Supplemental Calcium Regulates Proline Accumulation in NaCl-stressed Suspension Cultures of *Oryza sativa* L. At the Level of mRNA Translation

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**Abstract:** The influence of NaCl and CaCl_2 on growth, ion accumulation and proline accumulation was investigated in cell suspension cultures of rice (*Oryza sativa* L. cv. Taper-309). The relative growth rate of suspensions was significantly greater at high calcium level (8.0 mM) than at low (0.5 mM) in response to 150 mM NaCl. Salinity caused a large increase in Na^+ and K^+ contents of suspensions. K^+ content was not affected by the levels of calcium but Na^+ content was significantly reduced by a high calcium and showed a lower Na^+ /K^+ ratio. The proline level of cells increased in response to NaCl but the increase was 4.9-fold at high and 1.4-fold at low calcium levels respectively. Actinomycin-D had little effect on proline accumulation in stressed cells at high calcium. In contrast proline accumulation was severely inhibited by cycloheximide. It is suggested that in NaCl stressed suspension cultures of rice, calcium participate in the regulation of proline accumulation at the level of mRNA translation.

**Key words:** CaCl_2, cell suspensions, inhibitors, NaCl, *Oryza sativa* L., proline

**Introduction**
In arid and semi-arid regions salinity is the major problem in agriculture that impairs plant growth and productivity (Munns, 1993). Salt tolerance is a complex, quantitative polygenic trait. Plants have evolved many types of adaptations to salinity ranges from developmental and structural levels to physiological and biochemical levels (McCue and Hanson, 1989). Consequently, by classical breeding approach little commercial success has been achieved in cultivated species, despite several attempts. Alternatively pyramiding approach of crop improvement to accumulate physiological traits that contribute to tolerance in a single genotype has been proposed with the probability of more scope for maximizing tolerance improvement and their integration at various levels (Yao and Flowers, 1998; Noble and Rogers, 1992).

During salt stress, many plants including halophytes and glycophytes accumulate compatible cytosolutes such as glycinebetaine and proline. The accumulation of these compounds is believed to be of importance of adaptive significance in counteracting the effects of osmotic stress (Greenway and Munns, 1980; Wyn Jones, 1986). Among the organic cytosolutes, proline appears to be the most widely distributed osmolyte that accumulates under stress conditions in plants (McCue and Hanson, 1990). It has been suggested that proline accumulation under stress plays several roles, namely, as an osmoticum (Wyn Jones et al., 1977), nitrogen and carbon reservoir for post stress condition (Ahmad and Hellebust, 1989), hydroxy-radical scavenger (Smirnoff and Cumbes, 1989), redox buffer (Saradhi and Saradhi, 1991), mean of reducing the acidity (Venekamp et al., 1989) and compatible solute that protect enzymes and organelles (Palig et al., 1984, Nash et al., 1982). It is considered that proline accumulates mainly due to de novo synthesis from glutamate (Boggess et al., 1976, Rhodes et al., 1986, Voetberg and Sharp, 1991).

Elevated calcium concentration in medium has been reported to confer tolerance to plants subjected to NaCl stress (Lahaye and Epstein, 1971; Cramer et al., 1988; Lin and Kao, 1996).

Many data regarding the ameliorating influence of calcium on salinity stress is related to its importance for membrane integrity. The experimental evidences regarding the possible role of calcium in the accumulation of organic cytosolutes (e.g. proline) in stressed plant and cells is lacking. The present study was conducted to investigate the effect of calcium on proline accumulation in NaCl stressed cell suspension cultures of rice.

**Materials and Methods**

**Cell Cultures:** Seeds were dehulled and surface sterilized in 70% ethanol for 30 seconds followed by a 15 minutes washing with 70% sodium hypochloride. After 5 washings with distilled water, seeds were inoculated onto modified MS (Murashige and Skoog, 1962) medium supplemented with 2 mg liter^{-1}, 2,4-dichlorophenoxy acetic acid (2,4-D), 0.25 mg liter^{-1} kinetin, 30 g liter^{-1} sucrose and solidified with 9 g liter^{-1} agar. All cultures were incubated in dark at 28 ± 1°C.

