



Exogenous kinetin and putrescine synergistically mitigate salt stress in *Luffa acutangula* by modulating physiology and antioxidant defense

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Received: 1 August 2020 / Revised: 17 September 2020 / Accepted: 10 October 2020 / Published online: 1 November 2020
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Abstract Salinity is one of the most vicious environmental constraints that hamper agricultural production. Experiments were done to explore the significant role of sole and synergistic supplementation of kinetin (100 μ M KN) and putrescine (100 μ M PUT) on *Luffa acutangula* in NaCl (100 mM) treatment. The harmful effects of salinity on growth were manifested by decreased seedling length, biomass, and pigment contents. We studied the effect of KN, and PUT in preventing salt (NaCl) induced physiological disorders and oxidative damages in 20-day-old *Luffa acutangula* seedlings. The individual application of KN and PUT increased growth and biochemical parameters, whereas combined KN + PUT treatment showed significant enhancement in growth, photosynthetic pigment content, and osmolyte accumulation in salt-affected plants. Application of KN and PUT also prevented hydrogen peroxide and superoxide production as confirmed by inhibition in electrolyte leakage and lipid peroxidation. Kinetin and PUT application upregulated the antioxidant defense system by enhancing antioxidant enzymes and non-enzymatic contents. *Luffa* seedlings treated with NaCl + KN + PUT showed 79, 26, 74, and 73% rise in superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase enzymes, respectively, in comparison to NaCl-stressed *Luffa acutangula*. Findings revealed that

synergistic utilization of KN and PUT modulate growth and biochemical processes in seedlings efficaciously in comparison to the individual application under salt stress, and it may be due to a regulatory crosstalk mechanism.

Keywords Antioxidants · Phytohormones · Polyamine · Priming · ROS · Osmoprotection · Salinity

Introduction

Soil salinity is a serious problem that adversely influence crop production and sustainable development all over the world. Salinity, because of salt accretion in cultivable soil, is a major threat to agricultural activities (Negrao et al. 2017; D'Amelia et al. 2018; Liu et al. 2020). Application of fertilizers and excessive irrigation have enhanced salinity in crop fields out of which twenty percent of total global irrigated area is adversely impacted by NaCl stress, and more than half of fertile land is anticipated to be salinized by 2050 (Fischer et al. 2012; Shokat and Grosskinsky 2019). Salinity exerts a detrimental effect on the development of plants as the presence of excessive Na⁺ in soil alters the texture of the soil, checks soil porosity and aeration, reduces microbes and availability of nutrients on the earth surface, and negatively affects water uptake by plants (Vimal et al. 2017; Hmaeid et al. 2019). Soil containing salts gives rise to osmotic shock due to water deficiency, nutrient imbalance, ion toxicity, and oxidative stress, which may cause malfunctioning of metabolic pathways and finally, death of plant cells (Wu et al. 2013). Salinization increases reactive oxygen species (ROS) production, which degrades biomolecules and initiates lipid peroxidation and changes in nucleic acid (Yang et al. 2019). ROS induced oxidative stress hinders redox

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homeostasis which indirectly cause loss of photosynthetic efficiency, mineral absorption capacity, level of phytohormones, and gene expression in plants (Hasanuzzaman et al. 2013; Fallah et al. 2017; Ma et al. 2018). However, to combat the adverse impact of salt stress, plants develop complex antioxidant defense machinery composed of enzymatic and non-enzymatic components like ascorbate (AsA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), etc., which keep ROS under control and contribute to stress endurance mechanism (Hasanuzzaman et al. 2019b).

Phytohormone priming has been reported as a potent method for growth enhancement in plants in adverse environmental constraints (Syta et al. 2019). The growth hormones of plants take part in the modulation of key metabolic pathways in the state of adverse conditions as they crosstalk with other biomolecules to evoke protection mechanisms (Ahmad et al. 2016). Kinetin (KN) promotes growth of different crop plants exposed to salinity (Wu et al. 2012), heavy metals (Singh and Prasad 2014), and waterlogging (Younis et al. 2003). Kinetin has been reported to regulate cell division, morphogenesis, seed germination, nutrients absorption, and delay senescence in plants (Ahanger et al. 2020). Polyamines show significant functions against abiotic stresses, and because of the presence of cations, they preserve different biomolecules containing negative charges (Lopez-Gomez et al. 2017; Hasanuzzaman et al. 2019a). Polyamines have been found to linked with salt stress tolerance mechanisms as they help in scavenging of ROS and affect antioxidant activity at molecular and gene expression levels (Sang et al. 2016). The exogenous supplementation of polyamines protects cell membrane integrity, maintains ion balance in cells, minimizes inhibition of morphological parameters induced by NaCl stress, and restricts ROS generation by controlling the defense mechanism of plants under natural constraints (Lopez-Gomez et al. 2017). Putrescine (PUT; $C_4H_{12}N_2$), a ubiquitous low-molecular-weight polyamine, contains two amino groups (Baniasadi et al. 2018). It is a central product of the polyamine biosynthetic pathway and acts as a precursor of spermidine (Spd) and spermine (Spm; Xu et al. 2009). Putrescine inhibits accumulation of Na^+ and Cl^- ions in cells of *Atropa belladonna* under salt stress (Ali 2000) and also reduces superoxide levels in plant cells in order to check oxidative stress (Ali et al. 2020). PUT increased the growth and viability of *Oryza sativa* leaves under salt stress (Lutts et al. 1996). This may be due to the inhibition of ethylene biosynthesis as PUT checks NaCl-induced conversion of aminocyclopropane-1-carboxylic acid to ethylene.

Luffa acutangula L. belongs to the Cucurbitaceae family and is also widely used in traditional medicinal system to

treat various ailments. It exhibits several therapeutic properties and is used to cure dysentery, headache, jaundice, diabetes, fungal infections, and leprosy. However, during the initial growth phase, this plant is sensitive to salinity. Remediation of saline soil is a challenging task, but the application of phytohormones and polyamines can be an environmentally safe and sustainable approach for crop production under salinity. The function of KN and PUT in diminishing the adverse impact of abiotic stresses has been well documented, but the synergistic application by which they alleviate salinity-induced changes in *Luffa* plants has not been studied yet. It was hypothesized that the interaction between KN and PUT might trigger an effective defense mechanism against NaCl stress as observed by Ahanger et al. (2018, 2019). The present study may open new avenues by kinetin and putrescine synergistic application for the regulation of the salt tolerance mechanism in crop plants, specifically those cultivated in saline areas, and to increase the crop production via low-cost additives such as KN and PUT. Hence, the present investigation was executed to explore individual and synergistic effects of KN and PUT on *L. acutangula* grown under salt stress as a combined application can enhance salt tolerance capacity by modulating physiological, biochemical, and antioxidant enzyme responses.

