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Research article

Exogenous silicon alters organic acid production and enzymatic activity of TCA cycle in two NaCl stressed indica rice cultivars

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ABSTRACT

The activities of TCA cycle enzymes viz., pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase, succinate dehydrogenase and malate dehydrogenase as well as levels of different organic acids viz., pyruvic acid, citric acid, succinic acid and malic acid were studied in two rice cultivars viz. cv. Nonabokra and cv. MTU 1010 differing in salt tolerance grown under 25, 50 and 100 mM NaCl salinity levels. A contrasting response to salt stress on enzyme activities of TCA cycle and accumulation of organic acid was observed between two cultivars during twenty-one days period of study. Salinity caused enhanced organic acid production and increase in all five enzyme activities in cv. Nonabokra whereas in cv. MTU 1010 decrease in both organic acid production and enzymes activities were noted. Joint application of exogenous silicon along with NaCl, altered the organic acids levels and activities of enzymes in both cultivars of rice seedlings conferring tolerance against salt induced stress. Rice cv. MTU 1010 showed better response to exogenous silicon on parameters tested compared to cv. Nonabokra.

1. Introduction

The rapid increase in world population demands more food to be grown for human consumption. This can be fulfilled either by increase in cultivable land or by increase in crop productivity per unit area. While a concentration of 40 mM sodium chloride concentration in the soil is considered to be saline; hence making it unsuitable for agriculture (Acosta-Motos et al., 2017). Endeavours to increase arable land has led agriculturists to venture onto marginal, salinity affected lands. Moreover, the necessity of irrigation water for crop cultivated in semi-arid and arid environments has added to the salinity problem. Approximately 830 million hectares of soil is affected by salinity globally (Martinez-Beltran and Manzur, 2005). Population increase, as well as, land deterioration by salinization have forced scientists to explore ways to develop salt tolerant crops (Munns, 2005).

Rice which is considered as one of the most important food crops, both economically and nutritionally, is most sensitive to saline conditions (Maas and Grattan, 1999). India harbors the world largest rice growing areas (44.0 Mha) and is the second largest producer (106.29 million tons) next only to China (Bhambure and Kerkar, 2016). Adverse effects of salinity on rice plants are multifarious, affecting early seedling stages and later reproductive stages, culminating in decrease of germination rate and dry mass production, abnormal leaf development and sterility (Khatun et al., 1995; Asch and Woperies, 2001). According to a recent report, among all the states of India West Bengal contains the largest coastal saline land, and in fact some of the major rice growing districts of West Bengal, viz., 24-Parganas (North and South), Midnapur (East and West) and Howrah are affected by salinity (CSSRI, 2016).

Negative effects of soil salinity can be reduced by land reclamation and drainage, but this is a very costly process. A substitute for land reclamation could be the addition of silicon especially in those regions where irrigation water is saline. Interestingly, rice is considered to be a silicon accumulator. According to Ma et al. (2001) excess silicon accumulation in plants showed no deleterious effects due to its non-dissociative properties. According to Ma and Yamaji (2006), the positive effects of silicon were due to its accumulation on the cell walls of the stems, roots and leaves enhancing their strength and rigidity against the incoming stress factor. Application of sodium silicate ameliorated the

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detrimental effects of salt stress in soybean (Lee et al., 2010) and canola (Farshidi et al., 2012) and also in rice (Das et al., 2018).

The mitochondrial citric acid or tricarboxylic acid cycle (TCA Cycle) popularly known as Krebs cycle, is one of the major pathways of carbon metabolism in higher organisms that provides electrons during oxidative phosphorylation within the inner mitochondrial membrane (Schnarrenberger and Martin, 2002). Intermediates for amino-acid biosynthesis, and oxaloacetate for gluconeogenesis from succinate derived from fatty acids via the glyoxylate cycle in glyoxysomes is powered by TCA cycle itself (Martin and Schnarrenberger, 1997). TCA cycle is crucial to donate electrons from pyruvate to the membranes in mitochondria necessary for respiration, though not universal, some modified form is extensively present in eukaryotes (Yeo, 1998). The pathway was originally drafted by Krebs and Johnson (1937), had an endosymbiotic origin, and proved by gene to gene phylogenetic analysis (Huynen et al., 1999). Deprivation of electron flow in the TCA cycle during environmental stress be it abiotic or biotic (Petrovicˇ et al., 2002), cuts off the vital outer flows of molecules necessary for maintaining the amino acid synthesis and subsidiary processes thus cutting down on the wellbeing of the affected organism because carbon enters this chain as pyruvate. Any kind of perturbation in the TCA cycle influence the total carbon pool of the cell.

Mitochondria plays various pivotal roles in many fundamental process underpinning growth, development and death of plant. Its diversified activities in maintaining the cell's redox status sites of oxidative phosphorylation mitochondria are in a key position to study salinity response in plants. Status of mitochondria induces plant resistance to various stresses and modulate response to different stress signals (Millar et al., 2011). Mitochondrial respiration is considered to be the center of plant metabolic networks because TCA cycle links energy metabolism with nitrogen and carbon metabolism (Jacoby et al., 2013; Nunes-Nesi et al., 2013). Recent evidence proposed that mitochondria may behave as ‘sensors’ in plants against stress (Vanlerberghe et al., 2009).

The nine enzymes of the TCA cycle represent the major carbon metabolising machinery present in plant mitochondria. Pyruvate is produced through glycolysis in the cytosol (Tcherkez et al., 2012). Before entering the mitochondria, pyruvate is oxidized to acetyl- CoA in presence of the enzyme pyruvate dehydrogenase complex (PDC) (Lin et al., 2003). TCA cycle begins when acetyl -CoA condenses with oxaloacetate in the presence of the enzyme citrate synthase (CS) forming citrate (Remington, 1992). Through a reversible isomerisation reaction catalysed by aconitase, citrate is converted to isocitrate (Moeder et al., 2007). Isocitrate is then converted to 2-oxoglutarate by the enzyme isocitrate dehydrogenase (IDH) yielding one molecule of NADH (Galvez et al., 1999). Formation of oxaloacetate from 2-oxoglutarate requires subsequent action of 2-oxoglutarate dehydrogenase complex (OGDC), succinyl-CoA synthetase (S-CoAS), succinate dehydrogenase (SDH), fumarase and malate dehydrogenase (MDH).

Cation-anion impairment is one of the vital factors modulating organic acid level in plants. Organic acids like malate, citrate, malonate and aconitate provide negative charge that nullifies the positive charge due to over accumulation of cation through roots (Chang et al., 1991; Jones, 1998). Compensation of charge imbalance under salinity by organic acids was found in those plants where Na^+ was uptaken more than Cl-ions, for example Chenopodiaceae, or plants where translocation of Na^+ from root to shoot was more than Cl-ions, like Poaceae (Kinzel, 1982). Reports from field study indicate that organic acid contents decrease in glycophytes under salinity (Kinzel, 1982). Reduction of organic acid level in red grape cultivars in due to water deficit was already reported (Esteban et al., 1999).

The TCA cycle is highly flexible and tightly regulated in changing mitochondrial roles to adapt and survive under various kinds of stresses including NaCl stress (Fait et al., 2008). An altered TCA cycle activity is an unavoidable response of plants exposed to salt stress and is common for a number of plants like grapevine (Cramer et al., 2007), the halophyte *Limonium latifolium* (Gagneul et al., 2007), *Arabidopsis* (Gong et al., 2005; Sanchez et al., 2008b), *Lotus japonicus* (Sanchez et al., 2008a). Moreover, mitochondria play a vital role in mediating responses to a number of stresses and are also harbours a central role in activation of programmed cell death (PCD) (De Pinto et al., 2012). Salt induced changes in respiratory process are complex, because salt tolerant and sensitive species showed marked discrepancies in their rate of respiration (Jacoby et al., 2011). These variations are important criteria to differentiate between a sensitive and a tolerant crop against salinity. Salt stress resulted into increased ATP demand to operate different energy requiring adaptive mechanisms which include ion homeostasis, ROS defense etc. Ion toxicity is a marker of salinity stress which leads to membrane damage and alteration in enzyme activities (Hasegawa, 2013). According to Roy et al. (2014), some salt tolerant plants gain tissue tolerance i.e., able to grow under high saline condition by regulating intracellular ion homeostasis mediated by ion compartmentation and exclusion respectively. These two processes are ATP requiring processes. Plant salinity tolerance capacity depends on the efficiency of its metabolism of energy (Munns and Tester 2008; Jacoby et al., 2011). It has already been reported that salt tolerant species can fulfil this ATP requirement under salinity since they have more efficient ATP production mechanisms (Jacoby et al., 2013).

The responses of TCA cycle components to NaCl stress in rice was studied before but it is not clear whether these changes are the consequences of salt toxicity or provide tolerance to the plant. Moreover, the effect of silicon on respiratory cycle has seldom been documented. To explore the possibility to overcome adverse effects caused by salinity on rice seedlings with silicon, it is essential to study the intermediate substrates and key enzymes of the respiratory cycle. By silicon supplementation along with NaCl, we tried to encounter the problem of salt toxicity in rice. Such experiments will help to formulate a low-cost agronomic solution to combat the present alarming situation of salinity by the application of silicate fertilizers in saline rice fields.

2. Material and methods

2.1. Plant growth and treatment conditions

Rice seeds were procured from State Rice Research Station, Chinsurah, West Bengal, India. Two low land rice cultivars (cv.sMTU 1010 and Nonabokra) were subjected to surface sterilization prior to germination in wet petridishesfor 48 h at 30 ± 2 °C. The germinated seedlings were treated with different concentrations of sodium chloride solution (25, 50 and 100 mM) with or without 2 mM Sodium meta-silicate (Na_2O_3Si, $9H_2O$) solution (w/v). The seedlings were then exposed to 16 h photoperiod (260 $\mu mol\, m^{-2}\, s^{-1}$ PFD) for 21 days with addition of new modified Hoagland solution (Rafi et al., 1997) every alternate day. Throughout this period the pH of the nutrient solution was adjusted in the range of 5.5–6.0 using 0.1 M HCl to reduce polymerization of silicates. The harvested plants after 21 days, were washed with distilled water, dried, stored separately into shoot and root at −80 °C for further analyses (Das et al., 2018).

2.2. Qualitative estimation of mitochondrial membrane potential through confocal laser scanning microscopy (ΔΨm)

Fresh roots from control and all the treated sets (approx. 10 in number) were incubated in fluorescent stain Rhodamine 123 for 30 min in darkness (Ahmed et al., 2017), followed by a thorough washing in 1x Phosphate Buffered Saline to wash off excess stain (Manna and Bandyopadhyay, 2017a). The roots were scanned under a confocal microscope (1× CLSM 81, Olympus, Japan) and photographed using the software version Flouview FVV 1000 to document the changes in the mitochondrial membrane potential (ΔΨm). Bright-field images were simultaneously captured to document the shape of the affected root and shoot.

2.3. Quantification of sodium and potassium content

The sodium (Na^+) and potassium (K^+) contents were determined through ICP-OES spectroscopy following the protocol of Manna and Bandyopadhyay (2017) (b). Tissue from all the sets including control and treated samples were double acid digested and inductively coupled plasma-optical emission spectroscopy was performed using a Parkin Elmer Optima 5300 DV(USA).

