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SALT TOLERANT CALLUS LINES OF *DENDROCALAMUS STRICTUS* NEES UNDER SALT STRESS: GROWTH AND ION ACCUMULATION

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Abstract

Plant tissue culture technique offers a suitable opportunity for development of salt tolerant plants. The technique is also helpful in understanding physiological and molecular mechanisms of tolerance to salt stress under *In vitro* condition at the cellular, organ, and whole-plant level. This study was performed to understand the growth responses and accumulation of Na^+ and K^+ ions against the salt stress in NaCl tolerant callus of *Dendrocalamus strictus* Nees. The embryogenic callus tolerant to 100mM NaCl was screened out by exposing to increasing (0-200mM) concentrations of NaCl in 0.8% (w/v) agar gelled Murashige and Skoog medium having 3% (w/v) sucrose, 13.6 µM 2,4-Dichlorophenoxy acetic acid and 2.3µM Kinetin (Callus initiation medium). Stable growth of NaCl tolerant callus lines on NaCl-free as well as on NaCl-containing medium was recorded. The relative growth rate of NaCl sensitive callus decreased significantly with increasing concentrations of NaCl into the medium while, for tolerant callus was consistent on medium having 50 and 100mM NaCl and decreased further with increasing concentration of NaCl. The FW/DW of NaCl sensitive callus was higher than the NaCl- tolerant callus. The ratio K^+/Na^+ was recorded low when the callus was grown on medium supplemented with NaCl and among sensitive and tolerant callus this ratio was found least in tolerant callus when they were grown on medium having 100mM NaCl.

Key word: Bamboo; Callus; Growth; Sensitive; Tolerant

Introduction

Fecting many physiological and metabolic processes in plants (Zhu, 2001; Munns and Tester, 2008). The improper management practices of fertile lands is generally contributing in increasing the salinity in agricultural field of India and many other countries in the world (Liu and van Staden, 2000; Singh *et al*., 2003). The earlier studies demonstrated that plants differ in their tolerance of salinity and generally dicotyledonous species is known for greater salinity tolerance than in monocotyledonous species (Munns and Tester 2008). The understanding of the mechanisms that enable plants to adapt and grow under salt stress is necessary to utilise salt affected area in the world. Of the available methods for developing salt tolerant plants, the insufficient knowledge about mechanism (s) controlling the character and involvement of several genes, generally makes difficult breeding or genetic engineering for salt tolerance (Shanthi *et al*., 2010, Rai *et al*., 2011). However, plant cell and tissue culture offers an opportunity and are being used for the improvement and developing salt tolerance lines of economically useful plants (Gandonou *et al*., 2006, Mori-Gastelo *et al*., 2015; Hamdi *et al*., 2017). This technique also offers for studying the physiology of intact plantlets together with that of organs and single cells using homogenous plant material under uniform environmental conditions (Zhu, 2001; Munns and Tester 2008; Rai *et al*., 2011). Salt tolerant lines when compared with normal sensitive cells, can provide a useful means of measuring the capacity and range of stress tolerance and may be used to elucidate tolerance mechanisms at the level of cell (Liu and van Staden, 2000; Gandonou *et al*., 2006).

Bamboos, belongs to the family Poaceae, have long history of widely used plant resource (Singh *et al*., 2001). The different species of bamboo have great potential in reclamation of wasteland and in social forestry. The understanding the mechanism of salinity tolerance in *Dendrocalamus strictus* may be helpful in developing

salt-tolerance in other plant species and bamboos. Thus, the present investigation was undertaken with the objective of developing NaCl tolerant callus lines and comparing the response of salt-sensitive and selected salt-tolerant callus-line of *D. strictus* to salinity for understanding the mechanism involved in salt stress at callus level.

