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Calcium-alleviated salt tolerance in indica rice (*Oryza sativa* L. spp. *indica*): Physiological and morphological changes

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Abstract

Calcium (Ca) is a signaling molecule that plays an active role in regulating various mechanisms involved in recognition and response to abiotic stresses in plants. However, not much has been done to evaluate its role in regulating physiological and biochemical process in response to salt-induced stress. Two rice genotypes, Pokkali salt tolerant and IR29 salt susceptible, grown on liquid Murashige and Skoog medium (MS) supplied by 1.98 mM $CaCl_2$ (control) were compared to 2 (3.96 mM), 4 (7.92 mM) and 8 (15.84 mM) folds exogenous $CaCl_2$ pretreatment subsequently exposed to 200 mM NaCl salt stress. Thus, the present investigation evaluated the potential of exogenous calcium chloride ($CaCl_2$) supply in improving the growth performance and photosynthetic ability in salt stressed rice. In IR29 salt susceptible rice, leaf area of salt-stressed seedling was significantly recovered by exogenous application of 7.92 mM $CaCl_2$, which was greater by 1.38-folds over that in 1.98 mM $CaCl_2$ application. Exogenous $CaCl_2$ (7.92 mM) enhanced proline accumulation in both Pokkali (3.26 μmol g^{-1} FW) and IR29 (4.37 μmol g^{-1} FW) genotypes, and reduced relative electrolyte leakage thereby indicating its positive role in membrane stability. Treatment of 7.92 mM $CaCl_2$ significantly enhanced the photosynthetic abilities, including maximum quantum yield of PSII (F_v/F_m), photon yield of PSII (Φ_{PSII}), photochemical quenching (qP) and net photosynthetic rate (P_n), in two genotypes of salt-stressed rice seedlings, especially in salt susceptible IR29 genotypes. The study concludes that an exogenous application of 7.92mM $CaCl_2$ significantly enhanced the photosynthetic abilities and overall growth performances in the photoautotrophic growth of salt-stressed rice seedlings. Exogenous calcium in the culture media may absorb by root tissues, transfer to whole plant and function as salt defense mechanisms including calcium signaling in the abscisic acid (ABA) regulation system and calcium sensing in stomatal closure when plant subjected to salt stress.

Keywords: calcium, salinity alleviation, leaf area, photosynthetic abilities, salt stress.
Abbreviations: $CaCl_2$_calcium chloride; Chl_a_chlorophyll a; Chl_b_chlorophyll b; F_v/F_m_maximum quantum yield of PSII; MS_Murashige and Skoog; P_n_net photosynthetic rate; qP_photochemical quenching; Φ_{PSII}_photon yield of PSII; PPFD_photosynthetic photon flux density; REL_relative electrolyte leakage; C_{x+c}_total carotenoid.

Introduction

Rice (*Oryza sativa*) is a staple food of >2 billion people in Asia, Africa and Latin America (Khush, 2005). However, the crop productivity has been greatly affected by increasing salt concentration in the soil. It has been estimated that salinity affects ~7% of the world's land area and is responsible for >35% decrease in agricultural productivity world over (Tanji, 2002). Accordingly, the efforts are being made to screen crop cultivars for their tolerance towards salt. It has been reported that salinity impairs the metabolism in plant tissue that is manifested in terms of altered growth performance and physiological process to provide tolerance against salinity induced stress (Sairam and Tyagi, 2004; Mahajan and Tuteja, 2005). Plants have developed a well-organized mechanism of biochemical and physiological processes to protect themselves from the salinity-induced damages. These include antioxidant responses, ionic homoeostasis, and/or osmoregulation (Hasegawa et al., 2000; Parida and Das, 2005). Calcium (Ca) is a signaling molecule that plays a significant role in mediating mechanisms involved in recognition and response to abiotic stresses in plants. Under stress, plants either accumulate or release intracellular cytosolic Ca, which acts as a signal and regulate a range of physiological processes to adjust to these stresses (Kader et al., 2007; Kader and Lindberg, 2008; Kader and Lindberg, 2010). Additionally, Ca has been reported to restrict the entry of Na^+ into the plant cells (Kader and Lindberg, 2008; Hussain et al., 2010). In salt sensitive Jaya cultivar of rice, Ca application (4 mM Ca^{2+}) has been reported to improve the survival percentage and limit the cellular levels of Na^+ (Anil et al., 2005). Earlier, studies have reported that exogenous application of $CaCl_2$ alleviates stress in greengram (Manivannan et al., 2007), Jerusalem artichoke (Xue et al., 2008), soybean (Arshi et al., 2010), *Atriplex halimus* subsp. *schweinfurthii* (Nedjimi and Daoud, 2009), *Rumex* sp. (Chen et al., 2007) *Withania somnifera* (Jaleel and Azooz, 2009) and linseed (Khan et al., 2010). In addition, the gypsum ($CaSO_4$ $2H_2O$) has been exogenously applied to the saline soil for improving the soil chemical, leading to enhance the crop productivity (Cha-um et al., 2011). However, the studies

