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SELECTION OF SALT TOLERANT SOMACLONES FROM INDICA RICE THROUGH CONTINUOUS *IN VITRO* AND *EX VITRO* SODIUM CHLORIDE STRESS

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SUMMARY

This study aimed at the selection of salt tolerant somaclones from four Bangladeshi indica rice genotypes through *in vitro* and *ex vitro* NaCl stress applied through step wise and non-step wise methods. Callogenesis was initiated under four different levels of non-step wise NaCl stress (50, 100, 150 and 200 mM) and subsequent plant regeneration was observed under same levels of NaCl stress. Among the four genotypes Binnatoa and IR51491-AC5-4 produced fertile somatic embryos with relatively high NaCl (150 mM) stress. BRRI dhan29 and BRRI dhan40 produced fertile somatic embryos only from 50 and 100 mM NaCl stress conditions. *Ex vitro* (glass house) step-wise NaCl stress was applied to *in vitro* selected somaclones at the seedling (50, 100, 150 and 200 mM) and following booting stages with simultaneous acclimatization. Satisfactory seedling survival was observed for all genotypes up to 150 mM NaCl stress. Advanced flowering was observed mostly for all the genotypes when stress was applied at the seedling stage compared with combined stress at both seedling and booting stages. Fertile SC1 generations were observed for genotype IR51491-AC5-4 and Binnatoa up to 200 mM NaCl stress applied at the seedling and both seedling and booting stages. Information on transmission of somaclonal variation to sexual progeny is required for further exploitation of potentially useful variants.

Key words: **NaCl stress, rice, salt tolerance, somaclones**

INTRODUCTION

The productivity of many agricultural areas are limited by the accumulation of salt in the soils especially in the coastal regions of South and Southeast Asia. In Bangladesh, about 1 million hectare area is affected by varying degrees of salinity in 13 districts. Saline soils present a massive and increasing challenge to plant breeders and agronomists. Traditional breeding strategies might be supplemented by the production of plants by *in vitro* culture and subsequent regeneration of such cells in the presence of salts through selection of salt tolerance. The main problems in this selection process are metabolic adaptation by cultured cells, genotype-dependent characters, a dramatic decrease in the regeneration of embryogenic cultures (Binh and Heszky 1990, Lutts *et al*. 1999), phenotypic changes in regenerated plants and non-expression of salt-tolerant phenotypes in regenerated plants. There is also the difficulty of conserving other characteristics of the original varieties in plants selected for their tolerance to salts. Genetic variation for salt tolerance as defined by parameters such as survival and yield, has been reported in many crop species including rice.

Rice is considered moderately sensitive to salinity, mostly susceptible at the early seedling and flowering stages (Ponnamperuma 1984). Regenerated plants derived from tissue culture should normally result in clones that are phenotypically and genetically identical

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to the progenitor plants. However, in many cases regenerants deviate from the parental type (Brown *et al.* 1991). This phenomenon, widespread for a range of species, was defined as somaclonal variation (Larkin and Scowcroft 1981). *In vitro* culture systems have been exploited to obtain salt-resistant cell lines followed by utilizing somaclonal variation to obtain salt-tolerant rice plants (Mezencev *et al.* 1995, Winicov 1996, Lutts *et al*. 1999, Zhu *et al*. 1999). Somaclonal variation can result in undesirable changes or can be induced as a positive shift to generate new properties (Bregitzer and Poulson 1995).

When regenerated plants are transferred to *ex vitro* condition under standardized environmental variables, they can exhibit non-genetic or epigenetic changes as well as heritable and genetic variation, heritable but reversible (Karp 1991). Theoretically, salt tolerance of individual plants could be correlated with that of its isolated cells and tissues *in vitro* (Tal 1994) but in practice this correlation is not always absolute (Casas *et al.* 1991). Zhu *et al*. (1999) reported that tissue culture-derived plants might not be directly identified as improved in salt resistance/tolerance but need to be selected for one or several cycles. In the present study cells grown in the presence of high NaCl were subsequently differentiated into plantlets under the same or higher levels of NaCl stress. Many studies on regeneration of different salt tolerant plants do not focus on whether the induced salinity tolerance is retained in subsequent generations. In this study, *in vitro* selected materials were assessed at different growth stages by growing them under glasshouse conditions with or without periodic salt-stress. Targeting the condition of Bangladesh this study has been directed to determine the effect of NaCl on the restoration of callus induction, proliferation of cells and regeneration potential of Bangladeshi rice varieties. This study has also been extended to identify and characterize *in vitro* selected somaclones after *ex vitro* salt stress.

