Indian J. Plant Physiol., Vol. 13, No. 4, (N.S.) pp. 405-410 (Oct.-Dec., 2008)

SHORT COMMUNICATION

ETHYLMETHANESULPHONATE (EMS) INDUCED MUTATION AND SELECTION FOR SALT TOLERANCE IN SUGARCANE *IN VITRO*

MALLIKARJUN KENGANAL*, R.R. HANCHINAL AND H.L. NADAF

Sugarcane Tissue Culture Laboratory, University of Agricultural Sciences, Dharwad-580 005, Karnataka

Received on 11 Aug., 2008, Revised on 14 Nov., 2008

Sugarcane mutants against salt tolerance were derived using *in vitro* selection and regeneration under NaCl stress. Sugarcane cv. Co 86032 was used for callus development from meristematic leaf whorl on MS medium supplemented with 5mg/l 2,4-D + 0.2% sucrose. Four concentrations of NaCl were evaluated for salt tolerance level in sugarcane callus *in vitro*. NaCl 200mM did not allow any growth of calli and was adjudged most suitable selective pressure for salt tolerant sugarcane calli and shoots. Callus was treated with EMS, a potential mutagen at 0.5% for different time intervals of 1, 1.5, 2, 2.5 and 3 hours separately and regenerated on MS + 3mg/l 2- 4, D + 0.2% sucrose, fortified with 200mM NaCl. During four subcultures calli treated for 2.5 hours only could gain higher weight. Though treatment for 2 hours was capable of gaining calli weight but the growth rate was lower. Calli were regenerated on MS + 0.2mg/l BAP + 0.1mg/l NAA + 0.1mg/l Kinetin and 0.2% sucrose fortified with 200mM NaCl. Calli treated with EMS for 2 hours failed to induce any active regeneration. Maximum number of 15 healthy plants were successfully regenerated from the calli treated for 2.5 hours. Treatment of EMS for 3 hours induced regeneration at lower rate with three healthy plants only. Treatment for 2.5 hours was most ideal in deriving maximum mutants. However, lower period of time was less effective and higher was lethal. The *in vitro* regeneration via callus in itself causes selection and variation and treatment with EMS creates additional variation. The pressure of NaCl would be an ideal combination for development of true mutant salt tolerant sugarcane lines. The results offer cheap, less time and less labour intensive method for development of mutants for large samples.

Key words: **Callus, EMS, mutants, regeneration, salt tolerance**

Sugarcane (*Saccharum officinarum* L.) is an important commercial crop cultivated both in tropical and subtropical regions. It is the major source of raw material for sugar industries. Across the world, 70% sugar is manufactured form cane sugar. This cash crop occupies about 20.4 million hectares of land (2% of total cropped area of the world) with the production of 1392.4 million tons of the cane (FAO 2007). In India, it occupies 4.2 million hectares of land with the production of 281.17 million tons (Rao 2008). The crop has gained manifold importance in recent years. Apart from production of potable alcohol, acetic acid, butanol, paper, plywood, industrial enzymes and animal feed, cane has become raw material for production of ethanol (Arencibia 1998). However, crop productivity is declining in many places due to biotic and abiotic stresses. Salinity is one of the major factor among the abiotic factors responsible for the declining sugarcane yield globally. Non judicious use of water for sugarcane production has added additional burden of increased salinity in sugarcane area.

*Corresponding author, E-mail: mallikarjun_nss@rediffmail.com

Higher concentrations of salts in soils account for large decreases in the yield of a wide variety of crops all over the world (Tester and Davenport 2003). Approximately 5% of the cultivated land is affected by salt (Munns *et al*. 1999) (in India 6.65 mha). Understanding of the mechanisms that enable plants to adapt to salt stress and breeding for the resistant cultivars is necessary for exploiting saline soils. Sugarcane is a highly heterozygous and cross-pollinated in nature and requires very specific hot and humid climate for flowering. The ratio of vegetative seed multiplication by sets is also low (1:10). Therefore, the conventional breeding for incremental improvement in sugarcane is a time consuming and difficult task. Mutation induction has become a novel approach in crop improvement to supplement difficulties of conventional breeding in improving cultivars for specific traits. More than 2500 improved mutant varieties have been released for commercial cultivation in different crop species. The novelty of tissue culture techniques like *in vitro* improvement of vegetative propagated crops combined with mutation has been tried to develop the salt resistant sugarcane lines (Suprasanna and Bapat 2006).

