Novel screening protocol for precise phenotyping of salt-tolerance at reproductive stage in rice

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Abstract The present study reports an unequivocal and improved protocol for efficient screening of salt tolerance at flowering stage in rice, which can aid phenotyping of population for subsequent identification of QTLs associated with salinity stress, particularly at reproductive stage. To validate the new method, the selection criteria, level and time of imposition of stress; plant growth medium were standardized using three rice genotypes. The setup was established with a piezometer placed in a perforated pot for continuous monitoring of soil EC and pH throughout the period of study. Further, fertilizer enriched soil was partially substituted by gravels for stabilization and maintaining the uniformity of soil EC in pots without hindering its buffering capacity. The protocol including modified medium (Soil:Stone, 4:1) at 8 dS m⁻¹ salinity level was validated using seven different genotypes possessing differential salt sensitivity. Based on the important selection traits such as high stability index for plant yield, harvest index and number of grains/panicle and also high K⁺ concentration and low Na⁺–K⁺ ratio in flag leaf at grain filling stage were validated and employed in the evaluation of a mapping population in the modified screening medium. The method was found significantly efficient for easy maintenance of desired level of soil salinity and identification of genotypes tolerant to salinity at reproductive stage.

Keywords Salinity tolerance · Screening technique · Flowering stage · Rice · Modified system

Abbreviations

PH	Plant height (cm)
PL	Panicle length (cm)
DAF	Days to 50% flowering
PN	Panicle number/plant
PY	Plant yield (g)
GRAIN	Number of grains/plant
DEG	Spikelet degeneration (%)
STE	Spikelet sterility (%)
FET	Spikelet fertility (%)
HI	Harvest index
SW	Straw weight (g)
EC	Electrical conductivity (dS m ⁻¹)
YSI	Yield stability index
K ⁺	K ⁺ concentration (μg/ml)
Na ⁺	Na ⁺ concentration (μg/ml)
Na–K	Na ⁺ /K ⁺ ratio

Introduction

Rice is one of the most important cereal crops globally. As rice is cultivated in all agro-climatic situations, it is extremely prone to various abiotic stresses causing huge yield loss. Among the abiotic stresses, soil salinity severely affects rice cultivation and its yield worldwide (Molla et al. 2015). Compared to many cereals, rice is considerably sensitive to salinity stress, particularly at early seedling and reproductive stages (Munns and Tester 2008). After the discovery of Saltol-QTL, rice varieties have been developed through introgression of this major QTL, mainly for the seedling stage salt tolerance, which considerably

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diminishes upon attainment of reproductive stage (Islam et al. 2012). To cope with this problem, emphasis should be given to developing an improved cultivar with reproductive stage salt tolerance, so that the yield penalty could be minimized under salt stress. Impressive break through is yet to be made in this direction as we hardly have any highly tolerant source as well as gene/QTL responsible for imparting salt-tolerance at flowering stage.

Large diversity in *Oryza* genome hinted us about existence of differentially salt-responsive genotypes in cultivated varieties and local landraces in all over the world, which need to be explored systematically under controlled condition (Razzaque et al. 2017). The procedure for screening rice genotypes for salt tolerance at seedling stage has been well established and validated through number of experiments (Gregorio et al. 1997; Islam et al. 2012). But, unfortunately only a few protocols for screening salt tolerance at reproductive stage are currently in use, which are based on both in situ field evaluation and ex situ evaluation under controlled condition (Gregorio et al. 1997; Rao et al. 2008; Chattopadhyay et al. 2013; Vispo et al. 2015; Ahmadzadeh et al. 2016). Due to long growing period of rice plants and complexity in measuring tolerance level, no protocol has been found ideal for high throughput screening till date. Solution culture though creates uniform salinity stress but cannot maintain pH without intense intervention during the entire long growth period of plant. Moreover, results derived from hydroponic studies were found to be rarely reproducible in field condition. In contrary, the soil medium in pot culture experiment, is preferred for its higher buffering capacity (Federer and Hornbeck 1985), but also suffers from the problem of cumulative salt accumulation with time and low uniformity of soil EC, which hinders in getting reproducible screening results.

Due to adverse effect of climate change, development of cultivars resilient to different abiotic stresses had become very important. For this, high throughput screening/phenotyping of large number of germplasm and mapping population for identification of tolerant donors and QTLs/genes, respectively, is critical. In a recent study, among different available methods, hydroponics with sand was found most effective for large scale screening of germplasm for tolerance to low phosphorous stress (Yugandhar et al. 2017). Similarly, modification of screening for germination stage oxygen deficiency stress, frequently occurred in direct seeded rice, facilitated in identification of molecular and physiological clues for the tolerance against this stress (Vijayan et al. 2017). In the present study, we developed and standardized a protocol through modification of soil medium to attain higher uniformity and stability of salt stress level ideal for evaluating rice genotypes for prolonged period as required for reproductive

stage salt-tolerance studies. Here, we compared the existing standard protocol with our proposed method, which indeed found to be superior in terms of uniformity and stability in salinity stress and clarity in screening results at defined stress level. The key questions asked in the present study are (1) whether our proposed ‘modified screening method’ is superior than the existing one? (2) how best the modified method can sustain longer duration salt tolerance studies?, which was assessed using suitable selection criteria and salt-tolerant germplasms sources grown in coastal areas.

