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PHYSIOLOGICAL ASPECTS OF RICE CALLUS GROWTH AND PLANT REGENERATION IN A MODIFIED MS MEDIUM SUPPLEMENTED WITH NaCl

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ABSTRACT

Salt tolerance of four Iranian rice cultivars were evaluated *in vitro* using modified MS medium supplemented with six levels of NaCl (0, 20, 40, 60, 80 and 100 mM). Fresh weight, dry matter, water content, Na^+, K^+, Ca^{2+} and Cl^- ions and proline content of callus as well as plant regeneration percentage were measured. When NaCl content of the medium was increased from 0 to 20 mM fresh weight of callus and plant regeneration increased from 382.6 mg and 59% to 437.8 mg and 63%, respectively, and thereafter, rapidly decreased. The callus induction and growth as well as plant regeneration were found to be maximum on MS medium containing 20 mM NaCl supplemented with 2.0 mg l^{-1} 2, 4-D (9.04 µM) and 0.2 mg l^{-1} BAP (8.88 µM) (for callus induction) and medium with 3 mg l^{-1} NAA (16.11 µM), 2 mg l^{-1} kinetin (9.3 µM), 50 mg l^{-1} tryptophan, respectively. Also, results showed that proline accumulation in callus was negatively correlated with the fresh

weight and water content and was not correlated with Na^+ and Cl^- ion contents of the callus. Path coefficient analysis based on correlation matrix showed that Ca^{2+} and K^+ ions, which remained positively correlated with fresh weight and water content, affected fresh weight via their effects on water content. Proline, Na^+ and Cl^- ions had negatively influenced the water content of callus. It is concluded that 'Zayandeh Roud' cultivar due to its evaluated callus growth and plant regeneration on NaCl containing medium might be an appropriate candidate to be transformed by genes that enhance the rice salt tolerance.

Key words: Callus growth, MS medium, NaCl supplementation, *Oryza sativa* L., Regeneration efficiency, Salt tolerance.

Abbreviations: 2, 4-D- 2, 4-dichlorophenoxyacetic acid; BAP- 6-benzylaminopurine; IAA- indole-3-acetic acid; NAA- α-naphthaleneacetic acid.

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جنبه‌های فیزیولوژیکی بهبود رشد پینه و باززایی گیاهچه در محیط کشت MS تغییریافته دارای NaCl

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چکیده

تحمل شوری چهار رقم برنج ایرانی در شرایط درون شیشه ای با استفاده از محیط کشت MS تغییریافته‌ای که به آن شش سطح شوری (۰، ۲۰، ٤۰، ٦۰، ۸۰ و ۱۰۰ میلی‌مول NaCl) افزوده شده بود، مورد ارزیابی قرار گرفت. وزن تر، مادهٔ خشک، مقدار آب، یون‌های Na^+، K^+، Ca^{2+}، Mg^{2+} و Cl^- و محتوای پرولین در آنها و نیز درصد باززایی گیاهچه از پینه اندازه گیری شد. نتایج نشان داد که هنگامی که مقدار NaCl موجود در محیط کشت از صفر به ۲۰ میلی‌مول افزایش داده شد، وزن تر پینه و باززایی گیاهچه در ابتدا به ترتیب از ۳۸۲/٦ میلی‌گرم و ۵۹٪ تا ٤۳۷/۸ میلی‌گرم و ٦۳٪ افزایش یافت و از آن پس به سرعت کاهش یافت. انگیزش و رشد پینه و نیز باززایی گیاهچه از پینه به ترتیب در محیط کشت MS حاوی ۲۰ میلی‌مول NaCl که به آن ۲ میلی گرم در لیتر تو، فور-دی و ۰/۲ میلی گرم در لیتر BAP (برای محیط پینه‌زایی) افزوده شده بود و محیط کشت دارای ۳ میلی گرم در لیتر، NAA، ۲ میلی گرم در لیتر، کینتین و ۵۰ میلی گرم در لیتر تریپتوفان بهینه بود. تجمع پرولین با وزن تر و محتوای آب پینه همبستگی منفی داشت و با مقدار یون‌های Na^+ و Cl^- همبسته نبود. تجزیهٔ ضرائب مسیر بر مبنای ماترس همبستگی نشان داد که یون‌های Ca^{2+} و K^+ که با وزن تر و مقدار آب همبستگی مثبت دارند، اثر خود را از طریق افزایش مقدار آب پینه اعمال می‌نمودند. رقم 'زاینده رود' به خاطر بهبود رشد پینه و باززایی گیاهچه در محیط کشت حاوی NaCl رقم مناسبی برای انتقال ژن به منظور افزایش تحمل شوری در برنج است.

