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BRIEF COMMUNICATION

Salt tolerance of *in vitro* established salt-tolerant rice plants during further growth in soil

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Abstract

In vitro salt tolerant rice plants established by step up treatment with 0.5, 1.0, 1.5 and 2.0 % NaCl at 3-week intervals were examined to determine whether they could grow in potted paddy soil containing 0, 0.55 or 0.75 % NaCl till harvesting. All the control plants were necrotic by the 4th week in the culture. At the 10th week of culture, 100 % of the salt-tolerant plants subjected to 0 or 0.55 % NaCl survived, and 78 % of the plants at 0.75 % NaCl. The Na^+ and Cl^- contents in the leaves of salt-tolerant plants grown at 0.55 and 0.75 % NaCl were about 4 times of those without NaCl. The ion contents in non-tolerant plants and seedling plants were 10 to 12 times of those in 0 % NaCl treatment. One of the hypotheses to explain the present data is that the *in vitro* step up salt selection induces the capability to maintain no lethal concentration of NaCl in the leaves.

Additional key words: adaptation, cellular NaCl concentration, *Oryza sativa* L., step up salt treatment.

Demand for environmental stress tolerant plants is increasing with the increase in human population and environmental problems (*e.g.* Greenway and Munns 1980). When salt-tolerant plants of a given species were established, a procedure to regenerate plants from salt tolerant *in vitro* green cells or calli was attempted. Often regenerants did not keep stress tolerance in the field and the mechanism of salt tolerance of the *in vitro* salt tolerant plants was not reported (Smith and McComb 1983, Chandler and Vasil 1984, Oono and Sano 1986). Miki *et al.* (2001) made 100 % rice shoot bud clumps be 2 % NaCl tolerant plants by step up salt selection. The procedure may be a one to establish salt tolerant plants *in vitro*. However, it has not been examined whether the *in vitro* salt tolerant plants keep tolerant characteristics in soil.

Many papers deal with salt tolerance of young plants and limited papers deal with salt tolerance of reproductive stage of plants. Although salt tolerance of plants from the young stage to the reproductive stage should be determined, limited works were done (Iwaki 1970).

In this paper, the *in vitro* salt tolerant plants from single-seed derived shoot bud clumps of rice (*Oryza sativa* L.) were examined by growing them in saline potted paddy soil till harvesting. To estimate a possible mechanism of salt-tolerance, Na^+ and Cl^- ions were determined in the leaves.

Multiple shoot bud clumps were induced from a single seed of rice (*Oryza sativa* L. cv. Nipponbare) and amplified according to the procedures previously reported (Hisajima 1982, Miki *et al.* 2001). The shoot bud clumps were cultured in the medium containing 0.5 % NaCl in the first 3 weeks and then subcultured successively in 1.0, 1.5 and 2.0 % NaCl medium at 3 week intervals. Each shoot bud clump was cultured for 12 weeks in total and *in vitro* 2 % NaCl tolerant plants were established. Rooting occurred during the culture (Miki *et al.* 2001). *In vitro* non-salt tolerant plants were also established by culturing the above small shoot bud clumps in the same culture conditions except that the medium did not contain NaCl. *In vitro* plants were acclimatized by culturing in potted soil under shade in a greenhouse for a week.

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Seedling plants were prepared in the greenhouse by growing seedlings to be 4 - 5 leaves stage in small pots for 5 weeks. The above *in vitro* salt tolerant plants, *in vitro* non-salt tolerant plants and about 8 cm height seedling plants were used for the experiments.

On Aug. 8, 1999, rice plants were grown in pots containing soil and fertilizer (containing 0.5 g of potassium, 0.5 g of nitrogen and 0.5 g of phosphorus), and 0, 0.55, or 0.75 % NaCl, in the greenhouse till harvest. The plants were watered every day to maintain sufficient water supply and to maintain the same NaCl concentration as in the initial stage. Data were taken at one-week intervals from the 1st to 10th week, and at the harvesting time (12th week).

In the pots containing 0.55 % NaCl, 100 % of *in vitro* salt tolerant plants, 66.7 % of *in vitro* non-salt tolerant plants and 72 % of seedling plants survived at the 1st week. However, at the end of the 2nd week, the percentage survived were 100, 38.9 and 61.1 %, respectively. During the 2nd week, *in vitro* non-salt tolerant plants and seedling plants decolored rapidly and were necrotic, and both of them died off by the 10th week. On the other hand, 100 % of the *in vitro* salt tolerant plants survived at the 10th week (Table 1).