Materials and methods

Plant materials

For standardization of screening protocol three late maturing photosensitive rice varieties viz. Kalaputia, Ravana, and Sabita were taken for evaluation in *wet season* of 2014. Ravana was found tolerant to salinity stress at seedling stage, and Sabita and Kalaputia were tolerant to waterlogging in coastal areas of eastern India. For validation of the protocol, 7 genotypes (Pokkali:AC41585, Chettivirippu:AC39389, Dinkiasali, Luna Sankhi, Binadhan 10, Binadhan 8 and Naveen) with varying level of salt tolerance at flowering stage were grown in *dry season* of 2015. These genotypes belonging to the same maturity group (120–125 days) were selected from preliminary evaluation (data not shown) of 100 germplasm, collected from coastal saline areas, at simulation tank with $EC = 8 \text{ dS m}^{-1}$. For large scale validation of modified protocol, one hundred eighty-two BC_3F_5 lines belonging to a backcross population from IR64/Pokkali (AC41585) were grouped based on their maturity duration and evaluated in the *wet season* of 2015.

Experimental set up

Location and weather

Two experiments were conducted at the net house covered with transparent shading (> 80% light transmission) on the top at the ICAR-National Rice Research Institute, Cuttack (20.5°N latitude and 85.83° E longitude). The first experiment was conducted in *wet season* of 2014. The salinity stress was imposed before booting (from 12th October) and continued up to grain formation stage (6th December). On the other hand, the second experiment for validation was conducted in *dry season* of 2015. The stress was imposed starting from 13th March and extended up to 18th April. The weather data during the period of salinity stress for each experiment is presented in Supplementary Table 1.

Salinity stress was imposed by mixing NaCl with normal water, as it is the main constituent (80%) of sea water and thereby major salt of coastal saline zones of eastern India. Moreover, similar level of stress impact caused by NaCl solution and sea water was reported in earlier studies (Matoh et al. 1986).

Standard procedure

In the present study, we compared the standard salinity screening method with the modified method proposed by us using standard evaluation criteria. In the standard set up, plastic pots (12-inch diameter) were perforated using a drilling machine to create holes uniformly distributed to the side walls of the pot. The diameter of each hole was approximately 0.5 cm and the distance between two successive holes were kept 2–3 cm. One tightly netted nylon mess was fitted inside the pot and filled with well ground soil. Perforated pots were kept in plastic water tank of 0.5 m height and 1 m diameter. Standard procedure (Gregorio et al. 1997; Bhowmik et al. 2007) with requisite modifications was followed to salinize potted plants.

Modified procedure

In our modified setup, the composition of the growing medium was modified over and above the existing soil medium (Fig. 1). Here, 20% volume of the soil was substituted by three different sizes of gravels. The gravels were categorized as small (2–3 mm in diameter), medium (6–8 mm in diameter) and large (10–15 mm in diameter). After using nylon mess in perforated pot, one layer of large followed by one layer of medium and small gravels were placed one after another at the bottom of the pot. One layer of sand was placed atop of the gravels. One perforated pipe (piezometer) was placed inside the soil with its opening outside the soil zone. The rest of the pot was filled with well ground soil mixed with medium size gravels. The perforated pots were placed over a plastic frame kept on plastic bath to allow water to go inside the pots from the

bottom. Water from saturated soil was collected inside the piezometer. Regular in situ monitoring of salinity level of saturated soil extract inside this pipe was done using a hand-held EC meter. Step by step arrangement for development of modified medium, Soil:Stone (4:1) for screening of salinity stress at reproductive stage in rice is presented in Fig. 2.

Planting of seedlings and imposition of salt stress

The level of water in plastic bath was maintained at 2 cm below the soil surface of the perforated pots. For both the standard and modified method, 20–25 days old seedlings of were planted in perforated pots (as mentioned above) under respective experimental conditions. Potted plants were grouped according to the time of flowering (days to attain 50% flowering). One set of potted seedlings was salinized, while the other set was allowed to grow in normal condition in the net-house till the grain filling stage. Each set was having five replications for each treatment condition. Salinity treatment was started at three weeks prior to flowering, so that desired level of salt stress could be imposed well before booting (Fig. 3). For salinization, NaCl was dissolved to tank water to make water EC of $\sim 8 \text{ dS m}^{-1}$ and salt water was allowed to enter the porous pots to saturate the soil medium.

Nutrient management

The N:P:K was applied in pots at the rate of 100:50:50 kg/ha. For this, required amount of ammonium sulphate (NH_4SO_4) and potassium hydrogen phosphate (KH_2PO_4) were dissolved in water so that each pot could be fertilized with 30 mL solution. Half of the amount of NH_4SO_4 was applied at the time of planting and the rest amount was applied before panicle initiation.