INTRODUCTION

Plant productivity is strongly influenced by dehydration stress induced by high salt, drought and low-temperature conditions. It is estimated that up to two-thirds of the yield

potential of major crops could be lost due to unfavorable growing environments (4). Abiotic stresses such as salinity and drought are serious threats to sustainable food production. In rice (*Oryza sativa* L.), abiotic stresses affect cultivation more than biotic stresses caused by fungal, bacterial pathogens and insect pests (22). In arid and semi-arid areas of the world, increasing salt content of water and soils is a major limiting factor for crop production (3, 24). Plants use various simple and complex mechanisms to overcome or tolerate stresses at single cell or at whole plant levels. It has been demonstrated that salt tolerance mechanisms at whole plants are different from mechanisms that act at the single cells (2).

Plant tissue culture techniques are powerful approaches to study salt tolerance of plant cells. Many attempts were made to initiate callus production and regenerating plants from callus of different rice genotypes since the first plant regeneration in rice (18). Researchers have used many different media such as MS (17), N6 (7) B5 (10) and their modified composition to enhance callus induction, growth and plant regeneration rate (13, 25). This is mainly due to interaction between genotypes and media, although Indica rice cultivars have proved to be less amenable to *in vitro* culture (13). In addition, MS medium is commonly used as a basal medium for rice and supplements such as tryptophan (27) and proline (8) were used to enhance regeneration rate.

Application of NaCl during callus induction and plant regeneration processes offers a convenient way to study the effect of salinity on morphogenic steps of development (15). Presence of NaCl in regeneration medium reduced (32) or completely inhibited (30) regeneration of plantlets from rice callus. Lutts *et al.* (15) investigated the effects of polyethyleneglycol (PEG), tryptophan, proline, and plant growth regulators such as abscisic acid (ABA) and indole-3-acetic acid (IAA), in presence of 0, 50 and 100 mM NaCl on rice callus regeneration and survival of plantlets obtained from four cultivars. Their results showed that proline had no effect, tryptophan stimulated plant regeneration, and NaCl strongly reduced the regeneration percentage of all cultivars, but slightly increased the survival of regenerated plants. It was also hypothesized that the presence of NaCl in the regeneration medium would lead to selection of the most vigorous shoots; consequently the plantlets obtained would survive after transfer to greenhouse. It seemed logical that considering the wide range of NaCl concentrations short intervals could provide more information about the effects of NaCl on rice callus induction and plant regeneration. The objectives of the present study were to evaluate the salt tolerance of four Iranian rice cultivars (belonging to Indica group) and amenable to tissue culture (19), prior to being subjected to a choline oxidase (Cod A) gene transformation, and to determine an optimum NaCl level to enhance the efficiency of MS medium for callus growth and plant

regeneration. Effects of NaCl on callus induction, callus growth and plant regeneration in three aspects of water uptake, ion imbalance, ion toxicity and accumulation of proline were assessed and reported in this article.

MATERIALS AND METHODS

Plant Materials

Four Iranian rice (*Oryza sativa* L.) cultivars comprising 'Charam 2', 'Neamat', 'Tarom Molaie' and 'Zayandeh Roud' were used in this study. 'Charam 2' is a pure line selected from the landraces of southern Iran and is grown in the south and south-west of Iran. This cultivar was supplied by Gachsaran Research Center of Agriculture. 'Zayandeh Roud' is an aromatic pure line selected from a Lendjan landrace and was supplied by Isfahan Research Center of Agriculture. This cultivar is widely grown by farmers in central Iran. 'Tarom Molaie' is an aromatic pure line from northern Iran. 'Neamat' is a pure line selected from a cross between 'Sang Tarom' and 'Amol 3', both local cultivars, provided by the Rice Research Station of Amol at the north of Iran. All of these rice cultivars are classified as indica cultivars and indicated to be superior for callus induction, callus growth and plant regeneration in a previous study (19).

Callus Induction

Six levels of NaCl (0, 20, 40, 60, 80 and 100 mM equal to 0, 1.177, 2.354, 3.530, 4.707 and 5.884 g l^{-1}, respectively) were added to MS medium basal salts and vitamins (34). The media were enriched with 30 g l^{-1} sucrose, 2.0 mg l^{-1} 2, 4-D (9.04 µM) and 0.2 mg l^{-1} BAP (8.88 µM) as growth regulators and pH of medium was adjusted to 5.8 prior to adding 3 g l^{-1} phytagel as gelling agent and then autoclaved. A Merck dispenser dispensed the media uniformly with 35 ml medium in each petri dish.

Three hundred dehusked seeds of each cultivar were surface sterilized by 95% ethanol for 2 min and 2.5% sodium hypochlorite for 30 min. After each step, the seeds were washed three times with sterilized distilled water. Five seeds were cultured in each Petri dish so that the endosperms were located downward and their embryos were placed upwards in contact with the surface of medium. All Petri dishes were incubated in the dark at 26±1°C for 10 wk. Calli induced from seeds were sub-cultured once five weeks after culturing.

A factorial experiment involving two factors (four cultivars and six salt levels) in a completely randomized design (CRD) with 10 replications possessing five samples in each replicate was used. Contaminated Petri dishes were eliminated from the experiment and considered as lost samples within an experimental unit. Four weeks after the subculture of the calli, Petri dishes were divided in two parts and the calli from the first half were assigned for plant regeneration assessment and the calli from the second half were used for measuring fresh weight, dry matter, water content, Na^+, K^+, Ca^{2+} and Cl^- ions and proline content.