2.4. Quantification of chloride content

One gm of root and shoot samples were taken from each treatment. The samples were homogenised in 10 ml of deionized water, to which a small amount of activated charcoal was added and left undisturbed for 5 min. Then the charcoal was removed from the plant extract by filtration and centrifugation and were used for estimation of chloride (Cl-) by silver nitrate titration method (Sheen et al., 1938) and expressed as mg g-1 fresh weight.2.4. Quantitative estimation of organic acid contents:

2.5. . For estimation of the major organic acids of the TCA cycle

500 mg of freshly sampled root and shoot tissues were homogenised in 0.2 M phosphate buffer (pH 7.4), centrifuged at 4 °C, followed by deprotinization with 5% trichloroacetic acid (w/v) and filtered. The filtrate was used for the following quantitative estimations.

2.5.1. Pyruvic acid contents

Pyruvic acid contents were estimated according to Sadasivam and Manickam (2008). The reaction mixture constituted of filtrate, 0.2 M phosphate buffer, 0.02% DNPH (2,4-dinitrophenylhydrazine) was incubated for 30 min at 37 °C. Then 0.8 (N) NaOH was mixed with it and the mixture kept for a 10 min incubation period at room temperature. A blank sample without plant extract was similarly prepared. A mixture without any plant sample was used as a blank. The resultant brown coloured product was measured spectrophotometrically at 510 nm. Amount of pyruvic acid present in the samples was calculated from a standard curve using sodium pyruvate. The quantity of the pyruvic acid was expressed as mg g^{-1} fresh weight.

2.5.2. Citric acid contents

Citric acid was estimated using the method of Saffran and Denstedt (1948). The reaction mixture contained the filtrate, pyridine and acetic anhydride and was heated in a water bath at 32 ± 0.5 °C for 30 min. OD was taken with a Hitachi U-2000 spectrophotometer at 405 nm. Using known concentrations of citric acid a standard curve was drawn. The total amount of citric acid was expressed as mg g^{-1} fresh weight.

2.5.3. Malic acid contents

Malic acid was estimated with the protocol proposed by Hummel (1949). A reaction mixture was prepared with 0.5 ml of the filtrate, 1.0 ml of 1(N) HCl, 0.1 ml of 0.1% DNPH and 0.5 ml of 10% $CaCl_2$. After 30 min incubation at room temperature, 0.3 ml of 5(N) NH_4OH and 6 ml of absolute alcohol were added to each tube, which were then kept undisturbed at room temperature for 12 h to complete the precipitation. The tubes were centrifuged at 5000 g for 15 min and the supernatant was carefully poured off. The pellets were dried in an oven at 105 °C for 15 min, and 0.08% orcinol-sulphuric acid was added to each tube, the contents were mixed thoroughly with a stirring rod and the tubes were heated upto 100 °C for 10 min. The mixtures were cooled under tap water and diluted to 10 ml with concentrated H_2SO_4. The blue fluorescence thus produced was measured in a Hitachi-650-40 spectrofluorometer against blank. The amount of total malic acid was expressed as mg g^{-1} fresh weight using a standard curve.

2.5.4. Succinic acid contents

Succinic acid content was measured according to Valle (1978). Briefly, plant tissues were crushed in liquid nitrogen and homogenised in a buffer containing 0.4 M mannitol, 1 mM EDTA and 50 mM Tris (pH 8.2). The homogenate was fractionated by centrifugation at 0 °C for 10 min at 2000g.The resulting supernatant was centrifuged at 20,000 g for 20 min. The pellet containing a brown tightly packed lower layer was washed and re suspended in the homogenization buffer comprised of the mitochondrial fraction. The suspension was centrifuged for 20 min at 20,200 g. The washed pellet was re-suspended in potassium phosphate buffer pH 7.6.

The assay mixture comprised of buffer containing 0.1 M KH_2PO_4, 5 mM EDTA, 5 mM Sucrose, 0.0.5% INT, different concentrations of standard succinate solution or an experimental sample containing succinate and distilled water to produce a final volume of 0.9 ml. The tubes were placed on ice and 0.1 ml mitochondrial suspension was added followed by the unknown or standard succinate solutions to make a final volume of 1 ml. The tubes placed initially on ice for 15 min, were incubated at 37 °C for 60 min. The reaction was terminated by addition of 1 ml of 10% (w/v) trichloroacetic acid. The red formazan obtained was cooled on ice and extracted in ethyl acetate (4 ml). Absorbance was recorded at 490 nm. Amount of succinate present was calculated from the standard curve and was expressed as mg g^{-1}fw.

2.6. Quantitative estimation of enzyme contents

2.6.1. Assay of pyruvate dehydrogenase activity

Pyruvate dehydrogenase assay was performed according to Williams and Randalls (1979). 500 mg tissue samples were crushed in Tris HCl buffer (50 mM) (pH 7.8) containing sucrose (0.7 M), β-mercaptoethanol (57 mM), EDTA (2 mM) and 0.5% (w/v) BSA and centrifuged at 10,000 g for 20 min at 4 °C. 1.0 ml of the assay mixture was prepared the supernatant, 0.2 ml of 50 mM Tris HCl (pH 8.0) and 0.1 ml each of 5 mM $MgCl_2$, 0.12 mM CoA, 2.6 mM Cysteine-HCl and 1.5 mM pyruvate. The OD of the reaction mixture was measured in a Hitachi U-2000 spectrophotometer against a blank (340 nm). Then 0.2 ml of 1.4 mM NAD was added to the reaction mixture, mixed well and the absorption was further recorded for every 60 s for 2 min. The increasing OD indicated the amount of NADH produced per min. A standard curve was drawn with known concentrations of NADH from which the PDH activity was calculated and expressed as μmol NADH min^{-1} mg^{-1} protein.

2.6.2. Assay of citrate synthase activity

Citrate synthase activity was determined according to the method described by Srere et al. (1969). Samples were homogenised in a buffer containing 0.1 M Tris-HCl buffer (pH 8.0), 0.1% (v/v) Triton X-100 (Triton −100), 2% (w/v) PVP, and 10 mM iso-ascorbic acid. The extracts were centrifuged for 5 min at 15000 rpm at 4 °C and the supernatant was assayed for enzyme activity. The reaction mixture comprised of DTNB (1 mM), acetyl CoA (10 mM) and the supernatant. Initial absorbance was recorded followed by addition of 10 mM OAA and final absorbance was recorded at 412 nm in a Hitachi U-2000 spectrophotometer. Enzyme activity was calculated as μg citric acid formed mg^{-1} protein min^{-1}.

2.6.3. Assay of isocitrate dehydrogenase activity

Isocitrate dehydrogenase was assayed according to Zhou et al. (2012). 500 mg of root and shoot samples were homogenised in HEPES buffer (50 mM) (pH 7.5) containing β-mercaptoethanol (10 mM) and 5% polyvinylpolypyrrolidone (w/v). The homogenates were centrifuged at 14,500 g for 20 min at 4 °C. The assay mixture contained Hepes buffer (40 mM) (pH 8.2), sodium isocitrate (2 mM), NAD (800 μM), MnSO4 (200 μM) and enzyme in a final volume of 0.5 ml. The increase in absorbance of the mixture was measured in a Hitachi U-

2000 spectrophotometer at 340 nm for 2 min. The enzyme activity was expressed as μmol NADH min^{-1} mg^{-1} protein.

2.6.4. Assay of succinate dehydrogenase activity

The activity of succinate dehydrogenase was assayed according to Green and Narahara (1980). 500 mg of root and shoot samples were homgenised in Tris HCl buffer (4 mM) (pH 7.5) containing 0.19 M sucrose. The homogenates were centrifuged at 10,000 rpm for 15 min at 4 °C. The assay mixture contained 0.1 ml of distilled water, 0.05 ml each of sucrose (0.19 M), Tris HCl (0.1 M) (pH 7.5), sodium azide (10 mM), INT [2-(p-iodophenyl)-3-(p-nitrophenyl)-5-(phenyl tetrazolium chloride)] (8 mM), sodium succinate (0.5 M) and plant sample. The tubes were then incubated at 30 °C in a water bath for 10 min. Then 95% alcohol was added to each tube, mixed properly and kept in ice incubation for 10–15 min. Finally, the mixtures were centrifuged at 8,000 rpm at room temperature for 10 min and absorbance was recorded at 458 nm. Basal reduction of INT was determined in control tubes where succinate was omitted. The enzyme activity was expressed as μmol INT reduced min^{-1} mg^{-1} protein.

2.6.5. Assay of malate dehydrogenase activity

For determination of malate dehydrogenase activity (Dubey and Singh, 1999), 500 mg plant materials from each treatment were crushed in Tris HCl buffer (50 mM) (pH 8.0) containing $MgCl_2$ (50 mM), β-mercaptoethanol (5 mM) and EDTA (1 mM) and centrifugation was carried out at 10,000 g for 20 min at 4 °C. The assay mixture contained OAA (5 μM), $MgCl_2$ (10 μM), Tris HCl buffer (0.1 M) (pH 7.8) and 0.2 ml of the enzyme extract. The initial absorbance of the reaction mixture was measured in a Hitachi U-2000 spectrophotometer at 340 nm. Then 0.4 μM NADH was added to the reaction mixture and absorption was recorded every 60 s for at least 2 min. The enzyme activity was expressed as μmol NADH min-1 mg-1 protein.

2.7. Statistical analyses

All the experiments were done in triplicate sets to maintain reproducibility of the result, results are presented in the form of mean ± standard deviation. Each replica comprising a single petri dish containing an average of 50 seeds. The data were subjected to one way analysis of variance (ANOVA) and level of significance was established at ($p \leq 0.05$) throughout. In case of any significant interaction among the factors (varieties x dosage), Tukey's Test was used to compare the result (Manna and Bandyopadhyay, 2017a).

3. Results and discussion

ROS is deemed a necessary evil (Zhao, 2009). Mostly by-products of essential metabolic processes like photosynthesis and respiration, they are the primary signal transducing agents, critical for homeostasis and survival of any cell. At the same time, when their equilibrium changes, ROS accumulation becomes most harmful for a cell, halting cellular functions, ultimately killing it. Environmental disturbances, like salinity trigger this exact cascade. Plants try to manage ROS upsurge by engaging the Glutathione-Ascorbste cycle. However, the ROS scavenging does not suffice in certain cases leading to massive loss in ionic integrity of the cell, and eventually cell death (Das et al., 2018). Cultivation of rice being one of the leading agriculturally vital crops suffer massive loss as already mentioned owing to many extrinsic sources of salinity. Tolerance to salinity is a composite phenomenon involving a lot of metabolite alteration and metabolic pathways modulation. Study of TCA cycle enzymes and intermediates in two contrasting rice cultivars differing in their salt sensitivity contributed important evidence in understanding the role of mitochondria in governing stress tolerance under salinity in rice. Accumulation of ROS is an inevitable consequence of stress imposition in plants. A very common phenomenon of stress injury in plants is shifting the redox poise of ascorbate-glutathione to more oxidized states (Foyer and Noctor, 2011). Transgenic studies have proven the importance of mitochondria in regulating this phenomenon. Alteration of mitochondrial enzymes can modulate a plant redox balance and as a result impact stress tolerance (Dutilleul et al., 2003; Morgan et al., 2008; Tomaz et al., 2010).