Estimation and analysis of agro-physiological parameters

Agro-morphological parameters were recorded from each plant under two sets of experimental condition. Spikelet sterility (STE) was calculated by dividing number of unfilled grains to the total number of spikelets and designated in percentage. Vestiges of rudimentary rachis-branches left on the panicle were counted as degenerated spikelets (DEG) at maturity and expressed in percentage of the total spikelets (Saha et al. 1998). Yield and yield attributing traits were measured. Tolerance to salt stress at reproductive stage was estimated through Yield Stability Index (Bouslama and Schapaugh 1984) for yield and other important morpho-physiological traits using the following formula.

Fig. 1 Perforated pot and schematic arrangement of gravels, sand, soil and piezometer inside the perforated pot

Fig. 2 Step by step arrangement for development of medium, soil:stone (4:1) for screening salt stress at reproductive stage in rice

Fig. 3 Salinity stress ($EC = 8 \text{ dS m}^{-1}$) imposed on plants before booting in modified soil:stone (4:1) medium in wet season 2014

$$YSI \text{ (for plant yield)} = \frac{\text{Yield}_{\text{under stress}}}{\text{Yield}_{\text{under non-stress}}}$$

Na^+ and K^+ concentration in flag leaf, detected using Flame photometer by standard procedure (Yoshida et al. 1976). Statistical analysis was done using SPSS v.12 software and the least significant difference ($p < 0.05$) was considered to be statistically significant.

Results and discussion

Selection of screening medium and level of salinity stress

There were broadly three different methods and medium practiced for evaluating rice genotypes for salt tolerance at flowering stage. Out of them, one was hydroponics culture using nutrient medium with EC of 6 dS m^{-1} , where stone (gravels) were kept only to support the plants (Mohammadi et al. 2011). In this case, creating uniformity of stress was found to be easier, but there were problems in stabilization of pH, which can significantly affect the availability of nutrients for growing plants till its physiological maturity. Moreover, it is quite difficult to replicate the results obtained using hydroponics or in field conditions. On the contrary, another screening technique is being practiced

directly in target field situation, by irrigating the field with saline water (Iqbal et al. 2015). But, here the problem lied with uniform creation and maintenance of soil EC, even in a very small plot of 2 m × 2 m size. Therefore, reproducibility of results was very poor. The third and the most convincing option till date, was first standardized by Gregorio et al. (1997), using fertilized soil in perforated pots salinized by salt water. This method was found relatively more acceptable as it could more efficiently simulate salinity stress at targeted physiological stage (Bhowmik et al. 2007). Here, we propose a further modification of the method originally proposed by Gregorio et al. (1997) to obtain considerably better results (Fig. 2). One important modification is to use a perforated pipe (piezometer), which is kept in the centre of the pot inside the soil with its opening outside soil layer, which enabled us to regular in situ monitoring of EC and pH level to our advantage without disturbing soil of the pots for regular sampling. In the present study, the EC level was fixed at 8 dS m⁻¹ for screening at reproductive stage as the grain yield, panicle and 1000-grain weight were significantly reduced as the salinity increased from 4 to 6 dS m⁻¹ (Zaman et al. 1997). More so, in natural condition soil EC more than 6–7 dS m⁻¹ is very rarely found at flowering stage in the coastal rice growing regions. Bhowmik et al. (2007) and Razzaque et al. (2017) used perforated pots with fertilized soil salinized with saline water and EC was maintained at 6 dS m⁻¹. Rao et al. (2008) screened rice genotypes of varying tolerance (tolerant, semi-tolerant and sensitive) level in saline soil of electrical conductivity, ECe 4 and 8 dS m⁻¹ and found that grain yield was reduced by 26.7, 45.7 and 50.3% at EC 8 dS m⁻¹ in these three tolerance groups, respectively. Iqbal et al. (2015) also used salinized

soil prepared by adding salt water with EC 8 dS m⁻¹. We have also identified tolerant germplasm with QTL linked markers for salt tolerance at flowering stage using 8 dS m⁻¹ NaCl water (Chattopadhyay et al. 2013). To validate our protocol, rice genotypes for salinity tolerance at flowering stage were screened using saline water having EC of 8 dS m⁻¹, imposed before booting stage.

Buffering capacity of Soil:Stone (4:1) medium

It is well known that soil as a mixture of buffered systems, can neutralize the acids by bonding H⁺ and bases by the release of H⁺ (Federer and Hornbeck 1985). Therefore, soil medium is always preferable over hydroponics, simply because of better buffering ability. But, this can just be reversed for uniformity and stability of salinity stress. Hence, in the present study, we had modified the medium with the aim of generating more uniform nutrient availability and stabilization salt stress in the soil medium. From our earlier experiments, we observed that 20% soil when substituted by stone there is no significant differences of buffering capacity, but a significant improvement of porosity in the medium was observed. In the modified protocol, twenty percent of soil was substituted by chemically inactive gravels (stone) at the bottom of the perforated pot. Higher level of pH in gradually increasing order with significant fluctuation was observed in the tub water mixed with NaCl. But interestingly, in both the pots having both soil and Soil:Stone (4:1) culture, the pH level was maintained at neutral level (around 6.5) without significant fluctuations (Fig. 4). No significant difference ($p > 0.05$) was observed between the two media for mean pH value. This again indicated that the modified medium is as efficient as normal

Fig. 4 Graphical presentation of pH values in tub and individual pots starting from salinity treatment with EC = 8 dS m⁻¹ in soil and soil:stone (4:1) (soil + stone) medium in wet season 2014

Fig. 5 Graphical presentation of changes of piezometer readings (EC) starting from the day of allowing the salt water to enter in perforated pots in soil and soil:stone (4:1) in a *wet season* 2014 and **b** *dry season* 2015

soil medium to maintain stable pH during longer stress period, an advantage over the hydroponics culture for screening genotypes at later stages of growth.