Plant Regeneration

The calli allocated for plant regeneration assessment were weighted individually and transferred into a regeneration medium using a modified MS medium (34) containing one of the six NaCl levels and supplemented with 20 g l^{-1} sucrose, 3 mg l^{-1} NAA (16.11 µM), 2 mg l^{-1} kinetin (9.3 µM) and 50 mg l^{-1} tryptophan. The pH of the solution was adjusted to 5.8 prior to adding 3 g l^{-1} phytagel and then autoclaved. The petri dishes were placed in a 26±1°C phytotrone under a 12 h light period with 120 µmol $m^{-2}s^{-1}$ flourescent light intensity and relative humidity of 25%. After five weeks, regenerated shoots were transferred into vessels containing the rooting medium (the modified MS medium supplemented with 20 g l^{-1} sucrose, 3 g l^{-1} phytagel, and 1 mg l^{-1} IAA (5.7 µM)) and regeneration percentage was calculated.

Ions Extraction and Measurement

The calli of each petri dish assigned for ions extraction were bulked and fresh weight of callus was measured. Then, the calli were dried at 60 °C for 48 h and dry weight of the calli was measured. Dry weight percentage was calculated from callus dry weight divided by fresh weight. Water content percentage of callus was computed from difference between fresh and dry weights of callus divided by fresh weight.

Ions were extracted by using 10.0 mg of melted dried callus in a test tube. Two ml sulfuric acid were added to each tube and the tubes were heated in a microkjeldal apparatus upto 350 °C for 12 h or when the solution became colorless and most of the sulfuric acid evaporated (33). Then, deionized water was added until final volume of the solution reached 20 ml. Ca^{2+} and Mg^{2+} ions were measured using Perkin-Elmer Model 3110 atomic absorption apparatus. Na^+ and K^+ ions were measured using Corning Model 410 flame photometer apparatus. K^+/ Na^+ and

Ca^{2+}/ Na^{+} ratios were calculated from K^{+} and Ca^{2+} ion contents divided by Na^{+} ion content, respectively.

To measure chloride (Cl^{-}), 10.0 mg of dried callus of each replication were weighed in a test tube and 20 ml of deionized water were added. Test tubes were then shaken gently for 48 h and Cl^{-} ion was determined with ion selective electrode method using Jenway apparatus Model PCIM3.

Proline Extraction and Measurement

Proline was extracted by the method of Bates *et al.* (5) in which 20 mg melted dried callus was treated with 10 ml of 3% sulfosalicylic acid and the homogenous solution was centrifuged at 3,000 g for 20 min. Then 2 ml of the supernatant were added to 2 ml acetic acid and 2 ml acid ninhydrin and boiled for 1 h. Free proline dissolved in 4 ml toluene was measured by spectrophotometer using Perkin-Elmer apparatus Model 550 SE at 520 nm wavelength.

Statistical Analysis

Data were analyzed using SAS software (26) in a factorial model involving two factors with 5 replications possessing five samples in each replicate. Fresh weight, dry weight, water content percentage and regeneration percentage data were transformed using square root transformation (29).

RESULTS

Callus Induction and Plant Regeneration

Cultivars, salt levels and interaction of cultivar × salt levels differed significantly for both fresh weight of callus and plant regeneration percentage. Fig. 1 presents mean comparisons of cultivars and salt levels for these traits. Callus fresh weight and plant regeneration increased at the first salt level of 20 mM NaCl but rapidly decreased afterward. 'Zayandeh Roud' and 'Neamat' cultivars with 485.6 mg and 473.5 mg produced the highest and 'Tarom Molaie' and 'Charam 2' cultivars with 375.9 mg and 340.5 mg produced the lowest callus fresh weight, respectively. Furthermore, 'Zayandeh Roud' was found to be superior for plant regeneration percentage (54.4%) while 'Neamat' was inferior in this respect (20.6%).

