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RESEARCH ARTICLE

Acclimatory responses of seed pretreatment to modulate organic acid production associated with Krebs' cycle in NaCl-stressed legumes

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Abstract

Salinity hampers stand establishment of legumes at early growth stages that affect their yield. Seed pretreatment is an indispensable method for boosting tolerance to abiotic stresses and raising seed performance under salinity. Present study was aimed to evaluate whether seed pretreatment with mild dose of NaCl (sodium chloride) triggers ameliorative effects on growth and respiratory cycle of *Cajanus cajan* and *Vigna mungo* under salinity. Seedlings were raised hydroponically from non-pretreated and pretreated seeds under NaCl stress for three weeks. Non-pretreated *Cajanus* and *Vigna* seedlings exhibited reduction in root and shoot growth under salinity. Salt stress affected activities of enzymes associated with respiratory cycle specifically dehydrogenases, increased organic acid accumulation which played a versatile role to maintain cellular functions by balancing excess ions. Regardless of NaCl dose, the efficacy of seed pretreatment proved substantially beneficial to modulate the toxic effects of NaCl in both the test varieties. Lower perturbations of Krebs' cycle in pretreated seedlings caused lower accumulation of organic acids due to prior seed acclimation conferring improved defence to thrive under salinity. Conclusively, since seed pretreatment contributes to minimize salt stress in both the test varieties, present study could be extended to legumes grown on soil to confirm the eco-friendly, cost-effective and ameliorative role of seed pretreatment to combat salt stress in saline prone agricultural fields.

Keywords

amelioration; Krebs'cycle; legumes; salt stress; seed pretreatment

Introduction

Amongst the various abiotic stresses, salt stress predominantly affects crop productivity in arid and semi-arid regions. Arable lands of these regions are increasingly burdened by ions of various salts which affect growth of plants, especially glycophytes (1). Thus, enhancing crop tolerance to salinity is crucial to combat environmental adversities, sustain food productivity and feed increasing population (2).

Early developmental phases in legumes are salt-sensitive and therefore their yield is highly compromised under salinity (3). Growth of important dietary proteinaceous legumes like *Cajanus cajan* and *Vigna mungo* are also affected under salinity (4-6).

Various techniques are being employed at present to ameliorate detrimental effects of salt stress on plants. Seed pretreatment has emerged as a pragmatic and eco-friendly approach to invoke stress tolerance in plants. Very few studies to date have attempted to study the influence of seed pretreatment on respiratory cycle in legumes under salinity. Considering the high dietary value of *Cajanus* and *Vigna* and its

productivity loss in saline prone agricultural fields, understanding their physio-biochemical responses were considered important for development of partial salt tolerant legumes adopting seed pretreatment technology. Present study was designed to investigate the NaCl-induced damage on mitochondrial respiratory pathway and simultaneously determine the ability of seed pretreatment in alleviating such damage in the chosen legume seedlings. The supposition was whether seed pretreatment might modulate NaCl-induced stress by altering activities of key enzymes and levels of respiratory cycle intermediates, and if so, to probe the mechanisms by which pretreated *Cajanus* and *Vigna* seedlings adapt to high salt stress. Such study will help to grow legumes implementing seed pretreatment technique to combat the retarded growth of legumes in saline prone agricultural fields.

Materials and Methods

Plant materials and treatments

Seeds of *Cajanus cajan* L. and *Vigna mungo* (L.) Hepper were collected from Pulse and Oil Seed Research Institute Beherampore, West Bengal, India. The seeds were surface sterilized with 5% sodium hypochlorite solution for 15 mins followed by thorough washing in distilled water.

For preparation on non-pretreated sets, seeds were lined on sterilized glass plates containing moist blotting papers followed by their insertion into transparent packets containing suitable concentrations of NaCl (50 mM, 100 mM and 150 mM) supplemented to hydroponic solution where, only hydroponic solution served as control (7). The concentration of NaCl chosen for pretreatment was standardized by trial-and-error method for individual legume variety. It was determined that soaking seeds of chosen legumes in 50 mM NaCl for two hours followed by re-drying them to restore normal moisture content in the acclimatised seeds. Thus, for preparation of pretreated sets, seeds of each variety were soaked in 50 mM NaCl for two hours prior to their lining on glass plates covered with moist blotting papers. Pretreated sets were then exposed to different NaCl concentrations (50 mM, 100 mM and 150 mM) supplemented to hydroponic solution as done for the non-pretreated sets. Three replicates were prepared for each treatment and kept under controlled laboratory conditions (16/8h day/night photoperiod, temperature of 24±2°C, light of 200 µmol of photons $m^{-1}s^{-1}$ and a relative humidity of 55-65%) for three weeks. After 21 days, root and shoot samples were sampled, weighed and stored at -20°C for biochemical studies.

Evaluation of seedling growth

10 seedlings from each variety were randomly harvested from each of non-pretreated and pretreated sets after 21 days of growth, to determine influence of NaCl stress on root and shoot growth (8).

Estimation of TCA cycle intermediates

1g each of root and shoot sample from non-pretreated and pretreated seedlings were homogenized in 5 mL of 0.2 M phosphate buffer (pH 7.4) and centrifuged at 10,000g for 15 min at 4°C. The supernatant was deproteinized with 5% TCA (trichloro acetic acid) and filtered. The filtrate was used for estimation of organic acid contents.

Pyruvic acid content

For quantification of pyruvic acid, reaction mixture consisted of 1 mL deproteinized supernatant, 2 mL of 0.2 M phosphate buffer, 0.5 mL of 0.02% DNPH (2,4-dinitrophenylhydrazine) and incubated for 30 min at 37°C. Then 5 mL of 0.8 N sodium hydroxide was added followed by another incubation of 10 min at room temperature. Reaction mixture without plant sample served as blank. The absorbance of brown coloured product developed was measured in a Hitachi U-2000 spectrophotometer at 510 nm (9). Pyruvic acid contents were estimated from standard curve prepared using known concentrations of sodium pyruvate. Quantity of total pyruvic acid present was expressed as mg g^{-1} fresh weight.

Citric acid content

For determination of the amount of citric acid present, assay mixture consisted of 1 mL filtrate, 5 mL acetic anhydride (analytical grade) and 1.3 mL of pyridine (analytical grade) and kept in a water bath for 30 min at 32±0.5°C. Blank set was prepared usng 1 mL distilled water instead of the sample. The absorbance was measured spectrophotometrically at 405 nm (10). A standard curve was prepared using known concentrations of citric acid. Citric acid contents were expressed as mg g^{-1} fresh weight.

Succinic acid content

1g root and shoot samples each from non-pretreated and pretreated sets were cryocrushed and homogenized in a buffer containing 0.4 M mannitol, 1 mM EDTA (ethylene diamine tetraacetic acid) and 50 mM Tris (pH 8.2). Homogenate was fractionated by centrifugation at 0°C for 10 min at 2000 g. The supernatant obtained was centrifuged at 20,000g for 20 min. Obtained pellet was washed and re-suspended in homogenizing buffer containing mitochondrial fraction. The suspension was re-centrifuged for 20 min at 20,000g. The washed pellet was re suspended in potassium phosphate buffer (pH 7.6). The reaction mixture was composed of 0.5 mL buffer containing 0.1 M KH_2PO_4 (potassium dihydrogen phosphate), 0.05 mL of 5 mM EDTA, 0.05 mL of 5 mM sucrose, (pH 7.6), 0.2 mL of 0.5% INT (2-(p-iodophenyl)-3-{p-nitrophenyl}-5-{phenyltetrazoliumchloride}), different concentrations of standard succinate solution or 0.1 mL of experimental sample containing succinate and distilled water to get a final volume of 1 mL. The tubes were placed in ice and 0.2 mL mitochondrial suspension was added followed by the unknown or standard succinate solutions to make a final volume of 2 mL. The tubes were kept further in ice for 15 min followed by incubation at 37°C for 60 min. The reaction was terminated by addition of 1 mL of 10% (w/v) TCA. The red formazan obtained was cooled in ice and extracted in 4 mL ethyl acetate. Absorbance was recorded at 490 nm (11). Amount of succinate present was calculated from the standard curve and was expressed as mg g^{-1} fresh weight.

Malic acid content

For estimation of malic acid contents, assay mixture contained 0.5 mL of filtrate, 1.0 mL of 1 N HCl (hydrochloric acid), 0.1 mL of 0.1% DNPH (2,4-dinitrophenylhydrazine) and 0.5 mL of 10% calcium chloride. Assay mixture was incubated at room temperature for 30 min, followed by addition of 0.3 mL of 5 N

ammonium hydroxide and 6 mL of absolute alcohol. Samples were kept at room temperature for 12h for precipitation followed by centrifugation at 5000g for 15 min and supernatants were poured off. The tubes were oven-dried at 105°C for 15 min to remove moisture. To the dried pellets, 3 mL of 0.08% orcinol sulfuric acid mixture was added, thoroughly mixed and heated at 100°C for 10 min. The mixtures were cooled and diluted to 10 mL with concentrated sulphuric acid. The fluorescence was measured using a Hitachi -650-40 spectrofluorometer against blank (12). Malic acid contents were estimated from standard curve and expressed as mg g^{-1} fresh weight.

