Growth and Ion Content in Salt Tolerant and Normal Lines of *Vetiveria nemoralis* A. Camus under Salt Stress

S. Sritubtim[1], M. Nanakorn[2*] and P. Thanomchart[3]

[1] Department of Science, Faculty of Science and Technology, Rajamangala University of Technology Krungthep, Bangkok 10120, Thailand
[2] Department of Botany, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand
[3] Department of Packaging and Materials Technology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand

Introduction

As the world's population escalates, it is inevitable that agricultural land will decrease, with impacts routed from global climate change. The impact of these changes, alongside natural disasters, such as the Tsunami of 2004 and 2011, causes degradation of agricultural land, thus accelerating the expansion of salt affected areas (IPCC, 2003; UNFCCC, 2007; Hopmans and Maurer, 2008). To reduce this problem and generate more fertile soil, the area must be covered with salt-tolerant plants, which reduces the evaporation rate of soil water, and consequently suppresses the salt from spreading up towards the soil surface. Soil porosity and water permeability increases as the plants' roots penetrate through the soil. Finally, organic matter increases after plant decomposition, improving soil fertility.

Vetiver grass has a lesser ability to tolerate low salinity compared to other salt-tolerant grasses (Alshammary, 2004; Nanakorn *et al*., 2005) and its growth was progressively inhibited under 0.5-1.0% NaCl (85.5-171 mM NaCl) (Nanakorn *et al*., 2006). Nevertheless, the development of this species to become more tolerant to salt has become an interest for the improvement of salt-affected areas as the plant possesses large, elongated roots. These apparent characteristics not only improve soil structure and fertility, but also enable the fixing of the soil, thus preventing soil erosion. Moreover, vetiver grass is not invasive, nor difficult to remove. Due to these desirable properties, vetiver grass was selected to improve upon its salt tolerance *in vitro*. Although it has been reported that a salt-tolerant selected line of *V. nemoralis* was able to tolerate up to 4% NaCl in *in vitro* condition (Suwannachitr, 1997), it was unable to be maintained in a laboratory. In addition, its high salt-tolerance level may have been the result of an escape mechanism as its salt tolerance was not stable. Thus, it is important to carry on the experiment of salt-tolerant selection of vetiver grass for the future management of the salt affected areas, rehabilitation, and to enable the soil to become more fertile.

In the process of improving the plant's salt tolerance, it was found that the genetic variation, found in *in vitro* culture of somatic cells or so called somaclonal variation, is a valuable tool for the selection of salt-tolerant lines, as has been reported in *Vaccinium corymbosum* (Muralitharan *et al*., 1990), *Brassica juncea* (Kirti *et al*., 1991), rice (Winicov, 1996), and *Dactyloctenium aegyptium* (Nanakorn, 2005). In order to alleviate selection efficiency, somaclonal variation can be combined with colchicine. Polyploidy induction attributed by this chemical has been reported as an efficient way to improve abiotic stress tolerance,

* Coresponding author: fscimln@ku.ac.th

such as in *Coccinia palmata* (enhanced drought tolerance, Ntuli and Zobolo, 2008) and *Dendranthema nankingense* (enhanced cold-, drought- and salt tolerance, Liu *et al*., 2011). Therefore, in the present study, colchicine was used to induce polyploidy from callus, which is a source of genetic variability, in order to improve the salt tolerance of vetiver grass.

Materials and methods

Callus induction

Young inflorescences wrapped in a flag leaf of a Prachuab Kiri Khun germplasm (PC) of *Vetiveria nemoralis* A. Camus, were surface sterilized by dipped into a 70% ethyl alcohol solution, inflamed for surface sterilization, and then cut into 1 cm long. These explants were cultured for 4 weeks on a semi-solid MS medium (Murashige and Skoog, 1962) supplemented with 10 μM 2, 4-diclorophenoxyacetic acid (2, 4-D) for callus induction and for 4 weeks more on the same medium supplemented with 5 μM 2, 4-D for callus proliferation. The creamy white color nodular calli were selected and transferred onto hormone-free MS medium for 2 weeks to induce plantlet regeneration. The cultures were kept under 16 h light/ 8 h dark at 25 ± 3 ^{o}C.