The harmful effects of salinity on plants are attributed to the interference of salinity in the processes involved in energy production (photosynthesis and respiration), as well as water stress, ion toxicity, ion imbalance and nutritional deficiency (Muhammad and Hussain, 2010). The adverse effect of salinity on growth may be caused three ways: by reducing the available water in the root zone causing water deficit, by the phytotoxicity of ions such as Na^+ (sodium) and Cl^- (chlorine) and nutrient imbalance depressing uptake and transport of nutrients, and Na^+ competing with K^+ (potassium) for binding sites essential for cellular function (Munns and Tester 2008). Application of Si improved the growth of borage under normal and saline conditions. This effect may be due to the prominent role of Si in improving plant water status (Romero-Aranda et al., 2006). Also, the benefits of using Si are related to various in direct effects like increased capacity and efficiency of photosynthesis, decreased transpiration, and thus more shoot growth. Kamenidou et al. (2010) studied the effect of Si on morphological and developmental characteristics of gerbera (*Gerbera hybrid* L.) flowers in greenhouse culture conditions and reported the positive effects of Si as Na2Sio3 (sodium metasilicate) in medium on plant height, the thickness of stem, size of flowers and time of flowering. Similar to our results, Tuna et al. (2008) found a decrease in wheat biomass of 39 and 54% in two wheat cultivars when exposed to salinity stress. On the other hand, within each salinity concentration, applied Si alleviated salinity stress and increased wheat biomass and grain yield. Liang (1999) found an increase in biomass and grain yield when applied Si to barley (*Hordeum vulgare* L.) plants grown under salinity stress. Also, Hayat et al. (2014) found an increase in biomass and grain yield in wheat grown under salt stress and fertilized by K-silicate.

Excess salt deposition in soil causes a bottleneck effect for regular growth and development of plants by hindering the osmotic balance of tissues and also by specific ionic imbalance. Rice is considered to be a glycophyte, semi-aquatic plant that is generally grown in swampy lowland specifically adjacent to coastal regions. Salt accumulation in variable amounts and duration checks the normal growth and development of rice plants that eventually leads to considerable reduction of grain yield in different parts of South- East Asia (Gao et al., 2007). Moreover, rice genotypes show considerable degree of variation in their susceptibility to such adverse saline conditions and that characteristics are exploited in selecting resistant rice genotypes to such stressful conditions. Additionally, salinity seems to be a limit on rice plant growth and development compared to other crops because soil moisture stress/osmotic stress as well as oxidative overload work in tandem under such conditions. It is very clear from the results obtained in the present study that salt stress exerts a noticeable negative effect on the rice seedlings, irrespective of cultivars, i.e., MTU 1010 and Nonabokra. On addition, magnitude of the effect seems to be proportionate with the doses of salinity used in our experiments.

Silicon (Si), the second most abundant element in soils is present in the soil solution as silicic acid at concentration ranging between 0.1 and 2.0 mM (Epstein, 1994). In general it is absorbed by plants in the form of uncharged silicic acid, $Si(OH)_4$ (Ma and Yamaji, 2006) and is ultimately polymerised to form silica gel (SiO_2. nH_2O). Improvement of salt tolerance by the addition of Si has been reported in different plants like barley, maize, canola, spinach, cucumber and tomato (Liang et al., 2003; Al-aghabary et al., 2005; Gunes et al., 2007; Moussa, 2006; Tuna et al., 2008; Hashemi et al., 2010). Ameliorative effect of silicon on salt toxicity has been attributed to decreased oxidative damage (Al-aghabary et al., 2005). Si enhanced the growth of salt treated barley, by improving the chlorophyll content and photosynthetic activity of leaf cell organelles of barley (Bradbury and Ahmad, 1990). Matoh et al. (1986) suggested that silica deposition in the leaf decreased

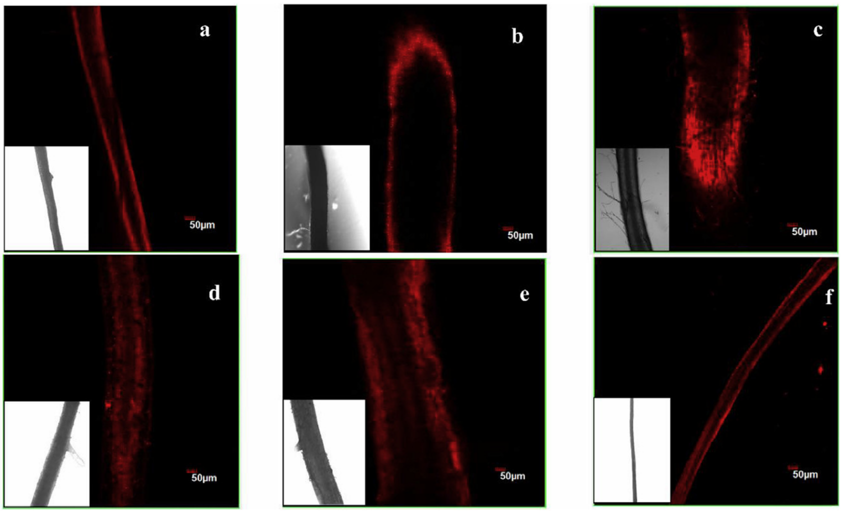

Fig. 1. Rhodamine 123 staining images showing change in mitochondrial membrane potential in roots of cv. MTU 1010 (a: Control; b: 25 mM NaCl; c: 100 mM NaCl; d: 2 mM Silicon; e: 25 mM NaCl + 2 mM Si; f: 100 mM NaCl + 2 mM Si) *Photos in the inset are the bright-field images of the subject studied*

transpiration and therefore decreased salt accumulation. Ahmad et al. (1992) suggested that silicon complexed sodium in the root, therefore decreasing sodium transport to the shoot, but no direct evidence was presented. Most of the beneficial effects of Si are attributed to its deposition in cell walls of the roots, leaves and stems (Ma and Yamaji, 2006).

3.1. NaCl induce changes in mitochondrial membrane potential (ΔΨm)

Salinity in the form of NaCl solution (25, 50 and 100 mM NaCl solution) affected mitochondrial membrane potential negatively which was documented through marked increase in fluorescence of the stained roots and leaves by absorption into the cytoplasm. There is a steep increase in fluorescence with increasing NaCl concentration at a dose dependent manner where the zone of elongation in roots and young leaf portions are most severely affected. On amelioration with silicon, fluorescence of the treated roots and leaves decreased mostly. Even the lowest dose of silicon altered the severity of the damage considerably (Figs. 1–4). Less colouration might be owing to less deposition on cytoplasm as the membrane were less severely damaged because the intact mitochondrion could quench Rh 123 within itself (Baracca et al., 2003).

Confocal images with Rh 123 staining of roots and young leaves showed an increase in fluorescence in the treated samples which is a direct indication of change in the mitochondrial membrane potential, which is an early indication of ROS induced damage leading directly to apoptosis, since mitochondria are the chief sites for electron cycle chains (complex I and II) and cataplerotic reactions. The earlier data of incidence of lipid peroxidation from the authors (Das et al., 2016) strengthen such proposition. Ahmed et al. (2017) and Faisal et al. (2013) working with metallic nanoparticles too came up with the same suggestion. Lower fluorescence in the control sets and ameliorated sets might be owing to intact mitochondrion with unretired proton pumps, hence Rh 123, were quenched by the mitochondrion and not dissipated into the cytoplasm. The complete opposite happened when the tissues were subjected to NaCl solution, the dye getting hypopolarized, were leached into the cytoplasm. Incrementing stress causes mitochondrion to either swell or shrink because of ruptured membranes and electron leakages, the inherent cause of peroxidation of lipid and uncoupling of mitochondrial proteins. However, silicon, was quite effective in protecting the mitochondrial electron chain (Figs. 1–4) thus refraining the cell from entering the state of cell death cascade. In the study sets, cv MTU 1010, showed such marked changes in fluorescence, however, in cv Nonabokra the change in fluorescence is less prominent, which is a

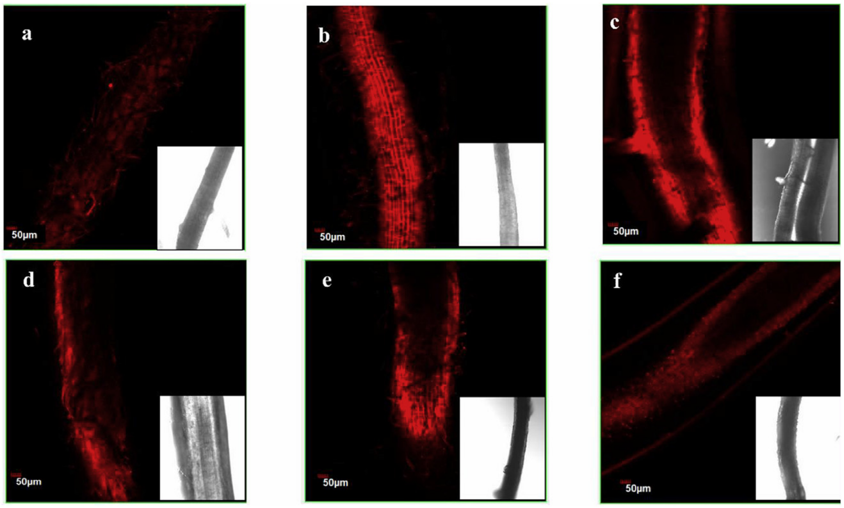

Fig. 2. Rhodamine 123 staining images showing change in mitochondrial membrane potential in roots of cv. Nonabokra (a: Control; b: 25 mM NaCl; c: 100 mM NaCl; d: 2 mM Silicon; e: 25 mM NaCl + 2 mM Si; f: 100 mM NaCl + 2 mM Si) *Photos in the inset are the bright-field images of the subject studied*

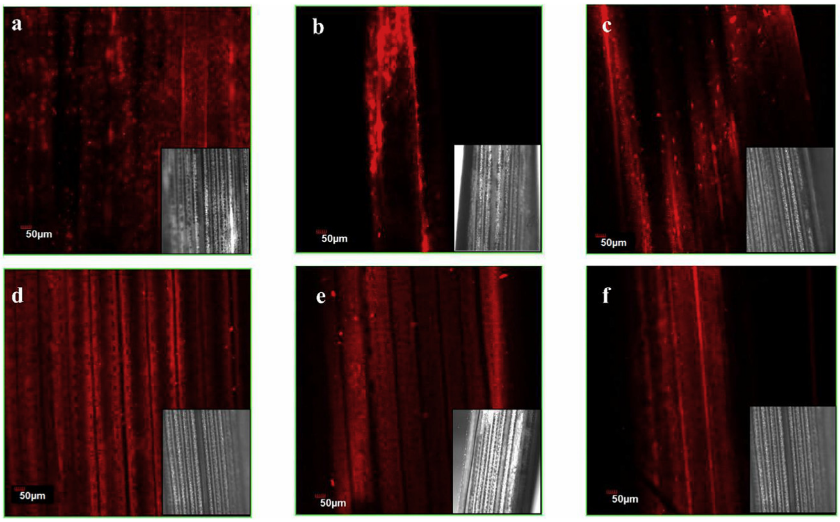

Fig. 3. Rhodamine 123 staining images showing change in mitochondrial membrane potential in leaves of cv. MTU 1010 (a: Control; b: 25 mM NaCl; c: 100 mM NaCl; d: 2 mM Silicon; e: 25 mM NaCl + 2 mM Si; f: 100 mM NaCl + 2 mM Si) *Photos in the inset are the bright-field images of the subject studied*

visual indication to the hypothesis that salinity resistant plants contain inherently higher level intracellular ROS pool to combat intruding abiotic changes.