Table 1. Shoot height (SH), root length (RL), leaf area (LA), fresh weight (FW) and dry weight (DW) per seedling of two rice cultivars treated by various concentrations of $CaCl_2$ for 7 days and subsequently transferred to 200 mM NaCl for 14 days. Mean error values represent by ±SE.

Rice cultivar	$CaCl_2$ (mM)	SH (cm)	RL (cm)	LA (mm^2)	FW (mg)	DW (mg)
Pokkali	1.98	26.9±2.2a	5.9±0.8a	438±12.7ab	227±21.6a	39±3.8a
	3.96	27.2±1.5a	6.2±1.2a	494±31.8a	230±41.6a	40±5.3a
	7.92	28.0±2.2a	6.3±1.3a	542±18.4a	248±20.9a	45±3.7a
	15.84	27.9±1.0a	6.1±1.1a	515±32.7a	244±28.6a	43±4.2a
IR29	1.98	18.5±0.7b	5.0±0.9b	338±47.5b	140±7.5b	28±1.7b
	3.96	18.5±0.8b	5.2±0.7b	422±22.5ab	156±11.5b	29±4.9b
	7.92	21.1±0.5b	5.3±0.1b	467±11.1a	166±7.0b	37±1.6ab
	15.84	19.2±0.9b	5.3±0.6b	463±11.2a	164±10.5b	33±2.5ab
Significant level						
Rice		**	**	**	**	*
$CaCl_2$		NS	NS	*	NS	NS
Rice × $CaCl_2$		*	*	*	*	NS

Means within a row followed by different letters in each column are significantly different at $p \leq 0.01$ (**) and $p \leq 0.05$ (*) by Duncan's New Multiple Range Test (DMRT). ^{NS} represented non-significant.

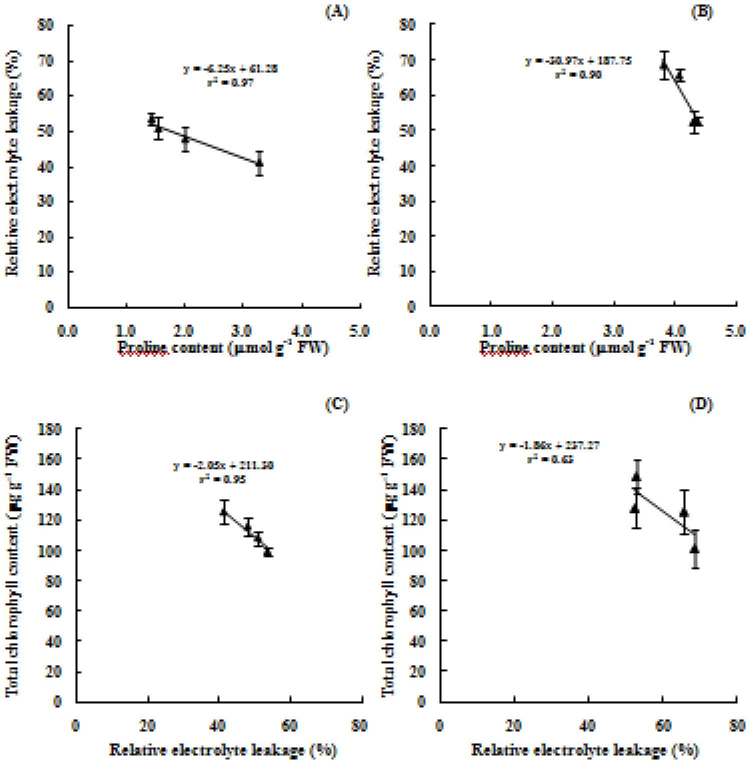

Fig 1. Relationship between proline content and relative electrolyte leakage in Pokkali (A) and IR29 (B) as well as relative electrolyte leakage and total chlorophyll content in Pokkali (C) and IR29 (D) treated by various concentrations of $CaCl_2$ for 7 days and subsequently transferred to 200 mM NaCl for 14 days. Error bars represent by ±SE.

