0.0491h$	0.6401 $\pm 0.038d$	0.4603 $\pm 0.02g$	0.4907 $\pm 0.019f$	0.5159 $\pm 0.016e$	0.6598 $\pm 0.017c$	0.6960 $\pm 0.020b$	0.7126 $\pm 0.013a$
AsA (nmol /mg FW)	302.8 $\pm 9.85f$	282.8 $\pm 9.12g$	314.1 $\pm 7.50e$	338.6 $\pm 7.76d$	365.0 $\pm 10.58c$	299.1 $\pm 9.32b$	305.6 $\pm 6.84b$	319.1 $\pm 7.76a$
GSH (nmol /mg FW)	276.9 $\pm 7.84h$	341.4 $\pm 8.05d$	296.2 $\pm 7.87g$	315.9 $\pm 8.23f$	328.1 $\pm 7.24e$	372.3 $\pm 9.00c$	390.5 $\pm 9.89b$	405.9 $\pm 12.04a$

Values presented are mean (\pm SE) of four replicates and different letters denote significant difference at $P < 0.05$

Discussion

Salinity stress is one of the damaging stress factors causing significant decline in yield of major crop plants. From time to time several strategies have been developed and tested for mitigating the salinity stress induced growth damage. Efficient management strategies like optimal availability of essential mineral nutrients and the growth promoting metabolites have been shown to improve plant growth and yield potential under extreme conditions. In present study the possible involvement of K supplementation and polyamine (spermidine, Spd) application in amelioration of salinity stress was studied in *Vigna angularis*. Salinity stress resulted in significant decline in growth in terms of plant height and dry weight. Supplementation of K and application of Spd resulted in significant enhancement in growth and dry weight under normal conditions. In addition, the salinity mediated decline was ameliorated significantly by K and Spd treatments. Salinity restricts growth by affecting cellular division through down-regulation of the cell cycle genes (West *et al.*, 2004), altering chromosomal and DNA integrity (Chatterjee and Majumder, 2010) and imparting osmotic and ionic stress (Ahmad *et al.*, 2018). Improvement in plant growth in terms of height and weight due to supplementation of K (Ahanger and Agarwal, 2017a) and Spd (Roychoudhury *et al.*, 2011) has been reported, however their interactive effect has not been studied. Plants need optimal K to re-enter the cell cycle and such processes also involve active participation of channel proteins (Sano *et al.*, 2007). On the other hand, optimal availability of polyamines like putrescine have been demonstrated to normalize the cell cycle kinetics and hence the progression of different stages (Nasizadeh *et al.*, 2005). Potassium and Spd mediated growth improved can be attributed to their influence on chlorophyll production and maintenance of optimal ion homeostasis. Reduction in Na accumulation due to application of K and Spd was evident thereby contributing to maintenance of low Na/K ratio. The maintenance of low Na/K is considered as key criterion for improved salinity tolerance in plants (Asch *et al.*, 2000). The maintenance of Na/K ratio involves proper functioning of transport proteins (Assaha *et al.*, 2017; Zhang *et al.*, 2018). Reduced Na accumulation due to application of K and Spd was much obvious in plants treated with K + Spd thereby averting the inhibitory effects of Na on key functions like enzyme activity, membrane functioning, chlorophyll synthesis and photosynthesis. Improved

uptake of K and reduced Na uptake due to K (Ahanger and Agarwal, 2017a) and Spd (Puyang *et al.*, 2016) has been reported, however reports discussing their combined effects are not available. Potassium is actively involved in regulation of key functions like enzyme activity, protein synthesis, carbon and nitrogen metabolism and tolerance to stresses (Marschner, 2012; Ahanger *et al.*, 2017a, b; Hasanuzzaman *et al.*, 2018). Maintaining optimal concentration of K due to supplementation of K and Spd could have contributed to growth maintenance under salinity significantly.