Polyploidy induction and salt-tolerant lines selection

For polyploidy induction, the embryogenic calli with developing somatic embryos, as indicated by green spots, were cultured on a semi-solid MS medium supplemented with 0, 0.01, 0.05 and 0.1% colchicine for 1 day, then transferred onto a hormone-free MS medium contained 1.5 and 1.75% NaCl for 4 weeks for selecting of salt tolerant lines. Four replications, each with four pieces of callus, were conducted in a completely randomized design (CRD). The number of regenerated plantlets was recorded.

The survived plantlets were analyzed for their DNA content with flow cytometer (Partec PA, Germany) following Cystain UV ploidy Partec protocol (Liu *et al*., 2007). Polyploidy plantlets were propagated by culturing on semi-solid MS medium supplemented with 10 μM BA for 6 weeks and sub-cultured three times, 4 weeks interval, on the same medium. Rooting of the polyploid shoots was induced on hormone-free MS medium for further studies on their physiological responses under *ex vitro* salt treatments.

Plantlets regenerated from non colchicine-treated calli, the normal line, were propagated by means of the same procedures as mentioned above.

Growth and ion content under salinity stress

Salt-tolerant and normal line plantlets were transplanted to an *ex vitro* sand culture and irrigated with half-strength Hoagland's solution. After 3 weeks, a full strength Hoagland's solution containing 0.25% NaCl was added for salt-stress treatment and regularly adjusted in 3 days interval to those required concentrations of 0.25, 0.5, 0.75 and 1.0% NaCl. Three replications, each with three plants, were carried out in CRD. After 5 weeks, the number of tillers per clump was recorded. The shoots, dry leaves, and roots were harvested separately, washed with distilled water, and then blotted dry and weighed. All samples were oven-dried at 60 ^{o}C until their dry-weights were constant.

For the K^+ and Na^+ analyses, the dried samples were digested in a conc. HNO_3: $HClO_4$ (2: 1) solution and examined with a flame-emission spectrophotometer. For the Cl^- analysis, dried samples were ashed, dissolved with boiled-deionized water, and the content was determined

with colorimetric method (Adriano and Doner, 1982).

Data was analyzed using IRRI Stat for analysis of variance and the significant difference between means was determined by Duncan's New Multiple Range Test at $p < 0.05$.

Results and Discussion

Polyploidy induction and salt tolerance of selected line

The calli with developing somatic embryos (SE) of the Prachuab Kiri Khun germplasm were used in this experiment to avoid the loss of regeneration capacity of the calli after selection on NaCl containing medium for a long period of time. Consequently, plantlets may not develop although salt tolerant calli were achieved. In this present study, after exposing to 0.05% colchicine for 1 day and subsequently to 1.5% NaCl for 8 weeks, only one SE developed to plantlet. However, chromosome doubling was not observed as determined by flow cytometry (Fig. 1). The result revealed that directly treated the calli with NaCl after colchicine treatment was not appropriate for the induction of polyploid-salt tolerant plantlets since the cell cycle usually take many hours to complete such as 36 h in *Datura*, 41 h in *Triticum* and 157 h in *Sinapsis* (Lyndon, 1990). Therefore, the developing somatic embryos may be killed by NaCl before chromosome doubling was taken place. On the other hand, it is possible that the one-day treatment of colchicine may not suitable for polyploidy induction in this germplasm of *V. nemoralis*. The range of colchicine concentrations used in this experiment was efficiently induced polyploidy in many plant species but with shorter or longer treatment duration. For example, the most efficient treatment for *Trigonella maritima* (legume) was 0.05% colchicine for 4 h (Haouala *et al*., 2009), for *Miscanthus* spp. (grass) was 0.0125% (313 μM) colchicine for 18 h (Glowacka *et al*., 2010), 0.005% colchicine for 8 h *Vicia villosa* (legume) (Eradi and Unal, 2010), *Gerbera jamesonii* was 0.1% colchicine for 8 h (Gantait *et al*., 2011).

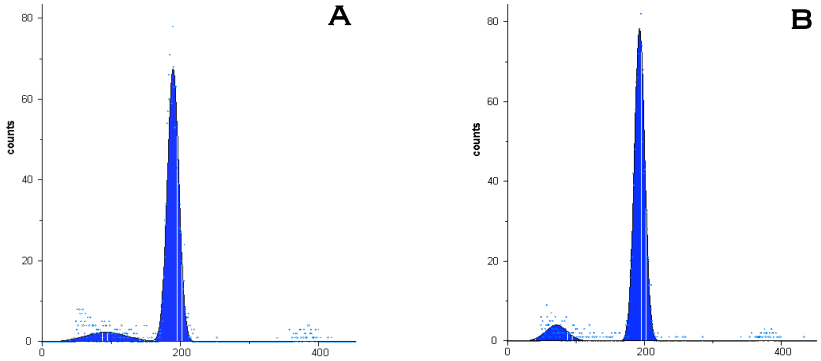

Fig. 1 Flow cytometry histograms of control diploid plant (a), and plant derived from 0.05% colchicine treated callus and selected on 1.5% NaCl (b).