In vitro culture is an ideal system for screening of salt tolerant mutants, as it is carried out under controlled conditions with limited space and time. Moreover unlike whole plant, large number of lines can be screened at one time for a desired trait. Salt-tolerant cell lines and plants have been derived by plant tissue culture technique in several species such as wheat (Barakat and Abdel-Latif 1996), rice (Lutts *et al*. 1999), barley (Sibi and Fakiri 2000), potato (Benavides *et al*. 2000) and sunflower (Alvarez *et al*. 2003). Compared with physical agents, chemical mutagens are perhaps more capable of leading to specific and predictable mutations (Bhagawat and Duncan 1998). EMS is a chemical mutagen of alkylating group and has been commonly used in plant breeding because it can cause high frequency of gene mutations and low frequency of chromosomal aberrations (Van Harten 1998). In the present study salt-tolerant lines were derived inducing chemical mutation and selection of calli *in vitro*.

*Explant collection***:** The sugarcane cultivar Co 86032 was used for inducing mutation. This variety is much preferred in many sugarcane growing belts of the country but is devoid of salt tolerance. Sugarcane tops measuring 8–10 cm were excised from eight month old field grown plants. Outer mature leaves were removed up to innermost spindle buds of 1cm diameter and 5–6 cm length. The buds were washed with detergent Tween 20 (2 drops per liter) and surface sterilized with 0.1% Bavistin for 8 minutes followed by three times washing with clean water. Second sterilization was carried out aseptically under laminar air flow cabinet with 70% alcohol for 30 seconds followed by three times washing with double distilled sterile water. Final sterilization was done with 0.1% $HgCl_2$ for 5 minutes followed by three washings with double distilled sterile water.

Callus initiation: The innermost intact 2- 3 meristematic leaf whorls were excised aseptically from sterilized buds, cut in to small pieces of 5mm length and used for inoculation. Murashige and Skoog (1962) basal medium supplemented with 5mg/l 2- 4, D containing 2% sucrose and 0.7% agar as solidifying agent was used for callus induction. The pH of the media was adjusted to 5.8 using 0.1N NaOH and autoclaved at 120^0C for 15 minutes at 15lbs pressure. Cultures were maintained at 23 ± 2^0C in dark condition.

Assessment of NaCl tolerance: The calli developed after 25 days was cut in to small pieces of 5mm size and re inoculated on callus initiation medium consisting NaCl of 50, 100, 150 and 200 mM concentrations separately. Three subcultures were done at 10 days interval. The rate of callus growth and survival was assessed and lethal dose of concentration was determined for selection of mutants.

EMS treatment: The friable callus induced after 25 days of inoculation was cut in to small pieces, weighed and treated with EMS 0.5% solution (prepared in sterilized distilled water and membrane filtered) for 1, 1.5, 2, 2.5 and 3 hours separately and inoculated on MS medium consisting 3mg/l 2- 4, D + 0.2% sucrose with the selection pressure of 200mM NaCl. Since at this concentration all the nonmutated calli tested failed to survive. Five replications with three clumps in each were used for individual treatment. Three subcultures were carried out with an interval of 10 days and finally the clumps were transferred for regeneration.

Regeneration and rooting: Regeneration of calli was accomplished on MS medium consisting 0.2mg/l BAP + 0.1mg/l NAA + 0.1mg/l Kinetin and 0.2% sucrose supported with selection pressure of 200mM NaCl on solid medium. The regenerated shoots were subjected to rooting on half strength MS medium consisting 0.2% sucrose + 0.01mg/l IBA and 200mM NaCl. Rooted seedlings were hardened in greenhouse under controlled conditions and further evaluated in field conditions after complete hardening.

Four concentrations of NaCl were used for assessing the lethal dosage of NaCl for selection of mutants regenerated, as many of the crops posses their own salt tolerance limit at field level. Sugarcane calli developed from meristematic leaf whorl was used for induced mutation using EMS. Calli showed tolerance of 58% at 50mM NaCl concentration and, rate of survival was very poor with decrease in calli weight gain at 100 and 150 mM NaCl concentration. 200mM concentration was found most ideal as no calli showed any survival or regeneration up to 30 days due to the toxic effects of NaCl. Rather shrinking and brown discoloration was found leading to death of the calli (Fig. 1). Non induced sugarcane calli were previously evaluated for stress tolerance by somaclonal variation at 50, 100 and 150 mM NaCl concentration (Errabii *et al.* 2007). However, the lower dosage of 50 and 100 mM were found not appropriate in deciding the change in behavior of cultures regenerated on stressed medium. Hence, higher dosage of 200mM concentration was used as a selection pressure for regeneration of mutated calli.