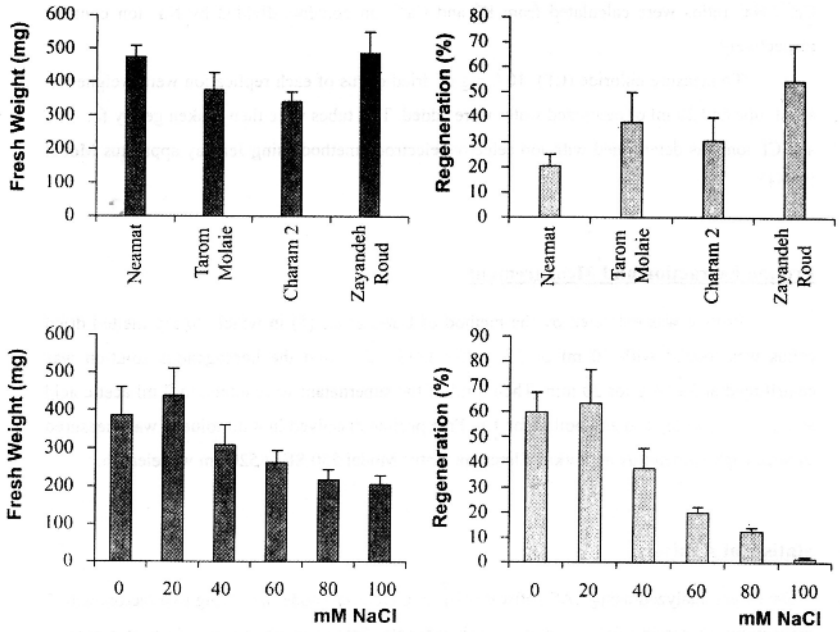

Fig. 1. Mean comparisons of cultivars (up) and salt levels (down) for callus fresh weight (right) and plant regeneration percentage (left) of calli produced and regenerated in media containing six salt levels.

The induction medium containing 20 mM NaCl produced the maximum callus fresh weight (438.7 mg) and also had the highest plant regeneration rate (63.3%). The medium containing 100 mM NaCl gave both the lowest callus fresh weight of 205.4 mg and plant regeneration (1.43%).

Mean comparisons of cultivar × salt interactions for fresh weight of callus and plant regeneration percentage are summarized in Fig. 2. 'Zayandeh Roud' cultivar had the maximum plant regeneration rate of 135% in medium containing 20 mM NaCl. 'Neamat' and 'Zayandeh Roud' cultivars produced the highest callus fresh weight of 587.2 mg and 586 mg at 20 mM NaCl, respectively. 'Neamat' did not regenerate at 80 mM NaCl containing medium and only 'Tarom Molaie regenerated poorly (4%) on medium containing 100 mM NaCl.

Callus Dry Weight, Water and Ions Contents

All characters were found to be significantly different between cultivars except for Mg^{2+} concentration. Salt level had also a significant effect on these traits. For cultivar × salt interaction only Mg^{2+} and Ca^{2+} concentrations were not significant traits. Tables 1 and 2 present results of means comparisons for four cultivars and six salt levels, respectively. 'Neamat' cultivar has highest value for all traits except for dry matter percentage and proline content. 'Charam 2' which accumulated highest proline (32.10 µmol g^{-1}), had lowest Na^{+} (15.46 mg g^{-1}) ion. However, 'Tarom Molaie' which ranked second for proline (27.330 µmol g^{-1}), has the highest Na^{+} ion content (20.37 mg g^{-1}).

The highest fresh weight, water content percentage, Ca^{2+} and K^{+} ions content and lowest proline were observed on medium containing 20 mMl^{-1} NaCl which had the best regeneration percentage too (Table 2 and Fig. 2). Meanwhile medium containing 100 mM NaCl showed lowest callus fresh weight, water percentage, $Ca2^{+}$ and K^{+} ions content but highest dry matter percentage, Na^{+} and Cl^{-} ions, and proline contents.

Mean comparison of significant interactions of cultivar × salt for nine traits is summarized in Table 3. Callus fresh weights of 'Neamat' and 'Zayandeh Roud' cultivars at medium containing 20 mM NaCl were superior. Also callus fresh weight of 'Zayandeh Roud' in medium containing 100 mM NaCl was 72.2% that of medium without salt and 55.6% of the best medium in this respect (20 mM NaCl). Furthermore, the least proline content belonged to 'Neamat' at 0 and 20 (9.17 and 10.88 µMol g^{-1} dry matter, respectively) and 'Zayandeh Roud' at 20 mM NaCl (10.30 µMol g^{-1} dry matter).

