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ORIGINAL ARTICLE

Relative salinity tolerance of rice cultivars native to North East India: a physiological, biochemical and molecular perspective

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Abstract Salinity is the second most prevalent abiotic stress faced by plants, and rice is not an exception. Through this study, it has been tried upon, to study the relative salinity tolerance of eight local varieties of North East India. Preliminary screening was based on their dose- and time-dependent physiological responses to salinity stress. Among the cultivars, Tampha was found to be relatively more tolerant, whereas MSE9 the most sensitive. To further ascertain their tolerance capacity, MDA and H_2O_2 content was determined, which also confirmed the tolerance level of the two cultivars. Histochemical assays for root plasma membrane integrity and leaf and root H_2O_2 and O_2^- content also showed more damage in Tampha in comparison to MSE9. Finally, gene expression analysis for Na^+/K^+ co-transporters, OsHKT2;1, OsHKT2;3 and OsHKT2;4, was performed to observe how the expression level of these transporters varies with the tolerance capacity of these two cultivars in leaves and roots under different time frames. The study reveals Tampha to be the most tolerant and MSE9 the most sensitive when compared to the other six screened cultivars for salinity stress.

Keywords *Oryza sativa* · Salinity · Abiotic stress · Physio-biochemical

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Introduction

Rice (*Oryza sativa* L. spp. *indica*) is considered the second most important crop after wheat, taken as staple food by nearly half of the world's population (Ghosh et al. 2016). Ninety percent of the world's total rice produce is consumed by Asian population (IRRI 2013). Rice as a crop is hampered by various abiotic stresses especially salt stress (Mittal et al. 2016). Salt stress is the second most prevalent abiotic stress to plants next to drought, limiting production and productivity of crops worldwide. Saline soil is characterised by high electrical conductivity of above 4 dS/m (approximately 40 mM NaCl; Chinnusamy et al. 2005). High salinity has adversely affected an estimated area of around 800 million hectares of the total land area of the world (Munns and Tester 2008). So, finding relative salt tolerance capability of genotypes will enable to achieve higher productivity of rice for meeting the demands of ever-increasing world human population, and the selected salt-tolerant genotypes shall go a long way for "omic" studies to decipher salt stress tolerance mechanisms.

Soil salinity negatively impacts plants generally by two mechanisms, osmotic stress and ion toxicity (Vaid et al. 2015). Osmotic stress is the result of increased amount of salt in growth medium that hampers capacity of plant to retain and absorb water (Morales et al. 2012). Whereas, ion toxicity is caused by ionic imbalance due to higher accumulation of Na^+ and Cl^- ions at toxic level thereby lowering the availability of calcium (Ca^{2+}) and potassium (K^{2+}) (Hussain et al. 2013). Excessive Na^+ may build up in apoplast thereby causing cell dehydration; they may also accumulate in cytosol thereby inhibiting enzymes responsible for metabolic processes including photosynthesis (Munns and Tester 2008). In case of rice, salinity induces biochemical and physiological alterations, causing growth inhibition and yield reduction (Ghosh et al. 2016). Growth reduction due to salinity differs greatly

with species and cultivars within a species (Khan et al. 2002). Shoot growth is found to be more effected by stress than root. It may be explained as plants' adaptive response to keep absorbing water by elongating roots even after the stress. As roots are in direct contact with saline growth medium, they need to elongate to access clean water. Whereas in case of shoots it is just the opposite, to adapt the osmotic stress due to salinity, they need to lower the transpiration pull for which there is reduction of foliage surface area. The significant biomass reduction is also due to impaired photosynthetic mechanisms and relatively decreased water content due to inability of plant for normal uptake of water. Severity and duration of the stress control the changes in stress-inducible parameters (Mishra et al. 2016).

Damage due to salinity is further accelerated due to overproduction of reactive oxygen species (ROS); like H_2O_2, O_2^- drastically hampers metabolic homeostasis and cell membrane integrity (Hussain et al. 2013). Significant higher build-up of ROS leads to lipid peroxidation thereby interfering membrane stability (Chunthaburee et al. 2016). Sensitive rice plants show higher generation of H_2O_2 and lipid peroxidation molecules which are quantified as malondialdehyde (MDA). Tolerant cultivars thrive to survive by generating antioxidant enzymes that will catalyse the removal of ROS.

Intracellular ion homeostasis is fundamental to the physiology of living cells, and K^+ and Na^+ homeostasis is more vital under salt stress (Mishra et al. 2016). Na^+ competes for uptake with K^+ into roots of rice plants after being exposed to stress. Low cytosolic Na^+ and the low Na^+/K ratio are required for osmotic and biochemical equilibrium in plant cells (Yao et al. 2010). Na^+/K^+ ratio is a key factor for salt tolerance in plants (Sun et al. 2014). Plants regulate the expression and activities of various membrane transporters to maintain this ratio. Among these, HKT (for high-affinity K^+ transporters) are integral membrane proteins that facilitate cation transport across the membrane (Waters et al. 2013). They are subdivided into two subgroups HKT1 and HKT2 based on phylogenetic analysis (Platten et al. 2006). Class 1 HKT shows more Na^+ transport activity, whereas the class 2 members show K^+ permeability in addition to Na^+. HKT1 are basically single-ion (Na^+) transporters preventing overaccumulation of Na^+ in the photosynthetic tissues, whereas HKT 2 transport both Na^+ and K^+ from the external medium depending on the concentration of each (Almeida et al. 2013). HKT2;1 is unique as it exhibits features of HKT class 1 transporters in having a serine residue instead of glycine in the "P loop", responsible for binding ions (serine depicts more Na^+ specificity and glycine more K^+). HKT2;3 and HKT2;4 show around 93% sequence homology but differ in their function. Comparatively less studies have been done on HKT2;3, and its expression was found to have no effect on varying dosages of K^+ and Na^+ in growth medium. HKT2;4 shows Na^+-independent K^+ transport and is found to be dependent on a wide range of divalent cations like Ca^{2+} and Mg^{2+} (Horie et al. 2011). Expression analysis of HKT genes is presumed to give a better insight into ion transport under salinity stress.