3.2. *Effect of NaCl and Si on sodium, Potassium and Chloride contents*

Internal ion homeostasis in plants is perturbed by higher salt concentration in the external atmosphere (Parida and Das, 2005). The accumulation of Na^+ was significantly ($p \leq 0.05$) increased in roots and shoos of both the varieties under 25, 50, 100 mM salt stress conditions. In cv. MTU 1010, Na^+ contents increased by about 117%, 225%, 362% in root and 82%, 181%, 286% in shoot after three doses of NaCl treatment (treatments being 25, 50, 100 mM NaCl solution). On the other hand, in cv. Nonabokra Na^+ contents increased by about 57%, 90%, 131% in root and 45%, 86%, 123% in shoot under 25, 50 and 100 mM NaCl treatments. The level of Na^+ was highest in root and shoot of cv. MTU 1010 seedlings compared to cv. Nonabokra after salt imposition. The $NaCl^+$ Si treatment significantly ($p \leq 0.05$) decreased the Na^+ level in both the cultivars. In cv. MTU 1010 silicon supplementation resulted into about 49%, 134%, 283% and 12%, 108%, 206% increment of Na^+ contents in root and shoot respectively, over control. Likewise, in cv. Nonabokra the rate of increment of Na + levels were reduced to about 11%, 47%, 101% in root and 10%, 49%, 92% in shoot compared to control (Fig. 5).

Potassium contents in all the salt treatments were significantly ($p \leq 0.05$) reduced in both root and shoot tissues of both the cultivars compared to control. The magnitude of decrease was higher in cv. MTU 1010 than cv. Nonabokra, where K^+ contents declined by 27%, 42%, 54% in root and 22%, 40%, 49% in shoot respectively compared to control under 25,50 and 100 mM NaCl. In cv. Nonabokra said decrements were about 14%, 25%, 36% in root and 12%, 22%, 33% in shoot under similar doses of NaCl. It was noteworthy that the level of K^+ was higher in root and shoot of cv. Nonabokra after NaCl stress. However, addition of silicon with NaCl resulted into better retention in K^+ level compared to only salt treatments. In cv. MTU 1010 silicon nutrition registered a decrease of K^+ level by about 14%, 35%, 48% and 5%, 25%, 40% in root and shoot respectively over control. On the other side, under similar treatment, cv. Nonabokra seedlings showed only about 8%, 17%, 27% and 3%, 13%, 23% reduction in K^+ level in root and shoot respectively from control (Fig. 5).

Chloride contents in all the salt treatments were significantly ($p \leq 0.05$) increased in both root and shoot tissues of both the cultivars

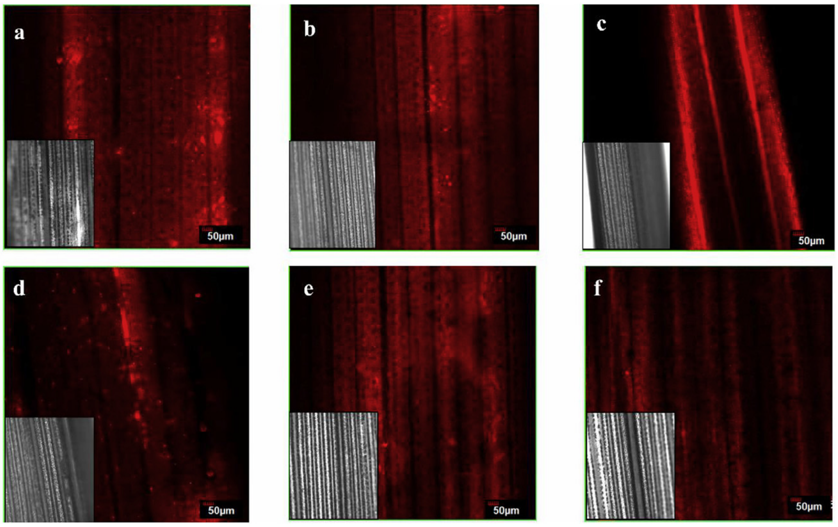

Fig. 4. Rhodamine 123 staining images showing change in mitochondrial membrane potential in leaves of cv. Nonabokra (a: Control; b: 25 mM NaCl; c: 100 mM NaCl; d: 2 mM Silicon; e: 25 mM NaCl + 2 mM Si; f: 100 mM NaCl + 2 mM Si) *Photos in the inset are the bright-field images of the subject studied*

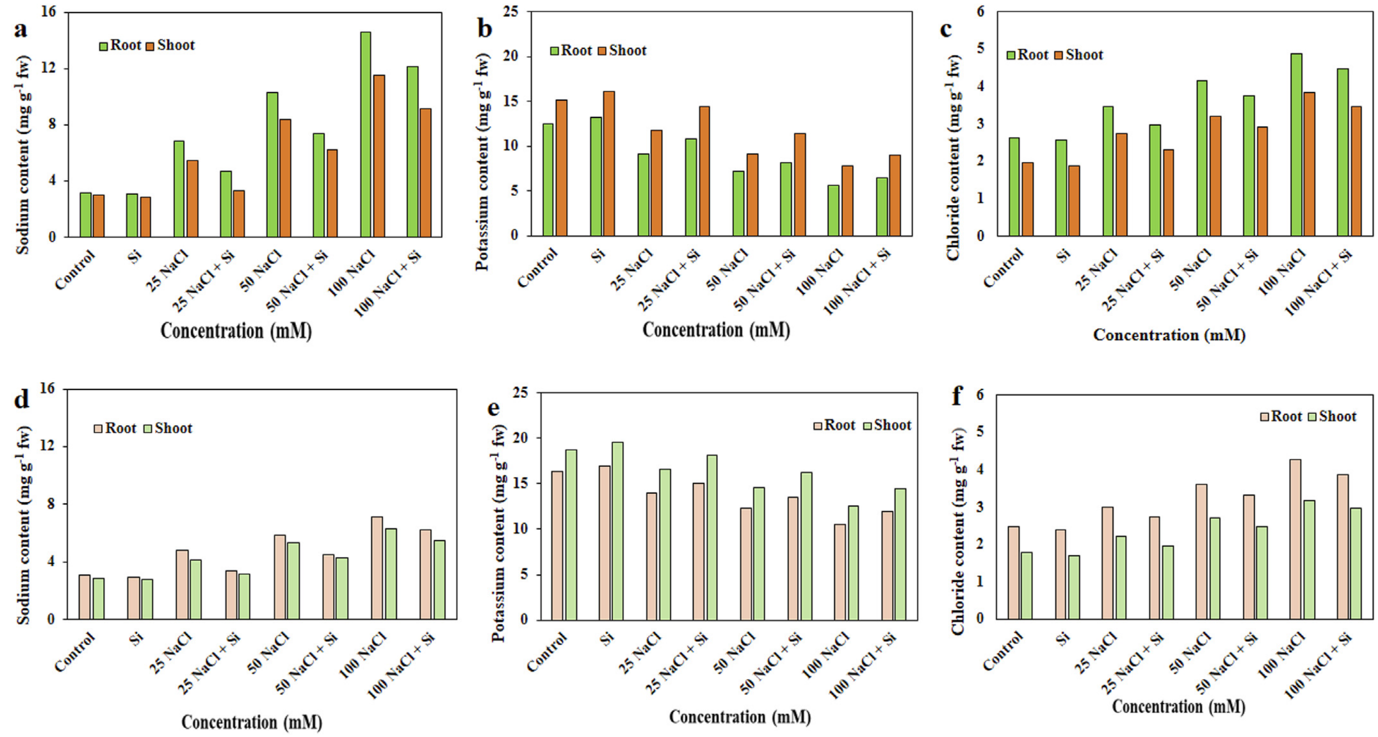

Fig. 5. Effect of Silicon (2 mM) and NaCl stress (25, 50 and 100 mM) on Sodium, Potassium and Chloride content (a–c: cv. MTU1010; d–f: cv. Nonabokra)

compared to control. The magnitude of increase was higher in cv. MTU 1010 than cv. Nonabokra, where Cl^- contents increased by about 33%, 58%, 87% in root and 41%, 64%, 96% in shoot respectively compared to control under 25,50 and 100 mM NaCl. In cv. Nonabokra said decrements were about 21%, 45%, 72% in root and 26%, 53%, 78% in shoot under similar doses of NaCl. The level of Cl^- was highest in root and shoot of cv. MTU 1010 seedlings compared to cv. Nonabokra after salt imposition. The $NaCl^+$ Si treatment significantly ($p \le 0.05$) decreased the Cl^- level in both the cultivars. In cv. MTU 1010 silicon nutrition registered an increase of Cl^- level by about 14%, 44%, 71% and 17%, 49%, 82% in root and shoot respectively over control. On the other side, under similar treatment, cv. Nonabokra seedlings showed only about 11%, 34%, 56% and 10%, 39%, 67% induction in Cl^- level in root and shoot respectively from control (Fig. 5).

