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Performance of *in vitro* selected NaCl-tolerant regenerants of four indica rice genotypes on further exposure to NaCl stress in glasshouse conditions

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ABSTRACT

In vitro selected NaCl-tolerant regenerants of four indica rice genotypes namely IR51491-AC5-4, BRRI dhan40, Binnatoa and BRRI dhan 29 were assessed in glasshouse condition by estimating their survival and comparing five agronomic characteristics at maturity after ex vitro step-wise NaCl stress compared with their original parents. Step-wise NaCl stress (50-200 mM) was applied in nutrient solutions by adding 50 mM NaCl at 2 day intervals at the seedling, both seedling and panicle initiation, stages. Two types of regenerants, derived from continuous step-wise post induction to regeneration stages (SC1a) and continuous step-wise induction to regeneration stages ($SC1_b$) were used in different experiments. Seedling survival was more for regenerants type $SC1_b$ compared with seed derived seedlings. Regenerants derived from Binnatoa with non-step-wise NaCl stress (150 mM) yielded fertile SC1 plants after exposure up to 200 mM NaCl stress applied at the seedling and booting stages. Salt stress resulted in advanced flowering. Salinity strongly increased sterility, particularly when applied at the panicle initiation stage whereas plants recovered best from stress induced at the seedling stage. The extent of somaclonal variation was observed among same type of somaclonal lines when same levels of stress were applied at different growth stages.

Key words: *Ex vitro, in vitro, NaCl-tolerant, regenerants, indica rice*

Regenerated plants derived from tissue culture should normally result in clones that are phenotypically and genetically identical 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 (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).

Most commonly, resistance like salinity is sought by imposing the selection pressure under controlled or natural environments. Entries showing the lowest mortality or least obvious damage are selected under saline environments (Yeo and Flowers, 1984). When regenerated plants are transferred to *ex vitro* condition under standardized environmental conditions, they can exhibit non-genetic or epigenetic changes as well as heritable and genetic variation (Karp, 1995). Theoretically, salt tolerance of individual plants could be correlated with that of its isolated cells and tissues *in vitro* (Tal, 1984) 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 this study, *in vitro* selected materials were assessed at different growth stages by growing them under glasshouse conditions with or without periodic salt-stress. Seedling survival was examined mainly with step wise salt stress compared with the original parental materials. Agronomic characters of *ex vitro* salt-stressed materials were compared with the original parent materials grown under *ex vitro* control conditions.

MATERIALS AND METHODS

Salt stressed regenerants derived from four indica rice genotypes namely, IR51491-AC5-4, BRRI dhan40, Binnatoa, BRRI dhan29 were used for *ex vitro* screening through NaCl stress. Two types of *in vitro* selected lines were screened under glasshouse conditions: a) somaclones derived from post-induction stress (continuous NaCl stress in subculture to regeneration media) following culture on salt-free shoot culture media (2-3 weeks) were further selected by an *in vitro* liquid sequential salinization method at the whole plant level, designated as type $SC1_a$; b) somaclones derived from induction to regeneration stage stress but without any *in vitro* salt stress at the whole plant level, designated as type $SC1_b$. Seed-derived plants were grown in glasshouse conditions for comparison.

Nutrient solution from 'Long Ashton Formula' designed by Hewitt (1968) with supplementation of different concentrations of NaCl was used for the study. In step-wise salinization, NaCl concentration of the nutrient solution was raised by 50 mM once every 48 h. In non-step wise salinization, stress commenced from the callus induction stage up to plant regeneration with 50, 100, 150 and 200 mM NaCl, respectively. Non-step wise salinization was mostly used under *in vitro* condition, whereas step-wise salinization was used under both *in vitro* and *ex vitro* conditions. In order to make osmotic adjustment in stressed seedlings, NaCl concentration decreased step-wise by 50 mM at 2-day intervals until it reached the level of the control and salinization continued for a week.

After removing phytagel, rooted plantlets were transferred to plastic pots (9-10 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 (~ 70%), pots on trays were either covered with ventilated lids for 4-5 days or transferred into a 'Fogging unit' followed by transfer on a 'Mist bench' for 4-5 days. After acclimatization, seedlings from these pots were transferred to bigger plastic pots (15 cm diameter) and placed into glasshouse cubicles until maximum tillering stage. To induce flowering, plants were transferred into growth cabinets (10-12 plants in each cabinet) with controlled environment (25/19 ± 1^0C day/night temperature 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.