This manuscript reports the relative salinity tolerance of eight *indica* rice genotypes, native to north eastern India using various physiological, biochemical and molecular strategies.

Materials and methods

Rice sample

Viable germplasms were collected from the Regional Agricultural Research Station, Karimganj, Assam and the Rice Research Centre, Thoubal, Manipur (Table 1).

Growth, treatment and relief

Seeds were surfaced-sterilised with 0.1% mercuric chloride and washed thrice thoroughly with distilled water. They were set for germination at 30 °C in dark. Uniformly germinated seeds were transferred to plastic pots containing Hoagland's nutrient medium (Hoagland Arnon 1950), and growth conditions of plants were set at growth chamber with photon flux density of 52 μmol s^{-1} m^{-2} (PAR) and 16 h photoperiod. On

Table 1 List of rice cultivars used in this study and salt tolerance groups

Sl. no.	Cultivar	Origin/source	Parental lines	Tolerance
1.	Tampha	CAU, Imphal, India	Leima Phou × BR-I	T
2.	Krishna	Regional Agricultural Research Station, Karimganj, Assam, India	Chandan × Samba Mahsuri	MT
3.	Pankaj	Regional Agricultural Research Station, Karimganj, Assam, India	Peta × Tongkai Rotan	MT
4.	Punsi	Rice Research Station, Thoubal, Manipur, India	Phouren × IR-661-1-140-1	S
5.	Luit	Titabor Regional Agricultural Research Station, Assam, India	Heera × Annada	S
6.	Sana	Rice Research Station, Thoubal, Manipur, India	Moirang Phou × Lawagin	S
7.	Leima	Rice Research Station, Thoubal, Manipur, India	Moirang Phou × Lawagin	S
8.	MSE-9	Regional Agricultural Research Station, Karimganj, Assam, India	Selection from Manoharsali	HS

T tolerant, *MT* moderately tolerant, *S* sensitive, *HS* highly sensitive

the sixth day, plants were treated with 150 mM of salt. After 24 and 96 h duration, both control and stressed seedlings were excised for analysis (Fig. 1). After the completion of the 96 h stress period, Tampha and MSE9 were re-watered with normal nutrient solution and allowed to grow for 6 days (Castillo et al. 2007). Differences in revival potential of the two varieties have been documented.

Determination of plant growth

Growth of plants scored in the form of length of root, shoot and fresh weights was measured after 24 and 96 h of the treatment (Fig. 1).

Estimation of RWC

Relative water content of both shoot and root was determined (Barrs and Weatherley 1962). Plant tissue samples were soaked in double distilled water for 4 h to get turgid weight after taking respective fresh weights. Later, they were oven-dried at 60 °C for 72 h to get the dry weight.

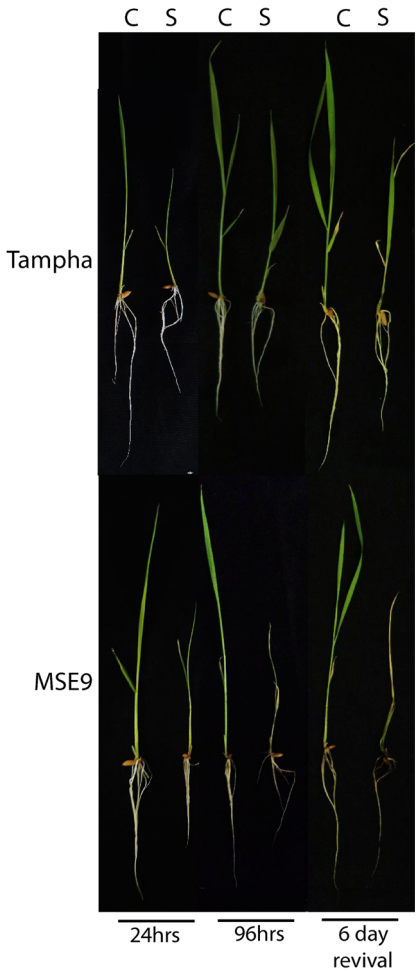

Fig. 1 Salt stress tolerance of contrasting rice varieties Tampha and MSE9 when subjected to 150 mM NaCl concentration for 24 and 96 h and then revival for 6 days. *C* control, *S* stressed

Na^+ and K^+ content

A total of 0.1 g of dry sample were weighed and ashed at 550 °C for 3 h in a muffle furnace (dry ashing method). The ash was dissolved in 2 N HCl and extracted for Na^+ and K^+ (Chapman and Pratt 1962). The content was measured by flame photometer (Flame photometer 129, Systronics, Ahmedabad, India).

Estimation of H_2O_2 and lipid peroxidation

Hydrogen peroxide contents of MSE9 and Tampha were estimated after 24 and 96 h of NaCl stress. For H_2O_2, 0.2 g of samples was extracted with 5% TCA and centrifuged at 12,500 rpm for 10 min (Sagisaka 1976). The reaction mixture contained 1.6 ml of supernatant, 0.4 ml of 50% TCA, 0.4 ml of 10 mM ferrous ammonium sulphate and 0.2 ml of 2.5 M KSCN. H_2O_2 content was measured at 480 nm against suitable blank. Lipid peroxidation was measured as MDA content (Khan and Panda 2008). A total of 0.1 g of sample were homogenised with 1 ml of 1% TCA and centrifuged at 15,000 rpm for 10 min. Then, 0.5 ml of supernatant was mixed with 1.5 ml of 0.5% TBA and incubated at 95 °C for 25 min. Absorbance was measured at 532 and 600 nm for non-specific turbidity.