Faster attainment of desired stress level and uniformity in maintaining that in Soil:Stone (4:1) medium over soil alone medium

The results obtained from the present study (*wet season* of 2014), clearly depicted that on an average the Soil:Stone

(4:1) medium took 7 days (from starting of the treatment), to attain the desired salinity level ($> 8 \text{ dS m}^{-1}$), whereas soil medium took more than 10 days to attain similar stress level (Fig. 5a). When the experiment was repeated with another set of genotypes in *dry season* of 2015, the results showed considerable and consistent statistically significant ($p < 0.05$) difference in time of attaining desired salinity stress level in two methods. Here, the Soil:Stone (4:1) medium took an average ~ 8 days, while soil alone medium took ~ 11 days to attain the desired EC level

Table 1 Mean values of rice genotypes for different yield related parameters under saline and non-saline condition in soil and soil:stone (4:1) medium in *wet season* 2014

Genotype	Medium	Condition	DAF	PH	PL	PN	DEG	STE	SW	PY	HI
Kalaputia	Soil:stone (4:1)	Saline	119.50	131.33	23.33	6.67	15.57	27.48	29.80	7.73	0.20
		Non-saline	117.67	144.33	24.06	21.83	0.00	13.57	41.12	37.78	0.48
	Soil	Saline	122.50	127.33	21.61	13.50	6.77	24.74	36.90	7.65	0.18
		Non-saline	120.50	146.17	23.44	22.83	0.00	25.84	64.57	53.45	0.45
	Mean		120.04	137.29	23.11	16.21	5.58	22.91	43.10	26.65	0.33
Ravana	Soil:stone (4:1)	Saline	125.00	144.33	20.94	7.17	8.26	43.46	34.58	10.05	0.21
		Non-saline	119.50	158.33	23.06	14.67	0.00	15.85	50.37	45.37	0.48
	Soil	Saline	143.33	151.17	20.28	7.67	6.75	52.71	56.70	4.22	0.07
		Non-saline	124.00	158.17	23.00	15.00	0.00	21.39	57.87	44.93	0.42
	Mean		127.96	153.00	21.82	11.13	3.75	33.35	49.88	26.14	0.30
Sabita	Soil:stone (4:1)	Saline	124.17	144.50	23.67	7.67	6.32	33.88	28.42	10.30	0.27
		Non-saline	121.33	160.67	24.67	19.00	0.00	15.23	45.70	45.88	0.50
	Soil	Saline	145.17	155.67	22.61	4.83	10.78	43.39	53.10	3.52	0.06
		Non-saline	127.17	163.33	24.11	19.83	0.00	10.66	48.75	46.63	0.48
	Mean		129.46	156.04	23.76	12.83	4.27	25.79	43.99	26.58	0.33
Mean	Soil:stone (4:1)	Saline	120.67	140.53	22.58	7.73	9.05	28.72	30.78	10.42	0.26
		Non-saline	120.33	154	23.95	17.87	0.00	14.98	46.5	44.48	0.49
	Soil	Saline	135.46	145.13	21.67	8.87	8.51	40.11	46.97	6.95	0.14
		Non-saline	124.06	154.86	23.42	19.07	0.00	14.74	56.95	47.55	0.46
CV	Soil:stone (4:1)	Saline	3.49	3.20	6.75	33.92	27.87	20.83	24.55	22.73	18.62
		Non-saline	1.91	4.8	6.02	15.68	0.00	21.66	25.25	18.41	13.02
	Soil	Saline	4.76	5.2	10.42	35.87	50.31	31.59	24.87	20.20	21.47
		Non-saline	2.1	6.7	5.42	16.39	0.00	28.24	24.83	20.11	10.12
General mean			125.13	148.73	22.91	13.38	4.39	24.64	45.30	27.35	0.34
SD			5.49	11.32	1.79	3.35	3.87	10.47	10.94	6.58	0.05
CV			4.40	7.60	7.80	20.10	24.00	18.50	20.10	14.00	0.11

PH plant height (cm), PL panicle length (cm), DAF days to 50% flowering, PN panicle number/plant, PY plant yield (g), GRAIN number of grains/plant, DEG spikelet degeneration (%), STE spikelet sterility (%), HI harvest index, SW straw weight (g)