MATERIALS AND METHODS

Materials used for in vitro and ex vitro culture: Mature seeds of four indica rice genotypes, namely IR51491-AC5-4, BRRI dhan29 BRRI dhan40 and Binnatoa were chosen for *in vitro* responses to salt stress. Salt stressed regenerants derived from above four indica rice genotypes were used for *ex vitro* screening through NaCl stress. Micro propagated young plantlets (10-14 day old) derived from *in vitro* cultures were used for *ex vitro* screening.

Callus induction and in vitro screening of cell lines through NaCl stress: Twelve sterilized seeds were cultured in plastic Petri dishes (9 cm diameter) containing 25 ml MS2 medium (MS + 2 mgl^{-1} 2,4-D, 3% sucrose and 0.3% phytagel) supplemented with different concentrations of NaCl (0, 50, 100, 150 and 200 mM). Sterilization was done with 70% (v/v) ethanol for 3 min followed by 25 min in commercial bleach followed by three washings with sterile distilled water. Cultures were placed in the dark at 25 ± 1°C. Eight Petri dishes represented 8 replications for each genotype. Callus induction frequency (CIF) was recorded 4 weeks after culture.

Primary calli from each concentration of NaCl were sub cultured on MS1 media (same as MS2 except 1 mg^{-l} 2,4-D) but supplemented with same concentrations of NaCl used for callus initiation. For the proliferation and selection of cell lines subculture was continued with same level of NaCl stress for two or three passages at three week intervals. Callus growth was recorded by visual observation.

Acclimatization and growing conditions in the glasshouse: Phytagel rooted plantlets after removing were transferred to plastic pots (9, 10 or 15 cm diameter) containing loam-based John-Innes compost No. 2. (Arthur Bower's, Firth Road, Lincoln, UK). For individual treatment, pots were placed randomly in large plastic trays maintained with 1-2 cm respective treatments at the bottom of trays. To retain a high humidity (approximately 70%) pots on trays were either covered with ventilated lids for 4-5 days or transferred into a 'Fogging unit' and following transfer, on a 'Mist bench' for 4-5 days, respectively. After acclimatization and survival seedlings from 9-10 cm diameter pots were transferred into 15 cm diameter plastic pots and placed into glasshouse cubicles until the maximum tillering stage. To achieve the flowering stage, plants were transferred into growth cabinets (10-12 plants in the each cabinet)

with controlled environments (25 ± 1^{0}C day and 19 ± 1^{0}C night with 70% humidity). A 10-14 h day/night photoperiod was maintained with no supplementation of artificial light from April to September. Plants were watered twice a day and fed weekly with nutrient solution until the maximum tillering stage.

Nutrient solution and ex vitro salinization procedures: Nutrient solution was from 'Long Ashton Formula' designed by Hewitt (1968) with supplementation of different concentrations of NaCl. In salinization procedure the NaCl concentration of the nutrient solution was raised by 50 mM once every 48 hours to attain the desired maximum concentration after a certain period of time. Plants were subjected to salt stress with NaCl dissolved in the nutrient solution at final concentrations of 0, 50, 100, 150 and 200 mM. In order for osmotic adjustment in stressed seedlings, NaCl concentration was also decreased step-wise, i.e. the defined NaCl concentration was decreased by 50 mM at 2 days intervals until the control condition. Hence, the total exposure of salt stress was 2 days for 50 mM treatment followed by 100, 150 and 200 mM after 6, 10 and 14 days respectively.