Histochemical detection of H_2O_2, O_2^- and plasma membrane integrity

Detection of hydrogen peroxide (H_2O_2) was done by 3,3-diaminobenzidine (DAB) staining according to the method of Ramel et al. (2009) and of superoxide radical (O_2^-) by NBT staining following the modified method of Rao and Davis (1999) in leaf segments and roots under both stressed and unstressed conditions. The leaf segments and roots were immersed and infiltrated under vacuum with 1 mg/ml DAB staining solution, pH 7.8, dissolved in H_2O for 6 h and 3 mg/ml nitro-blue tetrazolium (NBT) staining solution in 50 mM potassium phosphate buffer (pH 7.5) for 30 min at room temperature. Stained leaves were bleached in acetic acid:glycerol:ethanol (1:1:3 v/v) solution at 100 °C for 5 min and stored in glycerol:ethanol (1:4 v/v) solution until photographed.

The loss of plasma membrane integrity was evaluated using Evans blue staining method with slight modifications (Schutzendubel et al. 2001). Roots of intact seedlings were stained with 0.25% (w/v) Evans blue in 100 μM $CaCl_2$

(pH 5.6) for 30 min; then, the stained roots were washed with 100 μM $CaCl_2$ for 15 min. After rinsing with $CaCl_2$, root tips were cut with sharp razor blade for stereoscopic microscope observation.

Gene expression analysis

For total RNA extraction, 0.2 g of fresh tissue was homogenised in liquid nitrogen. Then, extraction and isolation were done as per manufacturer's instruction (Nucleopore RNA Sure Plant Kit, Genetix Biotech, New Delhi, India).

Total RNA extracted was processed for cDNA synthesis following manufacturer's instruction (First strand cDNA synthesis kit, Thermo Scientific, USA). Amplification for gene used for expression analysis was done by polymerase chain reaction (Takara PCR Thermal cycler, Japan) for 30 cycles using respective primers (Supplementary 1).

Statistical analysis

For statistical analysis, 10–15 seedlings per replicate per experiment were taken into account. Statistical comparison between the variances was determined by ANOVA (analysis of variance), and significant differences between mean values ($n = 3$), where n is the number of times experiments repeated, were determined by LSD analysis. $P \leq 0.05$ was deemed to show statistical significance.

Results

Changes in growth parameters

The effect of NaCl treatment on growth of plants in terms of root length and shoot length has been elucidated in Table 2. Salinity has caused reduction in the growth of roots of rice varieties except Krishna after 96 and 24 h of stress, respectively. Leima, Sana and Luit have been found to have root lengths significantly reduced from their respective controls. MSE9 has been observed to have the highest reduction in length of 27.83% over its control. Whereas root lengths of Krishna, Pankaj have not been significantly reduced showing tolerance to stress. The same trend has also been observed in Tampha after 24 h treatment and its decrease over control being 5.92 and 4.51% after 1st and 4th days of treatment (Fig. 1).

Shoot length of plants had demonstrated more susceptibility to salt stress as all the varieties have shown significantly reduced shoot lengths after 24 and 96 h of stress. Tampha had been least affected with an average percentage decrease of about 24% for both the treatment duration and MSE9 being

Table 2 Effect of time-dependent salinity on root and shoot length of different rice genotypes

Rice genotypes	Stress duration	Root length (cm)			Shoot length (cm)		
		Control	Salinity	Decrease over control (%)	Control	Salinity	Decrease over control (%)
Tampha	24 h	12.17 ± 0.66a	11.45 ± 0.56a	8.84	11.2 ± 0 .44	9 ± 1.16*	19.64
	96 h	13.3 ± 0.94b	12.7 ± 0.96*	4.51	12.87 ± 0.56	9.11 ± 0.83*	29.22
Krishna	24 h	10.85 ± 0.35	10.15 ± 0.21	6.45	11.55 ± 1.09	8.52 ± 0.80*	26.23
	96 h	12.26 ± 1.68	12.34 ± 1.9	−0.65	12.65 ± 0.82	9.11 ± 0.52*	27.98
Pankaj	24 h	10.48 ± 0.12	10.20 ± 0.19	2.67	12.45 ± 0.25	9.2 ± 0.16*	26.10
	96 h	11.20 ± 0.47	10.85 ± 0.28	3.13	13.15 ± 0.33	9.08 ± 0.65*	30.95
Punsi	24 h	10.32 ± 0.26	9.15 ± 0.46	11.36	13.67 ± 0.20	10.54 ± 1.10*	22.89
	96 h	9.65 ± 1.27	8.34 ± 0.55	13.58	14.82 ± 2.09	11.39 ± 2.79*	23.14
Luit	24 h	8.91 ± 1.11	8.25 ± 0.191	5.72	11.35 ± 0.84	9.21 ± 0.51*	18.85
	96 h	10.74 ± 0.70	9.5 ± 0.69*	11.55	13.19 ± 1.41	10.23 ± 1.47*	22.44
Sana	24 h	10.32 ± 0.53	9.15 ± 0.18*	11.34	11.15 ± 0.35	8.9 ± 0.27*	20.18
	96 h	11.62 ± 1.30	9.95 ± 0.67*	14.37	12.97 ± 1.37	9.69 ± 1.95*	25.29
Leima	24 h	9.2 ± 0 .41	8.35 ± 0.44	9.24	12.53 ± 1.84	9.53 ± 0.62*	23.94
	96 h	10.37 ± 0.71	9.02 ± 0.58*	13.02	14.83 ± 0.95	10.97 ± 1.57*	26.03
MSE9	24 h	9.53 ± 0.18	8.4 ± 0.68*	11.86	14.61 ± 1.14	10.7 ± 0.64*	26.76
	96 h	11.5 ± 0.39	8.3 + 0.32*	27.83	15.15 ± 1.31	8.61 ± 1.037*	43.17

Values sharing the * are significant from its respective control at $P \leq 0.05$ for each rice variety according to LSD analysis

the most affected as its treated shoot length has reduced by 57% in comparison to that of the control (Fig. 1). Krishna and Pankaj shoot lengths have been moderately effected (Table 2).