In the 0.75 % NaCl treatment, 100 % of salt tolerant, 33.3 % of non tolerant and 38.9 % of the seedling plants survived at the 1st week, but the percentage of survival decreased to 88.9, 22.2 and 6.7 %, respectively after 2 weeks. During the 2nd week, the *in vitro* non-salt tolerant plants and seedling plants decolored rapidly and were necrotic. In the 10th week all the non-salt tolerant plants died but 77.8 % of the salt tolerant plants survived.

Table 1. Effect of NaCl treatment on growth and yield components. Data were taken at 12th weeks. Means ± SE, n = 12. The same letter in each indicator indicates that there is no significant difference in 5 % significant level.

Plants	NaCl [%]	Leaf area [cm^2]	Survival rate [%]	Culm length [cm]	Ear mass [g plant^{-1}]	Number of tillers [plant^{-1}]	Number of earings [plant^{-1}]	Grain mass [mg]	Number of grains [plant^{-1}]	Ripened grains [%]	Yield [g plant^{-1}]
Tolerant	0.00	51.2±2.7a	100.0a	85.3±3.7a	14.1±32a	10.9±3.2a	10.7±3.5a	22.6±2.2a	648.2±160.0a	82.9±6.8a	36.35±9.5a
regenerants	0.55	40.2±3.7b	100.0a	76.1±5.6b	9.5±1.4b	9.7±2.0b	8.9±2.4b	18.3±1.2a	647.2± 54.9a	74.5±3.9a	26.55±3.9b
	0.75	32.4±2.1c	77.8a	47.14.4c	5.1±0.7c	6.2±1.2c	5.4±1.8c	11.8±0.6b	406.3± 39.6b	51.5±3.7b	7.65±2.1c
Non-tolerant	0.00	50.1±3.5a	100.0a	84.6±4.1a	14.2±2.6a	11.9±2.3d	11.5±2.7d	21.9±0.9a	660.9±134.2a	76.5±7.3a	33.15±7.3a
regenerants	0.55	0.0d	0.0b	0.0e	0.0d	0.0c	0.0c	0.0c	0.0c	0.0d	0.0d
	0.75	0.0d	0.0b	0.0e	0.0d	0.0c	0.0c	0.0c	0.0c	0.0d	0.0d
Seedlings	0.00	47.1±5.3e	100.0a	80.9±4.9a	12.9±1.5a	10.3±1.6a	9.3±2.2b	20.3±2.0a	639.9± 98.9a	77.9±6.5a	30.29±4.7ab
	0.55	0.0d	0.0b	0.0e	0.0d	0.0c	0.0c	0.0c	0.0c	0.0d	0.0d
	0.75	0.0d	0.0b	0.0e	0.0d	0.0c	0.0c	0.0c	0.0c	0.0d	0.0d

The average plant heights, leaf areas, Soil and Plant Analyzer Development (SPAD) number (chlorophyll content), numbers of tillers and earrings, and survival rates of *in vitro* salt tolerant plants, non salt tolerant plants and seedling plants grown in 0 % NaCl treatment at the 10th week were similar suggesting that their growth was similar (data not shown).

The difference in the average plant height between *in vitro* salt tolerant plants grown at 0 and 0.55 or 0.75 % NaCl were high (10 and 20 cm, respectively). The average leaf area, SPAD number, number of tiller, and number of earring of *in vitro* salt tolerant plants grown at 0 % NaCl were larger than those at 0.55 and 0.75 % NaCl (data not shown).

The culm length, ear mass, number of tillers, number of earing, grain mass, number of grains per plant, number of sinking grains per plant, percentage of bearing tillers, and percentage of ripened grains of *in vitro* salt tolerant plants, *in vitro* non salt tolerant plants and seedling plants grown in pot with 0 % NaCl were similar (Table 1). These characteristics decreased with increases in NaCl concentration in soil.

The Na^+ contents in leaves of *in vitro* salt tolerant plants increased with increase in the NaCl concentration in the pots and were about 3.3 and 4.6 times higher at 0.55 and 0.75 % NaCl than at 0 % NaCl. The Cl^- contents in leaves of *in vitro* salt tolerant plants grown at 0.55 and 0.75 % NaCl were about 2.4 and 4 times higher than at 0 % NaCl (Table 2).

The Na^+ and Cl^- contents in leaves of *in vitro* non-salt tolerant plants and in seedlings grown at 0.55 and 0.75 % NaCl were from 10 to 12 times higher than at 0 % NaCl (Table 2).

It was reported that rice plants became necrotic within a short time when cultured in 0.5 % NaCl potted paddy soil (Iwaki 1970). Therefore, 0.55 and 0.75 % NaCl employed in this experiment were high enough.