Electrical conductivity: Electrical conductivity was measured for nutrient solution supplemented with 0, 50, 100, 150 and 200 mM NaCl which represent 2, 8, 12, 15 and 18 dSm^{-1} respectively.

Salinity stress at different stages of growth: Salt stress was applied to young micro propagated plantlets beginning from *ex vitro* transfer using step-wise method. After imposing salt stress at the seedling stage, surviving plants were allowed to grow without any salt stress. Salt stress was applied again based on calculated booting stage (reproductive phase) of the original materials.

Assessment of agronomic characters at different stages of plant growth: The number of surviving plants was recorded two to three weeks after initial stress conditions. Different survival characteristics were scored on a scale of 1 to 5 in which survival with low shoot-root injury (1), moderate salt injury in roots and older leaves (2), moderate root damage but high salt injury in older leaf or leaves (3), moderate root damage in roots, young leaf or leaves (4), high root damage with high salt injury in both younger and older leaves (5). Plant height was measured from base to top of the flag leaf. Total number of tillers at the flowering stage was recorded. Days to flowering were recorded by the emergence of the panicle tip out of the flag leaf sheath. Emergence continued until 90% of the panicles were out of the sheaths. At harvest, among yield contributing characters, spikelet fertility was recorded on each fertile plant individually. At least three randomly selected samples were assessed on each individual replicated plant. Spikelet fertility (SF) was considered when plants produced at least one fertile grain and was expressed as a percentage (Zhu *et al.* 1999).

Statistical design and data analyses: Completely Randomized Design (CRD) with factorial arrangements was used in both laboratory and the glasshouse experiments. Data were analysed using the Genstat 4.1 Windows Computing Programme. Level of significance (P value) was determined using the standard analysis of variance (ANOVA). Differences among mean values were assessed by LSD test.

RESULTS AND DISCUSSION

Effect of NaCl on callus induction of different rice genotypes: The effect of NaCl on callus induction of mature rice embryos are shown in Table 1. Significant differences in callus induction frequency among genotypes were recorded and among these genotypes Binnatoa exhibiting the best response at 50 mM NaCl stress followed by genotype IR51491-AC5-4 (Table 1). Results in experiments with MS basal salts supplemented with 50, 100, 150 and 200 mM NaCl showed a declined callus induction with increasing salt concentration. BRRI dhan29 and BRRI dhan40 did not produce any callus with 200 mM NaCl stress. Similar trend of responses observed in an earlier study (Karim and Zapata 1994). There are also instances when the relative increase in salt in the induction and following subculture media, the callus growth was rapidly reduced (Arzani and Mirodjagh 1999, Shankhdhar *et al*. 2000). The present study supported this view because NaCl had an inhibitory effect on growth of callus with increasing NaCl concentrations indicating that the inability of plant cells and tissues to adjust with incremental increases of salt over sufficient time periods might be due to osmotic or ionic shock.

Table 1. Callus induction frequency (CIF) of four indica rices on MS based callus induction medium supplemented with different concentrations of NaCl. Values are mean ± SEM, n=3. Two way ANOVA was carried out; there was significant interaction between genotype and salt concentrations where P <0.001 and LSD at 5% (Genotype x salt concentrations) = 12.52.

	NaCl concentration (mM)				
Genotype	**0 (Control)**	**50**	**100**	**150**	**200**
BRRI dhan29	67.50 ± 7.50	55.0 ± 2.89	25.0 ± 2.89	12.5 ± 7.50	0.0
BRRI dhan40	75.00 ± 2.89	75.0 ± 2.89	50.0 ± 4.08	25.0 ± 8.66	0.0
IR51491-AC5-4	88.25 ± 4.25	82.5 ± 4.79	77.5 ± 2.5	72.5 ± 2.50	40.0 ± 7.07
Binnatoa	98.25 ± 2.50	100 ± 0	77.5 ± 2.50	77.5 ± 4.79	52.5 ± 4.79