Materials and methods

Cultivation of *Luffa acutangula* plants and treatment

Sponge gourd (*Luffa acutangula* cv. Pusa Sneha) seeds were sterilized with 10% NaOCl for 7 min, then proper cleaning was done with autoclaved distilled water. Kinetin (6-furfurylaminopurine, KN; molecular weight: 215.21 g mol⁻¹) and putrescine (PUT; molecular weight: 161.07 g mol⁻¹) were procured from Merck. Seeds of *Luffa* were immersed in distilled water and KN solution (100 μM) for 7 h. The sowing of seeds was done in plastic pots contained disinfected sand with a full-strength Hoagland solution. In a growth chamber, pots with germinated seedlings were placed under photo-synthetically active radiation of 150 μmol photons m⁻² s⁻¹ in 16:8 h day and night duration at 26 ± 2 °C with 90% moisture and sprayed with full-strength Hoagland solution after two days. After a week, two seedlings were kept in each pot. Application of modified Hoagland solution with NaCl (100 mM) was induced, and a mixture of 100 μM PUT with tween-20 (spreading agent) was applied on the foliage and the seedlings which were sprayed with distilled water considered as untreated. Total of eight groups were made: (1). Control (full-strength Hoagland solution with distilled

water) (2). 100 mM NaCl (3). 100 μ M KN (4). 100 μ M PUT (5). 100 μ M KN + 100 μ M PUT (6). 100 mM NaCl + 100 μ M KN (7). 100 mM NaCl + 100 μ M PUT (8). 100 mM NaCl + 100 μ M KN + 100 μ M PUT. Twenty day old seedlings, after 10 days of KN and PUT supplementation, and 100 mM NaCl treatment, respectively were used for the assessment of physiological processes.

Determination of growth parameters and relative water content

Growth parameters were examined in terms of seedling length and biomass. In relative water content assessment, the same size round shaped *Luffa* leaves were collected from control and treated samples and fresh weight was evaluated. After 12 h floating in distilled water at 25 ± 2 °C under dark conditions, turgid weight was analyzed for dry biomass measurement, and leaves were put at 80 °C in an electric oven for 2 days. RWC was analyzed with the formula: $RWC (\%) = \frac{Fw - Dw}{Tw - Dw} \times 100$.

Determination of pigment content

Luffa acutangula leaves (100 mg) were ground with 80% acetone and optical density of the extracted chlorophyll and carotenoid pigments were measured at 663, 646, and 470 nm by UV–visible spectrophotometer. Chlorophyll *a*, *b*, total chl and carotenoids were measured following the procedure of Lichtenthaler (1987).

Estimation of sugar, proline, and glycine betaine

Hedge and Hofreiter (1962) method was applied for the analysis of sugars. Leaves (0.1 g) were ground with 95% ethanol (5 ml), and after centrifugation, anthrone reagent (4 ml) was added in the supernatant (1 ml), and the mixture was kept for heating for 15 min. At 620 nm, absorbance was noted, and the sugar amount was analyzed by standard curve prepared from glucose.

The proline amount was calculated by Bates et al. (1973). *Luffa* leaves were treated with sulphosalicylic acid (3%), and acid ninhydrin and acetic acid were mixed in aliquot and boiled at 100 °C for 1 h. The absorbance was assessed at 520 nm after the addition of toluene (4 ml).

The content of glycine betaine was measured by the procedure proposed by Grieve and Grattan (1983). The periodide crystals were observed due to reaction with KI–I₂ reagent at low pH, absorbance was noted at 365 nm, and concentration was measured by standard curve.

Estimation of protein and nitrate reductase activity

The fresh *Luffa* leaves (10 mg) were grounded with 1 N NaOH at 100 °C for 5 min. After 10 min of adding alkaline copper reagent (5 ml) in the test tubes, Folin–Ciocalteu reagent (0.5 ml) was mixed in the sample. After 30 min, absorbance was taken at 650 nm, and protein amount was recorded by Bovine Serum Albumin standard curve (Lowry et al. 1951).

Nitrate reductase enzyme activity was measured following the protocol of Jaworski (1971). *Luffa* leaves (250 mg) were treated with the medium (4.5 ml) containing 100 mM phosphate buffer, 3% potassium nitrate and 5% propanol and the aliquot (0.4 ml) was treated with 3% sulphaniamide (0.3 ml) in 3 N HCl and 0.02% N-1-NEDD (0.3 ml). At 540 nm, absorbance was calculated and nitrate reductase activity was analyzed by sodium nitrite standard curve.

Estimation of electrolyte leakage, hydrogen peroxide, superoxide

Electrolyte leakage was estimated by the method of Dionisio-Sese and Tobita (1998).

Electrolyte leakage (%) = $\frac{EC_1 - EC_0}{EC_2 - EC_0} \times 100$.

Hydrogen peroxide was determined by the method of Velikova et al. (2000). At 390 nm, absorbance was taken and concentration of hydrogen peroxide was measured by the standard curve.

Superoxide concentration was measured by fusing fresh leaves with potassium phosphate buffer (65 mM, pH 7.8) and centrifugation for 10 min at 5,000 \times g. The hydroxylamine hydrochloride (10 mM) was added to the supernatant and sulphaniamide and naphthylamine were also added after 20 min. Absorbance was noted at 530 nm and concentration was assessed by standard curve prepared from sodium nitrite (Yang et al. 2011).

Lipid peroxidation and lipoxygenase activity

Malondialdehyde content was determined to identify oxidative damage of lipids in *Luffa* leaves (Heath and Packer, 1968). Two hundred milligrams *Luffa* leaves were mashed in trichloroacetic acid (0.1%) and centrifuged at 10,000 g for 10 min. Filtrate (1 ml) was added in 0.5% TBA (4 ml) and kept at 95 °C temperature for 30 min and after proper cooling, centrifugation was done. The optical density of filtrate was calculated at 532 nm and a correction was made by reducing non-specific absorbance at 600 nm. MDA concentration was determined by applying $155 \text{ mM}^{-1} \text{ cm}^{-1}$ extinction coefficient.

Doderer et al. (1992) method was applied for calculation of lipoxygenase activity. Absorbance was taken at 234 nm and the calculation was done with an extinction coefficient of $25 \text{ mM}^{-1} \text{ cm}^{-1}$.