Following third sub-culturing of 28 days each, rapidly growing friable calli were sub-cultured and used for suspension cultures. Suspension cultures were established by inoculation of calli into 50 ml liquid MS medium in 200 ml Erlenmeyer flasks. The cultures were incubated at 100 rpm at 16h day^{-1} photoperiod at 28 ± 1°C. After two weeks, dense suspensions of microcalli/cells were sub-cultured in medium by decanting to about 1/10 dilution every 7 days.

**Measurement of Growth:** The growth of the suspensions was calculated as the relative growth (RGR) rate of sediment cell volume (SCV) by the following formula of Shah et al. (1983)

**RGR:** \((\text{weeks}^{-1}) = \ln ISCV_{t=0} \div \ln ISCV_{t=\text{weeks}^{-1}} / \text{Weeks} \)

**Experimental Design and treatments:** The experiment was conducted using a complete randomized design containing two treatments of NaCl (0 and 150 mM) and two CaCl_2 levels (0.5 and 5.0 mM).
Determination of free proline: Free proline in cells was determined utilizing the method of Bates et al. (1973).

Extraction and measurement of Na⁺, K⁺ and Na⁺:K⁺ ratios: Suspension cultures were gently vacuum filtered. The cells/microcalli were washed with 10 ml of cold iso-osmotic D-sorbitol. Na⁺ and K⁺ ions in washed cell suspensions were extracted by boiling cells in distilled water in a boiling water bath for one hour and measured by atomic absorption flame spectro photometry (Chaudhary et al., 1989).

Cycloheximide and Actinomycin-D treatments: Cycloheximide (translation inhibitor) and actinomycin-D (transcription inhibitor) of the concentration range from 5 to 15 μg liter⁻¹, were added to the medium to investigate the regulatory role of supplemental calcium on proline regulation in NaCl- stressed suspensions. The cultures were incubated in the dark for 20 hours. The viability of microcalli was tested with fluorescein diacetate dye (Widholm, 1972). Free proline concentration was measured at every four hours during the treatment.

Data Analysis: Agstat package was used to analyze the data.

Results
Growth of microcalli/cells: The relative growth rates of suspensions are presented in Fig. 1a. Under non-stressed (0 mM NaCl) conditions calcium treatments showed no effect on growth. While a 150 mM NaCl concentration reduced growth by 18% and 42% of the control values at high (5.0 mM) and low (0.8 mM) calcium levels, respectively. Statistical analysis showed that the effects of NaCl and CaCl₂ and their interaction were highly significant on the growth of rice cells (Table 1).

Na⁺, K⁺ and Na⁺:K⁺ ratios: The Na⁺ and K⁺ contents of cells in both calcium treatments substantially increased when 150 mM NaCl was added in the medium (Fig. 1b and 1c). However, suspension grown at high calcium accumulated significantly lower Na⁺ than at low calcium level (Fig. 1b). The difference between two calcium treatments was statistically significant for Na⁺ but not for K⁺ concentrations in response to NaCl stress (Table 1). As a result of partial inhibition of Na⁺ uptake and exclusion of Na⁺ from suspensions a significantly low Na⁺:K⁺ ratio was observed at high calcium than that at low calcium (Fig. 1d).

Proline: The proline concentration in non-stressed cells at low and high calcium was similar. In case of NaCl stress, a large increase in proline concentration was observed in cells supplemented with high calcium, while there was a little increase in proline level of cells grown at low calcium (Fig. 2). The ANOVA test showed a highly significant effect of NaCl, CaCl₂ and their interaction on proline accumulation (Table 1).

Effects of Cycloheximide and actinomycin-D on proline accumulation: In non-stressed suspensions with no inhibitors the proline level remained stable (with little fluctuation) throughout the duration of experiment, while in stressed suspensions (i.e. 150 mM NaCl with or without cycloheximide / actinomycin-D the proline level began to increase gradually up to 8 hours (Fig. 3). It can be seen from Fig. 3 that the pattern of proline accumulation in actinomycin-D treated cells under stress was similar and almost parallel to the proline level of suspensions grown in NaCl only (i.e. a large and sharp increase in proline concentration of tissues from 8 to 24 hours of stress). While, at a similar stress (150 mM NaCl proline content of cycloheximide treated cells was substantially lower than the proline level observed in actinomycin-D treated cells.