At the end of second subculture on MS based callus induction media supplemented with NaCl at 0, 50, 100 and 150 mM, embryogenic calli with compact, whitish-yellow with globular structures (Fig. 1a and 1b) were transferred on MS based regeneration media supplemented with same concentration of NaCl salt. All genotypes performed better regeneration frequency in control regeneration media but regeneration frequency decreased with increased salt concentration (Table 2). Genotype IR51491-AC5-4 and and Binnatoa expressed their regeneration potential under all concentration of NaCl stress in regeneration media (Table 2). BRRI dhan29 and BRRI dhan40 did not produce any shoots in relatively high NaCl containing regenetaion media. In

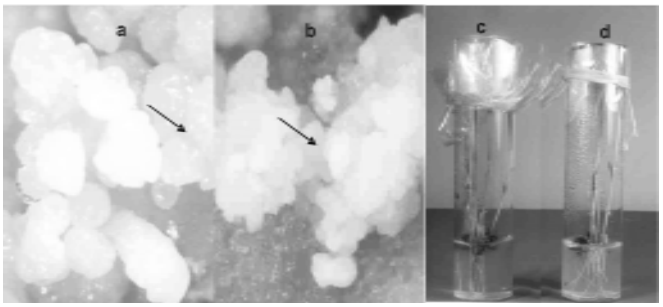

Fig. 1. ***In vitro*** **selection of secondary cell lines under continuous NaCl-stress at 150 mM [(a and b) x 500] concentrations and subsequent plantlet formation in same concentration of NaCl containing regeneration media (c and d). Arrows indicate pale yellow to whitish globular embryo-like structures. Tube diameter: 2.5 cm.**

Table 2. Regeneration frequency (RF) with the number of green shoots per culture (GSpc) in parentheses from 6 week old non-stepwise NaCl (0 to 150 mM) stressed calli of four indica rice genotypes on MS based regeneration media supplemented with 0, 50, 100 and 150 mM NaCl. Values are means ± SEM. There was no significant interaction between genotype and salt concentrations.

Genotype	**Regeneration media (RM) supplemented with different NaCl concentrations (mM)**			
	RM-0 (Control)	**RM-50**	**RM-100**	**RM-150**
BRRI dhan29	86.6 ± 6.2 (7.2 ± 0.8)	46.6 ± 8.1 (0.4 ± 0.1)	13.3 ± 2.1 (0.6 ± 0.1)	-
BRRI dhan40	60.0 ± 4.2 (1.7 ± 0.8)	33.3 ± 5.0 (0.3 ± 0.2)	20.0 ± 3.2 (0.5 ± 0.1)	-
IR51491-AC5-4	83.3 ± 4.1 (5.7 ± 0.9)	53.3± 5.9 (0.6 ± 0.2)	20.0 ± 4.1 (0.5 ± 0.1)	13.3 ± 0.1 (1 ± 0.1)
Binnatoa	93.3± 2.1 (5.0 ± 0.4)	60.0 ± 5.7 (0.8 ± 0.1)	40.0 ±5.2 (0.8 ± 0.1)	20.0 ± 0.2 (1.2 ± 0.1)

earlier reports it was found that salt pre-treatment had positive effect on plant regeneration (Yoshida *et al.* 1983, Karim and Zapata 1994). But in the present study only 50 mM stressed calli showed better regeneration frequency with good number of green plantlets (Table 2). However, it was also reported that the ability for differentiation of salt selected lines was strongly inhibited in the presence of NaCl in the regeneration media (Binh *et al.* 1992, Lutts *et al.* 1999). Many workers (Raval and Chatto 1993, Sahasrabudhe *et al.* 1999) used some catalizing agents (proline, tryptophan, ABA) in regeneration media for better regeneration responses. However, in the present study satisfactory regeneration was found in the presence of retatively high NaCl (100-150 mM) without any supplementation (Fig. 1 c and 1d). Since embryogenic calli were tolerant to many tissue culture systems and embryogenic cells showed the most tolerance in the present study, salt tolerance can be considered a characteristic of embryogenic cells of rice under *in vitro* culture conditions.