In the case of original parents stress was applied at germination followed by one week acclimatization in glasshouse conditions. Young plantlets, or mainly micro propagated axillary shoots (with two or three leaves) with minimum root systems were transferred to a glasshouse. Salt stress was applied 10-14 days after acclimatisation of seedlings of both type of regenerants ($SC1_a$ and $SC1_b$). After imposing salt stress at the seedling stage surviving plants were allowed to grow without any salt stress. Salt stress was applied again at reproductive phase.

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 and days to (50%) flowering were recorded. At harvest, among yield contributing characters, number of spikelets and spikelet fertility were recorded on each fertile plant individually. Spikelet fertility (SF) was considered when plants produced at least one fertile grain and was expressed as a percentage (Zhu *et al.*, 1999).

RESULTS AND DISCUSSION

All genotypes showed better survival with 50 mM NaCl stress compared with other treatments. Similarly, all seed-derived control plants survived under 50 mM stress except one control plant from BRRI dhan40 and BRRI dhan29 (Table 3). *In vitro* 200 mM NaCl stressed somaclones survived after *ex vitro* step wise NaCl stress until 200 mM for all genotypes except BRRI dhan29 (Table 1). However, for control plants the survival was noticed only in Binnatoa with 200mM NaCl stress (Table 3). This is in conformity with earlier findings (Narayanan and Sree Rangasamy 1989). They observed that only 25% or less of the seedlings were

resistant in R1 progeny (1st generation) lines, suggesting a recessive bias for the mutation conferring salt tolerance.

Micropropagated shoots were derived from the induction to regeneration stage stress with 150-200 mM NaCl for all genotypes except for genotype BRRI dhan 29 with 100 mM NaCl stress (Table 2). Somaclonal lines involved in this experiment showed better survival with *ex vitro* NaCl stress compared with control plants (Table 3) and somaclones type SC1a (Table 1). Genotype IR51491-AC5-4 and Binnatoa showed better survival with high (200 mM) *ex vitro* NaCl stress (Table 2), whereas no plants survived under the same level of NaCl stress imposed on control parental plants (Table 3). Both genotypes produced reasonable numbers of *in vitro* regenerants ($SC1_b$) from NaCl stressed conditions. The other two genotypes produced fewer of the same type of regenerants ($SC1_b$). One promising line was generated from the salt sensitive BRRI dhan29 and others from BRRI dhan40 by non-step-wise induction to regeneration stage stress at 100 and 150 mM concentrations, respectively. *Ex vitro* NaCl stress was applied step-wise (50-200 mM) at the seedling stage for both genotypes. In the case of genotype BRRI dhan40 seedlings survived from 50-150 mM NaCl stress and showed good recovery from 150 mM NaCl stress (Table 2). In the case of BRRI dhan29 moderate to high salt damage was observed one week after step-wise NaCl (50-200 mM) stress treatments although seedlings were survived upto 150 mM NaCl stress . Miki et al. (1999) suggested that *in vitro* salt tolerent rice plants grown in saline condition improved further tolerence to salinity by inducing the capability to maintain relatively low, non lethal concentrations of Na^+ and Cl^- ions in the leaves which

Table 1. Seedling survival of post-induction step wise NaCl-stressed derived plantlets ($SC1_a$) of four genotypes. Plantlets were further selected with *in vitro* step wise NaCl stress (50-200 mM) at the whole plant level.

Highest	IR51491-AC5-4		BRRI dhan40		Binnatoa		BRRI dhan29	
NaCl level (mM) reached	Number of plants tested	Scoring (Average)	Number of plants tested	Scoring (Average)	Number of plants tested	Scoring (Average)	Number of plants tested	Scoring (Average)
0	4	1.5	4	2.3	4	2	4	2
50	4	1.5	4	2 (1 dead)	4	2 (1 dead)	4	1.5 (1 dead)
100	4	2 (1 dead)	4	3 (1 dead)	4	2 (1 dead)	4	2.3 (1 dead)
150	4	2	4	3 (2 dead)	4	3 (2 dead)	4	2.3 (1 dead)
200	4	3 .5 (2 dead)	4	4 (1 dead)	4	3 (1 dead)	4	dead

· Sequential *ex vitro* stress was induced two weeks after *ex vitro* transfer. 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 two weeks after *ex vitro* NaCl stress.