Although polyploidy induction was not achieved, the plantlet survived on 1.5% NaCl was able to grow and tiller when cultured on 1.5% and 1.75% NaCl-containing MS medium for 8 weeks (Fig. 2), so called salt tolerant line (PCs). The normal line (PCn), which regenerated from non colchicine treated calli, capable of tolerating at a maximum concentration of 1.0% NaCl. The variation of PCs from its original PCn may result of somaclonal variation generated in *in vitro* by culture medium and environment (Bajaj, 1990) or colchicine treatment. Alam *et al*. (2011) reported that treatment of 0.05% colchicine for 2 h induced changing of many characters in local potato varieties of Bangladesh such as increasing in plant height, number of leaves per plant, plant fresh weight and tuber formation although no polyploidy induction.

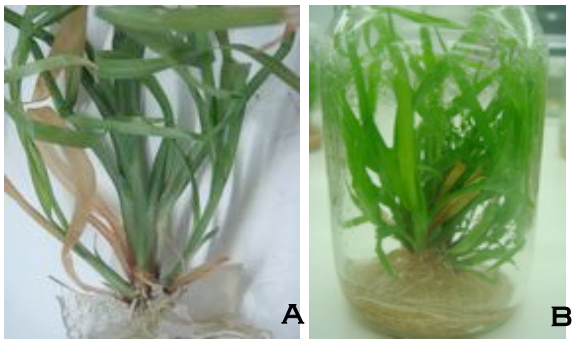

Fig. 2 Plant derived from 0.05% colchicine treated callus (PCs) cultured on MS medium supplemented with 1.5% (a), and 1.75% (b) NaCl for 8 weeks.

Growth and ion content under salinity stress

Under *ex vitro* treatment of 0-1.0% NaCl, growth of both lines was decreased as salinity increased except for root growth at some concentrations of NaCl (Fig. 3). However, the number of tiller/clump and shoot dry weight (as percentage of control) of PCs at 0.5-1.0% NaCl were higher than that of PCn, whereas, dry weight of dry-leaves or dead-leaves, which indicated leaf injury, was lesser. It was remarked that root growth of PCs was enhanced at 0.75% (165.7% control) and 1% NaCl (116.9% conrol) and significantly ($P<0.05$) different from PCn (table 1) whereas that of PCn was enhanced at lower concentration of 0.25% NaCl.