Discussion
In the present study the response of rice suspension cultures to interactive effects of NaCl and CaCl₂ has been investigated. Addition of 150 mM NaCl in growth medium caused reduction in growth but to a different degree in response to calcium treatments (Fig. 1a). The growth rate decreased to 83% and 58% of control at high and low calcium levels respectively. This indicates that additional calcium confer considerable tolerance to NaCl stressed suspensions of rice. These results are in agreement with reports of LaHay and Epstein (1971), Shah et al. (1990) and Zhong and Lauchli (1984). Growth is the consequence of cell division, elongation and salinity inhibits both of these processes while supplemental calcium in part reverses the NaCl-induced growth reduction (Hepler 1986, Binzel et al., 1988). In root cells of cotton Kruth et al.
Table 1: Summary of analyses of variance of the effects of NaCl and CaO₃ on relative growth rate, Na⁺/K⁺ ion concentration, 
Na⁺/K⁺ ratios and proline concentrations in cell suspension culture of rice (Oryza sativa L. cv. Taipi-309). Degree of 
freedom (df), mean square (MS), F-ratios (F) and exact probabilities (P-values) are given.

<table>
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<th>Source of variation</th>
<th>Relative growth rate</th>
<th>Na⁺/K⁺ Ion</th>
<th>Na⁺/K⁺ Ratio</th>
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<td></td>
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Fig 2: The effects of NaCl and CaCl₂ on proline 
accumulation in cell suspension culture of rice (Oryza 
sativa L. cv. Taipi-309). Data are means of 5 
replicates ± SE.

(1985) found decreased cell production and size at 150 mM 
NaCl with low calcium (0.4 mM), while no reduction was 
oberved at the same salinity at 10 mM calcium. On the other 
hand Zhong and Laughlin (1988) observed that 150 mM NaCl 
stress at 1 mM calcium caused a 50% reduction in glucose 
incorporation into the cellulose fraction of the cell walls of 
cotton roots, whereas 10 mM calcium completely counteracted 
the adverse effects of NaCl on glucose incorporation.
If we look at Fig. 1b, it is clear that under NaCl stress calcium 
plays a significant role in ion uptake and regulation, possibly 
by maintaining the stability and function of membranes. 
Supplemental calcium decreases the detrimental effects of 
NaCl by stabilizing the membranes through bridging phosphate 
and carboxylate groups of phospholipids and proteins at the 
membrane surface (Legge et al., 1992). Displacement of 
Ca²⁺ from plasma membrane by Na⁺ depends upon Na⁺ and 
Ca²⁺ concentrations of the medium (Cramer et al., 1989). 
While, Na⁺ displaces Ca²⁺ from plasma membrane. The 
displacement of Ca²⁺ from protein part reduces the membrane 
stability. At high external Ca²⁺, Na⁺ displacement of Ca²⁺ 
occurs only at phospholipid sites, which would not have the 
same physiological consequences as displacement from 
proteins (Cramer et al., 1988). Working with barley, rice and 
Arabidopsis Blas et al. (1996), Lin and Kao (1998) and Liu
and Zhu (1997) have suggested that calcium abates the toxic effects of Na⁺ rather than the osmotic effects of salt stress. Results presented in Fig. 2 and 1a reveals that calcium also has a role in alleviating the osmotic component of salt stress. This accord with the findings of Shah et al. (1990, 1997) and Colmer et al. (1998). In stressed cells at high calcium, Na⁺ content were 20% less as compared to cells grown at low calcium (Fig. 1b). It means that supplemental calcium reduces the toxicity component of NaCl stress. However, minimization of toxicity component of stress by ion exclusion leads to water deficit in cell i.e. osmotic stress (Mars and Nieman, 1978).

But in this study the cultures that had less Na⁺ ions, accumulated 3.9 times more proline than the cells having higher level of Na⁺ ions (Fig. 1b and Fig. 2). The higher level of proline accumulation probably facilitates osmotic adjustment and account for reduced inhibition of growth by NaCl.

Actinomyein-D (transcription inhibitor) had little effect on proline accumulation in response to NaCl stress, while cycloheximide (translation inhibitor) treatment resulted in a large decrease in proline content of suspensions (Fig. 3). It could be envisaged that supplemental calcium might participate in proline accumulation in NaCl-stressed suspension cultures mainly at the level of mRNA translation rather than at the level of DNA transcription. This provides for the first time a direct evidence for ameliorating the effect of supplemental calcium in mitigating the osmotic component of NaCl stress.

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References