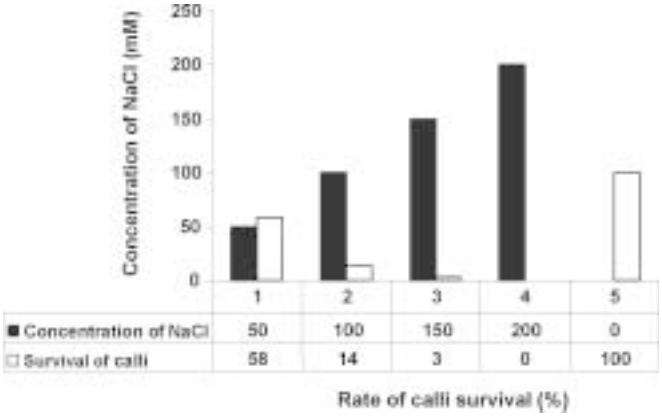

Fig 1. Influence of NaCl on growth and survival of sugarcane calli

Plate 1. Growth of callus on NaCl selection medium

Plate 2. Growth of mutant shoots on NaCl selection medium

Plate 3. Rooted mutant sugar seedlings

Observations of growth and regeneration of EMS treated calli on selection medium fortified with 200mM NaCl showed increasing trend of fresh weight gain (Plate 1) with increase in EMS treatment period, except at treatment for 3 hours (Table 1). Treatment of EMS for 1 and 1.5 hours did not yield regeneration of calli, because the time of treatment might be too short to cause mutation in the calli clumps treated. Higher period of 2.5 hours resulted in good survival of calli and regeneration and survival of maximum number of shoots (Plate 2 & 3) with three albinos. The survival of calli treated for 2 hours was poor resulting in no successful survival of shoots. Highest period of 3 hours treatment could support the growth of calli with lower rate of proliferation yielding 4 shoots successfully of which only three survived and one was albino. Treatment of 0.5% EMS for 2 and 2.5 hours has been found most successful in yielding salt tolerant sweet potato lines with higher rate of mutant regeneration (Luan *et al*. 2007). Results suggest that treatment of EMS for 2.5 hours is optimum for inducing desired mutation and can be effectively used for *in vitro* based mutation studies in sugarcane.

Creating salt tolerance in the cultivated varieties is always difficult task for vegetative propagated crops like sugarcane having longer crop duration. In such cases, development of salt tolerant lines through induced mutation *in vitro* has been practiced as a classical approach (Micke *et al*. 1990, Taylor *et al*. 1995, Hoy *et al*. 2003) where breeding methods pose difficulties in handling the material and offer poor results. In the present study similar approach was attempted using EMS.

Induction of mutation and *in vitro* selection shortens the time considerably for selection of desirable traits under selection pressure without having any adaphic influence. *In vitro* selection has been used for biotic stress tolerance like diseases (Jain 2000) and abiotic stress tolerance including salt (Bressan *et al.* 1985), drought and frost (Xing and Rajashekar 2001). Desirable mutations for wider stem girth and smut resistance were successfully obtained through *in vitro* gamma irradiation (Rodriguez *et al*. 2001) in sugarcane. However, development of salt tolerance was attempted using somatic variation only, but frequency of mutants was poor. Hence the use of 0.5 per cent EMS treatment for 2.5 hrs successfully induced desirable variation in sugarcane callus *in vitro.* Similarly sweet potato lines tolerant to NaCl were derived from EMS mutated calli as explants (Luan *et al*. 2007).

In the present study, explants were subcultured thrice at an interval of 8 days each for inducing a friable callus capable of regeneration. Phenol exudation was much from cut end surface of the explants. However, it is a sign of growth and development (Wolf *et al*. 1976) of cultures *in vitro*. In alfalfa, leaves used for callus formation developed phenolics, which decreased the proliferation of callus simultaneously with increase in

Table 1. Influence of EMS (0.5%) on development of mutants from sugarcane callus on NaCl (200mM) stressed medium *in vitro*

Treatment Hours	Weight gain in callus (gm) (± SE)	Callus growth reduction (%)	No. of shoots regenerated	No. of albinos	No. of plants survived
1.0	0.03 (0.0053)	95.58	0	0	0
1.5	0.07 (0.0096)	89.75	0	0	0
2.0	0.08 (0.0092)	88.23	1	0	0
2.5	0.12 (0.0067)	82.35	19	3	15
3.0	0.03 (0.0045)	95.58	4	1	3
0	0.01 (0.0016)	98.52	0	0	0
*	0.68 (0.0075)	0	216	1	212

* Without EMS and NaCl treatment

content of phenolics produced (Cvikrová *et al*. 1996). Hence, cultures were sub cultured frequently to avoid growth hindrance due to phenol accumulation.

Though the *in vitro* callus induction and selection in itself causes variation known as somaclonal variation. It may be desirable to enhance the chances of variation by additional physical or chemical mutagenic treatments. In the present study, the combinations of both the techniques have yielded a good number of salt tolerant sugarcane plants and the system may be useful for mutation induced improvement of sugarcane for other traits also.

REFERENCES

Alvarez, I., Tomaro, L.M. and Bernavides, P.M. (2003). Changes in polyamines, proline and ethylene in sunflower calluses treated with NaCl. *Plant Cell, Tissue Organ Cult.* **74:** 51-59.