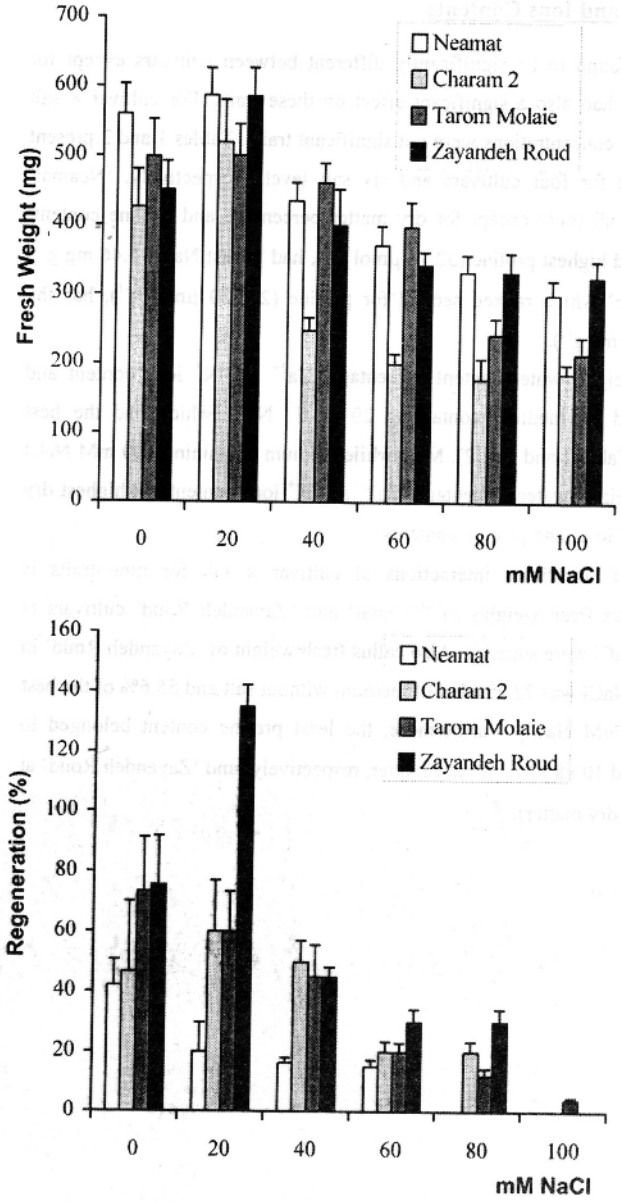

Fig. 2. Mean comparisons of cultivar × salt level interaction for callus fresh weight (up) and plant regeneration (down) in calli initiated and regenerated at different salt containing media.

Table 1. Mean comparisons of cultivars for ten traits measured in calli.

Traits	'Charam 2'	'Neamat'	'Tarom Molaie'	'Zayandeh Roud'	LSD (1%)
Fresh Weight (mg)	1702 [b]	2367 [a]	1857 [b]	2428 [a]	298
Water Content (%)	90.20 [b]	93.90 [a]	90.10 [b]	90.80 [b]	0.46
Dry Matter (%)	9.80 [a]	6.10 [b]	9.90 [a]	9.20 [a]	1.42
Ca^{2+} (mg g^{-1})	8.53 [ab]	9.68 [a]	4.63 [b]	7.87 [b]	1.52
Mg^{2+} (mg g^{-1})	5.91 [b]	7.58 [a]	5.79 [b]	5.64 [b]	0.69
Na^{+} (mg g^{-1})	15.46 [b]	18.27 [a]	20.37 [a]	20.31 [a]	2.63
K^{+} (mg g^{-1})	31.82 [b]	36.54 [a]	32.70 [b]	31.61 [b]	2.66
Ca^{2+}/Na^{+} Ratio	0.80 [b]	1.12 [a]	0.51 [b]	0.93 [b]	0.16
K^{+}/Na^{+} Ratio	1.54 [b]	2.06 [a]	1.35 [b]	1.87 [a]	0.22
Cl^{-} (mg g^{-1})	18.48 [c]	22.50 [a]	19.66 [a]	24.93 [a]	2.34
Proline (µmol g^{-1})	32.10 [a]	14.75 [d]	27.33 [b]	18.60 [c]	2.98

[a-d]: Means followed by same latter are not significant at related significant level.

Table 2. Mean comparisons six salt levels for ten traits measured in calli.

Traits	0	20	40	60	80	100	LSD (1%)
Fresh Weight (mg)	1934 ^{a}	2193 ^{a}	1544 ^{b}	1311 ^{b}	1086 ^{bc}	1027 ^{c}	264
Water Content (%)	93.30 ^{a}	92.70 ^{a}	91.40 ^{b}	91.00 ^{b}	89.70 ^{c}	88.50 ^{d}	0.57
Dry Matter (%)	6.70 ^{e}	7.30 ^{d}	8.60 ^{c}	9.00 ^{c}	10.30 ^{b}	11.50 ^{a}	1.07
Ca^{2+} (mg g^{-1})	9.51 ^{a}	9.31 ^{a}	8.57 ^{a}	6.68 ^{b}	5.59 ^{bc}	4.68 ^{c}	1.86
Mg^{2+} (mg g^{-1})	6.25 ^{a}	6.50 ^{a}	5.78 ^{a}	6.00 ^{a}	6.47 ^{a}	6.13 ^{a}	0.85
Na^{+} (mg g^{-1})	3.96 ^{e}	15.13 ^{d}	19.14 ^{c}	22.42 ^{b}	24.32 ^{b}	28.39 ^{a}	3.22
K^{+} (mg g^{-1})	36.87 ^{a}	36.12 ^{ab}	33.29 ^{bc}	33.20 ^{bc}	29.89 ^{cd}	28.33 ^{d}	3.26
Ca^{2+}/Na^{+} Ratio	2.07 ^{a}	0.79 ^{b}	0.68 ^{bc}	0.57 ^{cd}	0.51 ^{cd}	0.41 ^{d}	0.19
K^{+}/Na^{+} Ratio	3.95 ^{a}	1.58 ^{b}	1.38 ^{bc}	1.24 ^{cd}	1.13 ^{cd}	1.01 ^{d}	0.27
Cl^{-} (mg g^{-1})	9.00 ^{e}	15.95 ^{d}	21.89 ^{c}	23.44 ^{c}	28.24 ^{b}	31.6 ^{a}	2.86
Proline (μmol g^{-1})	19.96 ^{cd}	18.75 ^{d}	19.81 ^{cd}	23.58 ^{bc}	26.43 ^{b}	31.60 ^{a}	3.64

$^{a-d}$: Means followed by same letter are not significant at related significant level.