Material and Methods

Callus Induction

The seeds were dehusked and then surface sterilized by agitating in 1.0% (v/v) sodium hypochlorite solution for 10 min, followed by 10-12 min washing under running tap water. After washing all the seeds were dipped in 0.05% (w/v) mercuric chloride solution for 4 to 5 min and finally washed 3 times with autoclaved double distilled water. The surface sterilized seeds were aseptically inoculated for embryogenic callus induction on 0.8% (w/v) agar gelled Murashige and Skoog (1962, MS) medium containing 3% (w/v) sucrose 3.0mg/l 2,4-D, 0.5mg/l Kinetin (callus initiation medium). The pH of the medium was adjusted to 5.8 ± 0.02 before autoclaving at 1.06 kg cm^{-2} for 15 min. The callus produced on such medium were subcultured for 4 or 5 times on the same medium and the actively growing callus after subculture was selected for screening of NaCl –tolerant cell lines.

Screening of NaCl-tolerant callus

The actively growing callus was transferred to callus initiation medium with different concentrations (0, 50, 100, 150, 200 and 250 mM) of NaCl for screening of NaCl tolerant callus lines. The callus grew with necrosis patches on 0-200 mM NaCl containing medium. The healthy growing callus from each concentrations of NaCl-containing medium were separated and subcultured regularly on fresh media having same concentration of NaCl as was on the previous medium. In this way 100mM NaCl-tolerant callus was screened out. The stability test of 100 mM NaCl-tolerant callus was performed as reported by Singh *et al*., 2003.

Fresh weight and Dry weight measurement

The sensitive and 100 mM NaCl tolerant callus were taken out from the culture tube and blotted dry to remove the water droplets sticking to the external surface of the callus and immediately fresh weight of the sample was taken. For dry weight measurement, samples were oven dried at 60ºC for 48h and subsequently the dry weight was measured. The FW and DW measurements were carried out at 1, 7, 14, 21 and 28 day interval after the subculture of callus on fresh maintenance medium with 100mM NaCl.

Relative growth rate (RGR)

The relative growth rate (gg^{-1} $week^{-1}$) of control and NaCl tolerant callus lines determined were based on increase in FW over a period of 30 day, using the formula for exponential growth (Lutts *et al*., 1996).

$$R^{=x\ln W2-\Delta t^{In\ddot{u}Wiz\ddot{u}}}$$

Tolerance index (TI)

The tolerance index of control and 100mM NaCl tolerant callus lines was compared by measuring FW of embryogenic calli after 30 days of subculture on callus maintenance medium having various concentrations of NaCl and using the formula (Dutta Gupta *et al*., 1995).

$$TI=\frac{Fw\,on\,NaCl\,medium}{FW\,on\,NaCl\,\,free\,mdium}\times 100$$

Estimation of K^+/Na^+

To estimate K^+ or Na^+ ion in sensitive and tolerant callus, 1.0gm callus from each treatment was digested concentrated HNO_3 (20ml). The acid digested callus was the cooled and the final volume of the digest was maintained with double distilled water up to 50 ml. The Na^+ and K^+ in the acid extract were estimated with the help of a flame photometer (Clesceri *et al*., 1989) and the K^+/Na^+ ratio were calculated.

Experimental Design and statistical analysis

Experimental design was completely randomized. All the experiments were repeated three times and each experiments performed had 24 replications. Data were analysed using one-way analysis of variance and the comparisons between the mean values of treatments were made by least significant difference (LSD) test (Gomez and Gomez, 1984).

Results and Discussion

The tissue culture selection of NaCl tolerant callus lines has been successful with some plants such as *Morus* spp. (Vijayan *et al*., 2003), *Saccharum* sp. (Gandonou, 2006), *Solanum tuberosum* (Hamdi *et al*., 2017). In this study, screening of NaCl tolerant callus was performed by exposing the freshly grown embryogenic callus to medium having increasing concentration of NaCl, similar to our earlier studies as reported in Singh *et al*. (2003). The fresh weight measurement of control and tolerant callus on maintenance medium with or without NaCl did not show similar pattern (Table 1). The sensitive callus grown on maintenance medium without NaCl resumed their normal growth within seven days of culture and showed linear increase in FW with increasing culture

Table 1: Changes in fresh weight (FW) and ratio of fresh weight to Dry weight (FW/DW) of sensitive and NaCl-tolerant callus