investigating the role of Ca in regulating the physiological and biochemical process in response to salinity induced stress in rice are largely lacking. With this background, a study has been envisaged to understand the role of Ca in mediating salt stress-induced response in rice. Two cultivars of rice, including IR 29 (salt sensitive) and Pokkali (salt-tolerance), were selected as plant materials. The changes in terms of growth performance, photosynthetic ability (maximum quantum yield of PSII, F_v/F_m; photon yield of PSII, Φ_{PSII}; photochemical quenching, qP; and net photosynthetic rate, P_n), loss of electrolytes and proline accumulation were measured.

Results and discussion

Growth performance, proline accumulation and relative electrolyte leakage

Growth characters including shoot height, root length, leaf area, fresh weight and dry weight in Ca exposed Pokkali genotypes were unaffected in the salt tolerant ability as well as in IR29 rice cultivar, except leaf area in 1.98 mM $CaCl_2$ was significantly reduced when compared to other treatments. Overall, the growth performance in IR29-salt susceptible genotype was significantly dropped when

Table 2. Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (TC), total carotenoids (C_{x+c}) and proline contents of two rice cultivars treated by various concentrations of $CaCl_2$ for 7 days and subsequently transferred to 200 mM NaCl for 14 days. Mean error values represent by ±SE.

Rice cultivar	$CaCl_2$ (mM)	Chl_a (µg g^{-1}FW)	Chl_b (µg g^{-1}FW)	TC (µg g^{-1}FW)	C_{x+c} (µg g^{-1}FW)	Proline (µmol g^{-1} FW)
Pokkali	1.98	56.4±1.1c	42.6±2.0ab	99.0±3.0c	1.3±0.3b	1.42±0.16b
	3.96	58.3±1.3c	49.7±7.3ab	108.0±7.6bc	1.4±0.1b	1.53±0.03b
	7.92	59.8±0.9c	65.5±5.3a	125.3±4.5b	1.8±0.2b	3.26±0.24a
	15.84	57.6±1.7c	58.2±4.8ab	115.8±6.1c	1.5±0.1b	1.99±0.15b
IR29	1.98	64.1±6.9bc	37.0±5.7b	101.1±12.6c	3.2±0.5ab	3.80±0.28a
	3.96	79.3±6.7ab	46.4±9.2ab	125.7±14.8b	3.4±0.5ab	4.06±0.36a
	7.92	86.7±5.9a	62.4±7.3a	149.1±12.9a	6.3±0.4a	4.37±0.28a
	15.84	80.8±6.4ab	47.2±5.2ab	128.0±11.3b	5.0±0.6a	4.29±0.37a
Significant level						
Rice		**	*	*	**	**
$CaCl_2$		NS	NS	NS	NS	**
Rice × $CaCl_2$		*	NS	NS	*	*

Means within a row followed by different letters in each column are significantly different at $p \leq 0.01$ (**) and $p \leq 0.05$ (*) by Duncan's New Multiple Range Test (DMRT). ^{NS} represented non-significant.