In order to investigate and explain relationships between traits, which measured for calli, initiated and grew on media contained different NaCl levels, a correlation analysis was done (Table 4) which displayed that all traits except mg^{2+} were significantly correlated with fresh weight. Moreover, Table 6 shows that an increase in Na^{+} content due to increase of salt in the media, resulted in a decrease in Ca^{2+} and K^{+} uptake.

Path coefficient analysis based on correlation matrix (Table 4) showed that all ions and proline contents mainly affected fresh weight through water content. Water content itself stimulated growth directly and indirectly through accumulation of dry matter. On the other hand, dry matter severely affected water content, which stimulated uptake of solutes and in turn, increased dry matter and growth become possible.

DISCUSSION

The possibilities of obtaining somaclonal variants as well as adapted cells will increase with the number of subculture, and consequently, the probabilities of modifying the degree of salt tolerance of a genotype will also increase (31). Therefore, we used only one subculture to prevent somaclonal variation and to control experimental error in regeneration of plant from callus.

Salinity harms plant cells mainly through three major aspects; water deficiency, ion imbalance and ion toxicity (9). Calli of cultivars differed in response to salt levels in the present study. The results revealed that 'Zayandeh Roud' and 'Neamat' cultivars displayed the best growth in presence of different NaCl levels (Figs. 1 and 2), but 'Zayandeh Roud' cultivar had the maximum regeneration percentage. 'Neamat' accumulated minimum dry matter percentage and proline but the highest water content percentage, Ca^{2+}, Mg^{2+} and K^{+} were belonged to the 'Neamat' cultivar. There were not significant differences between 'Neamat' and 'Zayandeh Roud' cultivars in Na^{+} and Cl^{-} ions contents. Thus, the 'Neamat' cultivar used other mechanisms rather than accumulation of proline to regulate its osmotic pressure. More studies are needed to investigate precise mechanisms which these cultivars use to tolerate NaCl. Relative poor regeneration of the 'Neamat' cultivar probably was due to its poor genetic ability for regeneration. Genetic differences in callus induction, growth and regeneration in rice have been already reported (e.g. 1, 12). Relatively high callus fresh weight and regeneration of the 'Zayandeh Roud' cultivar and its other capacities and advantages, recommended it as a proper candidate for gene transformation, especially for salt tolerance.

Increase in NaCl content of cultured medium primarily increased fresh weight and regeneration of plant from callus and then decreased them rapidly (Fig. 1). This observation corroborates with the finding of Vajrabhaya *et al.* (32), Subhashini and Reddy (30), and Lutts *et al.* (15, 16). Also, our results showed that supplementation of modified MS medium (34) with 20 mM NaCl enhanced fresh weight of callus and regeneration of plant from callus (Fig. 1), but the significant cultivar × salt interaction shows that responses of different cultivars to NaCl supplementation is different (Fig. 2). Enhancement of growth and regeneration in presence of NaCl in rice has not been reported previously.

In the present study, proline negatively correlated with fresh weight and water content of callus, and did not correlate with Na^{+} and Cl^{-} ion contents (Table 4). This observation did not support the finding of Lin *et al.* (14) that showed both Na^{+} and Cl^{-} are involved in the increase of proline content induced by NaCl in detached leaves segments of rice. They also showed that

osmotic effect is unlikely to be a major factor contributing to the accumulation of proline. Furthermore, they observed that proline accumulation in detached leaf segments of rice plant was due to decrease in protein synthesis, or proline utilization, and increase in proline biosynthesis. However, they used detached leaf segments in their observations and mentioned that their result were not necessarily similar to other experiments even though in intact leaf of rice.

The actual role of proline accumulation remains unclear (23) but it has been speculated that it can serve as an osmotic regulator (21), a protector of enzyme denaturation (20) or a hydroxyl radical scavenger (28). However, some reports indicate no correlation between proline accumulation and stress resistance (11, 15). Lutts *et al.* (15) found that proline accumulation in rice plants was not related to proteolysis. Our results showed that proline accumulation in rice callus is a response to salt stress, but does not necessarily involve in increase of water content and decrease in Na^+ and Cl^- ions (Tables 3 and 4).