Relative water content

Relative water content (RWC) of eight rice varieties was found to be effected to different extent by salinity (Table 3), and RWC of each variety was significantly reduced due to stress compared to respective untreated condition. Among the varieties, Tampha showed least decrease having 80 and 74.12% RWC in roots and 84.92 and 74.52% RWC in shoots after 24 and 96 h of stress, respectively, while MSE9 variety showed highest decrease over control, i.e., 50% in case of both roots and shoots. Krishna and Pankaj gave better performance than the remaining varieties except Tampha. Punsi, Luit, Sana and Leima had higher value of RWC than that of MSE9 having around 70% RWC in roots and shoots after 24 h and 60% after 96 h. Time duration of the salt stress also negatively influenced RWC as we found that reduction after 96 h was more than that after 24 h stress in all varieties (Table 3).

Na^+, K^+ uptake and Na^+/K^+ ratio

All the varieties showed increased sodium uptake at the expense of decreased K^+ uptake after NaCl treatment. Na^+ content of all the plants significantly increased from their respective untreated condition at $P \leq 05$ after stress (Tables 4 and 5).

After 24 h, Na^+ content of rice shoot ranged from 12 to 26 μg/g while that of controls remained below 10 μg/g. MSE9 uptake was maximum, with a value of 26.65 μg/g of Na^+ while for Tampha it was the least, with 12.68 μg/g (Table 4). After 96 h, Tampha variety had accumulated 17.42 μg/g of Na^+ but MSE9 had 40.18 μg/g which was four times higher of its respective control. On the other hand, K^+ uptake had been significantly reduced due to treatment, lowest being recorded for MSE9 at 35.77 μg/g after 96 h treatment. Rice varieties Krishna and Pankaj demonstrated moderate Na^+ increase and K^+ decrease, which attributed to their relatively low Na^+/K^+ ratios when compared to other varieties except Tampha. Tampha showed the highest tolerance with least Na^+/K^+ ratio, its increase over control being minimal.

Though Tampha showed the least decrease, significant increase of Na^+/K^+ ratio in all the varieties after treatment has been recorded with respect to control. Decreased K^+ and increased Na^+ uptake have been manifested most prominently in MSE9 showing its extreme sensitivity to salinity. MSE9 Na^+/K^+ ratio has been observed to be the highest, after 96 h treatment (Tables 4 and 5).

H_2O_2 and MDA content

H_2O_2 and MDA content were analysed in Tampha and MSE9 varieties to confirm their tolerance level. MDA content in both the varieties had significantly increased even though Tampha had lower MDA than MSE9.

Table 3 RWC (%) of different rice genotypes

Rice genotypes	Stress duration	Root		Shoot	
		Control	Salinity	Control	Salinity
Tampha	24 h	91.23 ± 0.1.22	80.86 ± 0.435*	90.90 ± 1.100	84.92 ± 1.840*
	96 h	91.56 ± 0.905	74.12 ± 1.181*	91.36 ± 0.952	74.52 ± 2.308*
Krishna	24 h	90.44 ± 0.621	78.47 ± 0.670*	92.14 ± 0.747	76.59 ± 1.777*
	96 h	91.28 ± 0.479	69.97 ± 1.74*	91.73 ± 0.801	68.57 ± 0.351*
Pankaj	24 h	90.60 ± 0.585	78.12 ± 0.652*	92.39 ± 1.163	75.36 ± 4.133*
	96 h	88.21 ± 0.401	67.68 ± 2.673*	91.72 ± 0.500	68.23 ± 0.978*
Punsi	24 h	92.38 ± 0.801	72.07 ± 1.059*	91.316 ± 1.241	70.061 ± 0.914*
	96 h	91.605 ± 0.854	62.374 ± 1.994*	91.00 ± 1.305	62.65 ± 0.758*
Luit	24 h	90.62 ± 0.368	72.25 ± 0.564*	92.80 ± 0.959	72.48 ± 1.884*
	96 h	91.71 ± 0.724	62.43 ± 0.376*	91.52 ± 0.568	62.53 ± 2.378*
Sana	24 h	88.95 ± 2.205	70.92 ± 0.443*	91.20 ± 1.514	67.20 ± 1.544*
	96 h	87.68 ± 0.981	60.53 ± 1.019*	93.02 ± 1.740	61.74 ± 3.743*
Leima	24 h	90.19 ± 1.003	71.54 ± 1.045*	90.85 ± 1.068	69.88 ± 1.285*
	96 h	90.79 ± 0.380	61.47 ± 1.372*	92.00 ± 2.297	61.97 ± 5.894*
MSE9	24 h	90.42 ± 1.566	56.944 ± 0.464*	90.09 ± 4.502	62.16 ± 3.310*
	96 h	90.56 ± 2.685	50.202 ± 1.661*	91.32 ± 0.232	50.55 ± 2.182*