Assay of activity of Krebs'cycle enzymes

Pyruvate dehydrogenase activity (PDH; EC 1.2.4.1): For determination of PDH activity, 1 g each of plant root and shoot tissues were first homogenized in 1.5 mL of 50 mM Tris HCl buffer (pH 7.8) containing 0.7 M sucrose, 57 mM β-mercaptoethanol, 2 mM EDTA and 0.5% (w/v) BSA (bovine serum albumin). The extract was centrifuged at 10,000 g at 4°C for 20 min. The reaction mixture contained 0.2 mL of supernatant, 0.2 mL of 50 mM Tris-HCl (pH 8.0) and 0.1 ml of 5 mM $MgCl_2$(magnesium chloride), 0.1 mL of 0.12 mM CoA (coenzyme A), 0.1 mL of 2.6 mM cysteine HCl and 0.1 mL of 1.5 mM pyruvate. Absorbance of assay mixtures was recorded spectrophotometrically against a blank at 340 nm. Further, absorbance of assay mixtures was recorded for every 60s for 2 min after addition of 0.2 mL of 1.4 mM NAD. Increase in optical density of mixture showed the amount of NADH produced per min (13). A standard curve prepared with known concentrations of NADH helped to calculate pyruvate dehydrogenase activity which was expressed as µmol NADH formed mg^{-1} protein min^{-1}.

Citrate synthase activity (CS; EC 2.3.3.1): Activity was determined following the method of Srere (14). 1 g each of root and shoot samples were crushed in a buffer containing 0.1molL^{-1} Tris-HCl buffer (pH 8.0), 0.1% (v/v) Triton X-100, 2% (w/v) PVP (polyvinylpyrrolidone) and 10 mmolL^{-1} iso-ascorbic acid. Extracts were centrifuged at 15,000 g for 5 min at 4°C and supernatants were collected. Each reaction mixture consisted of 0.1 mL of 1mM DTNB (5′-dithiobis-2-nitrobenzoic acid), 0.03 mL of 10 mM acetyl CoA (coenzyme A) and 0.05 mL supernatant. Absorbance of the mixture was measured after the addition of 0.05 mL 10 mM oxaloacetate. Final absorbance was recorded at 412 nm spectrophotometrically. Enzyme activity was expressed as µg citric acid formed mg^{-1} protein min^{-1}.

Isocitrate dehydrogenase activity (IDH; EC 1.1.1.41): For determination of IDH activity, 1g each of test samples of were initially cryocrushed and homogenized in 1.5 mL of 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.5) containing 10mM β-mercaptoethanol and 5% PVP. The extracts were centrifuged at 14,500g for 20 min at 4°C. The reaction mixture contained 0.1 mL of 40 mM HEPES buffer (pH 8.2), 0.1 mL of 2 mM sodium isocitrate, 0.1 mL of 800 mM NAD, 0.1 mL of 200 mM manganese sulphate and 0.1 mL supernatant. Initial absorbance and the increase in absorbance of each reaction mixture was measured spectrophotometrically for 2 min at 340 nm and the enzyme activity was expressed as µmol NADH formed mg^{-1} protein min^{-1}(15).

α-ketoglutarate dehydrogenase activity (α-KGDH; EC 1.2.4.2): Root and shoot samples weighing 1 g from each treatment were cryocrushed and homogenized in 5 mL 50 mM MOPS (3-{N-morpholino} propanesulfonic acid) buffer (pH 7.5). Homogenates were centrifuged at 10,000g for 20 min at 4°C. The reaction mixture was composed of 0.6 mL 75 mM TES-KOH (n-tris hydroxymethyl methyl-2-aminoethanesulfonic acid) (pH 7.5), 0.3 mL 0.05% (w/v) Triton-X-100, 0.25 mL 0.5 mM $MgCl_2$, 0.2 mL 0.12 mM lithium Co-A, 0.14 mL 0.2 mM TPP (thiamine pyrophosphate) , 0.25 mLof 2.5 mM cysteine HCl, 0.2 mL 1 mM AMP, 0.25 mL 1 mM sodium 2-oxoglutarate, 3 units lipoamide dehydrogenase and 0.5 mL supernatant. The initial absorbance was recorded at 340 nm. Then 0.3 mL 2 mM NAD was added to it and the absorbance was measured at 340 nm for every 5 min after every 60s interval (16). α -KGDH activity was calculated using a standard curve with known concentrations of NADH and expressed as µmole NADH formed mg^{-1} protein min^{-1}.

Succinate dehydrogenase activity (SDH; EC 1.3.5.1): Assay of SDH activity was done according to Green and Narahara (17). 1 g each of root and shoot samples from each treatment were grounded in liquid nitrogen and homogenized in 1.5 mL of 4 mM Tris-HCl (pH 7.5) buffer containing 0.19 M sucrose. Homogenates were centrifuged at 10,000g for 15 min at 4°C. The assay mixture comprised 0.05 mL each of 0.19 M sucrose, 0.1 M Tris-HCl (pH 7.5), 10 mM sodium azide, 8 mM INT, 0.1 mL of 0.5 M sodium succinate, 0.1 mL of distilled water and 0.1 mL supernatant and incubated in a water bath at 30°C for 10 min. This was followed by mixing with 95% alcohol. The tubes were kept in an ice bath for 15 min and centrifuged at 8000g for 10 min. Absorbance was recorded at 458 nm spectrophotometrically. Basal reduction of INT was determined in control tubes where succinate was absent. The enzyme activity was expressed as µmol INT reduced mg^{-1} protein min^{-1}.

Fumarase activity (Fumarase; EC 4.2.1.2): For assay of fumarase activity, 1 g each of root and shoot samples were crushed in liquid nitrogen and homogenized in 100 mM potassium phosphate buffer (pH 7.6). The homogenates were centrifuged at 10,000g for 20 min at 4°C. Conversion of malate to fumarate by fumarase was determined by change in absorbance at 240 nm. The reaction mixture was comprised of 0.5 mL of 70 mM phosphate buffer (pH 7.4), 0.5 mL of enzyme extract and 50 mM malate. Increase in absorbance at 240 nm was noted at 10s interval for 60s and the said enzyme activity was expressed as μg^{-1} protein min^{-1}(18).

Malate dehydrogenase activity (MDH; E.C. 1.1.1.37): For determination of MDH activity, 1 g each of root and shoot samples from each set were homogenized in 3 ml of 50mM Tris-HCl (pH 8.0) buffer containing 50 mM magnesium chloride, 5mM β-mercaptoethanol and 1mM EDTA followed by centrifugation at 10,000g for 20 min at 4°C. The reaction mixture was composed of 0.5 mL of 5 mM oxaloacetate, 0.5mL of 10 mM magnesium chloride, 1.3 mL of 0.1 M Tris-HCl buffer (pH 7.8) and 0.2 mL of enzyme extract. Initial absorbance of the assay mixture was recorded spectrophotometrically at 340 nm followed by addition of 0.5 mL 0.4 mM NADH. The final absorbance was recorded at 60s interval for 2 mins (19). Enzyme activity was expressed as µmol NADH oxidized mg^{-1} protein min^{-1}.

Statistical analyses

All the experiments were carried out in completely randomized design (CRD), in three replicates and presented as mean ± SE (standard error) of three replicates. One-way ANOVA (analysis of variance) followed by Dunnett's multiple comparison test was carried out in Sigma Plot 14.0 software to examine whether difference between different NaCl doses were significant or not at $p \leq 0.05$ with respect to control. Pearson's bivariate correlation analysis was performed to determine correlation coefficient (r) using SPSS (Statistical Package for Social Science) software version 16.0.

Results and Discussion

NaCl administration reduced root and shoot lengths of both the test seedlings (Figs. 1, 2). Non-pretreated *Cajanus* recorded a reduction of about 31% and 27% respectively in root lengths and shoot lengths, whereas non-pretreated *Vigna*, documented a reduction of about 16% and 17% respectively in root and shoot lengths over control. Retarded growth of seedlings under salinity could be ascribed to probable deviation of energy destined for growth and development, to exclude excess ions from cellular absorption and accumulation of other compatible solutes during hypertonic saline conditions. Present results are consistent with previous reports showing retarded plant growth associated with salt-induced toxicity in *Lathyrus* (20). A plethora of related physiological and biochemical adaptive responses coordinate to lessen hyperosmolarity and prompt stress tolerance in plants. Therefore, pretreatment of *Cajanus* and *Vigna* seeds with 50mM NaCl for two hours prior to germination under salinity was found to be variably efficient in abrogating the injurious effect of NaCl on both root and shoot growth of test seedlings. In pretreated *Cajanus* and *Vigna*, inhibitions were decreased to about 7% and 5% respectively, for root lengths and to about

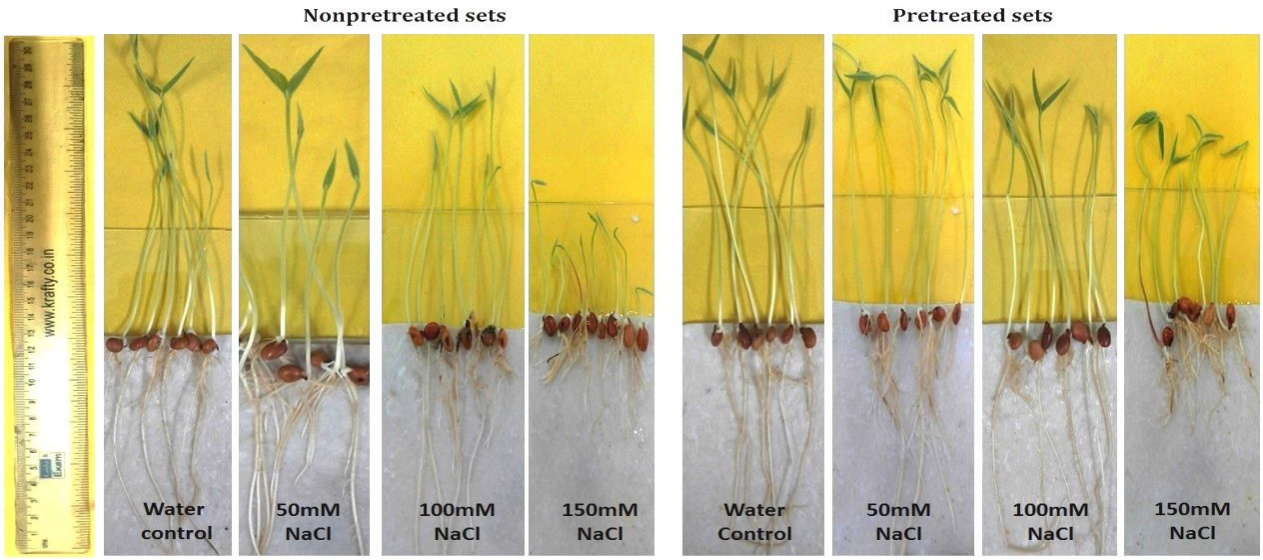

Fig. 1. Influence of NaCl on root lengths and shoot lengths of non-pretreated and pretreated seedlings of *Cajanus cajan* L.