Assay of antioxidant enzymes

The enzyme extracts were produced by fusing leaves of *Luffa* (500 mg) with 0.1 M sodium phosphate buffer with polyvinyl pyrrolidone. The mixture was centrifuged at $14,000g$ at 4°C for 30 min in a cooling centrifuge and filtrate was taken for analysis of various enzymatic components which are given below:

Superoxide dismutase was measured by nitroblue tetrazolium photochemical assay with the method of Beyer and Fridovich (1987). The enzyme (0.4 ml) was mixed with 20 mM methionine, 0.15 mM EDTA, 0.12 mM NBT, 13 μM riboflavin and 0.05 M sodium carbonate. Test tubes containing samples were kept under fluorescent lamps for 30 min and the same set which was unilluminated considered as blank. The purple-colored formazan shows NBT reduction under light, and it was calculated at 560 nm and analyzed with those samples which were without enzyme. One unit of enzyme activity is elucidated as the content of the enzyme needed to show 50% suppression in NBT reduction.

The activity of CAT was analyzed by separation of hydrogen peroxide at 240 nm for 1 min by applying an extinction coefficient of $39.4 \text{ mM}^{-1} \text{ cm}^{-1}$ (Cakmak and Marschner, 1992). Enzyme extract (0.2 ml) was mixed with 50 mM potassium phosphate buffer, 1 mM EDTA, and 10 mM H_2O_2 . The one unit of CAT activity is known as 1 nmol H_2O_2 dissociated min^{-1} .

Ascorbate peroxidase was determined by following Nakano and Asada (1981). Two millilitres of reaction mixture was composed of 25 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, enzyme extract (0.2 ml) and hydrogen peroxide (1 mM). Absorbance was calculated at 290 nm with an extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$.

Foster and Hess (1980) method was used for the analysis of GR. The reaction mixture (3 ml) was composed of potassium phosphate buffer (100 mM), EDTA (1 mM), NADPH (50 μM), oxidized glutathione (100 μM) and enzyme (100 μl) and change in absorbance was calculated at 340 nm.

Determination of ascorbate and reduced glutathione

The procedure of Mukherjee and Choudhuri (1983) was applied for the calculation of ascorbate content. The fresh leaves were crushed with 6% TCA and dinitrophenylhydrazine (2%) and thiourea (10%) were added. The samples

were kept in a water bath for 15 min, then after proper cooling 80% H_2SO_4 (5 ml) was added, and absorbance was measured at 530 nm, and a standard curve of ascorbate with known concentration.

The reduced glutathione content was analyzed by Ellman (1959). *Luffa* leaves (100 mg) were macerated with phosphate buffer and filtrate (500 μl) was mixed with 5,5-dithiobis(2-nitrobenzoic acid). Absorbance was measured at 412 nm and GSH content was analyzed by its standard curve.

Statistical analysis

Treatments were organized as a randomized block design with three replicates. The data were calculated using ANOVA and SPSS software and mean was calculated by using Duncan's multiple range test (DMRT) at $P < 0.05$.

Results

Growth parameters and relative water content

Luffa acutangula seedlings under the influence of NaCl stress, showed an obvious decrease in root and shoot length and weight in comparison to control. Under salt stress (100 mM), 49, 55, 60, and 64% decrease in lengths of root and shoot and fresh and dry weight, respectively, were observed over control (Fig. 1). Supplementation of KN and PUT reflected enhancement in seedling length and biomass and synergistic treatment of KN + PUT showed 32 and 38% rise in length of shoots and fresh weight in comparison to control. As compared to salt treated *Luffa* seedlings, NaCl + KN + PUT treatment reflected 66 and 137% stimulation in the length of roots and dry weight. Relative water status showed 25% reduction in NaCl treated seedlings, but RWC was elevated to 33, 34, and 36% in KN, PUT, and KN + PUT treated seedlings in comparison to salt treated seedlings alone. The kinetin priming and PUT spray improved relative water content in control along with salt stress. Relative turgidity was elevated by 25% in *Luffa* leaves treated with NaCl + KN + PUT in comparison to NaCl stressed seedlings (Fig. 1).

Pigment content

Pigments are indispensable component for the development of plants. NaCl stress adversely affected chl contents (*a*, *b* and total) and carotenoids. Seedlings either primed with kinetin or foliar sprayed with PUT showed an increase in chl and carotenoids contents. Supplementation of KN and PUT individually enhanced total chl by 5 and 14% and carotenoids content by 19 and 34%, respectively (Table 1).