Values sharing the * are significant from its respective control at $P \leq 0.05$ for each rice variety according to LSD analysis

Table 4 Effect of salinity on Na^+/K^+ of shoot of different rice genotypes

Rice genotypes	Stress duration	Na^+		K^+		Na^+/K^+		% increase of Na^+/K^+ over control
		Control	Salinity	Control	Salinity	Control	Salinity	
Tampha	24 h	9.83 ± 0.31	12.68 ± 0.113*	74.12 ± 0.274	63.03 ± 0.384*	0.133 ± 0.007	0.201 ± 0.001*	51.38
	96 h	9.60 ± 0.115	17.42 ± 0.499**	72.07 ± 0.265	55.21 ± 1.312**	0.133 ± 0.002	0.316 ± 0.016**	137.29
Krishna	24 h	9.45 ± 0.132	17.00 ± 0.98*	69.09 ± 1.227	57.15 ± 0.633*	0.137 ± 0.001	0.298 ± 0.019*	117.72
	96 h	9.99 ± 0.460	22.60 ± 1.185**	68.85 ± 1.295	49.93 ± 0.680**	0.146 ± 0.004	0.453 ± 0.025**	212.38
Pankaj	24 h	9.82 ± 0.423	15.77 ± 0.485*	69.05 ± 0.737	59.45 ± 0.465*	0.142 ± 0.055	0.265 ± 0.010*	46.42
	96 h	9.52 ± 0.139	25.97 ± 0.910**	68.8 ± 0.434	51.2 ± 0.653**	0.148 ± 0.003	0.507 ± 0.014**	242.11
Punsi	24 h	9.13 ± 0.12	21.66 ± 0.382*	67.70 ± 0.320	48.26 ± 0.706*	0.135 ± 0.002	0.449 ± 0.005*	232.62
	96 h	9.30 ± 0.115	33.56 ± 0.532**	69.03 ± 1.220	42.15 ± 0.756**	0.135 ± 0.001	0.797 ± 0.012**	491.18
Luit	24 h	8.5 ± 0.173	25.17 ± 2.355*	66.21 ± 1.123	49.53 ± 1.272*	0.128 ± 0.001	0.508 ± 0.045*	295.65
	96 h	8.63 ± 0.145	33.2 ± 0.808**	65.81 ± 0.786	42.4 ± 0.611**	0.131 ± 0.001	0.783 ± 0.017**	496.95
Sana	24 h	9.1 ± 0.159	21.65 ± 0.407*	69.45 ± 0.693	48.81 ± 0.280*	0.131 ± 0.002	0.444 ± 0.008*	237.80
	96 h	9.12 ± 0.121	33.24 ± 0.465**	70.42 ± 1.110	42.45 ± 0.501**	0.127 ± 0.001	0.785 ± 0.002**	505.4
Leima	24 h	9.25 ± 0.050	26.47 ± 2.293*	68.08 ± 0.874	48.91 ± 0.840*	0.136 ± 0.001	0.543 ± 0.054*	299.37
	96 h	9.13 ± 0.176	34.95 ± 0.993**	68.78 ± 1.064	42.40 ± 0.643**	0.133 ± 0.004	0.824 ± 0.014**	520.5
MSE9	24 h	8.88 ± 0.145	26.65 ± 0.414*	67.27 ± 1.484	41.48 ± 0.514*	0.132 ± 002	0.643 ± 0.002*	386.33
	96 h	9.15 ± 0.221	40.18 ± 0.785**	66.68 ± 1.315	35.77 ± 0.635**	0.137 ± 0.006	1.124 ± 0.090**	718.15

Different numbers of * indicates significance at $P \leq 0.05$ for a single variety after LSD analysis

Tampha had 23.097 and 26.624 μM/gfwt in leaf and root, respectively, after 96 h stress, while MSE9 had 25.419 and 28.860 in leaf and roots, respectively. H_2O_2 had also been found to be in decreased quantity in Tampha when compared to MSE9 (Fig. 2).

Histochemical detection of H_2O_2, O_2^- and plasma membrane integrity

H_2O_2, O_2^- production and plasma membrane integrity in stressed and unstressed rice leaf segments and roots were