The effect of NaCl on in vitro growth of somaclones on a fresh weight basis: Plantlets from all four genotypes were first exposed to 50 mM NaCl stress in liquid plain MS media (MS salts with no auxin) and subsequently to 100, 150 and 200 mM by the step wise method at 5 week intervals. The percentage weight gain was recorded at the end of each stress period. The highest gain was recorded for the genotype IR51491-AC5-4 at 50 mM followed by BRRI dhan29 at 100 mM NaCl. All the genotypes decreased in percentage weight gain as the salt concentration increased except BRRI dhan29. However, BRRI dhan29 also decreased in fresh weight at 200 mM NaCl stress. There was a significant interaction at the 5% level between genotype and salt concentration (Table 3).

The present study showed that NaCl stress does not inhibit shoot and root growth until 200 mM NaCl compared with callus growth and somatic embryo germination indicating that certain adaptive mechanisms of salt tolerance exist at the whole plant level to cope with these condition. Most of the studies made so far on regeneration capacity under *in vitro* salt stress are at the plantlet level but have not been focused whether the induced salinity tolerance is retained at the whole plant level either *in vitro* or *ex vitro* conditions. In contrast, the present study on *in vitro* growth of somaclones under NaCl stress indicates the inheritance of salt resistance from tissue tolerance to whole plant level.

Assessment of seedling survivability after ex vitro step-wise NaCl stress beginning from transfer : The results of seedling survivability were presented in Table 4. In each treatment for all genotypes 5 plantlets with good root system were tested and seedling survivability was recorded according to scale 1-5 (described in materials and method). *Ex vitro* step-wise NaCl stress was applied to *in vitro* stressed somaclones. The highest level of NaCl was similar to *in vitro* step-wise NaCl stress on *in vitro* grown somaclones.

Due to the small number of replicated samples, survival frequency has not been recorded. For all

Table 3. Percentage weight gain of *in vitro* grown somaclones of four genotypes after exposure to NaCl at different concentrations in liquid plain MS (without hormone) media. Two way ANOVA was carried out; there was a significant interaction between genotype and salt concentration where P = 0.05 and LSD at 5% = 62.43.

Genotype	NaCl concentration (mM)			
	50	100	150	200
BRRI dhan29	88.7 ±29.2	181.0 ± 12.9	94.5 ± 41.3	12.8 ± 9.76
BRRI dhan40	98.6 ± 22.0	58.6 ± 10.9	50.0 ± 21.2	16.5 ± 5.48
IR51491-AC5-4	127.1 ± 8.75	62.3 ± 13.1	57.2 ± 12.4	21.3 ± 5.11
Binnatoa	96.5 ±13.2	77.5 ± 53.9	34.4 ± 6.48	22.2 ± 7.94

Table 4. Seedling survivability of NaCl-stressed derived plantlets of four genotypes after different *ex vitro* sequential NaCl stress. Plantlets were selected with *in vitro* sequential NaCl stress (50-200 mM) at the whole plant level.

Highest NaCl level (mM)	BRRI dhan 29		BRRI dhan 40		IR51491-AC5-4		Binnatoa	
	Scoring (Av.)	No. of dead plants	Scoring (Av.)	No. of dead plants	Scoring (Av.)	No. of dead plants	Scoring (Av.)	No. of dead plants
0	1	0	1	0	1	0	1	0
50	1.5	0	2	1	1.5	0	1	0
100	2.3	0	3.3	1	2	0	1.5	0
150	3.3	2	3.5	2	2	0	2	2
200	0	5	4	3	3 6	1	3	2

* *Ex viro* stress was induced beginning from *ex vitro* transfer simultaneously with acclimatization in glasshouse condition. Highest concentration of NaCl reached for respective somaclones under glasshouse condition as they were developed from same levels of NaCl stress under *in vitro* condition.