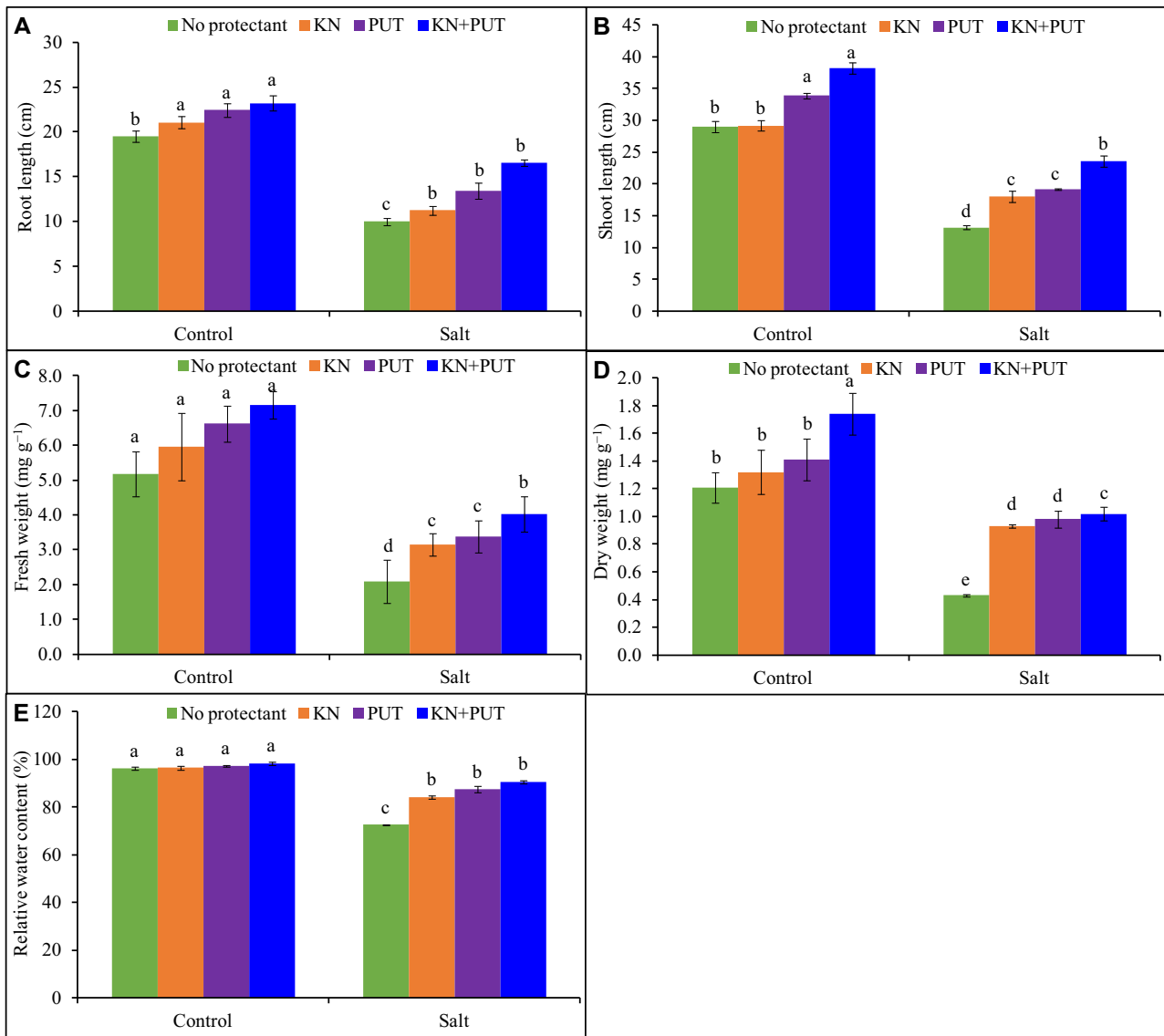


Fig. 1 Effect of salt stress on seedling length, biomass, and relative water content of *Luffa acutangula* L. with or without kinetin and putrescine. Data are mean ± standard error of three replicates. In a

bar, different letter(s) indicate a significant mean difference at $P < 0.05$ according to Duncan’s multiple range test (DMRT)

Table 1 Effect of salt stress on pigment contents of *Luffa acutangula* L. seedlings with or without kinetin and putrescine. Data are mean ± standard error of three replications. Values in a column with

different letter(s) show significant mean difference at $P < 0.05$ as per DMRT

Treatment	Chl <i>a</i> (mg g ⁻¹ FW)	Chl <i>b</i> (mg g ⁻¹ FW)	Chl (<i>a</i> + <i>b</i>) (mg g ⁻¹ FW)	Carotenoids (mg g ⁻¹ FW)
Control	2.98 ± 0.14 ^b	0.96 ± 0.01 ^b	3.94 ± 0.64 ^b	0.62 ± 0.08 ^b
NaCl	1.31 ± 0.12 ^c	0.54 ± 0.07 ^c	1.85 ± 0.19 ^d	0.39 ± 0.03 ^d
KN	3.10 ± 0.07 ^a	1.04 ± 0.10 ^b	4.14 ± 0.11 ^a	0.74 ± 0.08 ^b
PUT	3.24 ± 0.11 ^a	1.26 ± 0.10 ^a	4.50 ± 0.19 ^a	0.83 ± 0.03 ^a
KN + PUT	3.56 ± 0.13 ^a	1.38 ± 0.06 ^a	4.94 ± 0.13 ^a	0.88 ± 0.03 ^a
NaCl + KN	1.87 ± 0.11 ^c	0.66 ± 0.01 ^c	2.53 ± 0.24 ^c	0.45 ± 0.09 ^c
NaCl + PUT	2.15 ± 0.04 ^b	0.75 ± 0.09 ^c	2.9 ± 0.19 ^c	0.49 ± 0.03 ^c
NaCl + KN + PUT	2.43 ± 0.19 ^b	0.89 ± 0.07 ^c	3.32 ± 0.33 ^b	0.54 ± 0.11 ^c

The combined application of KN + PUT remarkably stimulated chl (a + b) and carotenoids by 25 and 42%, respectively, as compared to control. Thus, KN priming or PUT use inhibited reduction in pigment content with maximum improvement in salt affected *Luffa* seedlings exposed to KN + PUT.

Oxidative stress indicators

Salt stress increased H₂O₂, superoxide, electrolyte leakage, MDA, and LOX activity by 140, 145, 251, 358, and 157%, respectively, in seedlings in comparison to control. Kinetin and PUT application inhibited electrolyte leakage by 74 and 77%, respectively, over NaCl stress. Synergistic application of KN + PUT markedly decreased H₂O₂ and O₂^{•-} production and checked oxidative stress due to the lipid peroxidation and electrolyte leakage (Fig. 2).

Salinity exhibited notable stimulation in MDA content 358% and lipoxygenase activity 157% in *Luffa* leaves as compared to control. The exposure to KN or PUT reflected a reduction in LOX activity with 80% decline in KN + PUT administration over NaCl treatment in *Luffa* leaves (Fig. 2).

Protein content and nitrate reductase activity

Protein amount and NR activity were significantly affected by salt exposure. In protein content, 29% reduction was found in *Luffa* leaves with NaCl stress in comparison to control while it was stimulated to 3 and 8% with KN and PUT supplementation (Fig. 3). The 2 and 6% stimulation in NR activity was achieved in KN, and PUT treated seedlings in comparison to control. The 11% rise was recorded in NR activity in combined treatment, i.e., KN + PUT.

Activities of antioxidant enzymes

Enzymatic antioxidants showed different responses in *Luffa acutangula* during treatment with KN or PUT sole or in combination. Treatment of seedlings with KN or PUT individually and synergistically revealed enhancement in antioxidant activity over control and NaCl stress (Fig. 4). Salt stress promoted the activities of antioxidant enzymes as compared to untreated leaves, but stimulation was recorded when KN and PUT were applied to *Luffa acutangula* under NaCl stress. Seedlings exposed to NaCl + KN + PUT reflected augmentation of 79, 26, 74, and 73% in SOD, CAT, APX, and GR, respectively over salt-stressed leaves.

The synergistic impact of KN and PUT was more prominent in comparison to sole treatment. Salt stress inhibited AsA accumulation and enhanced GSH content,

both KN and PUT increased AsA and GSH in control and salt-stressed *Luffa* seedlings. The KN + PUT application improved 21% AsA and 19% GSH over control seedlings (Fig. 5).

Osmolytes content

Kinetin and PUT supplementation alone or, in combination, enhanced the levels of osmolytes (Fig. 6). The treatment of NaCl to *Luffa acutangula* increased contents up to 42, 79, and 50% of sugar, proline, and glycine betaine in comparison to control. Enhancement in osmoprotectants concentration was recorded by KN and PUT application with a significant rise 495, 155, and 330% was reported in *Luffa* seedlings treated with NaCl + KN + PUT in comparison to control (Fig. 6).