The data suggested *1*) that the present *in vitro* salt-tolerant plants maintained the salt tolerance in soil from 4 - 5 leaves stage till harvesting, and *2*) that the present *in vitro* salt treatment might be useful for rice biomass and grain production under saline conditions. If dense

planting of the present salt tolerant plants is employed in 0.55 and 0.75 % NaCl treatments, the yields in 0.55 % and 0.75 % NaCl treatments may be increased. By the present step up salt selection, 2 % NaCl tolerant pea and rice plants were established *in vitro*. Application of the same procedure for the establishment of different plant species is in progress (Saiki, personal communication).

Table 2. Effect of NaCl treatments of different duration on contents of Na^+ and Cl^- [mg g^{-1}(d.m.)] in tolerant or non-tolerant regenerants, and seedlings. Means ± SE. $n = 12$.

Ion	Plants	NaCl [%]	0 week	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
Na^+	tolerant	0.00	10.12±0.47	11.79± 0.49	13.00± 2.71	14.34± 0.38	16.42± 1.06	16.16± 0.13
	regenerants	0.55	10.15±0.48	18.71± 0.52	37.32± 0.31	54.84± 2.49	54.16± 0.73	57.74± 2.68
		0.75	10.11±0.42	40.04± 1.82	48.39± 4.18	76.58± 1.22	77.31± 1.06	73.88± 1.23
	non-tolerant	0.00	10.36±0.21	11.08± 0.04	12.63± 0.87	15.63± 0.87	16.36± 0.76	17.46± 1.08
	regenerants	0.55	10.39±0.23	106.77± 1.92	190.73±14.91	188.07± 1.76	199.54± 7.24	197.49± 6.32
		0.75	10.28±0.27	111.44± 2.85	191.88±11.93	196.30± 0.58	202.33± 4.26	203.50± 6.97
	seedlings	0.00	10.41±0.19	11.93± 0.31	12.69± 0.58	15.58± 0.53	16.36± 0.33	17.28± 0.61
		0.55	10.48±0.18	110.79± 1.63	184.26±12.74	186.15± 1.03	197.20± 5.65	192.37± 2.76
		0.75	10.44±0.17	120.81± 2.85	206.75± 7.70	205.40± 3.31	206.19± 1.40	211.80±11.89
Cl^-	tolerant	0.00	9.69±1.07	10.09± 0.93	11.89± 0.53	15.68± 0.31	16.56± 1.58	18.81± 0.20
	regenerants	0.55	9.64±0.91	19.01± 1.19	29.13± 0.41	42.43± 1.42	46.80± 5.23	45.73± 2.43
		0.75	9.67±1.19	36.95± 1.77	51.04±10.89	65.10± 3.15	66.36± 6.10	70.44± 4.61
	non-tolerant	0.00	9.15±0.20	9.69± 0.47	12.42± 0.05	15.40± 0.54	16.52± 0.80	18.32± 1.12
	regenerants	0.55	9.14±0.24	101.86± 6.02	191.52±13.55	199.18± 7.88	204.72±12.33	198.56± 8.69
		0.75	9.22±0.20	120.89±10.81	207.06±33.03	211.29± 8.60	222.74±14.18	223.61±14.67
	seedlings	0.00	9.56±0.31	10.09± 0.69	12.12± 0.41	15.47± 0.72	16.73± 1.23	18.41± 0.34
		0.55	9.54±0.37	101.52± 4.91	191.86±12.57	190.72±20.70	198.65±13.02	191.52± 7.84
		0.75	9.59±0.41	110.95±13.48	242.55±49.20	218.61±12.62	223.80±21.03	228.60±23.47

There are several mechanisms by which plants adapt to salt stress. These include production of solutes such as glycinebetaine, sugar alcohol, and proline, and regulation of ion concentration in cells (Gorham *et al.* 1985). A rice plant does not have the ability to synthesize glycine-betaine (Rathinasabapathi *et al.* 1993). There is no report yet where sugar alcohol was correlated with rice salt tolerance. During the *in vitro* salt selection process, excess proline was not detected (Nojiri *et al.* 1997).

Yamanouchi (1989) reported that the higher the NaCl contents in rice leaves, the lower the salt resistance of rice plants grown in field. In the present research, the Na^+ and Cl^- ion contents in the leaves of *in vitro* salt tolerant plants grown at 0.55 and 0.75 % NaCl were maintained almost constant during the growth and were about 4 times higher than those at 0 % NaCl. Conversely, all the *in vitro* non-salt tolerant plant and seedling plants were necrotic by the 4th week in culture, and the Na^+ and Cl^- contents in their leaves about 12 times higher than that at 0 % NaCl. One of the hypotheses to explain salt tolerance in the present *in vitro* salt tolerant rice plants grown in saline pot soil is that the present *in vitro* step up salt selection induces the capability to maintain relatively low, non lethal concentrations of Na^+ and Cl^- ions in the leaves.

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