(8 dS m⁻¹) (Fig. 5b). Soil with 20% gravels have considerably higher porosity and therefore, probably saturated much earlier than the soil alone medium. Due to evaporative losses, we observed a gradual increase in the EC level in the tub water, which finally reflected in increase in EC level inside the pot, with average EC of tub water found to be less than the EC in pot, which was mainly due to accumulation of salt inside the soil (Supplementary Fig. 1). But, the accumulation of salt in soil alone medium was relatively higher than the accumulation in Soil:Stone medium (Fig. 5a, b). Moreover, manual adjustment of EC level in pots with Soil:Stone (4:1) is easier and faster than soil alone medium as the EC level in Soil:Stone (4:1) medium was recovered a bit faster than the soil medium. Stabilization of salt stress was relatively improved as found in the present study. The average mean fluctuation of EC level, calculated taking absolute values of differences of readings, was found lower in Soil:Stone (4:1) medium (0.157) than the soil alone medium (0.341). The standard deviation also showed

significant differences ($p < 0.05$) between the two methods (Supplementary Table 2). These observations were validated by the second round of experimentation, where the average mean fluctuation of EC level again found to be lower in Soil:Stone (4:1) medium (0.276) as compared to the soil alone medium (0.406). The standard deviation also found to be significantly higher in soil medium ($p < 0.05$) in soil alone medium (Supplementary Table 3). Based on the consistency of results and faster attainment of desired stress level we suggest, the Soil:Stone (4:1) medium is much improved screening medium with better stabilized soil EC level than the soil alone medium.

Modified Soil:Stone (4:1) medium gives better accuracy, uniformity and reproducibility in long-term salinity stress studies

Analysis of variance (ANOVA) revealed significant ($p < 0.05$) variation for DAF, PH, PL, PN, STE and SW

Table 2 Mean values of rice genotypes for different yield related parameters under saline and non-saline condition in soil and soil:stone (4:1) medium in dry season 2015

Genotype	Medium	Condition	DAF	PH	PL	PN	GRAIN	DEG	STE	PY	SW	HI	Na+	K+	Na-K
Pokkali	Soil:stone (4:1)	Saline	85.00	122.56	26.92	6.11	75.11	22.34	26.91	6.65	10.24	0.37	52.67	78.00	0.70
		Non-saline	81.33	118.67	29.49	6.56	106.33	0.00	11.02	11.67	13.47	0.46	38.67	104.67	0.37
	Soil	Saline	87.33	118.23	27.14	6.11	54.28	26.33	37.93	6.88	11.67	0.33	49.67	47.67	1.05
		Non-saline	82.33	115.44	30.19	6.56	80.82	0.00	17.47	10.91	14.03	0.44	32.33	95.67	0.39
Chettivirippu	Mean		84.00	118.73	28.44	6.33	79.14	12.17	23.33	9.03	12.35	0.40	43.33	81.50	0.61
	Soil:stone (4:1)	Saline	89.00	92.00	24.88	4.67	51.56	21.12	47.04	2.17	10.87	0.17	84.33	46.67	1.80
		Non-saline	85.33	95.73	27.70	6.67	94.33	0.00	17.08	9.43	11.58	0.45	38.33	78.00	0.49
	Soil	Saline	92.00	96.67	24.67	5.78	41.11	25.28	62.93	3.32	10.38	0.20	96.67	42.33	2.29
		Non-saline	85.33	112.00	23.18	9.00	34.00	27.27	62.09	6.34	7.94	0.21	79.67	69.67	0.50
	Mean		87.92	99.10	25.11	6.53	55.25	18.42	47.29	5.32	10.19	0.26	74.75	59.17	1.27
Dhinkiasalli	Soil:stone (4:1)	Saline	94.67	112.00	23.18	9.00	34.00	27.27	62.09	2.12	7.94	0.21	79.67	51.33	1.56
		Non-saline	84.67	104.40	24.27	9.67	83.67	0.00	17.88	8.97	11.84	0.43	33.33	79.00	0.42
	Soil	Saline	96.33	107.00	24.30	7.89	36.67	19.52	60.68	1.73	9.41	0.16	67.00	48.67	1.37
		Non-saline	86.33	107.89	23.56	7.67	85.33	0.00	14.12	9.35	12.81	0.42	35.00	82.33	0.43
Naveen	Mean		90.58	107.82	23.83	8.56	59.92	11.70	38.69	5.54	10.50	0.30	53.75	65.33	0.94
	Soil:stone (4:1)	Saline	94.67	83.56	23.66	5.89	33.22	39.59	54.91	2.61	9.15	0.22	86.33	40.33	2.14
		Non-saline	89.67	101.00	26.71	7.00	94.00	0.00	12.12	11.41	12.83	0.47	28.33	85.00	0.33
	Soil	Saline	97.00	81.33	22.52	6.67	33.33	38.16	67.63	3.80	9.26	0.29	78.00	41.00	1.90
Luna Sankhi	Non-saline	Saline	91.33	102.33	26.63	5.56	93.00	0.00	18.52	11.37	12.13	0.49	29.00	85.33	0.34
		Non-saline	93.17	92.06	24.88	6.28	63.39	19.44	38.30	7.30	10.84	0.37	55.42	62.92	1.18
	Soil:stone (4:1)	Saline	90.33	97.33	23.84	4.56	30.33	34.71	62.31	4.01	9.92	0.29	86.67	46.00	1.89
		Non-saline	83.67	97.07	26.97	6.17	108.67	0.00	13.06	7.43	11.29	0.40	31.00	74.00	0.42
	Soil	Saline	92.00	94.22	25.01	5.67	56.33	32.89	47.08	4.75	11.37	0.29	93.33	46.00	2.03
		Non-saline	85.33	100.11	26.85	6.22	111.67	0.00	15.43	8.40	12.70	0.40	28.67	81.67	0.35
Binadhan 8	Mean		87.83	97.18	25.67	5.65	76.75	16.90	34.47	6.15	11.32	0.34	59.92	61.92	1.17
	Soil:stone (4:1)	Saline	96.33	96.44	26.29	5.22	47.44	19.68	49.55	3.69	8.84	0.29	65.67	47.67	1.38
		Non-saline	93.00	103.33	26.97	6.11	92.33	0.00	13.36	13.39	11.91	0.53	34.00	80.67	0.42
	Soil	Saline	100.00	98.00	23.92	4.89	38.00	19.92	56.66	3.20	9.13	0.26	61.67	48.67	1.27
Binadhan 10	Non-saline	Saline	95.33	103.00	26.56	6.00	105.78	0.00	16.35	12.02	13.48	0.47	31.67	85.00	0.37
		Non-saline	96.17	100.19	25.93	5.56	70.89	9.90	33.98	8.07	10.84	0.39	48.25	65.50	0.86
	Mean		96.17	100.19	25.93	5.56	70.89	9.90	33.98	8.07	10.84	0.39	48.25	65.50	0.86
	Soil:stone (4:1)	Saline	83.00	97.22	26.89	5.11	72.00	11.07	41.39	6.23	9.32	0.36	76.00	47.33	1.61
		Non-saline	78.33	108.44	28.58	6.11	102.33	0.00	14.19	14.13	13.37	0.51	34.00	82.88	0.42
	Soil	Saline	85.00	96.22	25.41	5.11	74.52	14.51	38.49	8.10	9.35	0.35	71.00	48.33	1.46
Mean	Non-saline	Saline	80.00	107.78	29.70	6.33	106.22	0.00	19.87	14.70	14.11	0.51	34.00	88.33	0.39
		Non-saline	81.58	102.42	27.65	5.67	88.77	6.39	28.49	10.79	11.54	0.43	53.75	66.72	0.97
	Mean		81.58	102.42	27.65	5.67	88.77	6.39	28.49	10.79	11.54	0.43	53.75	66.72	0.97
	Soil:stone (4:1)	Saline	90.43	100.16	25.09	5.79	49.06	25.11	49.17	3.71	9.47	0.27	75.9	51.05	1.58