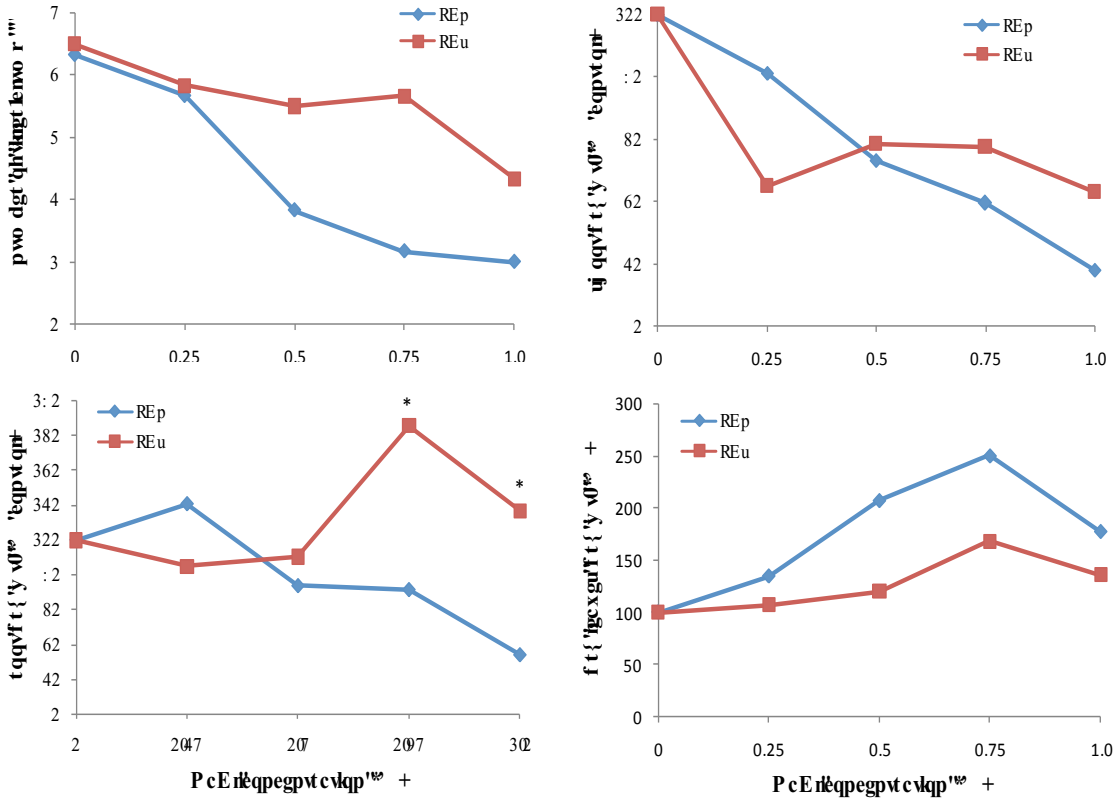

Fig. 3 Growth responses of normal (PCn) and salt tolerant line (PCs) of *V. nemoralis* subjected to 0-1% NaCl for 5 weeks. Values represent mean of four replicates and * shows significant difference ($P<0.05$) between the two lines at each concentration.

As external salinity ($NaCl_{ext}$) increase, both lines accumulated Na^+ and Cl^- in their shoots and roots with significantly differences (table 1). Although the accumulation in both lines was higher in shoots than in roots, a difference was observed. In PCs, Na^+ and Cl^- content (as % of fresh weight) in shoots increased progressively with slightly declined at 0.75% NaCl whereas in PCn, the content of these ions in shoots at 0.5-1.0% $NaCl_{ext}$ were controlled to a lower level than at 0.25% $NaCl_{ext}$ which salt ions content was maximum. K^+ content was progressively decreased in PCn whereas maintained close to control in PCs with slightly decline at 0.25% $NaCl_{ext}$ (Fig. 4). K^+ content of shoots was significantly different between lines (table 1).