Table 2. Seedling survival of regenerants ($SC1_b$) derived from induction to regeneration stage conditions followed by no *in vitro* NaCl stress at the whole plant level of four indica rice genotypes.

Highest	IR51491-AC5-4		BRRI dhan40		Binnatoa		BRRI dhan29	
NaCl level (mM) reached	Number of plants tested	Scoring (Average)	Number of plants tested	Scoring (Average)	Number of plants tested	Scoring (Average)	Number of plants tested	Scoring (Average)
0	4	1	4	2	4	1	4	1
50	4	2.5	4	2	4	1.8	4	1
100	4	3.2	4	2.5	4	2.4	4	2 (2 dead)
150	4	3.5	4	3 (3 dead)	4	2.8 (1 dead)	4	4 (2 dead)
200	4	4.2 (1 dead)	4	4 (2 dead)	4	3.0 (1 dead)	4	dead

Highest concentration of NaCl reached for respective somaclones under glasshouse condition as they were developed from same levels of NaCl stress on *in vitro* condition. Average scoring was recorded two weeks after *ex vitro* NaCl stress.

Table 3. Seedling survival of seed-derived control plants of four indica rice genotypes after *ex vitro* NaCl stress at different concentrations.

Highest	IR51491-AC5-4		BRRI dhan40		Binnatoa		BRRI dhan29	
NaCl level (mM) reached	Number of plants tested	Scoring (Average)	Number of plants tested	Scoring (Average)	Number of plants tested	Scoring (Average)	Number of plants tested	Scoring (Average)
0	4	1.5	4	1	4	1	4	1
50	4	1.6 (2dead)	4	2 (2 dead)	4	1.6 (1 dead)	4	1.6 (2 dead)
100	4	3 (3 dead)	4	2.5 (2 dead)	4	2 (2 dead)	4	3 (3 dead)
150	4	3 (3 dead)	4	4 (3 dead)	4	2.5 (3 dead)	4	3 (3 dead)
200	4	dead	4	dead	4	4 (3 dead)	4	dead

Table 4. Performance of different agronomic characters of *in vitro* step wise NaCl-stressed lines (SC1a) of different genotypes under *ex vitro* NaCl stress at different concentrations applied step wise at the seedling and both seedling and panicle initiation (PI) or seedling and booting (BT) stages. Parenthesis indicates *in vitro* NaCl stress (mM) supplemented with hormone free MS media (P)

Somaclonal lines	*Ex vitro* NaCl-stress at different growth stage	Plant height (cm)	Active tillers plant^{-1} (No.)	Days to flowering	Spikelets fertility (%)
IR51491-AC5-4 (Control)	*Ex vitro* control	94	13	125	51
IR51491-AC5-4-P(50)	Seedling-50 mM	75	8	125	0
IR51491-AC5-4-P (150)	Seedling-150mM and at PI-150mM	90	6	120	0
IR51491-AC5-4-P(200)	Seedling 50 mM and at PI-100 mM	97	4	124	0
IR51491-AC5-4-P(200)	Seedling 150mMand at Bt 200mM	98	6	124	0
BRRI dhan40 (Control)	*Ex vitro* control	103	12	129	68
BRRI dhan40 -P(50)	Seedling 50 mM	120	5	124	0
BRRI dhan40 -P(50)	Seedling 50 mM and at PI-50	111	5	127	0
BRRI dhan40 -P(150)	Seedling 100mM and at PI-100mM	108	5	124	17
Binnatoa (Control)	*Ex vitro* control	107	12	91	60
Binnatoa-P(200)	Seedling 50	130	6	95	32
BRRI dhan29 (Control)	*Ex vitro* control	95	13	131	68
BRRI dhan29-P(50)	Seedling 150 mM	72.5	5	87	77
BRRI dhan29-P(100)	Seedling 150mM and at PI-150mM	84	9	124	30
BRRI dhan29-P(150)	Seedling 150mM and at PI-150 mM	88	6	110	0
BRRI dhan29-P(200)	Seedling 50	83	9	90	0

P (50), P(100), P(150) and P (200) indicates 50, 100, 150 and 200 mM NaCl in MS media with no hormone where somaclones are grown under *in vitro* condition

were consistent with the present study.