Table 2 continued

Genotype	Medium	Condition	DAF	PH	PL	PN	GRAIN	DEG	STE	PY	SW	HI	Na+	K+	Na-K
CV	Soil	Non-saline	85.14	104.09	27.24	6.89	97.38	0.00	44.10	10.92	12.33	0.45	33.96	83.46	0.41
		Saline	92.81	98.81	24.71	6.01	47.75	25.23	53.06	3.84	10.08	0.27	73.95	46.10	1.63
	Soil:stone (4:1)	Non-saline	86.62	105.11	27.37	6.36	96.48	0.00	17.51	10.96	13.14	0.45	32.25	84.00	0.39
		Saline	2.90	3.77	4.61	17.53	24.04	26.7	15.24	20.60	9.80	14.02	8.45	4.9	10.67
General mean	Soil	Non-saline	1.48	3.12	2.64	9.47	9.60	0.00	17.95	5.69	6.04	4.02	8.37	4.35	5.09
		Saline	7.65	7.86	6.33	20.92	52.59	56.3	20.67	29.03	18.7	15.74	5.45	4.02	6.4
		Non-saline	3.94	3.36	3.15	10.33	15.62	0.00	30.02	10.65	7.63	6.23	9.69	4.49	12.72
SD		88.75	102.04	26.07	6.27	72.68	12.59	33.46	7.35	11.25	0.36	54	66.15	1.00	
		5.81	10.37	2.23	1.38	29.69	14.9	20.58	4.13	2.02	0.11	23.59	20.20	0.68	
		1.6	4.7	4.5	14.7	18.2	36.3	20.3	15.7	10.8	10.0	8.0	8.6	10.4	

PH plant height (cm), PL panicle length (cm), DAF days to 50% flowering, PN panicle number/plant, PY plant yield (g), GRAIN number of grains/plant, DEG spikelet degeneration (%), STE spikelet sterility (%), HI harvest index, SW straw weight (g), K + : K + concentration (μg/ml), Na + : Na + concentration (μg/ml), Na-K: Na +/K + ratio

(Supplementary Table 4) between control and treated plants in wet season 2014. Saline and non-saline treatments differed ($p < 0.05$) for all parameters. When the experiment was repeated with another set of genotypes and taking some additional important traits in *dry season* of 2015, in addition to the earlier traits, ANOVA showed significant ($p < 0.05$) variation for GRAIN, DEG, PY, HI, Na⁺, K⁺ and Na-K ratio in flag leaf (Supplementary Table 5). The interaction between genotypes and condition (saline vs. non-saline) revealed that the tolerant and susceptible varieties differed under stress condition for all parameters except panicle number/plant. Correlation analysis also revealed that PY had high positive association with PL ($r = 0.82$), GRAIN ($r = 0.85$), HI ($r = 0.99$) and K⁺ concentration in flag leaf ($r = 0.66$). PY showed high negative correlation with STE ($r = -0.85$), Na⁺-K⁺ ratio ($r = -0.69$) and Na⁺ concentration in flag leaf ($r = -0.67$). Some of these parameters such as PL, GRAIN, K⁺ concentration and FER/STE, were observed important contributors to sustain yield under salt stress (Shereen et al. 2005; Zeng et al. 2004; Hossain et al. 2015).