Our study showed that cv. MTU 1010 accumulated higher amount of Na^+ and absorbed a lesser amount of K^+ in root and shoot than cv. Nonabokra. Observations indicated that plant growth reduction in the test cultivars could be correlated with the magnitude of Na^+ accumulation in shoot and root. Cultivar Nonabokra showed better salt tolerance by preventing higher accumulation of Na^+ ions in root and shoot and thereby reducing Na^+ toxicity in tissues. Salinity induced increment in cytosolic Na^+ concentrations was documented earlier (Carden et al., 2003; Kader and Lindberg, 2005). Like our findings, Na^+ ion was found to be accumulated in the roots of both salt tolerant and sensitive green bean seedlings under salt stress (Yasar et al., 2006). Na^+ content was found to be negatively correlated to relative growth of shoot and root. Moreover, the correlation between Na + content and growth suppression were noted regardless of cultivars. A well-known consequence of sodium toxicity in plants is suppression of uptake of K^+. K^+ activates various cytosolic enzymes and Na + cannot perform those particular functions (Tester and Davenport, 2003). K^+ plays a vital role in osmotic adjustment, especially in older leaves (Jones and Turner, 1980). Cv. Nonabokra showed the capability to evade excess Na^+ ion accumulation and absorb K^+ ion to keep low Na^+/K^+ ratios, which could be a good tolerance mechanism for salt stress (Munns and Tester, 2008). K^+ content in the shoot and root of both cultivars were significantly decreased in a dose-dependent manner. Salt tolerance capacity includes not only adaptation to influx of Na^+ but also retention of K^+, the uptake of which is negatively affected by higher concentration of Na^+. Na^+ toxicity affects the ionic balance by binding with the K^+ transporter protein or by some unknown ways related to intercellular processes (Sairam and Tyagi, 2004). Accumulation of Na^+ in root was larger compared to shoot in both the cultivars, which is in accord with the results of Akita and Cabuslay (1990). On the other hand, suppression of K^+ concentration due to Na^+ was also reported earlier in barley (Flowers and Hajibagheri, 2001; Carden et al., 2003). Decrease in K^+ contents probably resulted in a cessation of plant capacity for osmotic adjustment, which finally led to growth reduction. Chloride ions exhibited a similar distribution pattern to Na^+ ions at all doses of salinity. Both Na^+ and Cl^- ions are readily water soluble and are up taken by plants. They act as osmotica upto moderate concentrations but above they retard plant growth and photosynthesis. Higher concentration of these two ions hinder the uptake of various other ions like Mg^{2+}, Ca^{2+} resulting in development of deficiency syndromes of other ions (Parida et al., 2004). A positive correlation between accumulation of Na^+ ion in root and shoot and reduced dry weight was reported in *Vigna subterranean* (Ambede et al., 2012) and *Cucurbita pepo* (Kurum et al., 2013) due to increasing salinity. Results showed a significant ($p \le 0.01$) increase in Cl^- concentration in roots and shoots of both cultivars; but Cl^- concentration in cv. MTU 1010 was higher than cv. Nonabokra. Cl^- had a negative correlation with K^+ and the pattern of Cl^- distribution was similar to Na^+. Cl^- accumulation in root tissue disrupts membrane uptake, and these led to enhanced translocation of Cl^- to shoot (Yousif et al., 1972). Our results are in accordance with Lacerda et al. (2003) who observed that under salinity Cl^- ion accumulation in shoot of salt-sensitive genotype was higher than salt-tolerant one. Na^+ and Cl^- accumulation in plant parts of cv. MTU 1010 and cv. Nonabokra led to suppression of shoot and root growth. The present results showed a higher accumulation of Na^+ and Cl^- in cv. MTU 1010 under all doses of salinity than cv. Nonabokra which may classify the former as more salt-sensitive. So, the results showed differential responses of these two rice cultivars against Na^+

and Cl^- contents in root and shoot and also clearly indicated that salt-tolerance is related to Na+ and Cl^- ion accumulation in an inversely proportional manner. On the other hand, greater loss of K^+ cv. MTU 1010 under salt stress might cause reduction in osmotic turgidity (Lee et al., 2001).

Inhibition of influx of Na^+ ion from outside and increase in efflux of the same ion from cell interior to exterior has been regarded as the primary salt tolerance mechanism in plants that maintains lower cytosolic Na^+ level (Munns and Tester, 2008). On the other hand, maintaining an adequate intracellular level of K^+ is vital for carrying out cellular functions (Kronzucker et al., 2013). In our study, Si addition reduced the uptake of Na^+ and Cl-ions and also inhibit the translocation of these ions to the shoot and increased K^+ ion contents in the salt-stressed rice seedlings of both cultivars. Similarly, Si nutrition reduced Na^+ contents in roots and its transport to shoot in salt-sensitive and tolerant genotypes of *Cicer arietinum* subjected to salinity and also increased K^+ uptake (Garg and Bhandari, 2016). One possible mechanism might be due to silicon polymerization and deposition in the exodermis and endodermis, thereby preventing apoplastic Na^+ uptake (Gong et al., 2006). Interactive effects of Si with NaCl was found in salt-stressed wheat and sugarcane that causes reduction in Na + level and increase in K^+ contents (Tuna et al., 2008; Ashraf et al., 2010). Previously, H^+-ATPase in the root plasma membrane and tonoplast was seen to be stimulated by Si supply in salt-stressed barley (Liang et al., 2005, 2006). This stimulation has been suggested to drive the Na^+ efflux and K^+ influx in the cell (Liang et al., 2005, 2006). Silicon deposition in root and shoot allows salt binding sites that finally checks their transport from root to shoot (Ashraf et al., 2010). However, Si induced alteration in ion contents were more prominent in the cv. MTU 1010 seedlings compared to cv. Nonabokra.

Na^+/H^+ antiporters are responsible for sequestering Na^+ into the vacuole (Liang et al., 2018) in many plants. Overexpression of the *Arabidopsis* plasma antiporter SOS1 has been reported to provide salt tolerance (Ward et al., 2003). On a similar note, rice OsbZIP71 gene infers higher level of resistance to salinity and drought by perusing the ABA pathway of regulation (Liu et al., 2014).

3.3. Exogenous NaCl application adversely affects substrates of TCA cycle in situ and addition of Si mitigates the damage

NaCl treatment brought about a drastic reduction in the pyruvic acid contents in the seedlings of cv. MTU 1010, when compared to the untreated controls, level of pyruvate decreased by about 31%, 36%, and 50% in the roots and by about 23%, 30%, and 41% in the shoots at 25, 50, 100 mM NaCl treatments respectively. When silicon was added along with NaCl, reduction in pyruvate contents in all the treated seedlings was in comparison to control seedlings however these levels were higher than those seen on of salt treatment alone. In roots, pyruvate contents decreased by about 16%, 29%, and 38% and in shoot that by about 17%, 27%, and 39% when exposed to 25, 50, 100 mM NaCl supplemented with Si treatments respectively. On the other hand in cv. Nonabokra, pyruvate levels increased by about 10%, 23% and 27% in roots and in shoots by about 4%, 8%, and 22% when seedlings were treated with 25, 50, 100 mM NaCl respectively. The values recorded in 50 and 100 mM NaCl were statistically significant ($p \leq 0.05$). Pyruvic acid levels in cv. Nonabokra was further enhanced to about 18%, 29%, 37% in roots and 6%, 12%, 22% in shoots under 25, 50, 100 mM NaCl concentrations supplemented with silicon respectively (Figs. 6 and 7).

Like pyruvic acid, citric acid contents were also differentially altered in the two rice cultivars subsequent to NaCl treatment. Compared to the untreated control, in roots of cv. MTU 1010, showed a decrease in citrate contents by about 31%, 37%, and 46% while in shoots the decrease was recorded to be about 22%, 32%, and 43% in 25, 50, 100 mM NaCl treatment respectively, though the changes were not always statically significant. Contrarily, in cv. Nonabokra, citrate contents increased by about 12%, 40%, and 64% in root and in shoot by about 3%, 34%, and 54% in 25, 50, 100 mM NaCl respectively, and changes in 50 and 100 mM treatments were statistically significant ($p \leq 0.05$). Silicon application with different concentrations of NaCl altered the citric acid content in roots and shoots of both cultivars of rice seedlings. In cv. MTU 1010, reduction in citrate level was narrowed down to about 12%, 31%, 37% in roots and about 5%, 25%, 36% in shoots upon application of 25, 50 and 100 mM NaCl with added silicon. However, in cv. Nonabokra, when the seedlings were jointly treated with 2 mM Si and NaCl, citrate levels increased to about 16%, 44%, 76% in roots and about 6%, 41%, 65% in shoots respectively over control (Figs. 6 and 7).

Succinic acid contents were found to decrease significantly in cv. MTU 1010 seedlings treated with 25, 50 and 100 mM concentration of NaCl, which was about 13%, 26%, 35% lower in roots and about 8%, 21%, 33% lower in shoots over control sets. Co-application of silicon with each of the NaCl concentrations partially retrieved the plummeted succinic acid levels in cv. MTU 1010 which was about only 5%, 18%, 27% and about 4%, 12%, 26% lower in root and shoot respectively, compared to control. In cv. Nonabokra succinic acid contents increased linearly with increasing salt concentrations. In roots, the level of promotion of succinic acid were about 5%, 14% and 22% whereas in shoot the promotion was about 4%, 13% and 21% in 25,50 and 100 mM NaCl treatments respectively, over control; changes in 50 and 100 mM concentrations were statistically significant ($p \leq 0.05$). Silicon addition with NaCl of three doses resulted in further increment in succinate level in cv. Nonabokra seedlings which were about 8%, 16%, 25% and about 8%, 19%–23% in roots and shoots respectively, compared to control (Figs. 6 and 7).

Significant ($p \leq 0.05$) decrease in malic acid contents were registered in root of cv. MTU 1010 that was about 16%, 20% and 29%, whereas, significant increase was noted in root of cv. Nonabokra which was about 8%, 17% and 24% compared to control in 25,50 and 100 mM NaCl treatments respectively. On the other-hand shoot of cv. MTU 1010 seedlings recorded a decrease of about 4%, 15%, 19%, while shoot of cv. Nonabokra recorded an increase of about 3%, 11%, 19% in malic acid level under 25,50 and 100 mM NaCl treatments respectively, where 50 and 100 mM treatments were statistically significant ($p \leq 0.05$) in both cultivars. During application of NaCl with Si, the malate contents also decreased in all treated seedlings of cv. MTU 1010 compared to control but less than NaCl treatment alone. In roots, malate contents decreased by about 6%, 17%, and 24% while in shoot by about 1%, 7%, and 16% in 25, 50, 100 mM NaCl along with Si treatment respectively. Si application with NaCl further increased malate contents in cv. Nonabokra seedlings, which was about 8%, 17%, 24% and about 4%, 12%, 20% over control in root and shoot respectively under the above-mentioned doses (Figs. 6 and 7).

Organic acids are precursors of amino acid biosynthesis and play an important role in energy production and modulating plant adaptation to environmental stress (Lφpez Bucio et al., 2000). There was a marked difference in the levels of organic acids between cv. Nonabokra and MTU 1010 even in untreated controls, the organic acid contents in both roots and shoots were generally higher in case of salt resistant cv. Nonabokra than that of susceptible cv. MTU 1010; following salt-stress the perturbation in the organic acid contents became more pronounced. In cv. Nonabokra, the levels of several organic acids, viz., pyruvic, citric, succinic and malic increased following treatment with NaCl. This increment points out to an increased flow of carbon from the glycolytic pathway to TCA cycle that could possibly result in the increased production of NADH, $FADH_2$ and ATP in this cultivar. This increased production was probably utilized to support growth in cv. Nonabokra even on exposure to salinity stress. *Au contraire*, levels of all the organic acids studied decreased both in roots and shoots of seedlings belonging to cv. MTU 1010. It can be argued that since levels of organic acids represent the metabolic status of the plant under examination, higher organic acid contents under stressed conditions may reflect the ability of a plant to survive and maintain growth in as evident in cv.