Discussion

High salt concentration in soil restricts seed germination, water, and nutrients uptake, reduces hydraulic conductivity, pigment synthesis, and other metabolic processes required for plant development (Gong et al. 2018). The inhibition in seedling length, as well as the reduction in biomass of *Luffa acutangula* because of NaCl stress was observed (Manai et al. 2014; Martinez et al. 2018). Salt stress impelled growth restriction might be due to inhibition in the elongation of plant cells (Queiros et al. 2011). Imbalance in the homeostasis of nutrients was another aspect of NaCl-induced damage in *Luffa* seedlings. Zhao et al. (2007) stated that alteration in the root plasma membrane by ion channels disruption and removal of nutrients by excessive Na⁺ influx was responsible for decreased nutrient uptake and imbalance in metabolic processes under NaCl stress. In this study, salinity induced decrease in growth was mitigated by KN priming or PUT or by their combined application (Ali et al. 2020). Our findings of the mitigation of salt-induced reduction in seedling length and biomass by kinetin are similar, as recorded by Kaya et al. (2010). Hamayun et al. (2015) observed kinetin increased resistance in soybean against salt stress by its interaction with growth hormones. Polyamines have been proved to be efficient to increase salt resistance by modifying membrane permeability, ion transport mechanism, ion channels, root and shoot growth (Lutts et al. 1996), Na⁺ intrusion and extrusion (Pottosin et al. 2012), photosynthesis (Shu et al. 2012), increase in nitrate reductase enzyme activity (Yadav et al. 2018) and antioxidative responses (Ikbal et al. 2014).

Relative water content is applied to check water content and osmotic adjustment by the plants under different environmental conditions (Ravikumar et al. 2014). More

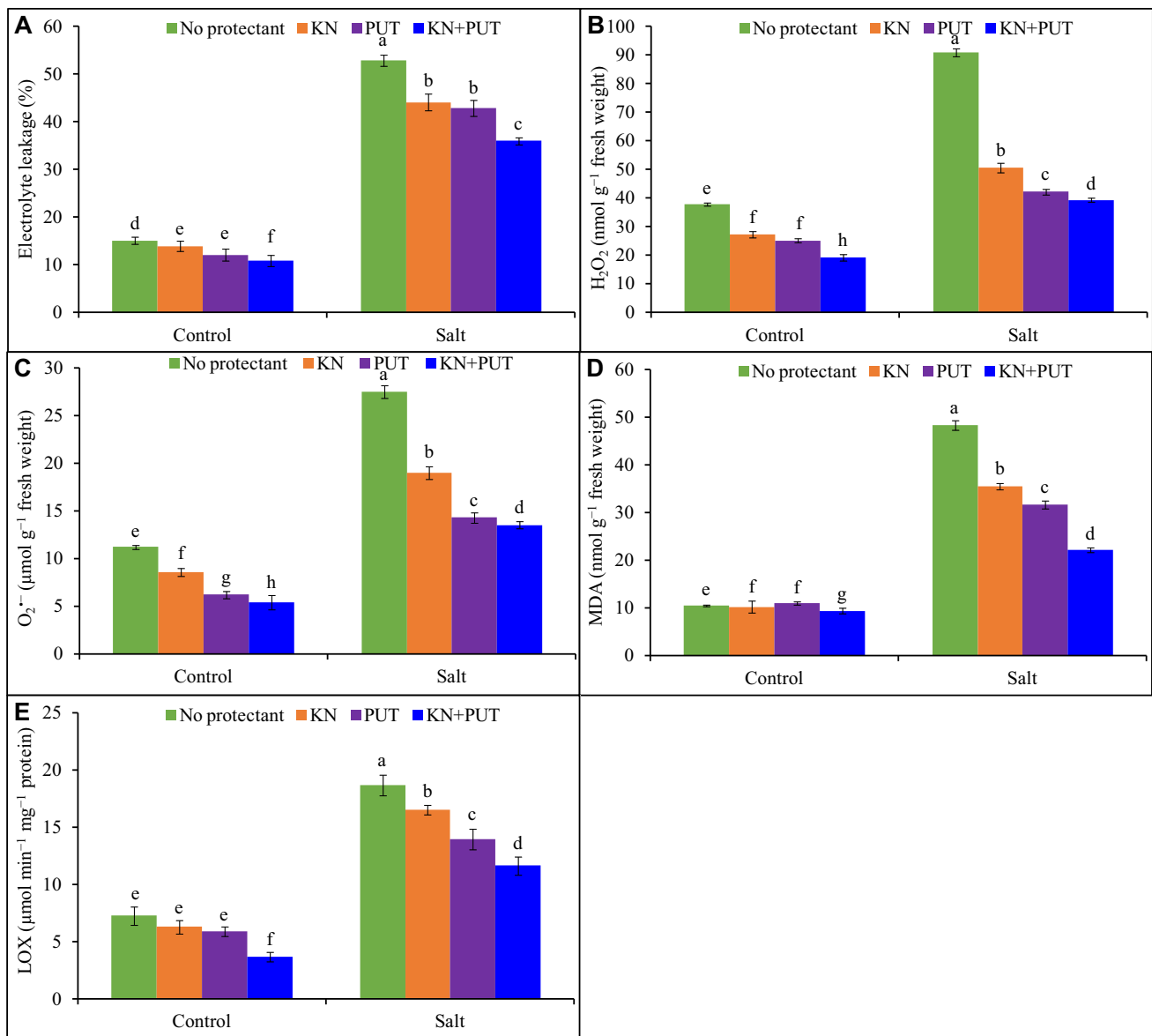


Fig. 2 Electrolyte leakage, hydrogen peroxide, superoxide, MDA content and lipoxygenase activity in *Luffa acutangula* seedlings grown under salt stress with and without kinetin and putrescine. In a

bar, different letter(s) indicate a significant mean difference at $P < 0.05$ according to DMRT

relative water content sustains the structure of proteins and improves photoassimilates mobilization. Gurmani et al. (2018) found that seeds of wheat primed with kinetin increased water status.

Salt enhances chlorophyllase activity and decreases the stability of the pigment–protein complex, whereas ROS produced due to NaCl stress degrades pigments (Shu et al. 2012; Martinez et al. 2018). Salt stress decreased pigment content might be due to decreased ribulose 1,5 biphosphate carboxylase/oxygenase activity, disorganization of chloroplast structure or thylakoid membrane and loss of membrane proteins in the chloroplast (Gengmao et al. 2015).