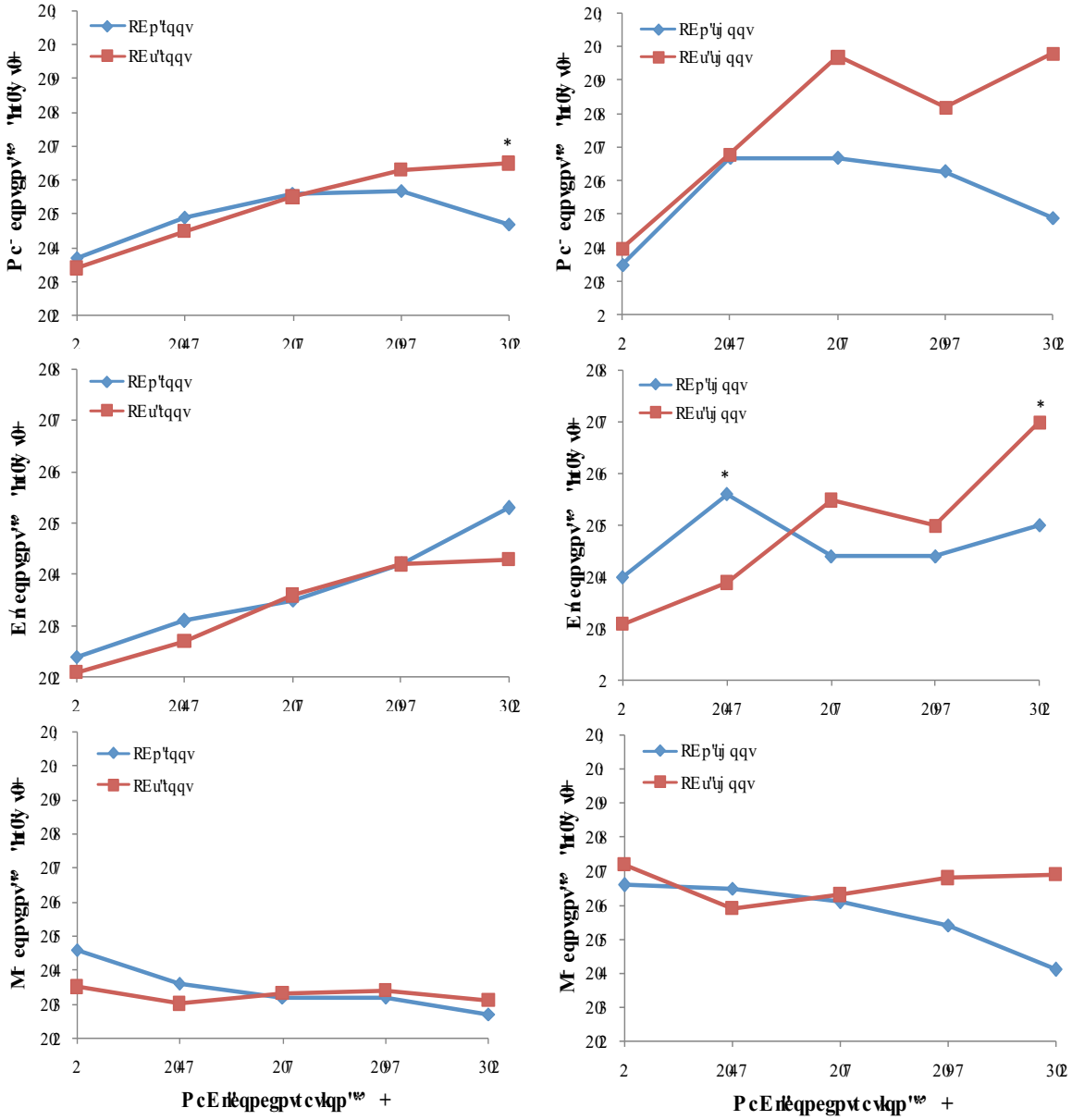

Fig. 4 Ions content in shoots and roots of normal (PCn) and salt tolerant line (PCs) of *V. nemoralis* subjected to 0-1% NaCl for 5 weeks. Values represent mean of four replicates and * shows significant difference ($P<0.05$) between the two lines at each concentration.

Since high salinity causes osmotic reduction of external solution and salt ions which penetrated into the cells cause toxic effect on metabolic processes (Orcutt and Nilsen, 2000; Munns, 2002; Tester and davenport, 2003; Hussain *et al*., 2008), growth of shoot was significantly decreased and dead leaves dry weight was significantly increased as salinity increased (Table 1). In contrast, salt ions accumulation in roots of PCs at some $NaCl_{ext}$ concentrations benefit to root growth. Stimulation of root growth by salinity were reported in many salt resistant grasses such as *Spartina patens* (Wu *et al*., 1998), *Distichlis spicata* (Alshammary *et al*., 2004), a salt tolerant selected line of *Dactyloctenium aegyptium* (Nanakorn *et al*., 2005). The stimulation effect of NaCl on root growth may be attributed to a capacity of root tissues to use Na^+ for osmotic adjustment and resulting of lower water potential than external solution, therefore, enable root tissues to absorb water for growth. Another beneficial effect of Na^+ is that it may be an essential element for plant (Cramer, 1997).

Table 1 Analysis of variance of plant response to salt treatment (S) and plant lines, salt tolerant and normal lines (L).

Variable	F value		
	S	L	SxL
Number of tiller/clump	10.45***	14.83***	2.21
roots dry weight (%control)	1.44	6.09*	3.72*
shoot dry weight (%control)	10.43**	0.13	2.25
dry-leaves dry weight (%control)	3.10*	4.95*	0.59
Ion content (% fresh weight)			
Na^+ in shoot	7.68***	14.29***	2.45
Na^+ in root	13.66***	1.93	2.90*
Cl^- in shoot	7.38***	0.57	5.57**
Cl^- in root	49.33***	5.42*	1.29
K^+ in shoot	1.55	5.6*	2.51
K^+ in root	26.15***	0.07	2.22

* = P< .05, ** = P< .01, *** = P< .001, ns = non significant.