Sodium ion (Na^+) had a negative correlation with both Ca^{2+} and K^+ cations in the present study. Sodium ion enters the cell via specific channels of both Ca^{2+} and K^+ ions (6, 9). Thus, potential of cell in preferable transport of K^+ and Ca^{2+} ions through cell membrane and their accumulation in cytoplasm represents salt tolerance potential. Also, assimilated Na^+ can be stored in vacuoles. Vacuolar compartmentation is an efficient strategy for plant cells to deal with salt stress because the stored Na^+ contributes to osmotic adjustment (9). The ability of cells to maintain low cytosolic sodium concentrations is an essential process associated with the ability of plants to grow in high salt concentrations (6). Na^+ ion content of the 'Neamat' cultivar increased about 20 folds. Study of compartmentation of Na^+ was not conducted in this investigation, but, the potential of the 'Neamat' cultivar to maintain its water content and to enhance its fresh weight in presence of NaCl may show that this cultivar can enclose Na^+ into its vacuoles. More experiments on compartmentation of Na^+ ion through cell sap are needed to determine the precise mechanism of Na^+ assimilation in this cultivar. Also, ability the 'Neamat' cultivar to absorb Ca^{2+} and K^+ (Tables 1 and 3) represents its capacity for salt tolerance manipulation. Moreover, ability of 'Charam 2' cultivar to exclude Na^+ and to prevent its absorption (Tables 1 and 3) is notable. Table 1: Analysis of variance for callus fresh weight and plant regeneration in media containing six salt levels^.

Table 3. Means comparisons of interaction of cultivar × salt for nine traits in callus initiated and grew in media contained NaCl levels.

Cultivar	Salt level	Fresh weight (mg)	Water content (%)	Dry matter (%)	Na^+ (mg g^{-1})	K^+ (mg g^{-1})	K^+/Na^+ ratio	Ca^{2+}/Na^+ ratio	Cl^- (mg g^{-1})	Proline (µmol g^{-1})
'Charam'	0	1876 ^{cdefg}	94.79 ^{a}	5.21 ^{h}	4.61 ^{j}	48.32 ^{a}	3.26 ^{b}	1.48 ^{b}	10.00 ^{ij}	18.45 ^{efgh}
'Charam'	20	2665 ^{abc}	91.47 ^{efgh}	8.53 ^{cdefg}	14.89 ^{ghi}	40.01 ^{b}	1.66 ^{cde}	0.79 ^{cd}	16.20 ^{fghi}	18.08 ^{efgh}
'Charam'	40	1270 ^{fgh}	90.82 ^{fghi}	9.17 ^{cdef}	14.93 ^{ghi}	31.73 ^{cdefg}	1.49 ^{cdef}	0.83 ^{cd}	16.50 ^{fghi}	22.50 ^{cdefg}
'Charam'	60	982 ^{gh}	89.91 ^{ij}	10.09 ^{bcd}	18.23 ^{efgh}	30.34 ^{cdefg}	1.31 ^{cdef}	0.69 ^{cde}	16.80 ^{fghi}	28.67 ^{cd}
'Charam'	80	940 ^{h}	88.19 ^{kl}	11.81 ^{ab}	15.59 ^{ghi}	24.17 ^{gh}	1.26 ^{def}	0.71 ^{cde}	21.60 ^{def}	43.43 ^{b}
'Charam'	100	870 ^{h}	86.83 ^{l}	13.16 ^{a}	21.58 ^{defg}	17.86 ^{h}	0.91 ^{f}	0.54 ^{cde}	33.00 ^{ab}	83.30 ^{a}
'Neamat'	0	2884 ^{ab}	94.71 ^{a}	5.29 ^{h}	1.82 ^{j}	36.36 ^{bcde}	4.71 ^{a}	2.86 ^{a}	11.20 ^{hij}	13.64 ^{gh}
'Neamat'	20	2958 ^{ab}	94.75 ^{a}	5.25 ^{h}	13.31 ^{hi}	37.82 ^{bcd}	1.77 ^{cd}	0.92 ^{c}	17.60 ^{efgh}	9.17 ^{h}
'Neamat'	40	2224 ^{bcde}	94.54 ^{ab}	5.45 ^{h}	22.68 ^{cdefg}	38.18 ^{bc}	1.33 ^{cdef}	0.72 ^{cde}	27.60 ^{bcd}	10.88 ^{h}
'Neamat'	60	1932 ^{cdef}	93.08 ^{bcd}	6.92 ^{bcdef}	24.29 ^{bcdef}	37.22 ^{bcd}	1.26 ^{def}	0.51 ^{cde}	26.00 ^{bcd}	13.87 ^{gh}
'Neamat'	80	1906 ^{cdef}	92.54 ^{cde}	7.45 ^{fgh}	27.64 ^{abcd}	37.76 ^{bcd}	1.18 ^{def}	0.48 ^{cde}	29.33 ^{bc}	15.78 ^{fgh}
'Neamat'	100	1520 ^{defgh}	91.7 ^{def}	8.29 ^{defg}	34.70 ^{a}	26.50 ^{fg}	0.87 ^{f}	0.46 ^{cde}	33.00 ^{ab}	21.49 ^{defg}
'Tarom molaie'	0	2425 ^{bc}	91.93 ^{cdef}	8.07 ^{defg}	9.37 ^{ij}	33.80 ^{bcdef}	1.94 ^{c}	0.80 ^{cd}	6.00 ^{j}	22.98 ^{cdefg}
'Tarom molaie'	20	2408 ^{bcd}	91.16 ^{efghi}	8.82 ^{cdefg}	15.92 ^{ghi}	34.53 ^{bcde}	1.48 ^{cdef}	0.62 ^{cde}	12.40 ^{ij}	25.24 ^{cdef}
'Tarom molaie'	40	2372 ^{bcd}	90.82 ^{fghi}	9.18 ^{cdef}	16.42 ^{fghi}	33.44 ^{bcdef}	1.50 ^{def}	0.60 ^{cde}	18.40 ^{efg}	25.34 ^{cdef}
'Tarom molaie'	60	2282 ^{bcde}	90.54 ^{efg}	9.46 ^{bcdef}	25.88 ^{bcde}	34.72 ^{bcde}	1.16 ^{ef}	0.47 ^{cde}	26.50 ^{bcd}	28.02 ^{cde}
'Tarom molaie'	80	1048 ^{fgh}	90.16 ^{ghij}	9.84 ^{bcdef}	26.32 ^{bcd}	30.70 ^{cdefg}	1.08 ^{ef}	0.30 ^{e}	25.60 ^{cd}	31.56 ^{c}
'Tarom molaie'	100	983 ^{gh}	86.86 ^{l}	13.14 ^{a}	26.07 ^{bcde}	30.15 ^{defg}	1.08 ^{ef}	0.37 ^{de}	26.33 ^{bcd}	30.46 ^{cd}
'Zayandehrood'	0	2254 ^{bcde}	92.39 ^{cde}	7.61 ^{efgh}	1.51 ^{j}	35.26 ^{bcde}	5.07 ^{a}	2.54 ^{a}	8.80 ^{j}	18.25 ^{efgh}
'Zayandehrood'	20	2930 ^{ab}	93.43 ^{abc}	6.57 ^{gh}	16.64 ^{fghi}	32.12 ^{bcdefg}	1.41 ^{cdef}	0.84 ^{cd}	17.60 ^{efgh}	10.30 ^{h}
'Zayandehrood'	40	2001 ^{cde}	89.81 ^{ij}	10.19 ^{bcd}	22.33 ^{cdefg}	28.56 ^{efg}	1.17 ^{def}	0.58 ^{cde}	24.50 ^{cde}	18.63 ^{efgh}
'Zayandehrood'	60	1711 ^{cdef}	89.95 ^{hij}	10.05 ^{bcde}	22.37 ^{cdefg}	31.62 ^{cdefg}	1.22 ^{def}	0.56 ^{cde}	25.60 ^{cd}	17.50 ^{fgh}
'Zayandehrood'	80	1666 ^{cdefgh}	89.02 ^{jk}	10.98 ^{abc}	30.23 ^{abc}	30.12 ^{cdefg}	1.02 ^{ef}	0.53 ^{cde}	39.00 ^{a}	22.39 ^{cdefg}
'Zayandehrood'	100	1628 ^{cdefgh}	89.79 ^{ij}	10.21 ^{bcd}	31.36 ^{a}	31.07 ^{cdefg}	1.01 ^{ef}	0.39 ^{de}	36.80 ^{a}	24.23 ^{cdef}
LSD $_{(\%1)}$		896	1.54	0.07898	7.91	8.02	0.65	0.48	7.042	10.02