For reproductive stage salt tolerance, an exhaustive screening method is indispensable for imposition of salt stress precisely coinciding with time of flowering in studied genotypes. Earlier reports suggested a considerable delay in time of flowering under the influence of salinity stress in rice and other plants. It was also reported that such delay was more pronounced in salt sensitive genotypes as compared to the tolerant ones (Lutts et al. 1995; Ryu et al. 2014; Cho et al. 2017). Sometimes, the genotypic differences for variation in flowering time is so wide that it becomes difficult to compare tolerant and sensitive genotypes in similar experimental setup. In the present study, differences in flowering time reduced significantly in Soil:Stone (4:1) medium as compared to soil alone medium especially for long duration photosensitive genotypes (Table 1) without having significant differences on Na⁺ and K⁺ uptake pattern under saline condition in both the medium. Apart from the cyto-toxic effect of higher tissue Na⁺ build-up under saline condition, non-uniformity of stress imposition process in soil alone medium (as evidenced by more fluctuating EC_{iw} and pH values) contributed more to the delay in flowering time in the studied genotypes, which shouldn't be the case when we specifically focus on studying the salinity effect. We found that high K⁺ concentration and low Na⁺-K⁺ ratio as good indicators of salt-tolerance at flowering stage under saline condition. Variation of data among the replication was considerably higher in soil alone medium than the Soil:Stone (4:1) medium as evidenced by higher CV in soil medium as compared to the Soil:Stone medium in both the experiments for most of the traits (Tables 1, 2). In *dry season* of 2015, the interaction between genotype and

Table 3 Yield stability index of three rice varieties for yield and yield attributing traits in *wet season* 2014 under soil and soil + stone medium and across the medium

Genotypes	Medium	YSI-DAF	YSI-PH	YSI-PL	YSI-PN	YSI-STE	YSI-SW	YSI-PY	YSI-HI
Kalaputia	Soil:stone (4:1)	1.01	0.90	0.96	0.36	1.58	0.69	0.22	0.48
	Soil	1.02	0.90	0.94	0.56	1.91	0.58	0.17	0.43
	Overall	1.01	0.90	0.95	0.47	1.74	0.63	0.19	0.46
Ravana	Soil:stone (4:1)	1.00	0.94	0.89	0.54	2.54	0.71	0.23	0.47
	Soil	1.14	0.97	0.89	0.57	2.38	0.89	0.13	0.25
	Overall	1.07	0.95	0.89	0.56	2.46	0.81	0.18	0.37
Sabita	Soil:stone (4:1)	1.00	0.90	0.98	0.43	1.73	0.58	0.25	0.61
	Soil	1.11	0.95	0.95	0.27	4.21	1.10	0.14	0.22
	Overall	1.06	0.92	0.96	0.35	2.97	0.84	0.18	0.42

YSI-DAF yield stability index for days to flowering, *YSI-PH* yield stability index for plant height, *YSI-PL* yield stability index for panicle length, *YSI-PN* yield stability index for panicle number, *YSI-STE* yield stability index for sterility, *YSI-SW* yield stability index for straw weight, *YSI-HI* yield stability index for harvest index, *YSI-PY* yield stability index for plant yield

medium revealed significant differences for GRAIN, STE, HI, Na^+ , K^+ and Na-K ratio. The coefficient of variance for PY, GRAIN, STE and DEG were significantly higher under stress condition in soil alone medium than the Soil:Stone (4:1) medium (Table 2). Therefore, under stress condition, Soil:Stone (4:1) medium guaranteed better reproducibility of results as compared to the soil alone medium, which was still being used for this kind of studies. Hence, we suggest that the modified Soil:Stone (4:1) medium could contribute to more accuracy in identification of tolerant sources especially in long-term salinity stress studies in rice.

Identification of tolerant genotypes is more precise in Soil:Stone (4:1) medium

In the first experiment, all three genotypes were found susceptible to salt stress at flowering stage in both the medium. This was realized from the low YSI for PY (0.18–0.19), PN (0.35–0.56) and HI (0.37–0.46) (Table 3). The non-significant interaction between genotype and treatment condition (saline/non-saline) for almost all traits also supported the uniformity of reaction of all these susceptible varieties to salt stress at reproductive stage (Supplementary Table 4). In the subsequent experiment for the validation of screening technique, Pokkali (AC41585), Luna Sankhi and Binadhan 10 were found moderately tolerant. The stability index for plant yield of Pokkali (AC41585), Luna Sankhi and Binadhan 10 were 0.60, 0.55 and 0.50, respectively (Table 4). Pokkali and Binadhan 10 were having high K^+ concentration in flag leaf. In addition, among the tested genotypes, highest K^+ concentration and lowest Na^+ concentration and lowest Na^+/K^+ ratio was observed in flag leaf of Pokkali (AC41585). The stability

index for GRAIN also found higher in tolerant genotypes, Pokkali (0.69) and Binadhan 10 (0.70).