The treatment of K and Spd resulted in increased δ -ALA, Proto IX, Mg-Proto IX, chlorophylls and carotenoid contents causing a concomitant increase in photosynthesis. Salinity stress adversely affects the synthesis of chlorophyll and the intermediates of chlorophyll biosynthesis pathway (Qin *et al.*, 2020). Metabolites including δ -ALA, Proto IX and Mg-Proto IX are the key intermediates of chlorophyll synthesis and stresses including salinity have been reported to alter the activity of key enzymes mediating synthesis of chlorophyll intermediates (Wu *et al.*, 2018). Earlier reports discussing the salinity induced decline in chlorophyll and carotenoids has been reported (Elkelish *et al.*, 2019; Soliman *et al.*, 2020). The mitigation of salinity mediated decline in chlorophyll and carotenoids due to K (Ahanger and Agarwal, 2017a) and polyamines (Hu *et al.*, 2016a) has been reported, however the combined effect of K and Spd has not been reported. Potassium application improves chlorophyll content and photosynthesis (Xu *et al.*, 2020), however salinity adversely affects the chlorophyll production and photosynthetic rate. Improved chlorophyll synthesis due to K and Spd application may be due to their affect on the activity of chlorophyll biosynthesising enzymes, however this has not been worked out yet. Moreover, stresses trigger decline in chlorophyll content by reducing uptake of Mg (Jan *et al.*, 2018; Zhao *et al.*, 2021) and also increasing degradation by up-regulating chlorophyllase activity (Todorov *et al.*, 2003). Declined photosynthesis and PSII activity due to salinity stress has been reported by Fatma *et al.* (2016) in *Brassica juncea*, Elkelish *et al.* (2019) in *Triticum aestivum*, Soliman *et al.* (2020) in *Glycine max*. Reduced stomatal conductance suppresses the intercellular CO₂ concentration and ultimately the Pn and hence the electron transport and functioning of photosystems is affected (Fatma *et al.*, 2016; Ahanger *et al.*, 2019a, b). Increased stomatal and non-stomatal attributes of photosynthesis due to K and Spd application justifies their beneficial role for improving photosynthetic function in *Vigna angularis*. Salinity alters the basic structure of photosynthetic apparatus and hence its functioning (Fatma *et al.*, 2016) besides the obvious effect of Na/K on the bioenergetic processes like photosynthesis (Sudhir and Murthy, 2004). Increase photosynthetic functioning due to K and Spd treatment can be attributed to improved RWC and redox homeostasis concomitant with significant reduction in oxidative damage thereby imparting structural and functional stability to photosynthetic apparatus. Potassium deficiency alters photosynthesis by reducing chlorophyll content, number of chloroplasts, grana and lamellae, concomitant with considerable increase in ROS (Qi *et al.*, 2019). Recently, Jiang *et al.* (2021) have demonstrated that Spd application protects photosynthesis by protecting PSII functioning through maintenance of endogenous polyamine concentration and the antioxidant system. From present study K and Spd proved most effective in preventing salinity induced damage to photosynthesis and further studies at molecular levels are required. Increased activity of nitrate reductase (NR) in Spd and K treated plants may have contributed to greater synthesis amino acids and subsequently protein synthesis. Increased nitrogen metabolism contributes to maintenance of sufficient N within cells. Greater nitrate reductase activity has significant contribution to nitric oxide production which can assist in integrating the signalling and response mechanisms under stressful condition (Agnihotri and Seth, 2016; Berger *et al.*, 2020). Earlier, Khan *et al.* (2014) have reported significant decline in NR activity under salinity stress resulting in reduced photosynthesis probably restricting the allocation of N for Rubisco synthesis. In present study Spd and K significantly mitigated the salinity mediated decline in NR with maximal amelioration in Spd + K treated seedlings.

In addition to this K and Spd treated seedlings maintained significantly lower concentrations of O₂⁻ and H₂O₂ thereby preventing damage to macromolecules like proteins and lipids. This resulted in considerable reduction in lipid peroxidation and hence protecting the structural and functional integrity of membranes. Earlier K (Qi *et al.*, 2019) and Spd (Nahar *et al.*, 2016) application has been reported to improve membrane