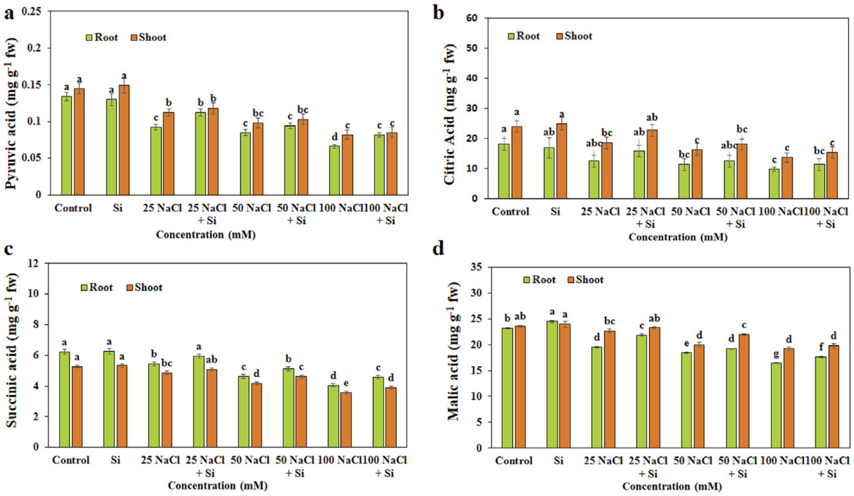

Fig. 6. Effect of Silicon (2 mM) and NaCl stress (25, 50 and 100 mM) on organic acids (a-pyruvic acid, b-citric acid, c-succinic acid, d-malic acid) in root and shoot of 21 days old rice seedlings of cv MTU 1010. Vertical bars represent the standard error (n = 3). Different letters at the same time point represent significant differences ($p \leq 0.05$) between the treatments.

Nonabokra when compared to cv. MTU 1010 growing under similar conditions of salinity. Our results are in accordance with Widodo et al., (2009) who reported increase in organic acid contents in the salt tolerant barley genotype Sahara, when the salt sensitive genotype Clipper opposite happened (organic acid contents decreased). Decrease in organic acid levels in cv. MTU 1010 is indicative of the inhibitory effect of salt stress on energy production that make this cultiver more vulnerable to saline environment, than cv. Nonabokra. Silicon application resulted in increase of organic acid contents in both the cultivars. Interestingly intermediate metabolites of TCA cycle, such as, citric acid, succinic acid and malic acid accumulated more in the silicon supplemented rice seedlings even in cv. MTU 1010, confirming that the repression of the TCA cycle by salt treatment was significantly relieved by silicon treatment. Optimum levels of malate synthesis is vital for growth of a plant as malate replenishes organic acids which in turn are converted into amino acids and other molecules leading to continuation of the TCA cycle (Yang et al., 2013). Thus, silicon enhanced the salt tolerance capacity of both the rice cultivars, by increasing energy production capacity and augmenting the levels of organic acids, intriguingly most prominently in the salt sensitive cv. MTU 1010. There is insufficient data regarding the effect of exogenous silicon on organic acid production under NaCl stress. Further work is thus needed to elucidate how Si modulates the organic acids metabolism in plants.

3.4. In situ analyses of TCA cycle enzymes reveal detrimental effect of exposure to NaCl which recovered upon Si application

Pyruvate dehydrogenase (PDH) (E.C. 1.2.4.1) is considered to be the primary target for many adverse environmental conditions. Pyruvate dehydrogenase, a component subunit of pyruvate dehydrogenase complex, is the largest known protein complex located in the mitochondrial matrix and has a molecular mass of about 9.5 KDa (Zhou et al., 2001). This enzyme complex plays important role in controlling the carbon entry in the TCA cycle (Randall et al., 1996). Suppression of pyruvate dehydrogenase activity thus causes insufficient carbon availability affecting lead energy requirements that ultimately result in cell damage and death (Samikkannu et al., 2003). In our study, we found that, PDH activity showed differential responses in the two rice cultivars under three doses of NaCl stress. In cv. MTU 1010, NaCl stress at 25, 50 and 100 mM concentrations resulted in significant ($p \leq 0.05$) decrease in PDH enzyme activity which were about 16%, 31%, 44% and about 14%, 29%, 40% lower than control in roots and shoots of seedlings respectively. Addition of silicate along with NaCl lowered this inhibition of enzyme activity in cv. MTU 1010 when compared to seedlings exposed to NaCl treatment alone. In roots, PDH activity decreased by about 8%, 24%, and 34%, while in shoots these were about 7%, 23%, and 33% in 25, 50, 100 mM NaCl and 2 mM silicate treatments respectively. In contrast, cv. Nonabokra in comparison to control exhibited enhancement of PDH activity; where the roots and shoots of seedlings displayed about 18%, 34%, 46% and about 13%, 29%, 41% elevation respectively under the above-mentioned doses of NaCl

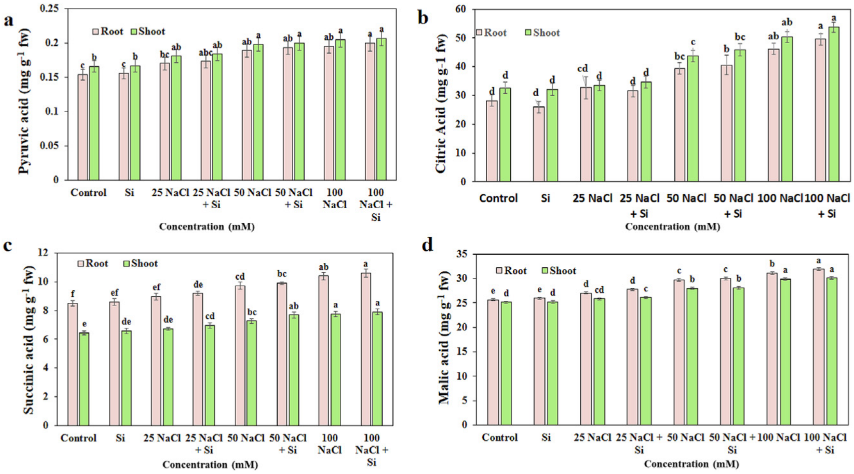

Fig. 7. Effect of Silicon (2 mM) and NaCl stress (25, 50 and 100 mM) on organic acids (a-pyruvic acid, b-citric acid, c-succinic acid, d-malic acid) in root and shoot of 21 days old rice seedlings of cv Nonabokra. Vertical bars represent the standard error (n = 3). Different letters at the same time point represent significant differences ($p \leq 0.05$) between the treatments.

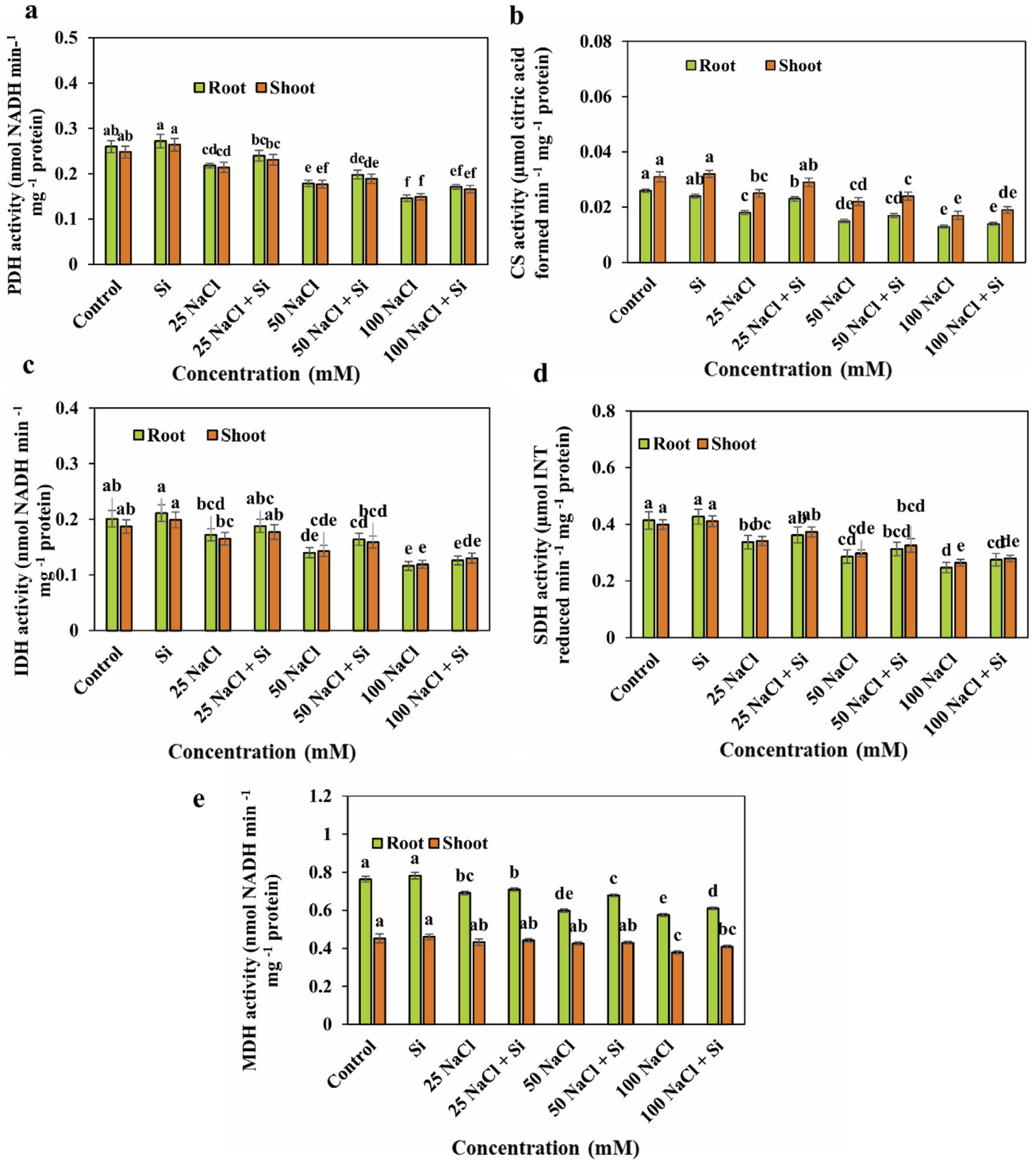

Fig. 8. Effect of Silicon (2 mM) and NaCl stress (25, 50 and 100 mM) on enzymes (a-pyruvate dehydrogenase, b-citrate sythase, c-isocitrate dehydrogenase, d-succinate dehydrogenase, malate dehydrogenase) in root and shoot of 21 days old rice seedlings of cv. MTU 1010. Vertical bars represent the standard error (n = 3). Different letters at the same time point represent significant differences ($p \leq 0.05$) between the treatments.

treatments; promotions in 50 and 100 mM NaCl treatments were statistically significant ($p \leq 0.05$). Co-application of silicate and NaCl resulted into further elevation of the said enzyme activity compared to the salt treated sets. In roots, enzyme activity increased by about 24%, 38%, and 53% while in shoots by about 22%, 34%, and 45% in 25, 50, 100 mM NaCl supplemented seedlings of cv. Nonabokra with 2 mM Si respectively (Figs. 8 and 9).