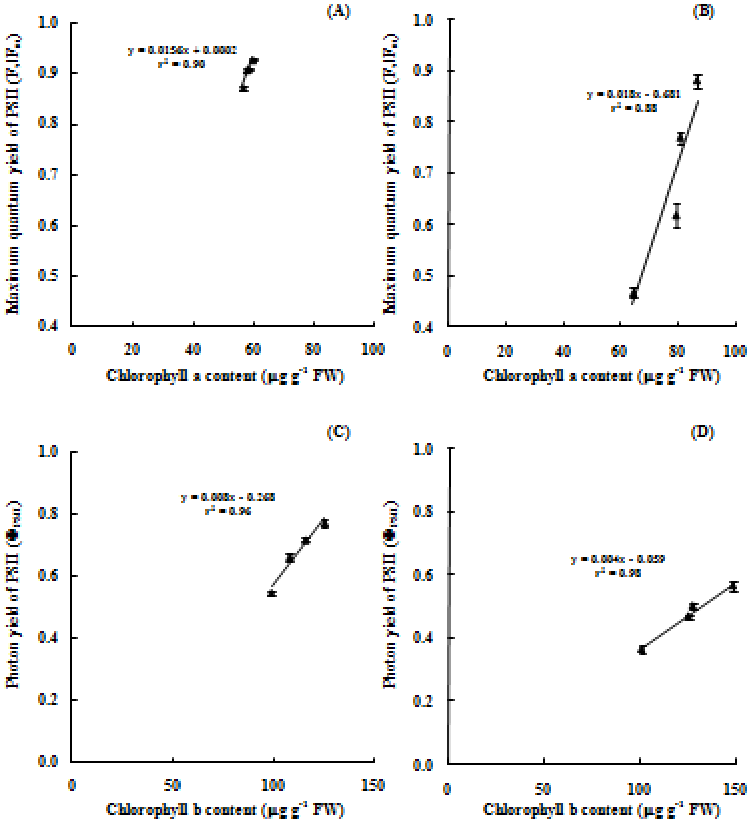

Fig 2. Relationship between chlorophyll a content and maximum quantum yield of PSII in Pokkali (A) and IR29 (B) as well as chlorophyll b content and photon yield of PSII in Pokkali (C) and IR29 (D) treated by various concentrations of $CaCl_2$ for 7 days and subsequently transferred to 200 mM NaCl for 14 days. Error bars represent by ±SE.

compared to salt tolerance Pokkali genotype (Table 1). In both Pokkali and IR29, there was a greater accumulation of proline content in the leaf tissues of rice seedlings pre-treated with high concentrations of $CaCl_2$ (Table 2), particularly in 7.92 mM $CaCl_2$. Proline content in salt-stressed IR29 seedlings was accumulated more than that in Pokkali genotype (Table 2). Relative electrolyte leakage (REL) was decreased in the rice seedlings treated with calcium (7.92 mM $CaCl_2$) and it correlated negatively to proline accumulation (Fig. 1A-B), especially in IR29. An increasing REL in the leaf tissues was also negatively correlated with total chlorophyll content (Fig. 1C-D). Exogenous/pre-treatment of Ca significantly improved the shoot height, root length, leaf area and biomass accumulation in both salt-tolerant (Pokkali) and sensitive (IR 29) rice genotypes. Our observations are in agreement to previous studies reporting an improvement of growth performance upon Ca addition to salt-stressed cowpea (Murillo-Amador et al., 2006), Jerusalem artichoke (Xue et al., 2008), linseed (Khan et al., 2010), *Rumex* (Chen et al., 2007), bean (Cabot et al., 2009), loquat and anger rootstocks (García-Legaz et al., 2008). Na^+ is a major toxicity causing the program cell death in rice crop, mainly in salt susceptible cultivars (Ferdose et al., 2009; Krishnamurthy et al., 2009). Earlier, Anil et al. (2005)

Table 3. Maximum quantum yield of PSII (F_v/F_m), photon yield of PSII (Φ_{PSII}), photochemical quenching (qP) and net photosynthetic rate (P_n) of two rice cultivars treated by various concentrations of $CaCl_2$ for 7 days and subsequently transferred to 200 mM NaCl for 14 days. Mean error values represent by ±SE.

Rice cultivar	$CaCl_2$ (mM)	F_v/F_m	Φ_{PSII}	qP	P_n (µmol m^{-2} s^{-1})
Pokkali	1.98	0.871±0.003b	0.548±0.004d	0.601±0.014d	1.27±0.14a
	3.96	0.910±0.003ab	0.666±0.013c	0.739±0.016c	1.50±0.15a
	7.92	0.927±0.002a	0.775±0.013a	0.854±0.013a	1.61±0.04a
	15.84	0.907±0.003ab	0.720±0.007b	0.813±0.003ab	1.54±0.012a
IR29	1.98	0.466±0.009e	0.363±0.009f	0.411±0.018f	0.43±0.02b
	3.96	0.618±0.023d	0.467±0.023e	0.507±0.014e	0.54±0.01b
	7.92	0.879±0.005b	0.564±0.005d	0.762±0.016bc	1.25±0.07a
	15.84	0.768±0.015c	0.500±0.015e	0.648±0.009d	0.69±0.05b
Significant level					
Rice		**	**	**	**
$CaCl_2$		**	*	**	**
Rice × $CaCl_2$		**	**	**	*

Means within a row followed by different letters in each column are significantly different at $p \leq 0.01$ (**) by Duncan's New Multiple Range Test (DMRT).