In general, the mechanism for salt tolerant plants to cope with salt ions is maintenance of ion homeostasis in the cytoplasm. Tolerant glycophytes maintain ion homeostasis by controlling uptake and transport of salt ions to maintain a low level in shoot or so called salt exclusion. On the other hand, halophytes exclude salt ions (salt exclusion), sequester salt ions that extrude to shoots into the vacuoles (ion compartmentation) and maintenance a high K^+ content or high K^+/Na^+ ratio. The compartmentation of salt ions into vacuole is mediated by active ion transport, Na^+/H^+ antiport systems (Orcutt and Nilsen, 2000; Munns, 2002; Tester and davenport, 2003; Hussain *et al*., 2008). In this study, PCn which was able to restrict Na^+ and Cl^- accumulation at high $NaCl_{ext}$ concentrations but shoot growth was progressively decreased. In contrast, PCs, which accumulated higher level of Na^+ and Cl^- in its shoot, had significantly higher average number of tiller/clump when compared to PCn whereas non-significantly different in their shoots dry weight. Moreover, root growth was maintained at 0.25-0.5% $NaCl_{ext}$ and stimulated at 0.75-1.0% $NaCl_{ext}$. These results indicated that PCs had

an ability to transfer salt ions into the vacuole to avoid the harmful of their toxicity. In addition, the ability of PCs to maintain K^+ content in both root and shoot at 0.5-1.0% $NaCl_{ext}$ closed to the non-stress plant indicated that K^+ uptake system was slightly interfere by high concentration of Na^+_{ext}. This response was very significance for salt tolerance since K^+ is an essential for osmotic regulation and activator of many enzymes in photosynthesis and respiration (Hopkins and Huner, 2004). Thus, the salt tolerance mechanism under salinity stress in this PCs line is partially contributed by the maintenance of K^+ content, compartmentation of salt ions and using these ions for osmotic adjustment.

The salt tolerant selected line is now propagating for study in salt affected area.

Conclusion

Polyploid plantlet of *Vetiveria nemoralis* was not achieved from embryogenic calli treated with 0.05-0.1% colchicine for 1 day and followed with 1.5% NaCl. However, the plantlet derived from 0.5% colchicine treatment was survived and its salt tolerance was improved. Some differential responses of these tolerant and normal lines under salt stress, 0.25 – 1.0% NaCl, were shown. These were the higher value of shoots and roots dry weight; number of tillers/plant; Na^+ and Cl^- content in shoots; K^+ content in both shoots and roots, and lower dry weight of dead leaves in the salt tolerant line compare to the normal line. These indicated that the variation in salt tolerance mechanism between the two lines was gained. Therefore, further study to find out the appropriate procedure of colchicine treatment to induce polyploid plantlets for the selection on salt tolerant lines is needed since the present study has clearly shown a promising result on salt tolerant induction in vetiver grass.

Referances

Adriano, D. C. and H. E. Doner. 1982. Bromine, chlorine, and fluorine, pp. 449-483. *In:* A. L. Page (ed.). Methods of Soil Analysis. The American Society of Agronomy, Inc. and Soil Science Society of America, Inc., USA.

Alam, M. M., M. R. Karim, M. A. Aziz, M. M. Hossain, B. Ahmed and A. Mandal. 2011. Induction and evaluation of polyploidy in some local potato varieties of Bangladesh. J. Biodiver. Environ. Sci. 1: 16-21.

Alshammary, S. F., Y. L. Qian and S. J. Wallner. 2004. Growth response of four turfgrass species to salinity. Agri. Water Manag. 66: 97-111.

Bajaj, Y. P. S. 1990. Somaclonal variation – origin, induction, cryopreservation, and implications in plant breeding. *In* Y. P. S. Bajaj (ed.). Biotechnology in Agriculture and Forestry. 11: Somaclonal Variation on Crop Improvement. Springer-Verlag, Berlin. pp. 3-48.

Cramer, G. R. 1997. Uptake and role of ions in salt tolerance. *In* P.K. Jaiwal, R. P. Singh and A. Gulai (eds). Strategies for Improving Salt Tolerance in Higher Plans. Science Publishers, Inc. USA. pp. 57-58.

Eradi, T. and M. Unal. 2010. Production of colchicines induced tetraploid in *Vicia villosa* Roth. Caryologia 63: 292-303.

Gantait, S., N. Mandal, S. Bhattacharyya, P. K. Das. 2011. Induction and identification of tetraploids using *in vitro* colchicine treatment of *Gerbera jamesonii* Bolus cv. Sciella. Plant Cell Tiss. Org. Cult. 106: 485-493.