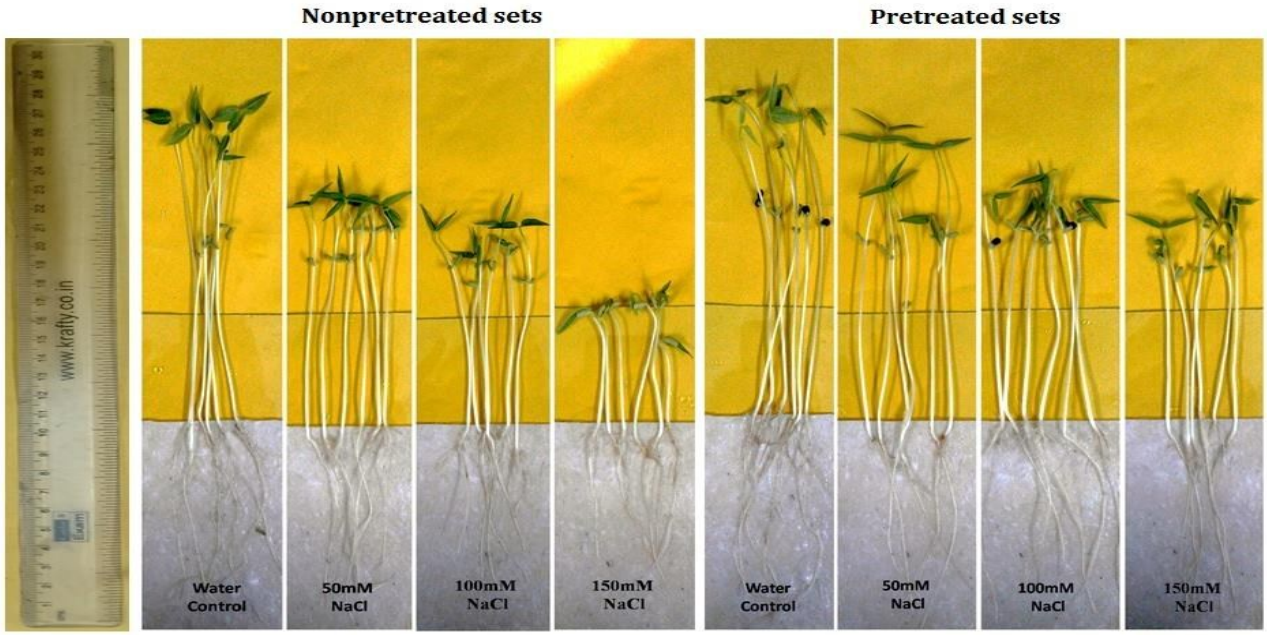

Fig. 2. Influence of NaCl on root lengths and shoot lengths of non-pretreated and pretreated seedlings of *Vigna mungo* L.

14% and 11% respectively for shoot lengths under salinity indicating stress release.

The Krebs' cycle produces more organic acids under stressful conditions to regulate cellular pH and osmotic potential (21). In present study, similar augmented production of organic acids in non-pretreated seedlings of *Cajanus* and *Vigna* likely specify their prerequisite to shield the biomolecules and cellular structures and simultaneously increase amount of energy probably needed to advance cell division for growth under stressful influence of NaCl. Similar increase in Krebs' cycle intermediates was observed in root elongation zone of barley cultivars under salinity (22).

Mitochondrial multi-enzyme complex, PDH connects glycolysis to Krebs' cycle. Salinity induced inactivation of PDH complex has been reported earlier (23). In line with such findings, our study showed linear decrease in PDH activity (to about 57% in root and 41% in shoot of *Cajanus*; 74% in root and 65% in shoot of *Vigna*) that invoked higher pyruvate accumulation (*Cajanus*: 74% in root and 21% in shoot; *Vigna*: 57% in root and 35% in shoot) under salinity in non-pretreated seedlings probably to support growth (Fig. 3a, 3b). Pretreated seedlings recorded lower pyruvate accumulation (to about 20% in root and 11% in shoot of *Cajanus*; 27% in root and 19% in shoot of *Vigna*) due to improved PDH activity (*Cajanus*: 19% in root and 49% in shoot; *Vigna*: 71% in root and 58% in shoot) conferring partial tolerance of both varieties under salt stress due to prior seed acclimation (Fig. 3a, 3b). It can also be argued that depletion in pyruvate production in salt stressed pretreated seedlings inhibited pyruvate transport to mitochondria or its conversion to acetyl-CoA resulting in decreased accumulation of downstream signalling organic acids like citrate, succinate and malate. Scatterplot matrices deduced from Pearson's correlation coefficient illustrate significant negative correlation between pyruvate accumulation and PDH activity under salt stress (r= -0.367, $p<0.05$) (Fig. 3c).

Citrate is the first organic acid synthesised from Krebs' cycle catalysed by CS. In the study, increase in CS activity (*Cajanus*: 105% in root and 45% in shoot; *Vigna*: 44% in root and 36% in shoot) along with decline in mitochondrial IDH activity (*Cajanus*: 73% in root and 50% in shoot; *Vigna*: 22% in root and 35% in shoot) was evident in NaCl-treated non-pretreated seedlings which corresponded to increased citrate accumulation meant to combat cellular toxicity. Greater inhibition of IDH activity and increased CS activity under NaCl stress resulted in higher citrate accumulation in non-pretreated seedlings (overall to about 17% in *Cajanus* and 21% in *Vigna*). Increased citrate production helped to combat ionic stress and sustain seedling growth under salinity (Figs. 4a, 4b, 4c). In contrast, seed pretreatment narrowed citrate accumulation in test seedlings (overall to about 10% in

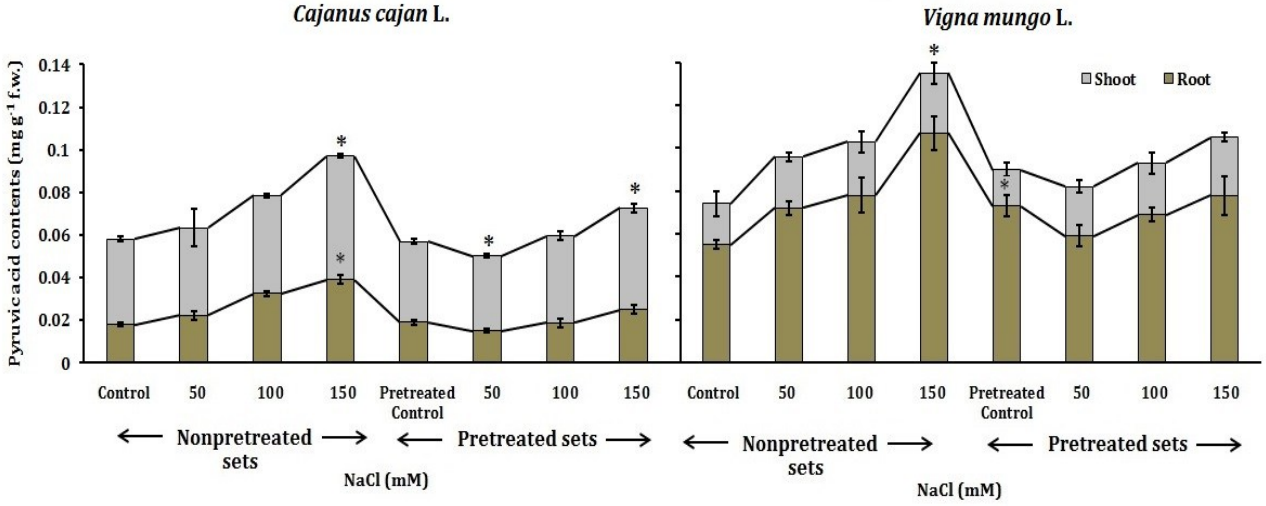

Fig. 3a. Influence of NaCl on pyruvic acid contents of non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