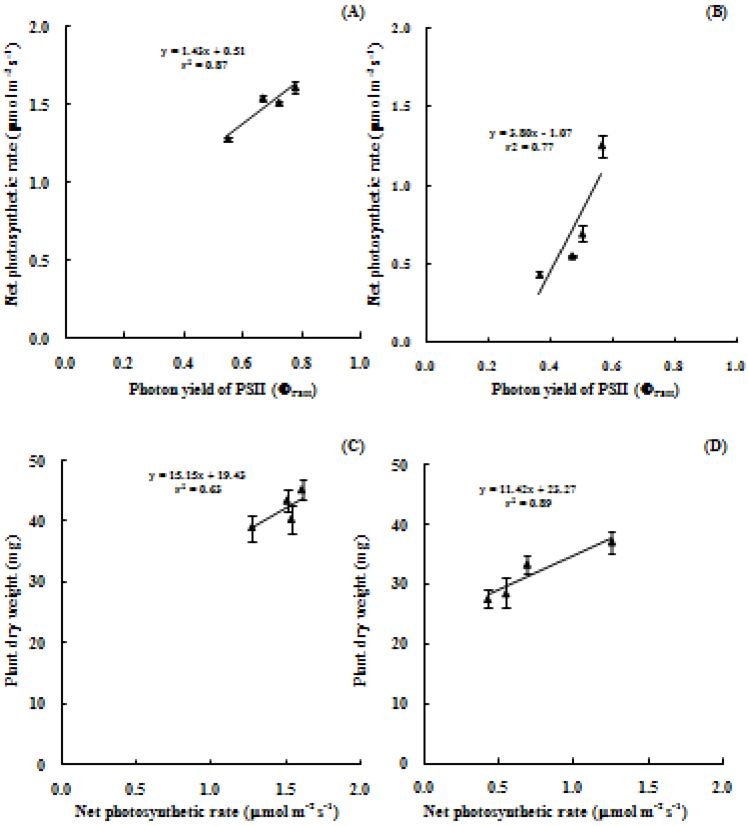

Fig 3. Relationship between photon yield of PSII and net photosynthetic rate of Pokkali (A) and IR29 (B) as well as net photosynthetic rate and plant dry weight of Pokkali (C) and IR29 (D) treated by various concentrations of $CaCl_2$ for 7 days and subsequently transferred to 200 mM NaCl for 14 days. Error bars represent by ±SE.

reported that Ca application restricts the intake of Na^+ in both salt tolerant Pokkali and susceptible Jaya rice cultivar. In addition, the osmotic and oxidative stresses derived from soil salinity also affect the plant metabolism, including osmolyte biosynthesis (proline, sugars and glycine betaine) for osmotic adjustment in the cells, and antioxidant enzymatic and non-enzymatic substances (Demiral and Türkan, 2005; Cha-um et al., 2007; Cha-um et al., 2009). Though we did not study the changes in oxidative metabolism in response to Ca pre-treatment to salt-stressed seedlings, yet, studies have reported that Ca treatment significantly induces antioxidant system in salt-stressed Jeruslaem antichoke (Xue et al., 2008 and linseed (Khan et al., 2010). In the present study, the leaf area of IR29 salt susceptible genotype pretreated with 7.92–15.84 mM $CaCl_2$ before exposure to 200 mM NaCl was significantly improved as in Pokkali and was even better than that in low calcium concentration (1.98–3.96 mM $CaCl_2$). Earlier, a similar improvement in leaf area has been reported in greengram (Manivannan et al., 2007), soybean (Arshi et al., 2010) and *Limonium bicolor* (Ding et al., 2010) in response to exogenous Ca.