Kinetin increases protochlorophyllide-synthesizing enzyme concentration (Demetriou, 2008). Significant stimulation in chl synthesis and number of chloroplasts have been reported in leaves of cowpea and maize by KN application (Tounekti et al. 2011; Acidri et al. 2020). Enhanced growth of *Luffa* seedlings with KN and PUT treatment was associated with its positive impact on photosynthesis. Ahanger et al. (2018) observed an increase in chl synthesis due to the foliar application of KN. Behera et al. (2002) reported that kinetin triggered carotenoids synthesis and protected photosynthetic apparatus from ROS by upregulating pigment synthesizing enzymes. Chattopadhyay et al. (2002) revealed that polyamine

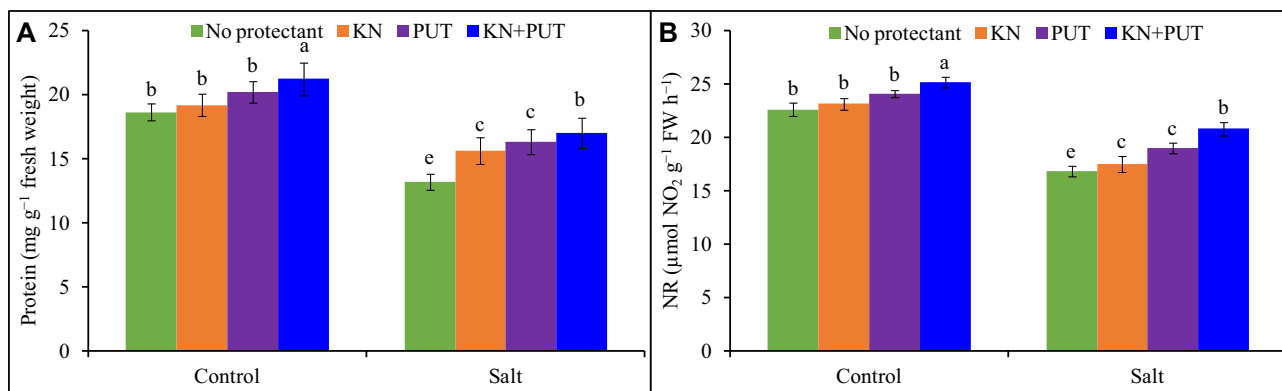


Fig. 3 Protein and nitrate reductase enzyme activity in *Luffa acutangula* leaves grown under salt stress with and without kinetin and putrescine. Data are mean \pm standard error of three replicates. In

a bar, different letter(s) indicate a significant mean difference at $P < 0.05$ according to DMRT

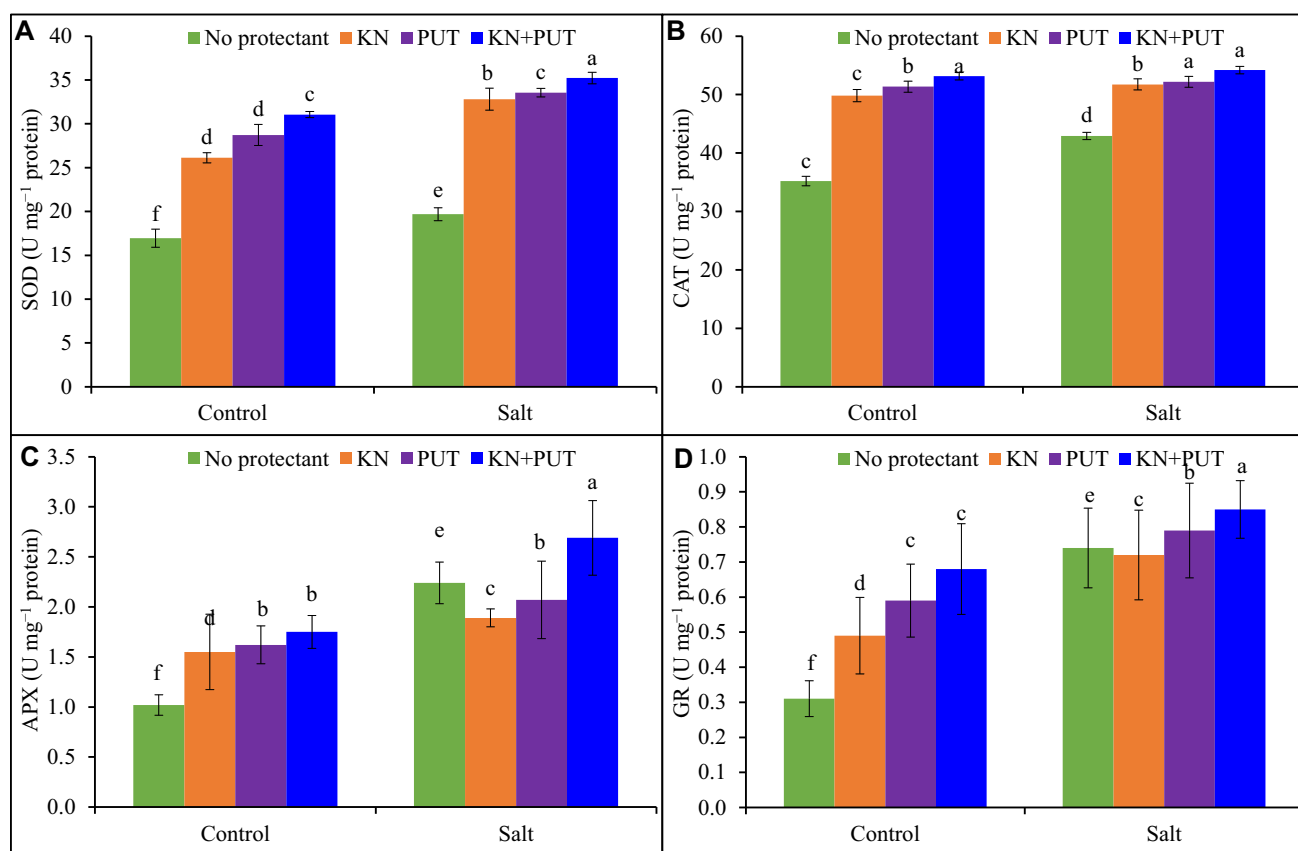


Fig. 4 Activities of SOD, CAT, APX and GR in *Luffa acutangula* leaves grown under salt stress with and without kinetin and putrescine. Data are mean \pm standard error of three replicates. In a bar, different letter(s) indicate a significant mean difference at $P < 0.05$ according to DMRT

application on rice plants inhibited chl loss and leaf senescence. Shu et al. (2013) observed that PUT increased polyamine levels in chloroplast and managed negative impacts of salinity on photosynthesis. The interaction of polyamines with PSII proteins stabilized protein structure and enhanced chloroplast function in cucumber under NaCl stress. Kinetin and PUT supplementation promoted

photoprotection, which influenced other attributes such as maintenance of water potential, antioxidant system, mineral assimilation, and metabolite accumulation (Oliveira et al. 2019). The role of KN and PUT might be oxidative stress reduction, maintenance of pH in the cells, protection of thylakoid membranes and pigment-protein complexes