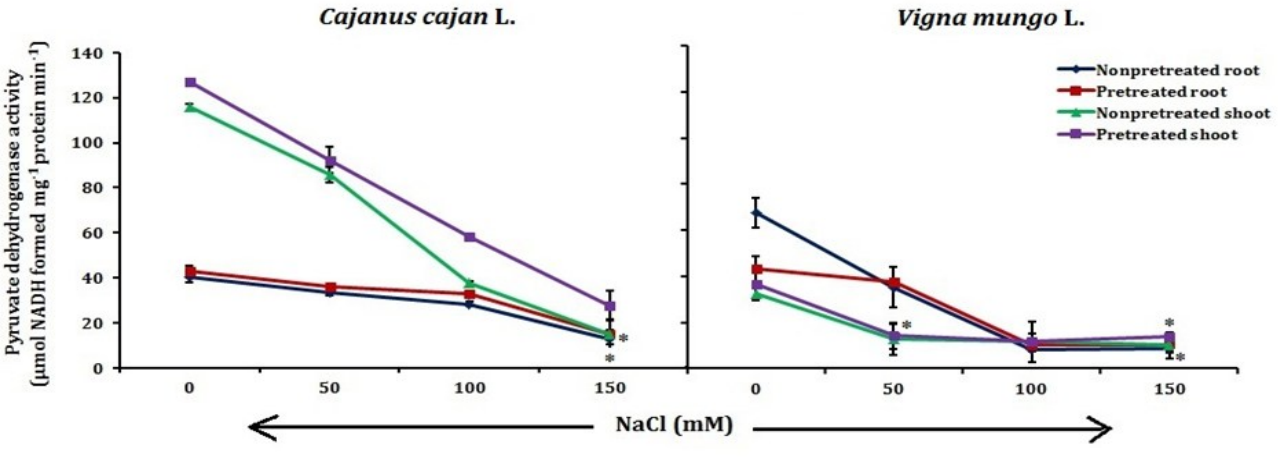

Fig. 3b. Influence of NaCl on PDH activity of non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

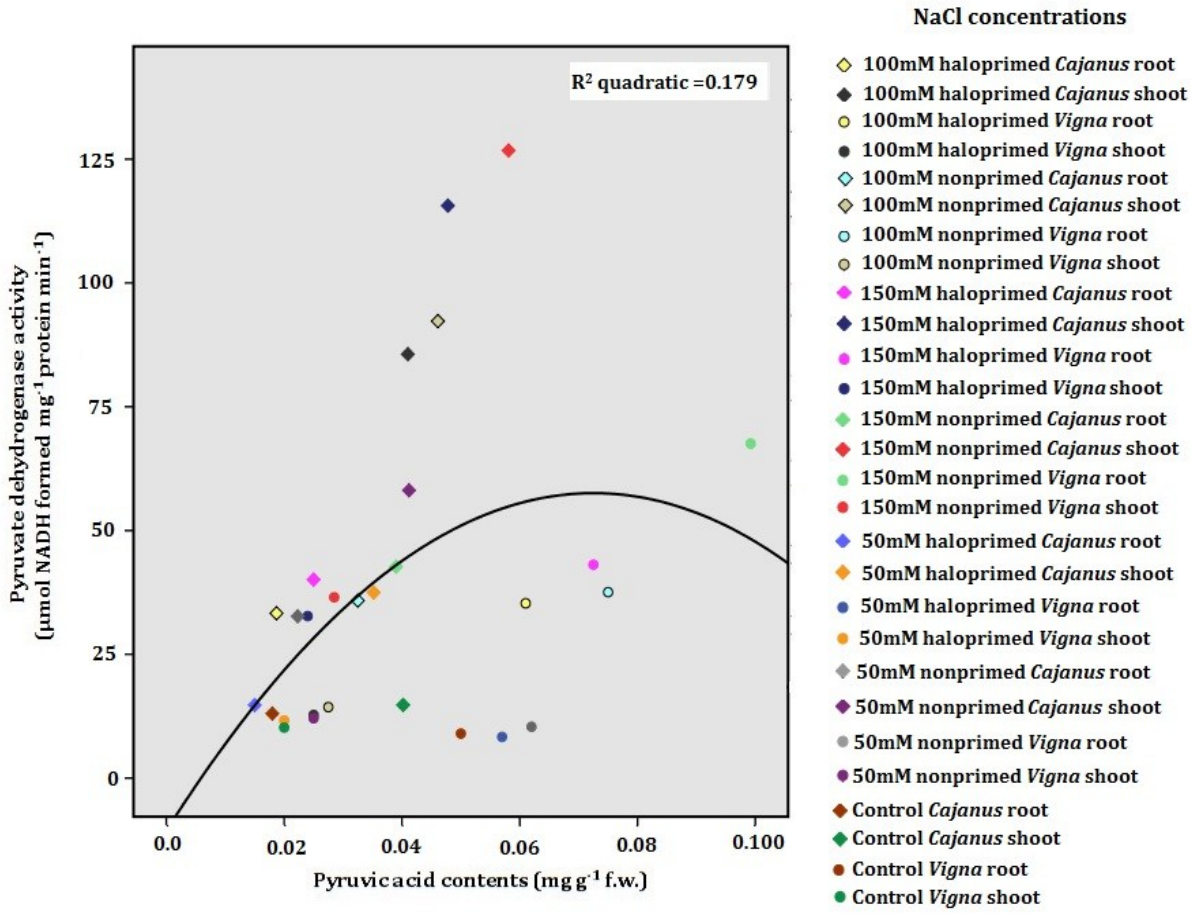

Fig. 3c. Influence of NaCl on PDH activity and pyruvate content of non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

Cajanus and 6% in *Vigna*) compared to their controls. Decrease in CS activity (*Cajanus*: 71% in root and 12% in shoot; *Vigna*: 30% in root and 25% in shoot) and improved IDH activity (*Cajanus*: 61% in root and 43% in shoot; *Vigna*: 15% in root and 31% in shoot) in pretreated seedlings triggered lower citrate accumulation indicating stress release (Figs. 4a, 4b, 4c). Since, IDH activity is known to unite carbon and nitrogen metabolism by supplying carbon skeletons for primary assimilation in plants, improved IDH activity in pretreated seedlings helped both the test seedlings to meet requirement for carbon skeleton favouring improved growth. Scatterplot matrices based on Pearson's correlation illustrate significant negative correlation between activities of CS and IDH (r= -0.863, p<0.01) and non-significant negative correlation between IDH activity and citric acid synthesis (r= -0.262, p<0.1). On the other hand, a strong positive correlation was obtained between CS activity and citrate synthesis (r=0.456, p<0.01) (Figs. 4d).

α-ketoglutarate dehydrogenase (α-KGDH) is a prime target of ROS (reactive oxygen species) in abiotically stressed cells (24). In the present study, NaCl stress decreased the activities of α-KGDH (*Cajanus*: 69% in root and 63% in shoot; *Vigna*: 60% in root and 51% in shoot) and succinate dehydrogenase (SDH) (*Cajanus*: 15% in root and 20% in shoot; *Vigna*: 15% both in root and 37% in shoot) in non-pretreated seedlings (Figs. 5a, 5b). SDH is a salt-sensitive enzyme which plays a crucial role in mitochondrial metabolism (25). NaCl induced decline in SDH activity corresponded to increased succinate accumulation (*Cajanus*: 60% in root and 53% in shoot; *Vigna*: 37% in root and 33% in shoot) in non-pretreated seedlings (Figs. 5b 5c). Also, in *Cajanus*, the magnitude of decrease in SDH activity was higher under salinity as compared to *Vigna* seedlings suggesting that salinity stress

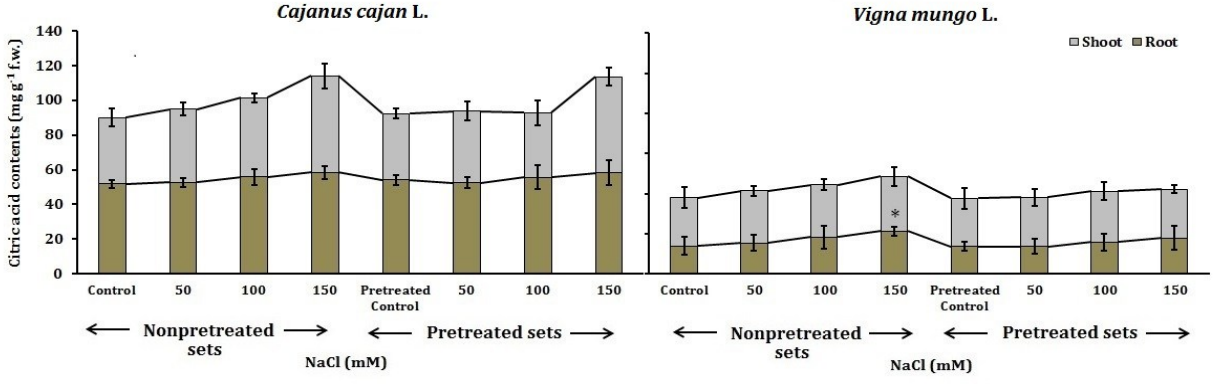

Fig. 4a. Influence of NaCl on citric acid contents of non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

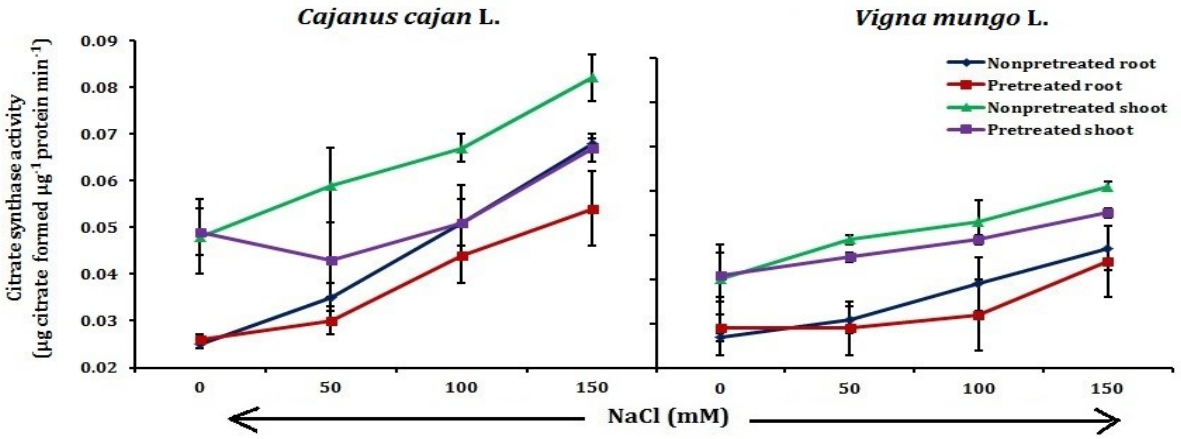

Fig. 4b. Influence of NaCl on CS activity of non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