* Average scoring was recorded for survived plants two weeks after *ex vitro* NaCl stress

'–' scoring was not done due to all plants were dead

genotypes no dead plant was observed for 0-100 mM NaCl stress except for BRRI dhan40 (Table 4 and Fig. 2a). Better survival score was recorded even with high (150 mM) NaCl stress for IR51491-AC5-4 (Fig. 2b) and Binnatoa while BRRI dhan29 and BRRI dhan40 showed high salt injury in older leaf/leaves. No plant survived with 200 mM NaCl stress for BRRI dhan29 while highest survival was observed for IR51491-AC5-4 followed by land race Binnatoa. Young seedlings and reproductive stage being more sensitive to salt than germination and tillering (Ponnamperuma 1984). Wilson *et al.* (1996) also reported that rice seedlings are extremely sensitive to salinity or excessive accumulation of soluble salts in salt affected soils. From a physiological point of view, resistance to seedling stage tolerance could be attributed to the restriction of toxic ion accumulation in the younger leaves. Vajrabhya and Vajrabhya (1991) reported that when rice plant showed injury from salt, it exhibited symptoms such as stunted growth, curled leaves and poor root growth which are consistent with the present study. In the present study cultivar differences were also observed in survivability.

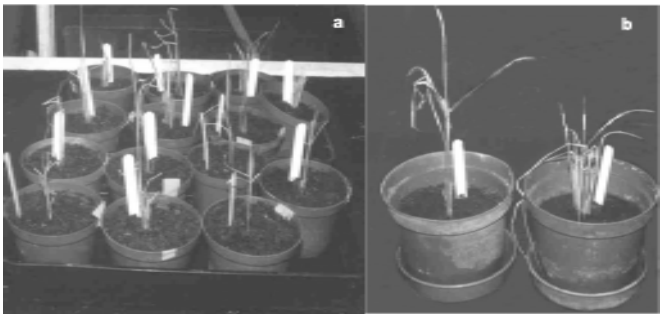

Fig. 2. Seedling survival (a) and salt damage (b) of *in vitro* NaCl stressed somaclones of indica rice genotypes two weeks after *ex vitro* step-wise NaCl stress up to 200 mM. Pot diameter: 10 cm (a) and 15 cm (b), respectively.

Performance of different agronomic characters at harvest: Seedlings which survived under *ex vitro* step-wise NaCl stress (50-200 mM) were allowed to grow or some of them were further subjected to *ex vitro* step-wise NaCl stress applied to the reproductive (either booting or reproductive) stages. Out of four genotypes only IR51491-AC5-4 and Binnatoa produced reasonable numbers of *in vitro* somaclones from NaCl stressed conditions. Therefore, it was possible to design experiments under *ex vitro* conditions only for these two genotypes. Agronomic performances after *ex vitro* NaCl stress at two different growth stages were assessed at harvest period [(Fig. 3 a-d and Fig. 4 a-d].

Since different treatments were induced at two different growth stages, agronomic performances after

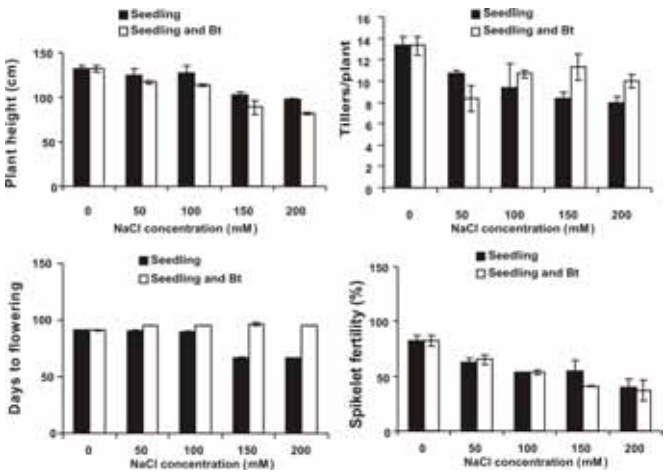

Fig. 3 (a-d). The effect of *ex vitro* NaCl stress on different agronomic characters applied step-wise either at the seedling or both seedling and panicle initiation (PI) stages of a somaclonal line derived from IR51491-AC5-4 from induction to regeneration stage stress with 150 mM NaCl stress. Bars represent standard error of mean.