Callus type	Concentration of NaCl in medium (mM)	Time period (in days)	Fresh Weight mg (Mean ± SE)	FW/DW (Mean ± SE)
Sensitive	0.0	1	170±10	6.29±0.46
		7	273.33±16.15	6.22±0.58
		14	435.11±54.06	6.10±1.17
		21	595.22±68.96	6.19±0.99
		28	667.55±27.28	6.23±0.41
Tolerant	0.0	1	170.00±10	6.51±0.14
		7	190.67±6.95	5.70±0.63
		14	337.78±15.98	5.33±0.39
		21	401.99±16.02	5.42±0.33
		28	501.78±78.57	5.20±0.48
Tolerant	100.0	1	170.00±10	5.08±0.11
		7	202.67±10.04	5.16±0.31
		14	317.22±39.19	5.56±0.47
		21	580.22±15.57	5.77±0.17
		28	640.56±67.12	5.63±0.19

time period but the FW of the tolerant callus grown on medium with or without NaCl was slow during initial 14 days of subculture and then resumed normal growth. On medium having 150 and 200 mM NaCl, callus growth could not be maintained after two months of transfer (Table 1). Such a pattern of growth was also seen in *Solanum melongena* (Hassan and Wilkins, 1988; Mori-Gastelo *et al*., 2015). The initial lag in growth of tolerant callus could be due to diversion of metabolites used for normal developmental process towards the process associated with various adaptive mechanisms like maintenance of higher level of osmotic solutes, in avoidance of the uptake of the toxic ions and translocation of these ions and their accumulation at specific sites within the cell (compartmentalisation) and to absorb the inorganic ions like Na^+ for maintaining a high osmotic level (Chinnusamy *et al*., 2005; Rai *et al*., 2011). The FW of the tolerant callus on NaCl supplemented medium was more or less equal to the sensitive callus grown on medium without NaCl, thus, showing towards adaptation to salt stress. The FW of the tolerant callus measured on 28th day of transfer on medium without NaCl was low as compared to their growth on NaCl Medium. This shows that the tolerant callus requires NaCl for its optimal growth and similar observation has been reported earlier in sugarcane (Gandonou *et al*., 2006), rice (Mori-Gastelo *et al*., 2015). The ratio of FW to DW was higher for sensitive callus transferred to medium without NaCl as compared to tolerant callus grown on medium with or without NaCl (Table 1). The low FW/DW ratio in tolerant callus observed during their growth on medium with (100mM) or without NaCl, may be due to accumulation of various solutes associated with osmotic adjustment which were perhaps essential for balancing the water potential inside the cells as compared to the surrounding media (Zhu, 2001; Chinnusamy *et al*., 2005; Rai *et al*., 2011).

Relative growth rate (RGR)

The RGR of tolerant callus was significantly higher than sensitive callus at each concentration of NaCl tried and also with increasing concentration of NaCl, the RGR of both control and tolerant callus decreased but the decrease observed in RGR of tolerant callus between 50 and 100 mM NaCl was insignificant, while, at higher concentrations the decrease was significant. As compared to tolerant callus, the decrease observed in RGR of control callus was significant at each concentrations of NaCl studied (Fig. 1). This observation on decrease in RGR of sensitive and tolerant callus with increasing NaCl stress could be due to non adjustment of callus cells to external stress. Decrease in RGR of rice callus by presence of stressing agent like NaCl, KCl, Na_2SO_4, artificial sea water and mannitol in the medium has been observed by Lutts *et al*. (1996).

Tolerance Index

The tolerance index of both sensitive and tolerant callus decreased with increasing concentrations of NaCl in to the medium. The TI of tolerant callus was significantly high as compared to sensitive callus on medium supplemented with 50, 100 and 150 mM NaCl and at 200 mM NaCl though the TI of tolerant callus was higher than sensitive callus but the difference was insignificant (Fig. 2). The higher TI of NaCl tolerant callus than sensitive callus observed in the present study demonstrates former to be more resistant to NaCl than

Table 2: Accumulation of Na^+ and K^+ ions in NaCl Sensitive and Tolerant callus

Callus Type	Conc. of NaCl in medium (mM)	K^+/Na^+ (m molesg^{-1} FW) Mean ± SE	
		Initial Value	Final Value
Sensitive	0.00	1.75±0.26	1.87±0.28
	100.00	1.75±0.25	0.59±0.05
Tolerant	0.00	0.68±0.12	2.04±0.38
	100.00	0.68±0.12	0.46±0.05