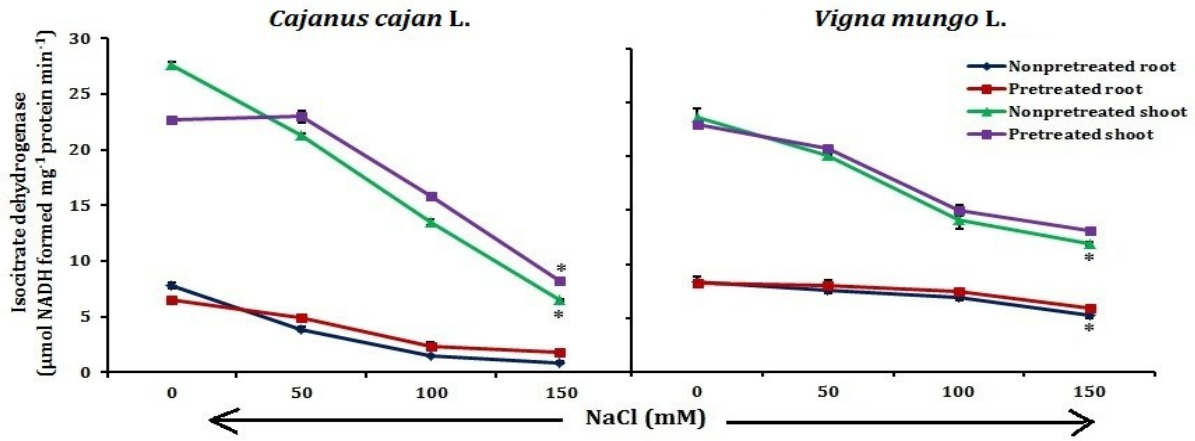

Fig. 4c. Influence of NaCl on IDH activity of non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

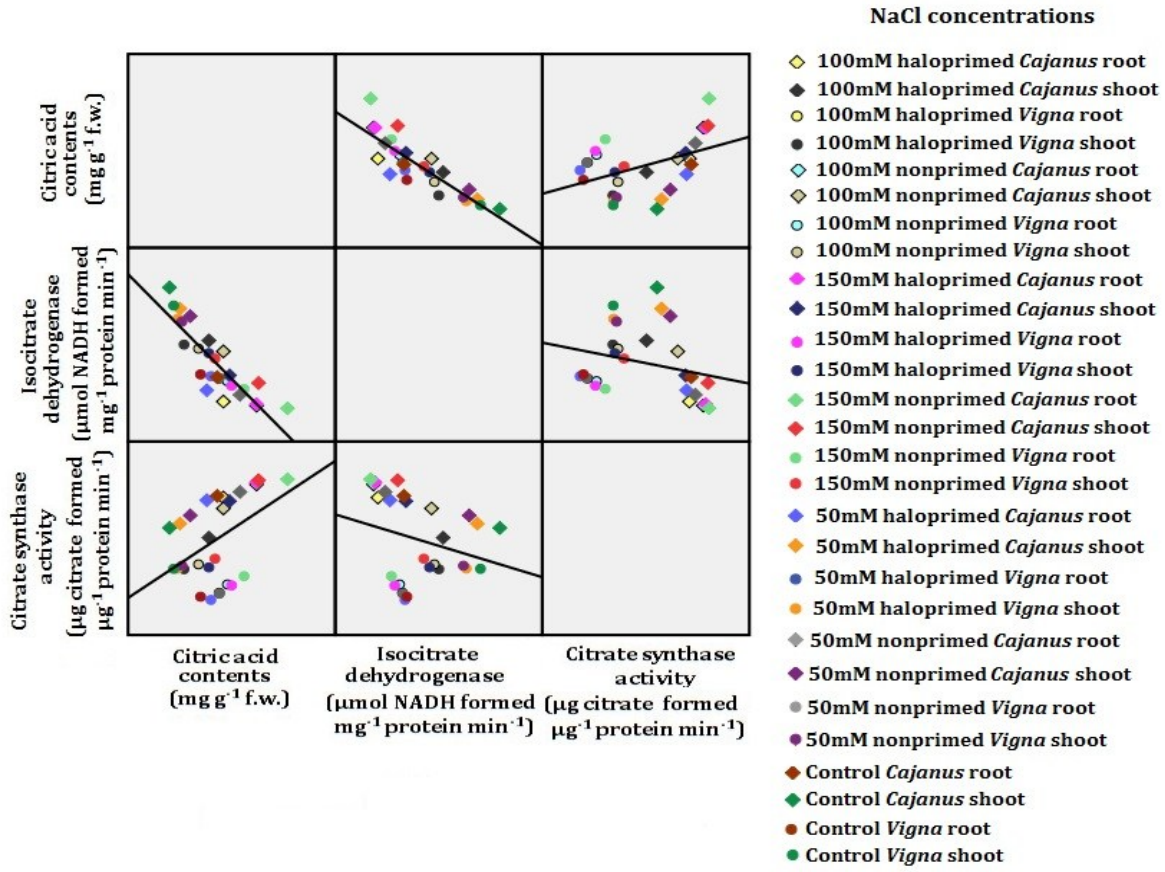

Fig. 4d. Scatterplot matrix showing correlation between activities of CS and IDH and citrate accumulation under NaCl stress in non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

exerted a greater toxic effect on growth of *Cajanus* seedlings. Pretreated seedlings of *Cajanus* and *Vigna* documented improved activities of α-KGDH (to about 34% in root and 31% in shoot of *Cajanus*; 23% in both root and shoot of *Vigna*) and SDH (*Cajanus*: 12% in root and 1% in shoot; *Vigna*: 27% in root and 1% in shoot) which corresponded to lower succinate accumulation (*Cajanus*: 23% in root and 25% in shoot; *Vigna*: 14% in root and 11% in shoot) (Figs. 5a, 5b, 5c).

Probable improvements in metabolic flux of pretreated seedlings due to enhanced α-KGDH activity helped to grant more NADH for respiratory chain enabling better energy balance and seedling growth. Pearson's correlation matrix depicts significant negative correlation between α-KGDH activity and succinate production (r= -0.752, p<0.01), SDH activity and succinate production (r= -0.625, p<0.01) whilst a strong positive correlation was obtained between activities of α-KGDH and SDH (r=0.536, p<0.01) (Fig. 5d).

Malate generation is crucial for plant nourishment, nitrogen fixation and phosphorus accretion (26). Its formation is catalysed from fumarate by fumarase enzyme. Activity of malate dehydrogenase (MDH), a key enzyme of Krebs' cycle was decreased (*Cajanus*: 15% in root and 11% in shoot; *Vigna*: 19% in root and 13% in shoot) along with increase in fumarase activity (*Cajanus*: 73% in root and 57% in shoot; *Vigna*: 67% in root and 15% in shoot) under salinity that caused higher accumulation of malate (to about 67% in root and 247% in shoot of *Cajanus*; to about 10% in root and 16% in shoot of *Vigna*) in non-pretreated seedlings of *Cajanus* and *Vigna* (Figs. 6a, 6b, 6c). Malate generation does not follow any unique way for its formation as MDH can act in opposite direction in Krebs' cycle generating malate. So, in present work, malate registered highest concentration after citrate among all four organic acids estimated in both the non-pretreated seedlings. Similar increase of malate in corn has been reported to confer salinity tolerance (27). Low-key activity of malate dehydrogenase (MDH) impairs several physio-biochemical processes in plants (28). On the contrary, in pretreated seedlings of *Cajanus* and *Vigna*, improved MDH activity narrowed levels of malate accumulation that favoured physiological processes to confer better seedling growth. Improved MDH activity (*Cajanus*: 6% in root and 5% in shoot; *Vigna*: 7% in root and 7% in shoot) along with decline in fumarase activity (*Cajanus*: 46% in root and 23% in shoot; *Vigna*: 27% in root and 74% in shoot) in pretreated seedlings could thus be correlated with reduced malate accumulation (*Cajanus*: 7% in root and 137% in shoot; *Vigna*: 12% in root and 8% in shoot) in pretreated seedlings indicating stress release. Pearson's correlation matrix depict insignificant negative correlation between MDH activity and malate production (r= -0.204, p<0.2) and MDH activity and fumarase activity (r= -0.066, p<0.7) under salt stress. Also, an insignificant positive correlation between activities of fumarase and malate production was noted (r=0.160, p<0.4) (Fig. 6d).