Table 5 Effect of salinity on Na^+/K^+ of root of different rice genotypes

Rice genotypes	Stress duration	Na^+		K^+		Na^+/K^+		% increase of Na^+/K^+ over control
		Control	Salinity	Control	Salinity	Control	Salinity	
Tampha	24 h	10.91 ± 0.157	13.71 ± 0.485*	77.22 ± 0.734	65.26 ± 0.623*	0.141 ± 0 .003	0.210 ± 0.008*	48.77
	96 h	11.24 ± 0.153	18.87 ± 0.512**	77.63 ± 0.684	53.59 ± 0.327**	0.146 ± 0.001	0.352 ± 0.008**	143.15
Krishna	24 h	11.02 ± 0.494	18.23 ± 0.512*	70.46 ± 0.952	59.51 ± 1.543*	0.156 ± 0.005	0.306 ± 0.001*	96.07
	96 h	11.01 ± 0.724	23.58 ± 0.448**	72.47 ± 0.657	51.47 ± 0.39**	0.152 ± 0.*010*	0.458 ± 0.005**	201.43
Pankaj	24 h	10.84 ± 0.024	19.65 ± 1.039*	70.9 ± 0.424	60.14 ± 0.378*	0.156 ± 0.002	0.356 ± 0.016*	128.97
	96 h	11.12 ± 0.032	27.33 ± 0.655**	71.42 ± 0.300	51.71 ± 0.556**	0.164 ± 0.004	0.542 ± 0.013**	231.22
Punsi	24 h	10.18 ± 0.636	28.95 ± 2.113**	70.57 ± 0.23	51.05 ± 1.303*	0.144 ± 0.008	0.569 ± 0.490*	294.67
	96 h	10.55 ± 0.378	36.24 ± 0.527**	72.13 ± 0.541	40.25 ± 0.644**	0.146 ± 0.006	0.90 ± 0.007**	515.30
Luit	24 h	10.00 ± 0.144	28.22 ± 3.56*	68.3 ± 0.612	53.43 ± 0.726*	0.146 ± 0.003	0.527 ± 0.059*	259.55
	96 h	10.23 ± 0.421	37.83 ± 0.03**	67.13 ± 1.22	42.65 ± 0.555**	0.152 ± 0.005	0.887 ± 0.013*	482.47
Sana	24 h	9.40 ± 0.109	24.05 ± 0.155*	71.19 ± 0.859	49.75 ± 0.606*	0.132 ± 0.001	0.396 ± 0.001**	201.70
	96 h	10.19 ± 0.387	37.31 ± 0.697**	72.44 ± 0.465	41.78 ± 0.309**	0.141 ± 0.007	0.893 ± 0.010*	534.82
Leima	24 h	10.52 ± 0.12	27.51 ± 1.657*	69.78 ± 0.376	53.66 ± 0.693*	0.151 ± 0.001	0.513 ± 0.031*	240.30
	96 h	10.20 ± 0.535	40.30 ± 0.654**	69.10 ± 0.528	44.65 ± 0.321**	0.148 ± 0.007	0.90 ± 0.011**	511.78
MSE9	24 h	9.617 ± 0.280	29.44 ± 1.00*	70.80 ± 0.983	44.79 ± 1.179*	0.136 ± 0.002	0.657 ± 0.007*	384.06
	96 h	10.28 ± 0.451	48.014 ± 0.254**	72.29 ± 0.600	36.31 ± 0.299**	0.142 ± 0.007	1.135 ± 0.195**	697.49

Values having different number of * are significant to each other for every single variety at $P \leq 0.05$ according to LSD test for each rice variety

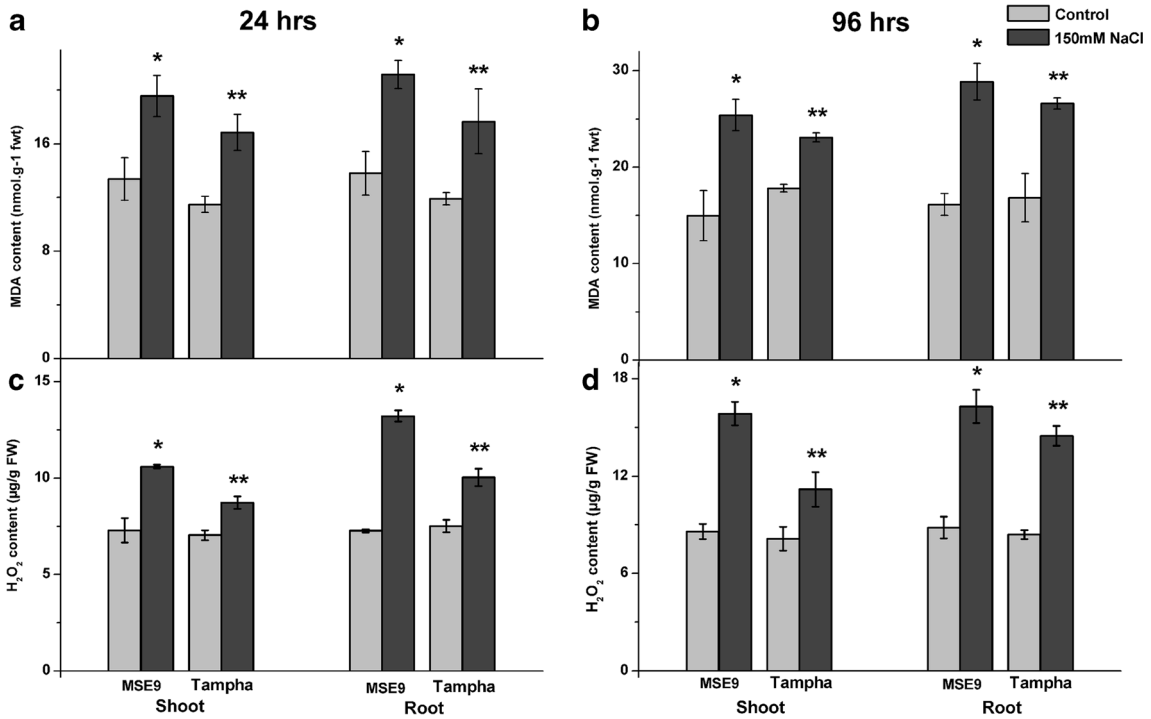

Fig. 2 Quantitative assay for H_2O_2 accumulation and lipid peroxidation (MDA) of shoot and root tissue samples at 24 and 96 h. Data represents mean values (n = 3) ± SE, where n is the number of times experiment repeated and SE denotes standard error. Statistically significant values at $P < 0.05$ using LSD analysis are indicated by *star marks*

investigated qualitatively using DAB, NBT and Evans blue histochemical staining, respectively (Fig. 3). Under normal physiological conditions, both Tampha (tolerant) and MSE9 (sensitive) showed low O_2^- and H_2O_2 accumulation and almost intact plasma membrane. However, under salinity stress, rice leaf segments and roots of Tampha exhibited marked lower NBT, DAB and Evans blue staining than MSE9 which is an indication of less ROS production and less oxidative damage in Tampha (Fig. 3).