Seedlings which survived two weeks after transfer from *ex vitro* step wise NaCl (50-200 mM) stress were further subjected to *ex vitro* step wise NaCl stress applied at either the panicle initiation (PI) or booting (Bt) stages. Somaclonal variations were observed for all agronomic characters in different lines with or without *ex vitro* salt stress. Comparing all agronomic characters with the original parents (Table 3) a mostly negative shift of variation was observed in which spikelet fertility was reduced with different levels of *ex vitro* NaCl stress (Table 4).

The number of panicle bearing tillers decreased when stress was applied at the panicle initiation stage (Table 4). Somaclones derived from BRRI dhan29 also showed less fertile panicles when 150 mM NaCl stress was applied at either the seedling or both seedling and PI stages. Advanced flowering was observed for genotypes BRRI dhan40 and BRRI dhan29 with *ex vitro* salt stress compared with control plants

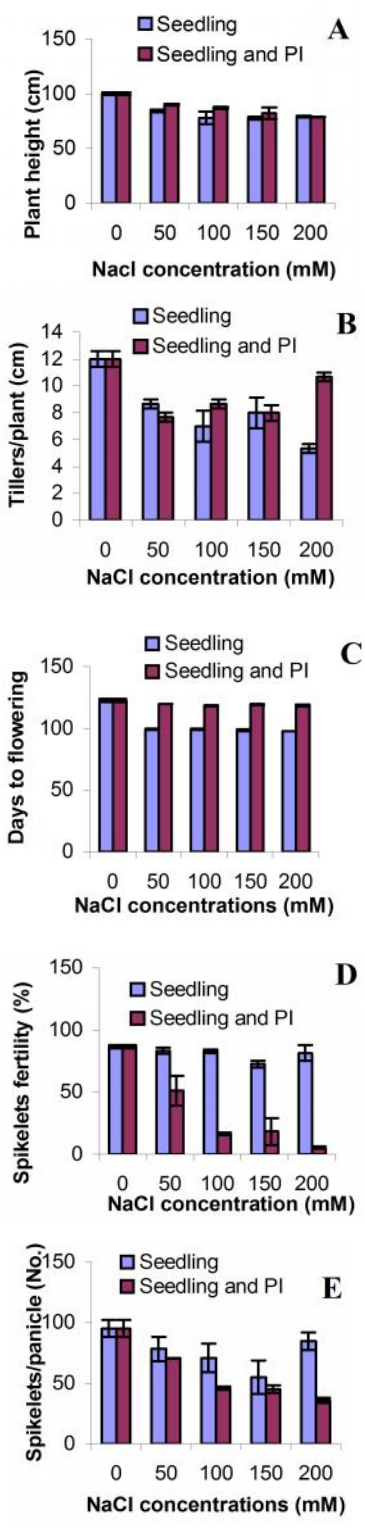

Fig. 1. The effect of *ex vitro* NaCl stress on different agronomic characters applied sequentially either at the seedling or both seedling and panicle initiation (PI) stages of a somaclonal line derived from IR51491-AC5-4 from induction to regeneration stage stress with non-step-wise 150 mM NaCl stress. Bars represent standard error of mean.