Striking increase in organic acid production in non-pretreated seedlings of *Cajanus* and *Vigna* under salt stress was an

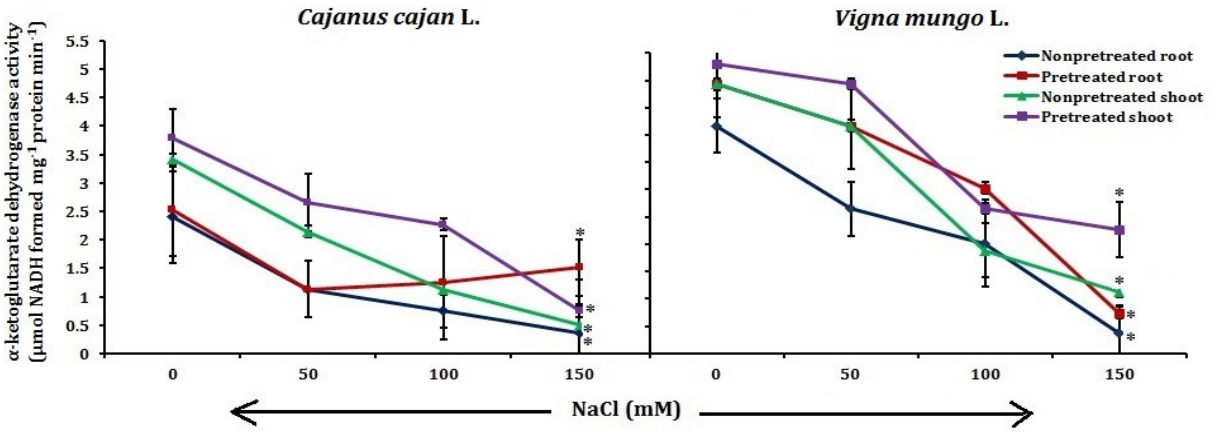

Fig. 5a. Influence of NaCl on α-KGDH activity of non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

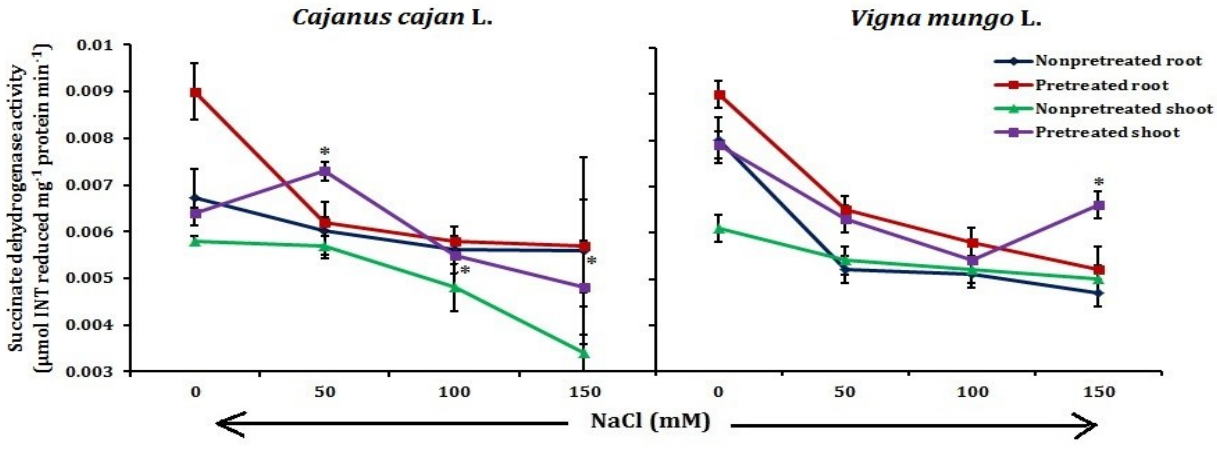

Fig. 5b. Influence of NaCl on SDH activity of non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

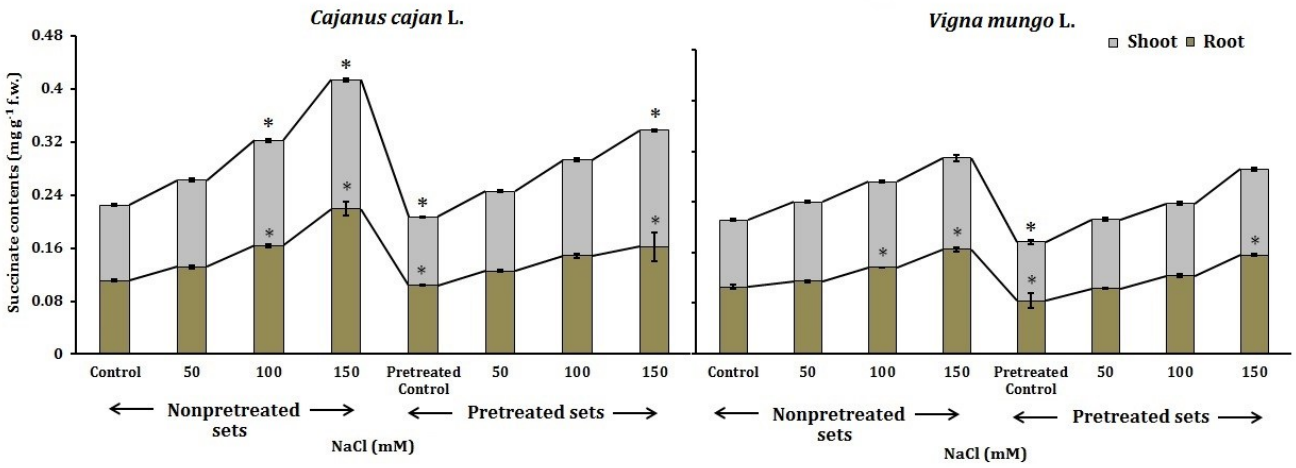

Fig. 5c. Influence of NaCl on succinic acid contents of non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

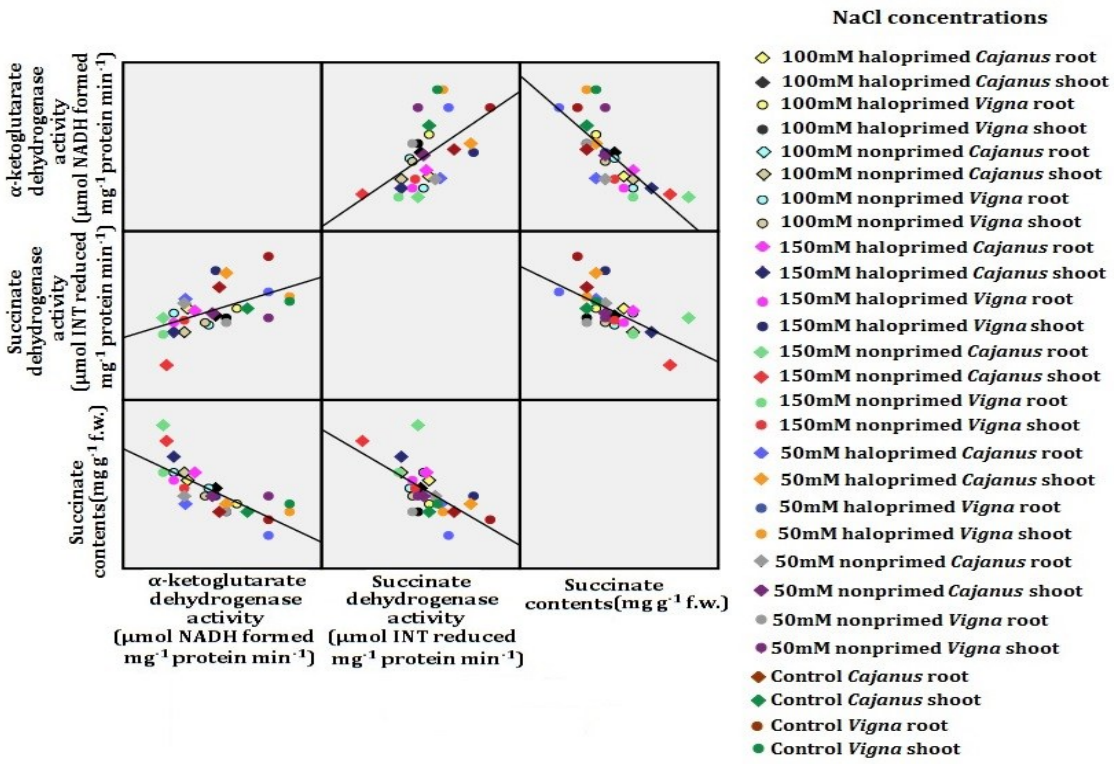

Fig. 5d. Scatterplot matrix showing correlation among activities of α-KGDH, SDH and succinate accumulation in non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

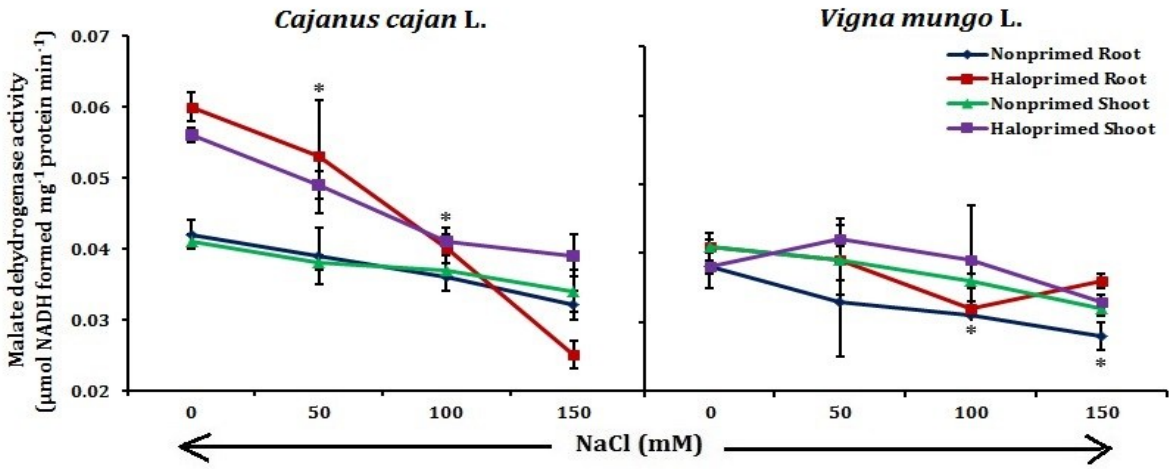

Fig. 6a. Influence of NaCl on MDH activity of non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