Gene expression analysis

For *Os*HKT2;1, in leaves, first, there was increase in expression level with increase in stress duration then again decrease, but the intensity was more in the case of MSE9 in comparison to Tampha; whereas, in case of roots, there is gradual decrease throughout. *Os*HKT2;3 expression pattern did not show any deviation with increase in stress duration for both the varieties except in case of MSE9 leaves where there was a gradual increase. HKT2;3 expression was less in roots in comparison to leaves. No specific pattern was observed in case of *Os*HKT2;4. In some cases, there was gradual increase, whereas in others, there was increase and then decrease with increment of stress duration. All of these analyses were done after normalising the cDNA concentration with *Os*Actin primers (Fig. 4).

Discussions

In this study, we have analysed physiological, biochemical and molecular characteristics in rice varieties of North East India under salt stress to show relative tolerance. Plant growth scores in the form of root length and shoot were found to be reduced implying that salt stress represses the growth of rice plants. Similar findings were earlier reported in rice (Jia et al. 2015; Yeo et al. 1990; Hussain et al. 2013). All throughout, shoot growth has been more susceptible compared to roots as it has been significantly reduced for all plants to different extent (Fig. 1, Table 2). It might be explained citing the fact that relative shoot length reduction compared to root would be helpful to plants for decreasing the water use, being already osmotically stressed (due to increased Na^+ uptake) by salt (Munns and Tester 2008; Munns et al. 2006). Lower water potential in the cell causes stomatal closure and limits CO_2 assimilation mounting single direct negative impact on photosynthesis which also causes growth reduction (Pattangul and Thitisaksakul 2008).

RWC is considered an appropriate measure of plant water status as well as osmotic adjustment under stress (Baisakh et al. 2012). It is known that osmotic stress due to salinity disturbs plant water status (Amirjani 2010) causing significant reduction in RWC of rice plants in both root and shoot (Qin et al. 2010; Rodriguez et al. 1997). Under such circumstances, plant responds by osmotic adjustment by increased uptake of

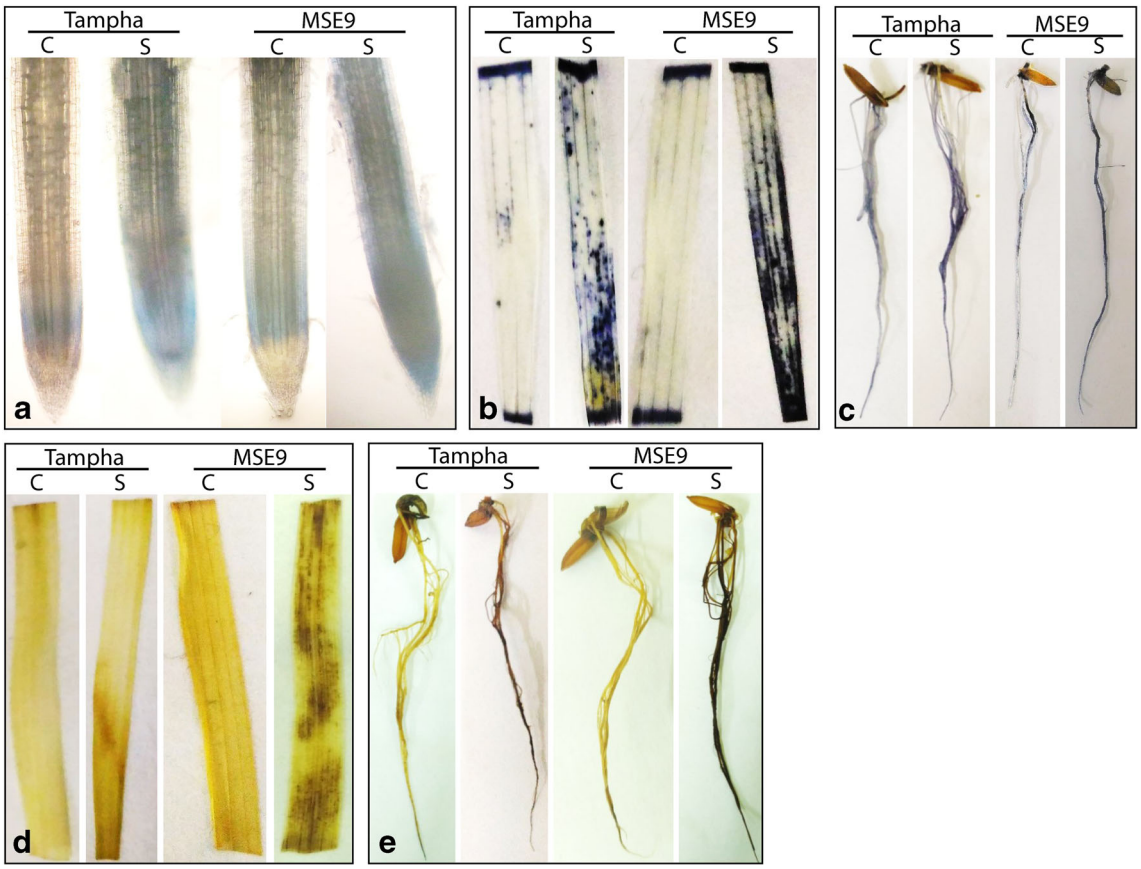

Fig. 3 Histochemical assay. **a.** Evans blue staining of roots to ascertain cell membrane integrity; **b**, **c**. NBT stained leaf segments and roots depicting O_2^- accumulation; **d, e.** DAB stained leaf segments and root depicting H_2O_2 accumulation. *C* control, *S* stressed

Na^+ and Cl^- which is readily available in saline or treated growth condition. This, in turn, causes ion toxicity and inactivates various metabolic functions like photosynthesis and electron transport chain. But such adaptation is always not adequate enough, resulting in observation of decreased RWC in treated plants (Table 3; Pattangul and Thitisaksakul 2008).