functioning by reducing the production of toxic ROS molecules. Salinity stress resulted in increased O_2^- and H_2O_2 accumulation and hence causing increased lipid peroxidation (Elkelish *et al.*, 2019; Soliman *et al.*, 2020; Naliwajski and Skłodowska, 2021). Stresses trigger increased generation of ROS by activating the ROS producing sites in chloroplast, mitochondria and membranes (Huang *et al.*, 2019). Increase ROS production up-regulates the lipoxygenase therefore intensifying the peroxidation of membrane lipids and hence affecting their stability (Nahar *et al.*, 2016; Soliman *et al.*, 2019). Application of polyamines including spermidine (Roychoudhury *et al.*, 2011) and spermine (Ahanger *et al.*, 2019b) has been reported to reduce the lipoxygenase activity and the production of ROS under salt stress however reports discussing the interactive effect of K and Spd are not available. Nevertheless, K deficiency has been demonstrated to increase ROS production hence causing oxidative damage (Ahmad *et al.*, 2014). Reduced oxidative damage in K and Spd (individual and combined) treated seedlings can be due to the up-regulation of antioxidant system, secondary metabolite accumulation and the maintenance of osmolyte accumulation resulting in better salinity stress amelioration through mediation of quick ROS elimination (Ahanger *et al.*, 2017a, b). Activation of antioxidant system by the application of K (Ahmad *et al.*, 2016) and Spd (Jiang *et al.*, 2021) has been reported to protect growth and other major cellular pathways like photosynthesis by maintaining optimal ROS levels. Application of Spd to rice has been demonstrated to increase the activity of SOD and GR thereby causing significant reduction in O_2^- and H_2O_2 (Liu *et al.*, 2015). In another study Ahanger and Agarwal (2017a) have demonstrated significant up-regulation of SOD, APX and GR in wheat due to K application reflecting in alleviation of salinity induced oxidative effects. SOD neutralises the superoxide radical thereby protect electron transport in chloroplast and mitochondria while as APX, MDHAR and GR along with AsA and GSH are the key components of ascorbate-glutathione cycle mediating the elimination of H_2O_2 . Optimal functioning of ascorbate-glutathione cycle has been reported to have significant influence on the functioning of plant at cellular and while plant level under normal as well as stressful conditions (Soliman *et al.*, 2019; Hasanuzzaman *et al.*, 2019). The efficient functioning of ascorbate-glutathione cycle reflects in maintenance of redox homeostasis and thereby protecting the photosynthetic electron transport (Pandey *et al.*, 2015; Soliman *et al.*, 2019). Application of K and Spd alleviated the decline in AsA content and also up-regulated the activities of APX, MDHAR and GR resulting in quick elimination of H_2O_2 and the impact was much obvious due to their combined application. Ascorbic acid and glutathione are powerful antioxidants and are key to normal plant functioning and the stress adaptation (Foyer and Noctor, 2011).

Increased accumulation of total phenols and total flavonoids due to application of K and Spd was evident under normal and the salt stress conditions. Secondary metabolites including phenols and flavonoids contribute to ROS elimination, protection of structural and functional integrity of membranes and stress tolerance (Wink, 2018; Austen *et al.*, 2019; Yadav *et al.*, 2021; Jan *et al.*, 2021). Recently, Begum *et al.* (2021) has demonstrated a significant modulation of the secondary metabolite profile actively imparting tolerance to stress in tobacco. The concentration, type and form of secondary metabolites exhibit a considerable variation with genotype, developmental stage, physiology and the growth conditions (Isah, 2019). In present study, salinity, Spd and K treatments showed a significant effect on the phenol and flavonoid accumulation, with K and Spd application further enhancing their accumulation thereby providing improved protection against the salinity mediated growth damage. Secondary metabolite accumulation and associated signalling events can protect plant metabolism from adverse effects of stress for example they can trigger stomatal closure through vascular to guard cell signalling (Yadav *et al.*, 2021; Anjitha *et al.*, 2021). The accumulation of secondary metabolites is controlled by key genes coding transcription factors like MYB, ERF, CBL etc (Patra *et al.*, 2013; Yadav *et al.*, 2021) and molecular insights about this can be interesting to study. The transcriptional regulation of secondary metabolite biosynthesis involves the expression of activators as well as repressors in response to changes in environmental conditions thereby forming a dynamic regulatory for fine tuning with change in time, amplitude and growth condition (Patra *et al.*, 2013). Earlier salinity induced increased in secondary metabolites has been reported in soybean (Soliman *et al.*, 2020) and wheat (Ahanger *et al.*, 2019a). Application of K has been reported to improve secondary metabolites in *Stevia rebaudiana* resulting greater chlorophyll and biomass