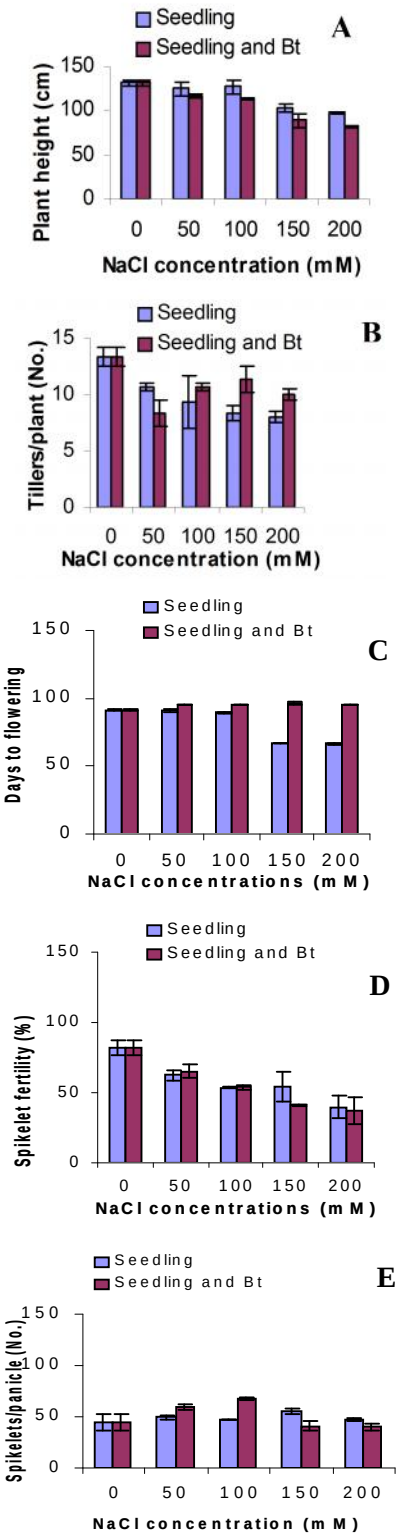

Fig. 2. The effect of *ex vitro* NaCl stress on different agronomic characters applied sequentially either at the seedling or both seedling and booting (Bt) stages of a somaclonal line derived from Binnatoa from induction to regeneration stage stress with non-step-wise 150 mM NaCl stress. Bars represent standard error of mean.

(Table 4). Early flowering was also observed by Taeb *et al.* (1992) and Khatun and Flowers (1995). An improvement was also noted for spikelet fertility in case of BRRI dhan29 when high NaCl stress was applied at the seedling stage only (Table 4). These high yielded somaclonal variants could be further grown for observing inheritance pattern of high-yielding capacity.

Among the four genotypes, IR51491-AC5-4 and Binnatoa produced reasonable numbers of *in vitro* somaclones ($SC1_b$) from NaCl stressed condition. The other two genotypes did not produce sufficient somaclones ($SC1_b$). Plant height and tiller number decreased due to NaCl stress (Fig. 1, 2). Days to flowering also decreased due to salt stress. Spikelet fertility did not decrease especially in IR51491-AC5-4 when stress was applied at seedling stage (Fig. 1D). However, in Binnatoa the decrease of number of fertile spikelets occurred even when the stress was applied at seedling stage (Fig. 2 D). Somaclonal variations result mainly from modifications during callogenesis (Skirvin *et al.*, 1994; Lutts *et al.*, 2001). In the present study somaclonal variation was observed among genotypes and even within different cell lines (derived from different stress conditions) of individual genotypes. For all genotypes tissue culture-derived plants exhibited a higher heterogeneity than those of initial cultivars. In the current study spikelet fertility was always decreased when 100-150 mM salinity stress was again imposed at the panicle initiation or booting stage (Fig. 1D and Fig.2D) after periodic seedling stage stress, a finding similar to that of Asch and Wopereis (2001). The number of spikelets panicle^{-1} either decreased or increased due to salt stress (Fig. 1E, 2E). Zhu et al. (1999) reported that populations differed greatly for the number of spikelets produce per fertile plant which was consistent with the present study. The better behaviour of these somaclonal lines in the presence of relatively high NaCl (150-200 mM) is attributable to an increase in the general vigour of these materials. This confirms the views of Mezencev *et al.* (1995) and Winicov (1996) that useful individual somaclones could be identified among materials regenerated from cultured cells of rice.

One of the hypotheses to explain salt tolerance in the present study is that *in vitro* grown NaCl tolerant regenerants further exposed to salt stress by step-wise method in *ex vitro* condition induces the capability to maintain relatively improved resistance in salt tolerance through seedling survival and spikelet fertility. In conclusion, the present study demonstrates the usefulness of *in vitro* selection techniques to obtain somaclonal variants which could be successfully integrated in a conventional breeding programme through crosses with available cultivars. Information on transmission of variation to sexual progeny is required for further exploitation of potentially useful variants.

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