$^{a-l}$: Means followed by same latter are not significant at related significant level.

Table 4. Correlation coefficient between eleven traits measured from calli initiated and grew on media contained six different NaCl levels.

Variables	Fresh weight	Water content	Dry matter	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	K^{+}/Na^{+} ratio	Ca^{2+}/Na^{+} ratio	Cl^{-}	Proline
Fresh weight	1.0000										
Water content	0.9990 ***	1.0000									
Dry matter	0.7558 ***	0.7261 ***	1.0000								
Ca^{2+}	0.3018 **	0.3220 ***	-0.0765 ns	1.0000							
Mg^{2+}	0.0409 ns	0.0603 ns	-0.2565 **	0.1118 ns	1.0000						
Na^{+}	-0.2980 **	-0.3112 **	-0.0262 ns	-0.3360 ***	-0.0690 ns	1.0000					
K^{+}	0.5633 ***	0.5725 ***	-0.2806 **	0.0695 ns	0.1767 ns	-0.2479 *	1.0000				
K^{+}/Na^{+} ratio	0.3362 ***	0.3517 ***	0.0194 ns	0.3348 ***	0.1542 ns	-0.7669 ***	0.3373 ***	1.0000			
Ca^{2+}/Na^{+} ratio	0.3051 **	0.3220 ***	-0.0250 ns	0.5731 ***	0.1164 ns	-0.7263 ***	0.1679 ns	0.9291 ***	1.0000		
Cl^{-}	-0.2898 **	-0.2997 **	-0.0690 ns	-0.2766 **	-0.0471 ns	0.7884 ***	-0.2760 **	-0.5743 ***	-0.5269 ***	1.0000	
Proline	-0.3210 ***	-0.3302 ***	-0.1010 ns	-0.2511 *	0.2176 *	-0.0489 ns	-0.3208 ***	-0.0997 ns	-0.1308 ns	0.0298 ns	1.0000

ns, *, ** and *** not significant, significant at P<0.05, significant at P<0.01, and significant at P<0.001, repectively.

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