Salt induced inactivation of pyruvate dehydrogenase complex is said to occur due to the dissociation of the component subunits (Katz et al., 2007). Role of pyruvate dehydrogenase in *Arabidopsis* subjected to chilling or to drought stress (Taylor et al. 2005) as well as under salinity (Rapala-Kozik et al., 2012) was reported earlier. Increased PDH activity under salt stress in the roots and shoots of mungbean (*Vigna radiata* L. *Wilczek*) was reported earlier (Saha et al., 2012). A linear increase in PDH activity was noted in cv. Nonabokra, whereas a decrease in enzyme activity was seen in the cv. MTU 1010 under NaCl stress. Salt induced reduction in PDH activity in cv. MTU 1010 may have resulted in decreased carbon supply to the TCA cycle through pyruvate metabolism. Co-application of silicon and NaCl increased the said enzyme activity irrespective of cultivars, but the rate of increment was much more pronounced in cv. MTU 1010. Increase in PDH activity upon silicon addition helps to feed carbon to the TCA cycle resulting in augmented production of ATP under saline condition. A slight increase in the enzyme activity was noted in the cv. Nonabokra under joint application of NaCl and Si. This may be because under saline conditions Nonabokra had already upregulated PDH activity feeding its energy demand (Figs. 8 and 9).

Citrate synthase (CS) (E.C. 2.3.3.1) is the enzyme ubiquitous to all living cells that catalyzes the first condensation step of the TCA cycle. It acts as a quantitative marker for healthy mitochondrion and is encoded

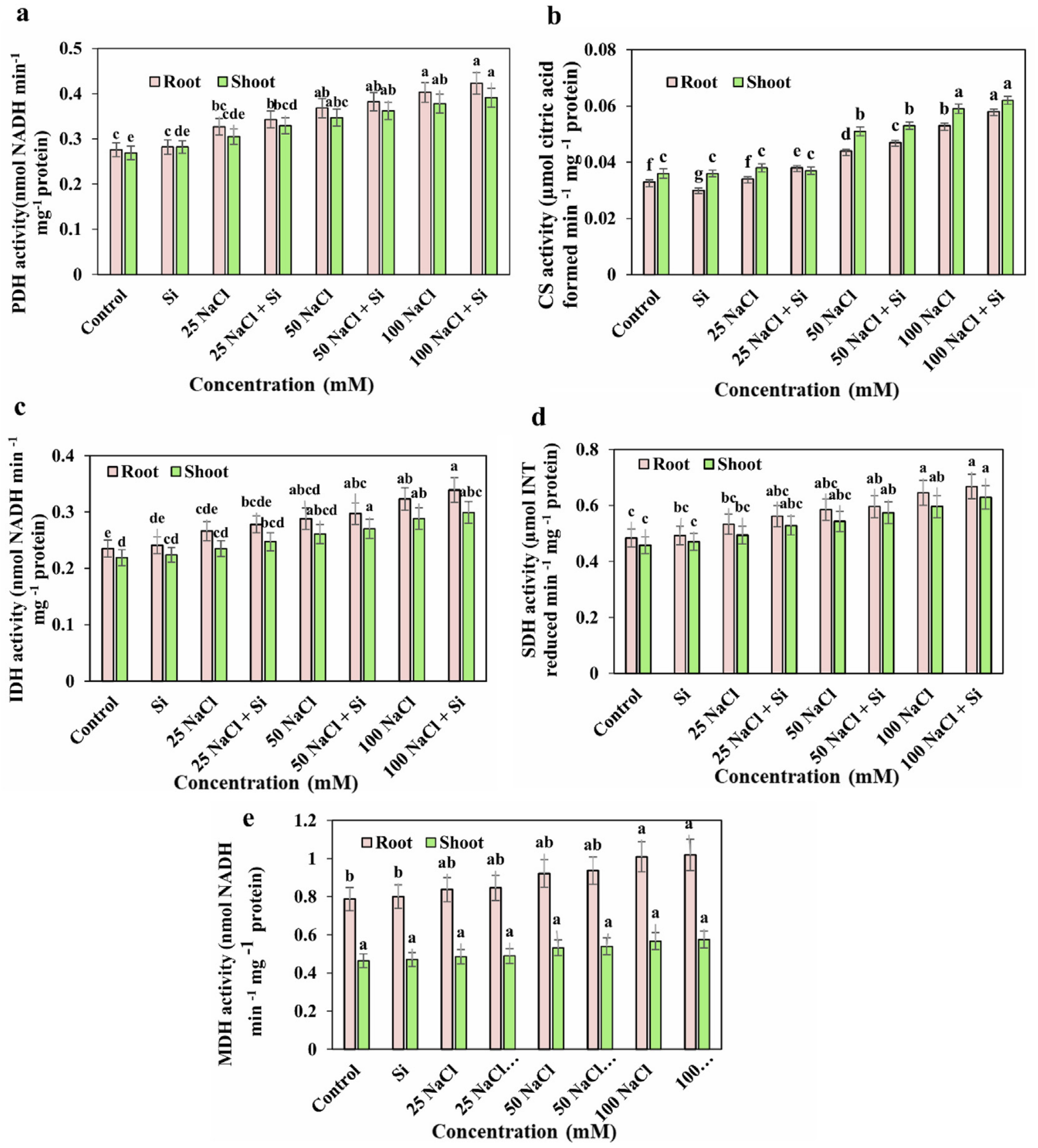

Fig. 9. Effect of Silicon (2 mM) and NaCl stress (25, 50 and 100 mM) on enzymes (a-pyruvate dehydrogenase, b-citrate sythase, c-isocitrate dehydrogenase, d-succinate dehydrogenase, malate dehydrogenase) in root and shoot of 21 days old rice seedlings of cv. Nonabokra. Vertical bars represent the standard error (n = 3). Different letters at the same time point represent significant differences ($p \leq 0.05$) between the treatments.

by the nuclear DNA (Sienkiewicz-Porzucek et al., 2008). Roots of cv. MTU 1010 recorded about 30%, 41% and 48% decline in citrate synthase (CS) activity while the shoots registered a decline of about 19%, 29% and 45% over water control under 25 mM, 50 mM and 100 mM NaCl treatments respectively. The decline in enzyme activity both in root and shoot in all the doses were, however, statistically significant ($p \leq 0.05$). On the contrary, roots of cv. Nonabokra reported about 3%, 32% and 58% elevation in CS activity while the shoot registered an upregulation of about 3%, 38% and 59% over water control under similar doses of NaCl treatments respectively, however only changes under 50 and 100 mM NaCl treatments were statistically significant. Addition of 2 mM silicate along with NaCl resulted in partial retrieval of CS enzyme activity in cv. MTU 1010. In roots of seedlings, CS activity decreased by about 11%, 33%, and 44% while in shoots that were about 6%, 23%, and 39% in 25, 50, 100 mM NaCl and 2 mM silicon treatments, respectively compared to control. On the other hand, co-application of silicate along with above mentioned doses NaCl in cv. Nonabokra resulted in further elevation of enzyme activity by about 15%, 41%, 74% in roots and about 5%, 43%, 68% in shoot respectively in comparison to water control (Figs. 8 and 9).

Citrate synthase activity was found to be differentially regulated in two cultivars under consideration under similar concentrations of NaCl treatment. Increment in enzyme activity was noted in cv. Nonabokra whereas a gradual reduction was observed in cv. MTU 1010 under salt stress. In cv. Nonabokra elevated citrate synthase activity could be correlated with increased citrate production, whereas in cv. MTU 1010 reduction in citrate synthase activity resulted in decreased citrate production. Silicon application increased the said enzyme activity irrespective of cultivars. On similar note, Citrate synthase overexpression in phosphorous deficient medium helped to enhance growth in *Arabidopsis* by increasing excretion of citrate from roots to increase availability of inorganic phosphate (Koyama et al., 2000); hence plants follow similar mechanism to cope most kinds of abiotic stresses.

Isocitrate dehydrogenase (IDH) (E.C. 1.1.1.41) activity helps in maintaining the 2-oxoglutarate level thereby modulate assimilation of nitrogen (Lemaitre et al., 2007; Foyer et al., 2011). IDH also has an important role in regulating mitochondrial redox balance and confers protection against oxidative damage caused by radiation (Jo et al., 2002). The roots and shoots of seedlings of cv. MTU 1010 experienced about 14%, 30%, 42% and about 12%, 24%, 36% decline in isocitrate dehydrogenase (IDH) activity under 25 mM, 50 mM and 100 mM treatments of NaCl respectively over water control, where changes in 50 and 100 mM concentrations were statistically significant ($p \leq 0.05$) both in roots and shoots. Addition of 2 mM silicate in three doses of NaCl resulted in some revival of the enzyme activity in the said cultivar. In roots, activity of the said enzyme was decreased by about 6%, 18%, and 37% while in shoots that were about 5%, 15%, and 30% in 25, 50, 100 mM NaCl along with silicate treatments respectively. In contrast, cv. Nonabokra in comparison to control sets exhibited promotion of IDH activity where the root and shoot displayed about 13%, 23%, 37% and about 7%, 20%, 32% elevation respectively under three doses of NaCl treatment, where up-regulation at 50 and 100 mM concentrations in root and only 100 mM NaCl in shoot were statistically significant ($p \leq 0.05$). On the other hand, co-application of silicate with NaCl resulted into further elevation of enzyme activity which was about 19%, 26%, 44% and about 13%, 23%, 37% higher in roots and shoots respectively under the above-mentioned doses in comparison to water control.

Although increase in amino acid and nitrogen containing compounds under salinity is very common in different plant species (Jorge et al., 2016), marked variation in IDH activity was noted in response to salinity. Proteomic analysis showed increase in IDH abundance in *Solanum lycopersicum* roots (Gong et al., 2014) whereas decreased in *Lupine luteus* embryos (Wojtyla et al., 2013) and *Arabidopsis thaliana* roots (Jiang et al., 2007). From these findings, it is clear that the regulation of IDH activity under salt stress is dependent on the particular plant species. Similarly, in the present study a remarkable decrease in IDH activity was observed in cv. MTU 1010 whereas in cv. Nonabokra IDH activity increased in a dose dependent manner. Decreased IDH activity in cv. MTU 1010 may cause a depression of the TCA cycle under salt stress. Application of Si along with NaCl increased the IDH activity irrespective of cultivars tested, leading to an increased production of isocitrate, that can be isomerized to citrate thus coinciding with the increased production of citrate in rice seedlings.

Succinate dehydrogenase (SDH) (EC 1.3.5.1), plays an important role in mitochondrial metabolism and participates in both TCA cycle and electron transport chain. Succinate dehydrogenase (SDH) activity exhibited differential response in the two rice cultivars on imposition of salt stress. Compared to control, cv. MTU 1010 showed significant ($p \leq 0.05$) decrease in SDH activity that were about 19%, 31%, 40% in root, and about 15%, 26%, 33% in shoot in 25, 50 and 100 mM NaCl treatments, respectively. On the contrary, in cv. Nonabokra increased SDH activity increase by about 10%, 21%, 33% in root and 8%, 19%, 30% in shoot under 25, 50 and 100 mM NaCl concentrations respectively, where only changes in 100 mM NaCl treatment was statistically significant ($p \leq 0.05$) both in roots and shoots. Addition of silicate with NaCl partially relieved the downregulation of enzyme activity in cv. MTU 1010 compared to salt treatment alone. In the roots, SDH activity decreased by about 13%, 24% and 34% and in shoot decreased by about 6%,18%, and 30% in 25, 50, 100 mM NaCl plus silicon treatments respectively. On the other hand, the said enzyme activity in cv. Nonabokra was further escalated which was about 16%, 23%, 38% higher in root and about 15%, 25%,37% higher in shoot under 25, 50, 100 mM NaCl concentrations supplemented with silicon respectively.