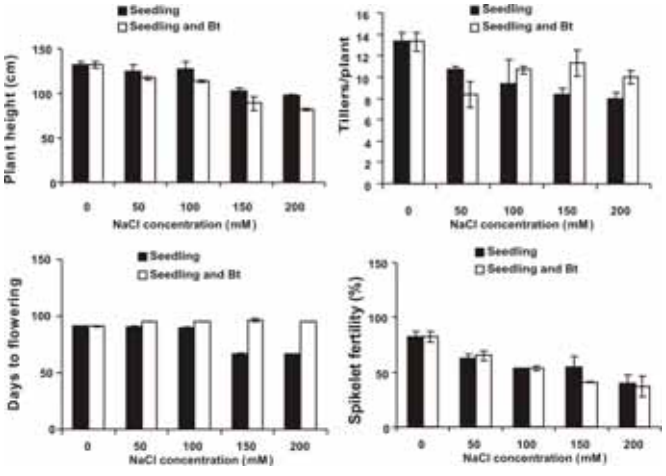

Fig. 4. (a-d). The effect of *ex vitro* NaCl stress on different agronomic characters applied step-wise either at the seedling or both seedling and booting (Bt) stages of a somaclonal line derived from Binnatoa (land race) from induction to regeneration stage stress with 150 mM NaCl stress. Bars represent standard error of mean.

each NaCl stress treatments were assessed at harvest period. For both genotypes, plant height reduced after *ex-vitro* stress applied at the seedling and both seedling and reproductive stages compared with no *ex vitro* salt stress (Fig 3a and 3b). Tiller number also decreased in both the genotypes but IR51491-AC5-4 showed good number of tillers even at 200 mM NaCl stress. This indicates that salt treatment did not affect tiller number when stress was applied at the reproductive stages.

Advanced flowering was observed for all the treatments in case of genotype IR51491-AC5-4 when stress was applied only at seedling stage compared with control (Fig. 3c). In contrast, approximately 3 weeks advanced flowering was observed for genotype Binnatoa when high NaCl stress applied at the seedling stage (Fig. 4c). Flowering time did not change much when stress was applied at the reproductive stage for both the genotypes (Fig. 3c and 4c). This indicates that imposing stress at early growth stage induces signals for early flowering and completion of life cycle. Spikelet fertility was severely affected for genotype IR51491-AC5-4 (Fig. 3d) than Binnatoa (Fig. 4d) with high NaCl stress applied at the booting stage. Spikelet fertility was also less for genotype Binnatoa but similar spikelet fertility trends was observed after different NaCl stress at seedling and both seedling and booting stage. The results indicate the existence of genetic variability for salt tolerance in these rice genotypes as indicated by others for different crops (Karp 1995, Lutts *et al.* 1998). It could be hypothesized that the responsiveness of plants to salt tolerance is linked with parent material as well as a feature distinct to *in vitro* culture for different lines. Surprisingly true that spikelet fertility was not remarkably reduced when 200 mM NaCl stress was applied at the seedling stage as compared with control (Fig. 4d). It is thus evident that after recovering from stress period plants attempt to mobilize all it's available resources to produce fertile seeds and complete their life cycle.

Comparing the overall performance of both the genotypes, IR51491-AC5-4 was better in case of plant height, spikelet fertility while Binnatoa showed early flowering suggested underlying somaclonal variation for individual characters. Early flowering was also observed by Khatun and Flowers (1995). The earlier the flowering the shorter the growth cycle with good spikelet fertility resulted the better behaviour of these somaclonal families in the presence of relatively high NaCl (150-200 mM) stress. This result supported the view of Winicov (1996) that useful somaclonal lines could be identified among materials regenerated from cultured cells of rice. In the

current study spikelet fertility always decreased when salinity stress was imposed at the booting stages after periodic seedling stage stress. This finding was similar to the study conducted by Asch and Woopereis (2001). It is important to screen and evaluate the full spectrum of genetic variability available for tolerance to salt stress.

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