Photosynthetic pigments, chlorophyll fluorescence and net photosynthetic rate

Photosynthetic pigments, Chl_a, Chl_b, total chlorophyll (TC) and C_{x+c}, in the salt-stressed leaf tissues of Pokkali, were unchanged in both high and low calcium treatments. In contrast, photosynthetic pigments in salt-stressed leaf tissues of IR29 were significantly decreased in 1.98 mM $CaCl_2$ when compared to 7.92 mM $CaCl_2$ (Table 2). Chl_a and Chl_b contents in salt stressed seedlings were positively related to F_v/F_m and Φ_{PSII}, respectively (Fig. 2). The F_v/F_m, Φ_{PSII} and qP in both Pokkali and IR29 rice cultivars cultivated under salt stress were significantly improved by 7.92 mM $CaCl_2$ pre-treatment (Table 3) and there was a positive correlation between Φ_{PSII} and P_n in both the genotypes (Fig. 3A-B). The P_n in IR29 rice seedlings treated with 7.92 mM $CaCl_2$ was better than that in other cases (Table 3). In addition, a positive relationship was observed between P_n and plant dry weight (Fig. 3C-D). In our study, Ca pre-treatment improved the photosynthetic ability, including chlorophyll fluorescence and net photosynthetic rate (P_n) in salt-stressed rice thereby suggesting a protective effect of Ca on photosystems. Such an observation is strengthened by an earlier report that Ca prevents the toxic effects of Na^+ on tomato leaf photosynthesis (Montesano and van Iersel, 2007). REL is an indicator of membrane damage that occurs due to membrane peroxidation resulting from an oxidative burst (Bajji et al., 2002). In our study, REL in salt susceptible Pokkali genotype pretreated with 7.92 mM $CaCl_2$ was lower than that in 1.98 mM $CaCl_2$ and it was positively related to proline accumulation. Earlier, Nedjimi and Daoud (2009) also observed an ameliorative effect of exogenous Ca on membrane permeability in salt-stressed *Atriplex halimus* subsp. *schweinfurthii*. Proline is a known osmolyte that provide osmolytic adjustment, tolerance and protection against abiotic stresses-induced damage to photosystems and membranes (Ain-Lhout et al., 2001). In the present study, the reduction in REL in response to Ca pre-treatment was associated with enhanced proline accumulation. The salt sensitive Pokkali cultivar accumulated greater amount of proline when exposed to 200 mM NaCl and enriched in 7.92 mM $CaCl_2$ pre-treatment compared to non-Ca treatments. It indicated greater role of proline in maintaining osmotic adjustment and thus membrane stability in response to NaCl. A similar enrichment of proline was earlier reported in NaCl-stressed linseed upon Ca treatment (Khan et al., 2010)

Material and methods

Plant materials and treatments

Seeds of two genotypes of indica rice (*Oryza sativa* L. spp. *indica* cv. Pokkali-salt tolerance and IR 29-salt susceptible) were manually dehusked and pre-sterilized with 70% alcohol. These were sterilized in 5% Clorox® for 60 min, then in 30% Clorox® for 30 min, and rinsed three times with sterile distilled-water. Surface-sterilized seeds were germinated on 0.25% Phytagel®-solidified MS media (Murashige and Skoog, 1962) with 3% sucrose (photomixotrophic condition) in a 250 mL glass vessel. The media were adjusted to pH 5.7 before autoclaving. Rice seedlings were cultured *in vitro* under 25±2°C, 60±5% relative humidity (RH) and 60±5 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density (PPFD) provided by fluorescent lamps with a 16 h d^{-1} photoperiod. The Ca content in the culture medium was adjusted to 1.98 mM, 3.96 mM (control), 7.92 mM and 15.84 mM with exogenous $CaCl_2$. Seven-day rice seedlings were aseptically transferred to modified MS-liquid sugar-free medium (photoautotrophic conditions). The number of air-exchanges in the glass vessels was adjusted to 2.32 $\mu mol\ CO_2\ h^{-1}$ by punching a hole on plastic cap (∅ 1 cm) and covering the hole with a microporous filter (0.20 µm pore size) (Cha-um et al., 2010). Rice seedlings were subjected to salt stress (as NaCl) for 14 days by adjusting to 200 mM NaCl in the culture media. Thereafter, growth performance, photosynthetic ability, electrolyte leakage and proline content, were determined.