Initial Value : Analysis performed on the day of subculture
Final Value : Analysis performed on 28th day of subculture

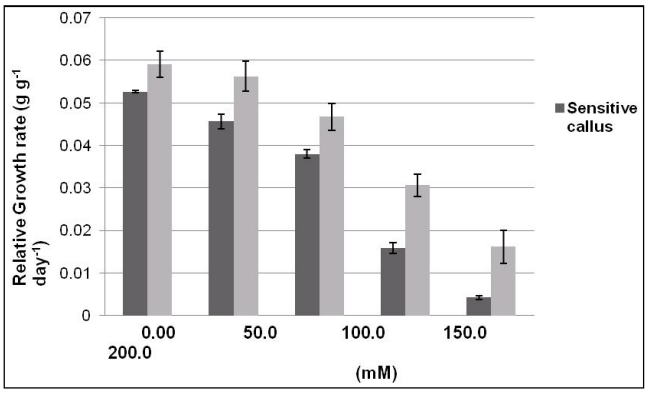

Fig. 1: Relative Growth Rate (RGR) of NaCl-tolerant and sensitive callus under salt stress

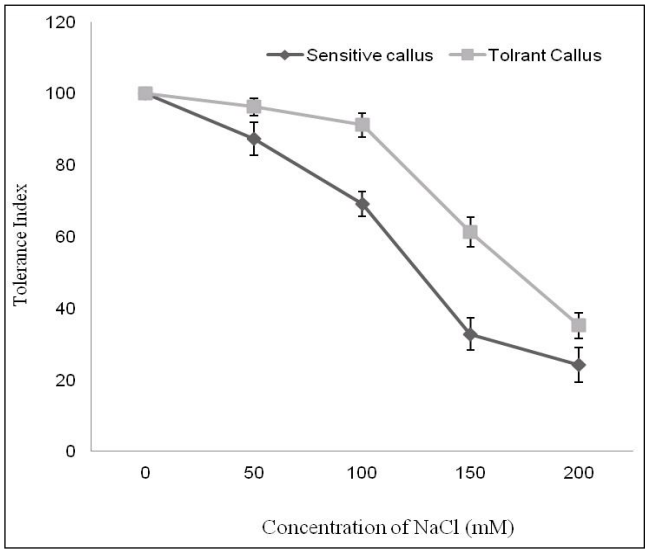

Fig. 2: Tolerance index (TI) of NaCl tolerant and Sensitive callus under salt stress

the later. Similar observation was also recorded by Dutta Gupta *et al*. (1995) in *Dactylis glomerata* L.

Ion accumulation

Accumulation of inorganic ion is a common osmoregulatory response to salt stress. In the present investigation the ratio K^+/Na^+ was about 3 fold high when the sensitive or 100mM NaCl tolerant callus was grown on medium without NaCl as compared to their growth on medium having NaCl. This variation in K^+/Na^+ ratio is due to increase in Na^+ level in the presence of NaCl in the medium and decline in K^+ concentration (Blumwald, 2000; Munns and Tester 2008; Deinlein *et al*., 2014). In the sensitive callus the ratio was significantly higher when the callus was grown on medium without NaCl than on NaCl supplemented medium. Similarly, about 3 fold increase in the K^+/Na^+ ratio was recorded when the tolerant callus was grown on medium without NaCl as compare to their growth on 100mM NaCl supplemented medium. A significant difference was recorded in K^+/Na^+ ratio in tolerant callus during their subculture on medium with 100 mM and on medium without NaCl (Table 2). The ability of plant cells to maintain low cytosolic sodium concentrations is an essential process associated with the ability of plants to grow in high salt concentrations (Blumwald, 2000). On unstressed conditions, a high cytosolic K^+/Na^+ ratio is maintained in plant cells. But on increase in NaCl concentration around surrounding environment, Na^+ may establishes a large electrochemical gradient and favouring the passive transport of Na^+ ions into the cells through K^+ transporters (Blumwald, 2000; Roy *et al*., 2014).

Thus, this can be concluded that *In vitro* culture can be used for understanding the mechanism of salt stress and in improvement and developing salt tolerance lines useful plants.

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