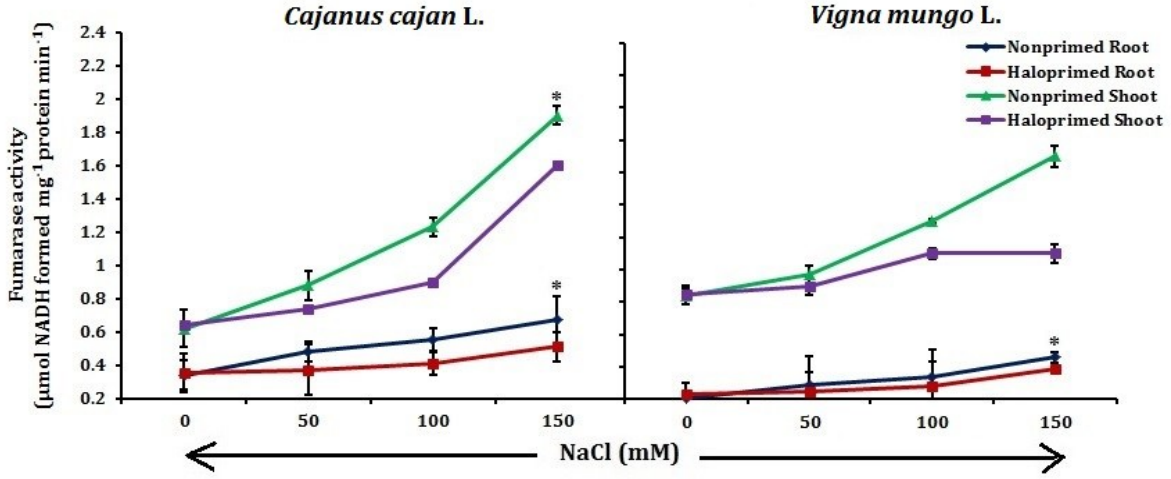

Fig. 6b. Influence of NaCl on fumarase activity of old non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

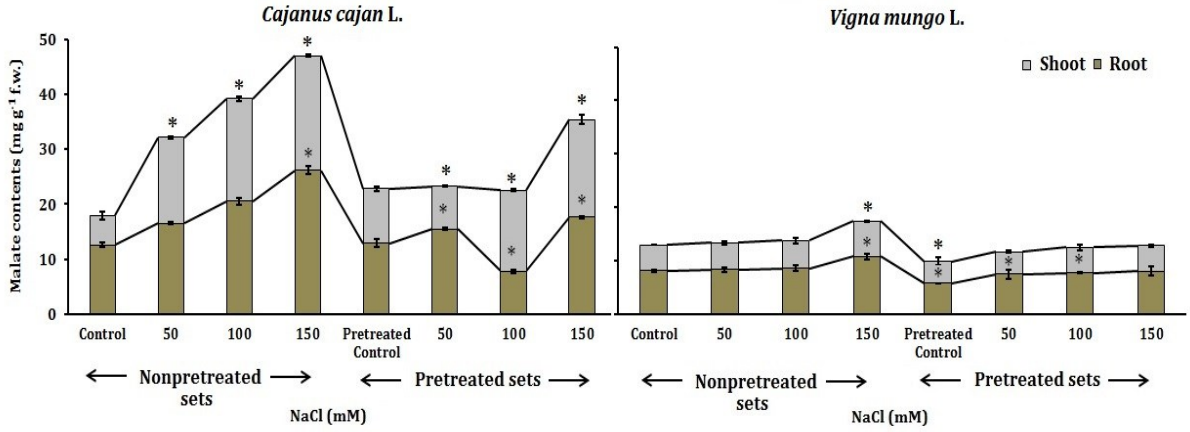

Fig. 6c. Influence of NaCl on malic acid contents of non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

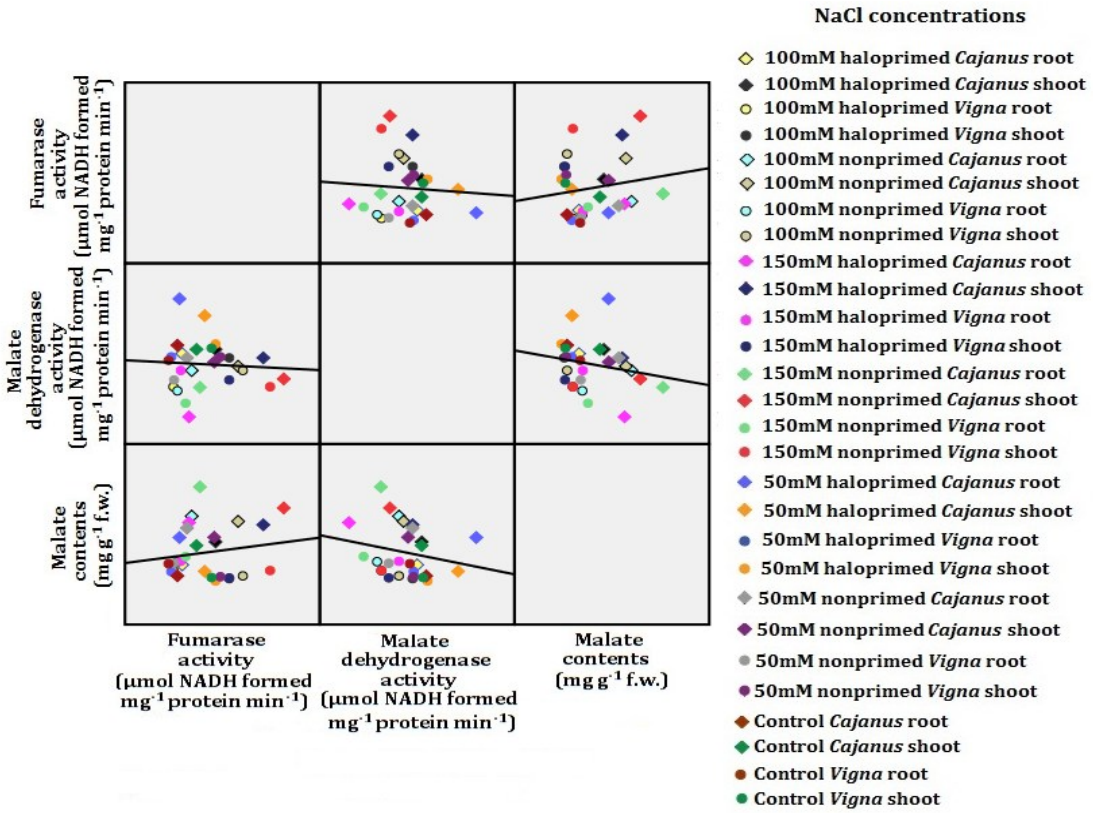

Fig. 6d. Scatterplot matrix showing correlation among activities of MDH, fumarase and malate accumulation in non-pretreated and pretreated seedlings of *Cajanus cajan* L. and *Vigna mungo* L.

outright mechanism to sustain intracellular ion balance aiding seedling survival under salinity. Amongst all crucial enzymes of Krebs' cycle, the dehydrogenases demand substantial consideration in generation of reducing power. Subdued functioning of dehydrogenase enzymes are known to affect plant growth (29). Concomitant with above view, depreciation in activity of all dehydrogenases must have been one of the possible reasons for growth retardation of non-pretreated seedlings under salinity. However, seed pretreatment with 50mM NaCl, documented improved activities of dehydrogenases in both *Cajanus* and *Vigna* seedlings attributing to improved seedling growth under salinity (Fig. 7). Thus, seed pretreatment with 50mM NaCl invoked alterations in enzymatic activities favourably helping to ameliorate the detrimental consequences imposed by NaCl.

Conclusion

Salt stress imposed notable reductions in root and shoot growth of non-pretreated *Cajanus cajan* and *Vigna mungo* seedlings. Present study focuses on the defence strategies overtaken by NaCl pretreated seedlings to curb the hazard of salinity by modulating respiratory cycle. From the responses of *Cajanus* and *Vigna*, we hypothesize that both varieties promisingly responded to seed pretreatment showing considerably higher salinity tolerance as compared to their non -pretreated seedlings. Thus, both varieties can be utilized in breeding program of legumes to enhance salinity tolerance. Lower perturbations of Krebs' cycle prompted defence in pretreated seedlings to thrive under salinity. Decreased organic acid accumulation and improved activities of dehydrogenases due to prior seed acclimation favoured growth of pretreated seedlings under salt stress. Further analyses is still required to explicate the participating genes and mechanisms of salinity tolerance observed in the two studied legumes. Overall current findings suggest that seed pretreatment with NaCl appeared as a suitable eco-friendly and cost-effective option to ameliorate growth of *Cajanus cajan* and *Vigna mungo* to salt stress. So, the present study could be extended to said legumes grown in salt laden agricultural fields to confirm the efficacy of seed pretreatment in abrogating salt stress.