Although no significant difference for PY was noted, but SW was found higher and HI was found lower in soil medium than in Soil:Stone (4:1) medium under both saline and non-saline conditions in wet season 2014 (Table 1). Similarly, in dry season 2015, a little higher value of YSI in Soil:Stone (4:1) medium was noted. Due to imposition of uncontrolled, non-uniform and slightly higher level of salinity stress in the soil alone medium, the plants were recorded to have slightly higher vegetative growth, higher biomass, lower harvest index and higher spikelet sterility in this medium. Higher fluctuation in soil saturation with salt particle resulting in stiff increase of soil EC contributed to prolonged vegetative stage in salt stressed plants grown in soil medium, which delayed flowering in some photosensitive genotypes as reported in our earlier experiment, where Kamini did not flower under salt stress in soil medium (Chattopadhyay et al. 2013). In the present study, our modified protocol identified 3 already tolerant genotypes, Pokkali (AC41585), Luna Sankhi and Binadhan with greater degree of tolerance as evidenced from higher values of YSI for PY, HI, GRAIN and lower values of STE.

Conclusion

Taken together the experiments conducted in the present study clearly demonstrated that the modified protocol was much efficient for screening of salt tolerance level of rice germplasm at reproductive stage. Our result suggested that in longer duration salt tolerance studies Soil:Stone (4:1) medium is comparative better in terms of (1) time taken to attain desired level of salt stress (2) uniformity and homogeneity in maintaining the appropriate level of salt

Table 4 Yield stability index of seven rice varieties for yield and yield attributing traits in *dry season* 2015 under soil and soil:stone (4:1) medium and across the medium

Genotypes	Medium	YSI-PL	YSI-PN	YSI-GRAIN	YSI-HI	YSI-PY
Pokkali	Soil	0.91	0.93	0.71	0.80	0.57
	Soil:stone (4:1)	0.90	0.93	0.67	0.76	0.63
	Overall	0.91	0.93	0.69	0.78	0.60
Chettivirippu	Soil	0.90	0.70	0.55	0.37	0.23
	Soil:stone (4:1)	1.06	0.64	1.21	0.96	0.52
	Overall	0.98	0.67	0.88	0.67	0.38
Dhinkiasalli	Soil	0.96	0.93	0.41	0.49	0.24
	Soil:stone (4:1)	1.03	1.03	0.43	0.37	0.19
	Overall	0.99	0.98	0.42	0.43	0.21
Naveen	Soil	0.89	0.84	0.35	0.46	0.23
	Soil:stone (4:1)	0.85	1.20	0.36	0.60	0.33
	Overall	0.87	1.02	0.36	0.53	0.28
Luna Sankhi	Soil	0.88	0.74	0.44	0.72	0.54
	Soil:stone (4:1)	0.93	0.91	0.50	0.74	0.57
	Overall	0.91	0.82	0.47	0.73	0.55
Binadhan 8	Soil	0.97	0.85	0.51	0.56	0.28
	Soil:stone (4:1)	0.90	0.81	0.36	0.55	0.27
	Overall	0.94	0.83	0.44	0.55	0.27
Binadhan 10	Soil	0.94	0.84	0.70	0.69	0.44
	Soil:stone (4:1)	0.86	0.81	0.70	0.69	0.56
	Overall	0.90	0.82	0.70	0.69	0.50

YSI-PL yield stability index for panicle length, *YSI-PN* yield stability index for panicle number, *YSI-GRAIN* yield stability index for number of grain/plant, *YSI-HI* yield stability index for harvest index, *YSI-PY* yield stability index for plant yield

stress for a longer duration of study (especially important for salt-tolerance studies at reproductive stage) and (3) more reproducibility in studied selection trait resulting in more precise phenotyping for reproductive stage salt-tolerance in rice. The protocol was validated over two growing seasons with a total of 10 genotypes, which confirmed better accuracy and better level of uniformity in our modified medium over the existing medium (soil alone) for reproductive stage salt-tolerance screening. The development would greatly facilitate to identify potent genetic resources for reproductive stage salinity tolerance. This modified medium has been employed in the large scale high throughput evaluation of mapping population (Chattopadhyay et al. 2017) for the identification of the QTLs for the reproductive stage salt tolerance in rice. Therefore, this modification of the screening method for salt tolerance at reproductive stage could accelerate resistant breeding for the devastating abiotic stress.

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Authors' contributions KC, AKN, BCM, AP and RKS performed the experiments. KC, AKN, KoC, and RKS discussed the results. KC,

KoC and AKN wrote the article. All authors have approved the manuscript.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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