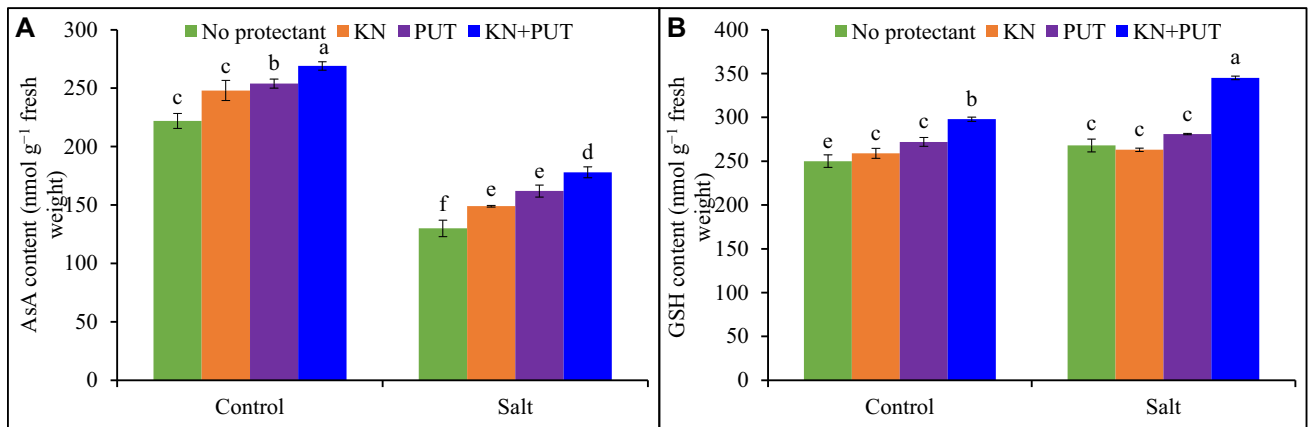


Fig. 5 Ascorbate and reduced glutathione contents in *Luffa acutangula* leaves grown under salt stress with and without kinetin and putrescine. Data are mean \pm standard error of three replicates. In a bar, different letter(s) indicate a significant mean difference at $P < 0.05$ according to DMRT

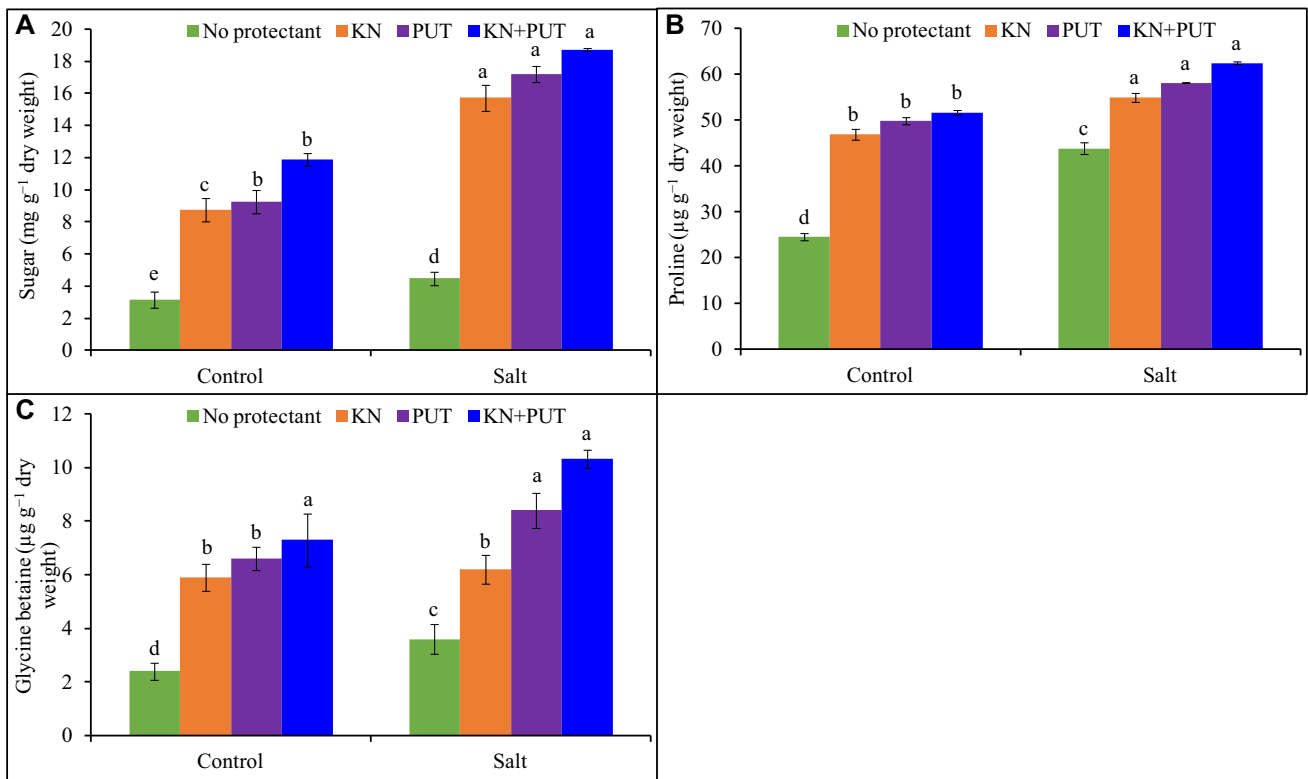


Fig. 6 Effect of salt stress on sugar, proline, and glycine betaine contents of *Luffa acutangula* L. seedlings with or without kinetin and putrescine. In a bar, different letter(s) indicate a significant mean difference at $P < 0.05$ according to DMRT

and maintenance of pigment synthesis and catabolic processes.