Growth performances

Shoot height, root length, leaf area, fresh weight and dry weight of rice seedlings were measured 7-days after exposure to NaCl stress. Seedlings were dried at 80°C in a hot-air oven for 2 days, and then incubated in desiccators before the measurement of dry weight. The leaf area of rice seedlings was measured using a Root/Leaf Area Meter DT-scan (Delta-Scan Version 2.03, Delta-T Devices, Ltd, Cambridge, UK).

Photosynthetic pigments

Chlorophyll a (Chl_a) and chlorophyll b (Chl_b) were analyzed following the methods of Shabala et al. (1998), and total carotenoid (C_{x+c}) concentrations were assayed according to Lichtenthaler (1987). Leaf tissue (100 mg) was placed in a 25 mL glass vial, along with 10 mL 95.5% acetone, and blended using a homogenizer. The glass vials were sealed with parafilm to prevent evaporation, and stored at 4°C for 48 h. Thereafter, the absorbance was measured at 470 nm, 662 nm and 644 nm against a blank of 95.5% acetone. Amounts of Chl_a and Chl_b and C_{x+c} were calculated.

Photosynthetic ability

It was measured in terms of maximum quantum yield of PSII (F_v/F_m), photon yield of PSII, (Φ_{PSII}), photochemical quenching (qP), and net photosynthetic rate (P_n). Chlorophyll fluorescence emission from the adaxial surface of the leaf was measured using a fluorescence monitoring system (FMS2, Hansatech Instrument Ltd., Norfolk, UK) in the pulse amplitude modulation mode as previously described by Loggini et al. (1999). A leaf, adapted to dark conditions for 30 min using leaf-clips, was initially exposed to the modulated beam of far-red light, and initial (F_0) and maximum (F_m) fluorescence were measured. The variable fluorescence yield (F_v) was calculated by the equation, F_m–F_0. The maximum quantum yield of PSII was calculated at F_v/F_m. Φ_{PSII} was calculated as $\Phi_{PSII} = (F_m'-F)/F_m'$ (Maxwell and Johnson, 2000). P_n was calculated by comparing the different concentrations of CO_2 inside (C_{in}) and outside (C_{out}) the glass vessel containing the rice seedlings. The CO_2 concentrations at steady state were measured by Gas Chromatography (GC; Model GC-17A, Shimadzu Co. Ltd., Japan). The P_n of *in vitro* cultivated plantlets was calculated according to the method of Fujiwara et al. (1987).

Relative electrolyte leakage

Relative electrolyte leakage (%) was determined according to the Dionisio-Sese and Tobita, (1998) method. Leaf tissue (100 mg) was incubated in distilled water at 25°C for 2 h in test tubes and initial conductivity (E_1) of the bathing medium was measured. The tubes were then boiled for 30 min to release all the electrolytes. These were cooled to 25°C and the conductivity (E_2) was measured again. The electrolyte leakage was calculated as follows: $REL = (E_1/E_2) \times 100$.

Proline content

Proline in the leaf tissue was extracted and analyzed as per Bates et al. (1973). Fresh tissue (100 mg) was ground with liquid nitrogen in a mortar. The homogenate powder was mixed with 1 mL aqueous sulfosalicylic acid (3% w/v) and filtered through filter paper (Whatman #1, England). The extracted solution was mixed with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 mg ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M H_3PO_4) and incubated at 95°C for 1 h. The reaction was terminated by placing the container in an ice bath. The reaction mixture was mixed vigorously with 2 mL toluene. After cooling to 25°C, the chromophore was measured by spectrophotometer DR/4000 at 520 nm using L-proline as a standard.

Statistical analysis

The experiment was arranged as 2×4 factorials in a completely randomized design with 5 replications ($n = 5$). The data were subjected to one-way ANOVA and mean values in each treatment were compared using DMRT (Duncan's New Multiple Range Test) using SPSS software. The correlations between growth characters, biochemical, and physiological parameters were evaluated using Pearson's correlation coefficients.

Conclusions

Calcium treated seedlings (7.92 mM $CaCl_2$) of salt-sensitive rice (IR29) plays as alternative way to enhance the salt-tolerant ability, as indicated by low REL, maintained photosynthetic pigments, stabilized photochemical system in PSII, resulting in high net photosynthetic rate and overall growth characters. An exogenous Ca application may further be applied to improve the salt tolerant abilities of rice crop grown in salt affected soil.

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