In the present investigation, SDH activity declined in NaCl treated seedlings of the cv. MTU 1010, whereas in cv. Nonabokra it increased (Figs. 8 and 9). However, supplementation of silicate along with NaCl led to an increase in SDH activity in both the varieties. In a previous study, the activities of IDH, MDH and SDH decreased in *Lotus japonicas* under waterlogging condition (Rocha et al., 2010), which has a systemic similarity to that of salinity stress *i. e*, both causes shutdown of osmotic mechanism (Munns, 2002).

Mitochondrial malate dehydrogenase (MDH) (E.C. 1.1.1.37) is responsible for reversible transformation of malate into oxaloacetate. It leaks out into the cytoplasm that acts as an important marker for cellular damage. In the NaCl treated seedlings of cv. MTU 1010, activity of MDH was decreased by about 9%, 22%, 25% in root and 4%, 6%, 16% in shoot in 25, 50 and 100 mM NaCl treatments respectively over water control. All the values were statistically significant for root while in shoot only 100 mM NaCl was statistically significant ($p \leq 0.05$). Addition of 2 mM silicate along with NaCl resulted in partial revival of the enzyme activity in the said cultivar. In root, MDH activity decreased by about 7%, 11%, and 20% while in shoot that were about 2%, 5%, and 10% in 25, 50, 100 mM NaCl along with 2 mM silicon treatments, respectively compared to control. In contrast, seedlings of cv. Nonabokra in comparison to control exhibited upregulation of MDH activity where the root and shoot displayed about 6%, 17%, 28% and about 5%, 15%, 22% elevation respectively on three doses of NaCl treatment respectively. On the other hand, co-application of silicate along with said concentration of NaCl resulted into further elevation of enzyme activity by about 7%, 19%, 29% in root and about 5%, 16%, 24% in shoot in comparison to water control (Figs. 8 and 9).

Recent experiments on TCA cycle mutants have demonstrated the role of TCA cycle enzymes in anaplerotic delivery of organic acids for various processes in plants like, amino acid metabolism, photorespiration, photosynthetic performance and even in regulating stomatal physiology. Antisense mutation of aconitase and malate dehydrogenase (MDH) in tomato resulted in increased photosynthetic carbon assimilation as well as enhanced ascorbate levels (Nunes-Nesi et al., 2005).

Deficiency of the mitochondrial MDH expression in growing tomato plants resulted into dwarf phenotype (Nunes-Nesi et al., 2008). In the present study, a marked decrease in the activity of mitochondrial MDH was observed in cv. MTU 1010 when exposed to NaCl, whereas the said enzyme activity increased in cv. Nonabokra under salinization. In the cytosol MDH catalyzes formation of malate from OAA. High levels of MDH activity resulted in better functioning of the TCA cycle because OAA which is produced in the reaction reacts with another molecule of acetyl CoA to start the next turn of TCA cycle. Thus, optimum level of MDH activity is vital for all growth processes (Salisbury and Ross, 1986). Increased MDH activity in leaves of *Halimione portulacoides* under salt stress was reported earlier (Kalir et al., 1984). Mitochondrial MDH activity decreased at lower salinity level in seedlings of cv. MTU 1010, whereas in cv. Nonabokra, even a higher salinity level of 100 mM NaCl caused an increase in MDH activity. This result suggests differential behavior of MDH activity in the two rice cultivars differing in their salt tolerance capacity. It is clear that salt tolerance ability in rice under salinity is correlated primarily with increased MDH activity. Co-application of Si with NaCl increased enzyme activity and helped to maintain higher activity of TCA cycle leading to restoration of growth in both the cultivars. Enhanced activities of isocitrate dehydrogenase and malate dehydrogenase have been attributed to increased salt tolerance in rice seedlings (Ritambhara and Dubey, 1995). According to these authors, salt induced inhibition of pentose phosphate pathway might be overcame by production of NADPH via isocitrate dehydrogenase activity. The up-regulation of malate dehydrogenase in the shoot provides evidence for its role in regulating the redox poise which is crucial for mediating photosynthesis and respiration in the light (Tomaz et al., 2010).

Acetyl CoA, starting block of the TCA cycle, is a product of the Pyruvate Dehydrogenase Complex that initiates Krebs Cycle, thus linking glycolysis and TCA cycle. Methylglyoxal is a toxic intermediate in the glycolytic pathway, accumulation of which leads to various developmental errors through the degradation of various essential amino acids, changing binding pattern of DNA and breaking up the antioxidant defence mechanism of the plant (Yadav et al., 2005). Glyoxalase system is responsible for producing GSH by isomerisation and hydrolysis into GSH. Reduced glutathione-GSH, inadvertently reduces methylglyoxal content, and the authors too have already reported that AsA-GSH pathway remains compromised during salinity stress (Das et al., 2018). In fact, great alteration in the proteome level under salt responsive rice varieties were reported, the highly affected pathways beings-photosynthesis, ATP generation, glycolysis and defence proteins (Sobhanian et al., 2011). Glyoxalase system and triosephosphate isomerase have been reported to be upregulated in salt sensitive rice (Sobhanian et al., 2010). It is a general deduction that alternative pathways using the similar substrates are responsible for salt resistive nature in different plants, upregulated glycolysis mechanism is used to bypass TCA cycle and strengthen the ATP biosynthetic pathway as stress response helps cultivers like Nonabokra to be comparatively unperturbed during high level of salinity stress response. Calmodulin-dependent glyoxalase I and defence proteins like osmotins in high amount provide a certain degree of advantage against salinity related disorders. Higher levels of antioxidant enzymes and sugar alcohols helps resistant plants in bypassing the stressed condition. Several glycolytic enzymes like that of triosephosphate isomerase and enolase, vital for stress remediation, are upregulated in rice during NaCl stress. According to reports, in rice, Cytochrome c Oxidase is also upregulated, atleast one subunit of this multimeric protein were induced by salinity in order for remediation helping in energy generation in respiratory cycle. During salinity stress glycolysis is further activated in sensitive *Vigna* while normal phosphorylation mechanisms were compromised (De Melo et al., 1994; Minhas and Grover, 1999). High salinity often leads to O_2 depletion, so this pathway of nominal energy production becomes essential for survival (Minhas and Grover, 1999). Glycolytic mechanism is sufficient to generate necessary amino acids (Plaxton, 1996). Similarly, pentose phosphate pathway (PPP) too gets upregulated during salt stress that also converts glucose in the cytosol of cells. Generated NADPH can be to produces precursors for the synthesis of ribose and deoxyribose sugars during synthesis of RNA and DNA and phenolic compounds. (Misra and Dwivedi, 2004; Krishnaraj and Thorpe, 1996).

4. Conclusion

From the present study, it was noted that salt stress altered the enzymatic machinery in mitochondria (Fig. 10). Differential activities of the TCA cycle enzymes along with organic acid accumulation under similar doses of NaCl in two rice seedlings can be attributed to difference in salt sensitivity of the cultivars that finally resulted into altered energy metabolism. The parameters studied in our experiments to understand the response of TCA cycle enzymes and intermediates under NaCl stress has revealed the salt tolerant nature of the cultivar Nonabokra. Redox system operated by the mitochondria of cv. Nonabokra was efficient in the development of resistance against NaCl induced oxidative stress. The activities of PDH, CS, IDH, SDH and MDH as well as pyruvate, citrate, succinate and malate contents were enhanced in cv. Nonabokra under all doses of NaCl. This may be attributed to the fact that bypass mechanisms like that of glycolysis and pentose phosphate pathways are enhanced in cv. Nonabokra to provide ATP required to maintain optimum growth under saline environment. On the other hand, decrease in the said enzymes activities as well as organic acids level indicated the impairment of vital energy yielding and phosphorylation process under salinity that made MTU 1010 a salt sensitive rice cultivar because of inherent programing of energy producing mechanisms and buildup of toxic byproducts like methylglyoxal. According to literature, no previous reports exists on the influence of exogenous silicon on mitochondrial enzymes, organic acids production and respiration in rice subjected to salinity. Based on our results we conclude that exogenous silicon increased carbon fluxes into TCA cycle, enhanced the activity of TCA cycle enzymes and decreased ROS production in mitochondria resulting in increased tolerance of the rice seedlings under salinity. The effect of silicon was much more pronounced in salt sensitive rice cv. MTU 1010 than the tolerant cv. Nonabokra. Although a short time effect of NaCl on rice plants in laboratory condition may not elucidate the exact scenario that the plants face in field condition, but this study on two contrasting rice cultivars helps to decipher the role of mitochondria in salt tolerance.

Author contribution

The authors have made equal, substantial, direct contribution to the work and approved of publication and they would like to declare that there is no conflict of interest. IM, PD and PS designed and carried out the experiments, prepared the manuscript and did data analyses. MB and AKB checked and rectified the manuscript, made vital contribution and analysed data as well.

Conflicts of interest

The authors declare that there is no conflict of interest. The research was done in the absence of any commercial or financial relationship that could pose as a potential conflict of interest.

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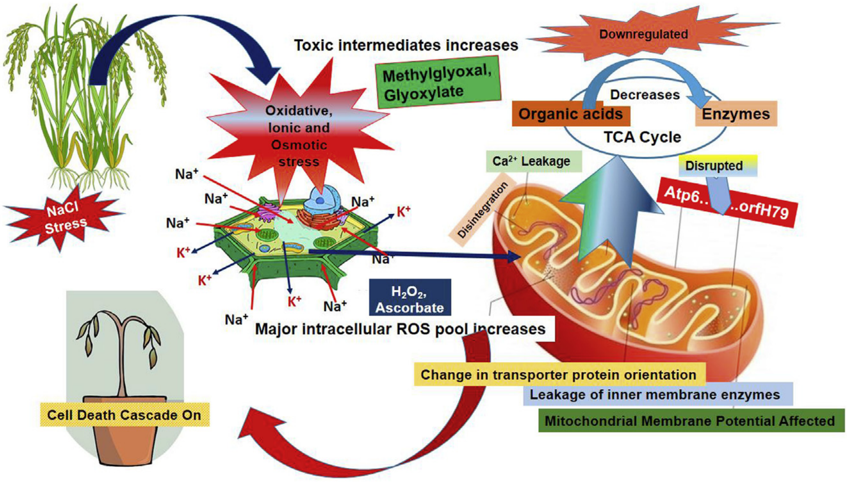

Fig. 10. Schematic representation of NaCl induced alteration of TCA Cycle in rice seedlings.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.12.026.

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