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Authors' contributions

PC and SB contributed equally towards the manuscript. PC carried out experiments and participated in data acquisition. SB drafted the manuscript, participated in data acquisition and prepared graphs. BP statistically analysed all data with the help of SB. AKB conceived the study, helped to design experiments, analysed data and finalized the manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

Ethical issues: None

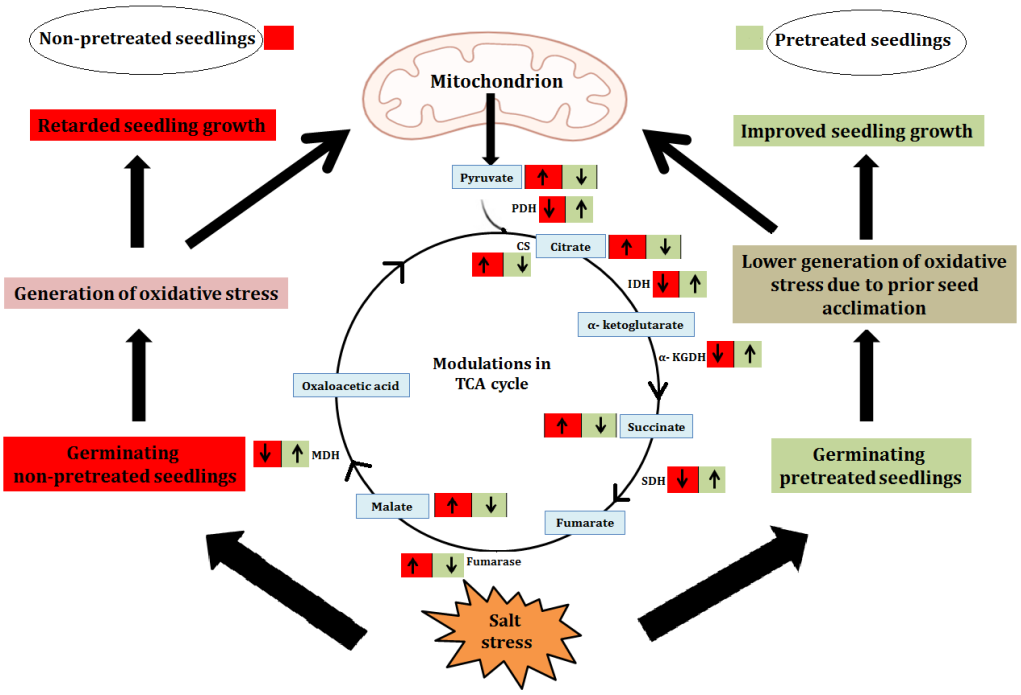

Fig. 7. Schematic representation depicting alterations in Krebs' cycle events for alleviation of NaCl-induced toxicity in *Cajanus cajan* L. and *Vigna mungo* L. seedlings by seed pretreatment.

References

1. Richter JA, Behr JH, Erban A, Kopka J, Zörb C. Ion-dependent metabolic responses of *Vicia faba* L. to salt stress. Plant Cell Environ. 2019;42(1):295-309. https://doi.org/10.1111/pce.13386
2. Liu C, Mao B, Yuan D, Chu C, Duan M. Salt tolerance in rice: Physiological responses and molecular mechanisms. Crop J. 2021;10(1):13-25. https://doi.org/10.1016/j.cj.2021.02.010
3. Tlahig S, Bellani L, Karmous I, Barbieri F, Loumerem M, Muccifora S. Response to salinity in legume species: An insight on the effects of salt stress during seed germination and seedling growth. Chem Biodiversity. 2021;18(4):e2000917. https://doi.org/10.1002/cbdv.202000917
4. Deorukhkar A, Ananthanarayan, L. Consumption of decorticated pulses ensures the optimum intake of isoflavones by the urban Indian population. *Nutrition and Cancer*, *71*(5), 870-80. https://doi.org/10.1080/01635581.2018.1559936
5. Tayyab A, Azeem M, Ahmad N, Ahmad R, Qasim M. Salt Stress responses of pigeon pea (*Cajanus cajan*) on growth, yield and some biochemical attributes. Pak J Bot. 2016;48(4):1353-60.
6. Hassan MJ, Ali Raza M, Khan I, Ahmad Meraj T, Ahmed M, Abbas Shah G, Ansar M, Awan SA, Khan N, Iqbal N, Peng Y, Li Z. Selenium and salt interactions in black gram (*Vigna mungo* L.): Ion uptake, antioxidant defense system, and photochemistry efficiency. Plants. 2020;9(4):467. https://doi.org/10.3390/plants9040467
7. Widodo P, Patterson JH, Neubigin E, Tester M, Bacic A, Roessner U. Metabolic responses of salt stress of barley (*Hordeum vulgare* L.) cultivars, Sahara and Clipper, which differ in salinity tolerance. J Exp Bot. 2009;60(14):4089-4103. https://doi.org/10.1093/jxb/erp243
8. Saha P, Kunda P, Biswas AK. Influence of sodium chloride on regulation of Krebs cycle intermediates and enzymes of respiratory chain in mungbean. Plant Physiol Biochem. 2012;60:214-22. https://doi.org/10.1016/j.plaphy.2012.08.008
9. Sadasivam S, Manickam A. Biochemical methods. New Delhi:New Age International Publishers; 2008
10. Saffran M, Denstedt O. A rapid method for determination of citric acid. J Biol Chem. 1948;175(2):849-55.
11. Valle ABF, Panek AD, Mattoon JR. Colorimetric determination of succinic acid using yeast succinate dehydrogenase. Ann Biochem. 1978:91(2):583-99. https://doi.org/10.1016/0003-2697(78)90545-6
12. Hummel JP. The fluorometric determination of malic acid. J Biol Chem. 1949;180(3):1225-28.
13. Williams M, Randall DD. Pyruvate dehydrogenase complex from chloroplasts of *Pisum sativum* L. Plant Physiol. 1979;64(6):1099-1103. https://doi.org/10.1104/pp.64.6.1099
14. Srere PA. Citrate synthase: [EC 4.1.3.7. Citrate oxaloacetate-lyase (CoA-acetylating)]. Methods Enzymol. 1969;13:3-5. https://doi.org/10.1016/0076-6879(69)13005-0
15. Zhou J, Tian X, Qiao L, Qin P. Respiratory enzyme activity and regulation of respiration pathway in seashore mallow (*Kosteletzkya virginica*) seedlings under water logging conditions. Aust J Crop Sci. 2012;6(4):756-62.
16. Millar AH, Hill SA, Leaver CJ. Plant mitochondrial 2-oxoglutarate dehydrogenase complex: Purification and characterization in potato. Biochem J. 1999;343(Pt 2):327-34. https://doi.org/10.1042/0264-6021:3430327
17. Green JD, Narahara HT. Assay of succinate dehydrogenase activity by the tetrazolium method: evaluation of an improved technique in skeletal muscle fractions. J Histochem Cytochem. 1980;28(5):408–12. https://doi.org/10.1177/28.5.6966645
18. Bergmeyer HU, Gawwehn K, Grass M. Enzymes as Biochemical reagents. In: Bergmeyer HU,editor. Methods of Enzymatic Analysis. New York: Academic Press; 1974. p. 425-556
19. Kumar RG, Shah K, Dubey RS. Salinity induced behavioural changes in malate dehydrogenase and glutamate dehydrogenase activities in rice seedlings of differing salt tolerance. Plant Sci. 2000;156(1):23-34. https://doi.org/10.1016/S0168-9452(00)00224-7
20. El-Serafy RS, El-Sheshtawy AA, Atteya A, Al-Hashimi A, Abbasi AM, Al-Ashkar I. Seed priming with silicon as a potential to increase salt stress tolerance in *Lathyrus odoratus*. Plants. 2021;10(10):2140. https://doi.org/10.3390/plants10102140
21. López-Bucio J, Nieto-Jacobo MF, Ramírez-Rodríguez VV, Herrera-Estrella L. Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. Plant Sci. 2000;160(1):1-13. https://doi.org/10.1016/s0168-9452(00)00347-2
22. Shelden MC, Dias DA, Jayasinghe NS, Bacic A, Roessner U. Root spatial metabolite profiling of two genotypes of barley (*Hordeum vulgare* L.) reveals differences in response to short-term salt stress. J Exp Bot. 2016;67(12):3731–45. https://doi.org/10.1093/jxb/erw059
23. Katz E, Fon M, Lee YJ, Phinney BS, Sadka A, Blumwald E. The citrus fruit proteome: insights into citrus fruit metabolism. Planta. 2007;226:989-1005. https://doi.org/10.1007/s00425-007-0545-8 \
24. Tretter L, Adam-Vizi V. Alpha-ketoglutarate dehydrogenase: a target and generator of oxidative stress. Philos Trans R Soc Lond B Biol Sci. 2005;360(1464):2335-45. https://doi.org/10.1098/rstb.2005.1764
25. Hamilton EW, Heckathorn SA. Mitochondrial adaptations to NaCl. Complex I is protected by anti-oxidants and small heat shock proteins, whereas complex II is protected by proline and betaine. Plant Physiol. 2001;126(3):1266-74. https://doi.org/10.1104/pp.126.3.1266
26. Schulze J, Tesfaye M, Litjens RHMG, Bucciarelli B, Trepp G, Miller S, et al. Malate plays a central role in plant nutrition. Plant Soil. 2002;247:133-39. https://doi.org/10.1023/A:1021171417525
27. Richter JA, Erban A, Kopka J, Zörb C. Metabolic contribution to salt stress in two maize hybrids with contrasting resistance. Plant Sci. 2015;23:107-15. https://doi.org/10.1016/j.plantsci.2015.01.006
28. Tomaz T, Bagard M, Pracharoenwattana I, Lindén P, Lee CP, Carroll AJ, et al. Mitochondrial malate dehydrogenase lowers leaf respiration and alters photorespiration and plant growth in *Arabidopsis*. Plant Physiol. 2010;154(3):1143-57. https://doi.org/10.1104/pp.110.161612
29. Bouthour D, Hajjaji-Nasraoui A, Saafi L, Gouia H, Chaffei-Haouari C. Effects of NaCl on growth and activity of enzymes involved in carbon metabolism in leaves of tobacco (*Nicotiana rustica*). Afr J Biotech. 2012;11(63):12619-29. https://doi.org./10.5897/AJB11.1103