Osmolytes act as signaling molecules that reduce unfavorable effects of NaCl stress on biochemical pathways by preserving enzyme activities, photosynthetic oxygen-evolving complex and control redox balance and energy status during salt-induced stress (Khan et al. 2014; Ahanger and Agarwal 2017). Gharsallah et al. (2016) and Ahanger

and Agarwal (2017) reported an increase in osmolyte concentration under NaCl stress. Plants with a higher amount of osmolytes show better stress withstanding ability and fast free radicals elimination (Slama et al. 2015; Ahanger et al. 2019). High relative water content improves photoassimilate mobility and stabilizes protein structure. More osmolytes accumulation exhibited maintenance of water potential in tissues which leads to lowering of water

potential to facilitate continuous uptake of water (Ahanger et al. 2020). Application of KN and PUT significantly enhanced sugar, proline, and glycine betaine contents in NaCl-treated seedlings by inhibiting the efflux of K^+ by improving ROS scavenging and preventing photoinhibition (Cuin and Shabala 2007).

The high electrolyte leakage indicates damage in the membrane as noted in *Luffa* plants during treatment with salt stress (Hussain et al. 2017). The rise in MDA level because of salinity indicates oxidative stress owing to excessive reactive oxygen species production in *Luffa* seedlings but KN and PUT application significantly decreased the lipid peroxidation.

Seedlings treated with NaCl showed H_2O_2 production which was responsible for the peroxidation of lipids, however KN and PUT treatment significantly mitigated salt incited lipid peroxidation by decreasing the concentration of H_2O_2 (Ahanger et al. 2018). Kinetin and PUT protect the photosynthetic electron transport chain by modulation in $NADP^+$ and NADPH contents and check electrons movement to molecular oxygen and restricts $O_2^{\cdot-}$ production as observed in our results (Wu et al. 2012). Under NaCl stress, LOX enzyme activity increased, but a decline in LOX activity was found in plants exposed to KN and PUT (Sofa et al. 2004). The reduction in LOX activity was an indication of enhanced proteins and lipids stability in KN and PUT treated seedlings and improved oxidative stress tolerance capacity.

The decrease in protein content in seedlings with NaCl treatment was due to the decrease in protein synthesis or increased protein degradation (Hussain et al. 2017). Nitrogen metabolism is related to carbon metabolism. In the form of nitrate, nitrogen is absorbed by the plants which promotes development. Nitrate reductase, a substrate-incited enzyme, converts nitrate to nitrite. Significant reduction in NR activity in leaves under NaCl stress may be due to a reduced rate of carbon fixation or reduced nitrate uptake by roots (Kleinhofs and Warner 1990). The notable escalation was reported by the supplementation of KN and PUT may be due to improved photosynthetic ability and NO_3^- uptake by roots (Yadav et al. 2018).

Salt stress causes a reduction of osmosis, ion cytotoxicity, and overproduction of free radicals which causes oxidative stress and hampers biochemical processes in plants (Kamran et al. 2020). The enzymatic and non-enzymatic components are an integral components of plant defense mechanism and can scavenge ROS in order to stabilize membrane structure and prevent membrane lipid peroxidation. SOD converts $O_2^{\cdot-}$ to H_2O_2 , whereas CAT removes the H_2O_2 by converting H_2O_2 to H_2O . APX scavenges H_2O_2 in cytosol and chloroplast and prevents its entry into different cells to avoid injury. The supplementation of KN and PUT either individually or synergistically

prevented salt-induced changes by triggering of antioxidant enzymes (Ahanger et al. 2018). Ascorbate and GSH are vital constituents of AsA-GSH radical scavenging cycle. This cycle contains APX and GR enzymes which play a significant role in AsA and GSH production. Ascorbate is water-soluble, low molecular weight antioxidant, and helps in removal of H_2O_2 . The maintenance of AsA and GSH by upregulation of APX and GR activities results in inflow of electrons to oxygen in the chloroplast, thereby preventing superoxide radical production (Noctor and Foyer 1998). Polyamines function in the regulation of the AsA-GSH cycle and antioxidants might contribute to stress resistance (Nahar et al. 2016). Supplementation of spermidine to salinized nutrient solution stimulated SOD and CAT activities and mitigating NaCl-incited membrane injury and pigment synthesis reduction and polyamine content was enhanced (Duan et al. 2008). The increased *Luffa* seedling resistance against salt stress was correlated with enhanced antioxidant activity. Wu et al. (2012) and Siddiqui et al. (2015) stated that kinetin incited the upregulation of antioxidants. Acidri et al. (2020) observed that KN-treated coffee plants upregulated the non-enzymatic antioxidants and it was associated with enhanced free radicals scavenging capacity. Kinetin and PUT interact with other molecules and provide coordinated action towards stress resistance (Hasanuzzaman et al. 2019a). Ahanger et al. (2018) observed that kinetin treated tomato plants exhibited an increase in osmolyte content, regulation in AsA-GSH cycle with a robust antioxidant system. Nahar et al. (2016) observed that polyamine supplementation to salt-stressed mung bean increased GSH and AsA contents and antioxidant enzymes activities and inhibited oxidative stress. Hence, synergistic KN and PUT application induced up-regulation of osmolytes and antioxidant components which strengthens endurance strategies in *Luffa acutangula* against salt stress.

Conclusion

Salt stress markedly reduced the morphological parameters of *Luffa acutangula* seedlings. It reduced the contents of pigment and protein and nitrate reductase enzymes. It stimulated the accumulation of osmolytes and caused electrolyte leakage and lipid peroxidation. Significant enhancement in seedling length and biomass has been observed with KN, PUT, and their combined treatment, i.e. KN + PUT. Application of KN and PUT alleviated unfavorable effects of salt stress on morphological and biochemical components such as pigment and protein contents and NR activity of *Luffa acutangula* by triggering the up-regulation of osmolytes and antioxidants. The increase in antioxidant enzymes due to kinetin and

putrescine supplementation exhibited a defensive role against salt stress. Thus, KN and PUT ameliorate the unfavorable impact of salt stress by strengthening the defense system of *Luffa* seedlings. Kinetin and PUT mediated growth promotion in *Luffa* seedlings under NaCl stress may be due to the development of a detoxifying system against ROS by incorporation between various signaling constituents. The present study shows probable crosstalk between KN and PUT in preventing NaCl stress. Further investigations are required to unravel the molecular mechanism in relation to KN and PUT application for plant stress resistance.

Acknowledgements Dr. Kapoor is grateful to Amity Institute of Biotechnology, Amity University Uttar Pradesh, for providing the laboratory facilities to carry out the work.

Author contributions RTK and MH designed and planned the experiment. RTK conducted the experiment. MH analyzed the data. RTK and MH prepared the manuscript draft. MH edited the manuscript. Both authors approved the final version of the manuscript.

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