Abiotic stress including salt stress leads to oxidative damage due to rapid and uncontrolled ROS production (Miller et al. 2010; Saha et al. 2016). The primary effects of salinity like membrane damage, ion toxicity and imbalance decrease assimilation of CO_2 and reduce antioxidant enzyme activity leading to higher H_2O_2 production. Tampha variety after treatment showed lesser H_2O_2 accumulation compared to MSE9 even though both the varieties generate significantly higher H_2O_2 than their respective control (Fig. 2). This certainly concludes that Tampha is comparatively more tolerant and MSE9 is most sensitive. MDA, produced due to membrane lipid peroxidation, is often used as an indicator to differentiate between sensitive and tolerant cultivars (Dhanyalakshmi et al. 2013). In this study, Tampha contained less MDA than MSE9.

Low Na^+/K^+ ratio is an indicator of ionic homeostasis in plants (Rao et al. 2013). Under salt stress, Na^+ ion competes with K^+ due to its small size and abundant availability to be taken up by root through epidermal cells. The rice cultivars

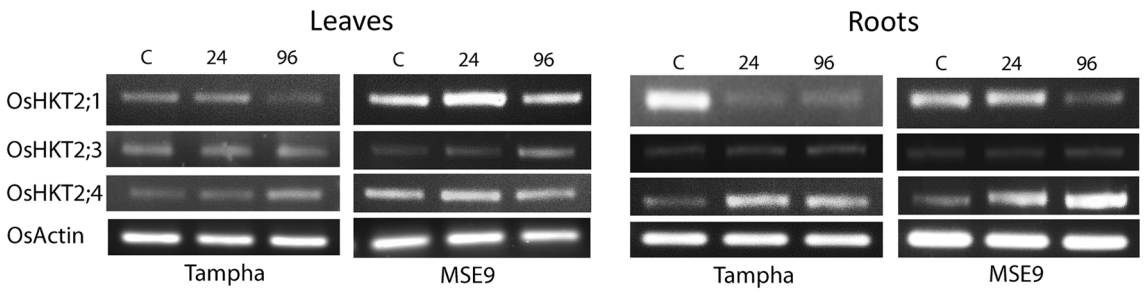

Fig. 4 Relative expression of *Os*HKT2;1, *Os*HKT2;3 and *Os*HKT2;4 under 24 and 96 h stressed root and shoot samples of Tampha and MSE9. The cDNA quantity was normalised with *Os*Actin

when arranged in decreasing order of Na^+ content are as follows: MSE9, Leima, Sana, Luit, Punsi, Pankaj, Krishna and Tampha; the reverse is true for K^+ uptake (Tables 4, 5). Lower accumulation of K^+ and higher Na^+ accumulation impart the higher Na^+/K^+ ratio in all varieties after treatment than the control. While comparing among the varieties, the lesser the ratio, the higher the salt tolerance adaptation (Kanawapee et al. 2013; Rao et al. 2013; Chunthaburee et al. 2016).

Accumulation of ROS (H_2O_2, O_2^-) was histochemically observed in leaf segments and roots of both unstressed and stressed plants exposed to salinity stress (Fig. 3). It was observed that Tampha showed lesser accumulation of H_2O_2 and O_2^- indicative of lesser oxidative damage and more tolerance to salinity stress in comparison to MSE9 (Saha et al. 2016). Also, Evans blue uptake exclusively at the root tip which was more in the case of MSE9 was observed. Evans blue uptake has largely been used as a marker for loss of plasma membrane integrity thus depicting excessive loss of integrity in MSE9 due to salinity stress (Fig. 3; Yamamoto et al. 2001; Zhang et al. 2016; Yang et al. 2016; Awasthi et al. 2017).

Expression analysis through semi-quantitative PCR was performed for HKT group of Na^+ and K^+ transporters, viz. HKT2;1, HKT2;3 and HKT2;4, in order to ascertain the implications of salinity on these transporters. HKT2;1 showed gradual increase in expression at 24 h in comparison to control, but at 96 h, it decreases. Tampha showed lesser expression when compared to MSE9 (Fig. 4). Depending upon exterior Na^+ and K^+ concentrations, HKT2;1 acts as symporter or uniporter (Jabnoune et al. 2009). Since in case of salinity, stress exterior Na^+ concentration is excessive, HKT2;1 acts as Na^+ uniporter leading to its increased accumulation in cells. Tampha showed lesser expression of HKT2;1 which might had led to lesser accumulation of Na^+ hence greater tolerance. No change in expression pattern of HKT2;3 was observed with stress and increase in time frame which indicated of its being independent of stress. Wu et al. (2009) reported similar results. HKT2;4 showed no pattern in expression; so, it remains inconclusive.

Conclusions

In this present study, we demonstrated relative salt-tolerant capacity of eight rice varieties of North East India based on physiological parameters. Tampha and MSE9 were found to be most tolerant and most sensitive, respectively, among the others. The two varieties were further analysed based on lipid peroxidation, H_2O_2 content and HKT transporter gene expression. Based on the findings, we conclude that differences in salinity tolerance mechanisms might be partially due to differences in regulation of gene expression of HKT2 transporter proteins and plants' metabolomic adaptations to resist damage caused by oxidative stress.

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Author contribution TO and SKP conceived and designed the experiment. TO and SS procured seeds for the experimental work. TO and BS performed all the experimental works. TO, BS and SKP contributed to writing of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest in the present investigation.

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