production under salinity stress (Mahajan *et al.*, 2020). Liu *et al.* (2020) have demonstrated increase in specific secondary metabolites in cucumber due to Spd application resulting in increased salinity tolerance and greater protection to energy metabolism. However combined effect of K and Spd on secondary metabolite accumulation has not been evaluated. Further the significant enhancement in secondary metabolites has been reported to impart greater antioxidant functioning in plants (Ahanger and Agarwal, 2017a, b; Begum *et al.*, 2020, 2021). Therefore, combined treatment of K and Spd can be beneficial in strengthening the tolerance mechanisms for lessening the deleterious effects of salinity on key metabolic pathways including photosynthesis and nutrient metabolism (Babar *et al.*, 2014; Ibrahim *et al.*, 2014). Secondary metabolites and osmolytes have significant contribution to redox maintenance and ROS scavenging (Dey and Bhattacharjee, 2020).

The accumulation of compatible osmolytes was significantly improved by the application of Spd and K individually as well as combinedly. The compatible osmolytes have been reported to protect the plant metabolism from the adverse effects of stresses (Elkelish *et al.*, 2019; Ahanger *et al.*, 2020; Dey and Bhattacharjee, 2020). Osmolytes potentially contributes to maintenance of cell turgor by maintaining the driving gradient for better water uptake, osmotic adjustment, neutralisation of ROS and maintenance of redox potential thereby contributing to protection of cellular machinery (Zaid and Wani, 2019; Ghosh *et al.*, 2021). Considerable research studies have that over-expression of osmolyte accumulation leads to better stress adaptation and tolerance (Miranda *et al.*, 2007; Mattioli *et al.*, 2008; Nguyen *et al.*, 2019; Mbambalala *et al.*, 2020; Ugarte *et al.*, 2021). Earlier improved salinity tolerance due to increased accumulation of proline and free sugars in K treated wheat has been demonstrated by Ahanger and Agarwal (2017a). Similarly, polyamines including Spd (Roychoudhury *et al.*, 2011), spermine (Ahanger *et al.*, 2019b) and putrescine (Islam *et al.*, 2021) have been reported to improve the accumulation of compatible osmolytes reflecting in significant improvement of growth and photosynthesis. In present study maximal accumulation of compatible osmolytes was observed in seedlings treated with both Spd and K, thereby imparting greater salt stress tolerance which was obvious as increased growth, biomass accumulation, photosynthesis and yield productivity. Increased accumulation of osmolytes results due to the differential regulation of their biosynthesis and catabolism pathways as has been reported for proline (Khan *et al.*, 2015; Elkelish *et al.*, 2019). Combined Spd and K application may have fine regulated the functioning of enzymes regulating synthesis and catabolism of osmolytes and further studies in this direction can be worthwhile. Increased accumulation of osmolytes protects enzyme functioning, elicit stress signalling and can contribute to maintenance of ion homeostasis. Both osmolyte accumulation as well as maintenance of toxic ion exclusion are key to salinity tolerance (Rad *et al.*, 2021), and in present study Spd and K application resulted in significant decline of Na accumulation in addition of lesser ROS accumulation thereby contributing to protection of major cellular pathways including photosynthesis.

Conclusions

In conclusion, Spd and K application affectively mitigated the salinity induced damage. Reduced generation of ROS and oxidative damage in Spd and K treated seedlings significantly improved the photosynthetic performance. Combined application of Spd and K proved much affective in mitigating the salinity mediated growth and photosynthetic decline. The positive influence of Spd and K was related with the up-regulated antioxidant functioning, osmolyte and secondary metabolite accumulation in them, thereby lessening the salinity damage. Potassium proved much affective than Spd while as potentiated the beneficial effect of K under their combined application. Future studies should focus on identifying the regulatory mechanisms involved in strengthening the tolerance mechanisms against salinity stress.

Authors' Contributions

The author read and approved the final 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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