Głowacka, K., S. Jeżowski and Z. Kaczmarek. 2010. *In vitro* induction of polyploidy by colchicine treatment of shoots and preliminary characterisation of induced polyploids in two *Miscanthus* species. Indust. Crops Prod. 32: 88-96.

Haouala, R., S. Ouerghemmi, A. archoune and N. Boughanmi. 2009. Improvement of *Trigonella maritima* Delilee X. Poir. germination by polyploidization. Pak. J. Bot. 41: 3001-3008.

Hopkins, W. G. and N. P. A. Huner. 2004. Introduction to Plant Physiology. 3rd ed. John Wiley & Sons, Inc. New York.

Hopmans, J. W. and E. P. Maurer. 2008. Impact of climate change on irrigation water availability, crop water requirements and soil salinity in the SJV, CA. Technical Completion Reports, University of California Water Resources Center, UC Berkley.

Hussain, T. M., T. Chandrasekhar, M. Hazara, Z. Sulan, B. K. Saleh and G. M. Gopal. 2008. Recent advances in salt stress biology – a review. Biotech. Molec. Biol. Rev. 3: 8-13.

Kirti, P. B., S. Hadi, P. A. Kumar and V. L. Chopra. 1991. Production of sodium chloride tolerant *Brassica juncea* plants by *in vitro* selection at the somatic embryo level. Theor. Appl. Genet. 83: 233-237.

Liu, G., Z. Li and M. Bao. 2007. Colchicine-induced chromosome doubling in *Platanus acerifolia* and its effect on plant morphology. Euphytica 157:145–154

Liu, S., S. Chen, Y. Chen, Z. Guan, D. Yin and F. Chen. 2011. *In vitro* induced tetraploid of *Dendranthema nankingense* (Nakai) Tzvel. shows an improved level of abiotic stress tolerance. Scientia Hortic. 127: 411-419.

Lyndon, R. F. 1990. Plant Development: The Cellular Basis. Unwin Hyman, London. p. 27-36.

Munns, R. 2002. Comparative physiology of salt and water stress. Plant Cell Environ. 25: 239-250.

Muralitharan, M. S., R. F. M. Van Steveninck and S. F. Chandler. 1990. Growth characteristics and ion contents of non-selected and salt-selected callus lines of highbush blueberry (*Vaccinium corymbosum*) cultivars Blue Crop and Denise Blue. Plant Cell Rep. 9: 151-155.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Nanakorn, M., P. Thanomchat, S. Suwanawong, C. Wongwattana and I. S. Shim. 2005. Comparative Physiology of normal and salt tolerant selected line of *Dactyloctenium aegyptium* (L.) P. Beauv. under salt stress. Thai J. Agri. Sci. 38: 27-35.

Nanakorn, M., S. Suwanwong, C. Wongwattana, K. Kobayashi and K. Usui. 2006. Physiological changes under salt stress of some grass. J. Weed Sci. Tech. 51: 196-197. (sup.)

Ntuli, N. R. and A. M. Zobolo. 2008. Effect of water stress on growth of colchicine induced polyploidy *Coccinia palmata* and *Larenaria sphaerica* plants. Afr. J. Biotechnol. 7: 3548-3652.

Orcutt, D. M. and E. T. Nilsen. 2000. The Physiology of Plants under Stress: Soil and Biotic Factors. John Wiley & Sons, Inc. New York.

Suwannachitr, S. 1997. *In vitro* screening and selection for salt tolerance in vetiver grass. Master Thesis, Kasetsart University, Thailand.

Tester, M. and R. Davenport. 2003. Na^+ tolerance and transport in higher plants. Ann. Bot. 91: 503-527.

The Intergovernmental Panel on Climate Change (IPCC). 2003. How Will Global Warming Affect My World? Available source: http://www.unep.org/dec/docs/ipcc_wgii_guide-E.pdf

United Nation Framework Convention on Climate Change (UNFCCC). 2007. Climate Change: Impacts, Vulnerabilities and Adaptation in Developing Countries. Available source: http://unfccc.int/resource/docs/publications/impacts.pdf

Winicov, I. 1996. Characterization of rice (*Oryza sativa* L.) plants regenerated from salt-tolerant cell lines. Plant Sci. 13: 105-111.

Wu, J., D. M. Seliskar and J. L. Gallagher. 1998. Stress tolerance in the marsh plant *Spartina patens*: Impact of NaCl on growth and root plasma membrane lipid composition. Physiol. Plant. 102: 307-317.