Arencibia, A. (1998). Gene transfer in sugarcane. In: T. Hohn, K.M. Leisinger (eds.), Biotechnology of Food Crops in Developing Countries, pp. 79-104. Springer-Verlag, New York.

Barakat, M.N. and Abdel-Latif, T.H. (1996). *In vitro* selection of wheat callus tolerant to high levels of salt and plant regeneration. *Euphytica* **91:** 127-140.

Benavides, P.M., Marconi, L.P., Gallego, M.S., Comba, E.M. and Tomaro, L.M. (2000). Relationship between antioxidant defence systems and salt-tolerance in *Solanum tuberosum*. *Aust. J. Plant Physiol*. **27:** 273-278.

Bhagawat, B. and Duncan, E.J. (1998), Mutation breeding of banana cv. Highgate (Musa spp., AAA Group) for tolerance to *Fusarium oxysporum* f. sp. Cubense using chemical mutagenesis. *Scientia Horti*. **73:** 11-22.

Bressan, R.A., Singh, N.K., Handa, A.K., Kononowicz, A. and Hasegawa, P.M. (1985). Stable and unstable tolerance to NaCl in cultured tobacco cells. In: M. Freeling (ed.), Plant Genetics: Proc. Third Annual ARCO Plant Cell Research Institute –UCLA symposium on Plant Biology*:* New Series, pp. 755-769. A.R. Liss, New York.

Cvikrova, M., Mala, J., Eder, J., Hrubcova, M. and Vagner, M. (1998). Abscisic acid polyamines and phenolic acids in sessile oak somatic embryos in relation to their conversion potential. *Plant Physiol. Biochem*. **36:** 247-255.

Errabii, T., Gandonou, C.B., Essalmani, H., Abrini, J., Idaomar, M. and Senhaji, N.S. (2007). Effects of NaCl and mannitol induced stress on sugarcane (*Saccharum* sp.) callus cultures, *Acta Physiol. Plant.* **29:** 95-102.

F.A.O. (2007). Food and Agricultural Organization of United Nations. http://apps.fao.org.

Hoy, J.W., Bischoff, K.P., Milligan, S.B. and Gravois, K.A. (2003). Effect of tissue culture explant source on sugarcane yield components. *Euphytica* **129:** 237-240.

Jain, S.M. (2000). Tissue culture-derived variation in crop improvement. *Euphytica*. **118:** 153-166.

Luan, Y.S., Zang, J. and Gao, X.R. (2007). Mutation induced by ethylmethanesulphonate (EMS), *in vitro* screening for salt tolerance and plant regeneration of sweet potato (*Ipomoea batatas* L.). *Plant Cell, Tissue and Org. Cult.* **88:** 77-81.

Lutts, S., Bouharmont, J. and Kinet, J.M. (1999). Physiological characterization of salt-resistant rice (*Oryza sativa*) somaclones. *Aust. J. Bot.* **47:** 835-849.

Micke, A., Donani, B. and Maluszynski, M. (1990). Induced mutations for crop improvement. Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna.

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

Munns, R., Hare, R.A., James, R.A. and Rebetzke, G.J. (1999). Genetic variation for improving the salt tolerance of durum wheat. *Aust. J. Agric. Res.* **51:** 69-74.

Rao, R. (2008). General Studies for ARS and Other Competitive Examinations. Jain Brothers, New Delhi.

Rodríguez, G.L., Orellana Pérez, P., García Rodríguez, L., Pérez Ponce, J.N., Veitía Rodríguez, N., Bermúdez Caraballoso, I., Clavero García, J. and Romero Quintana, C. (2001). Use of the mutagenesis to improve the girth of the stem in the somaclone IBP 89-169 of sugarcane. *Biotechnología Vegetale* **1&2:** 71-75.

Sibi, M.L. and Fakiri, M. (2000). Androgenese et gynogene‘ se, sources de vitrovariation et de tolerance a la salinite´ chez l’orge *Hordeum vulgare*? *Se´cheresse* **11:** 125- 132.

Suprasanna, P. and Bapat, V.A. (2006). Advances in the development of *in vitro* culture systems and transgenics in sugarcane. Int. Symp. Technologies to improve sugar productivity in developing countries, pp. 629- 636. China.

Taylor, P.W.J., Geijskes, J.R., Ko, H.L., Fraser, T.A., Henry, R.J. and Birch, R.G. (1995). Sensitivity of random amplified polymorphic DNA analysis to detect genetic change in sugarcane during tissue culture. *Theor. Appl. Genet.* **90:** 1169-1173.

Tester, M. and Davenport, R. (2003). Na^+ tolerance and Na^+ transport in higher plants. *Ann. Bot.* **91:** 503-507.

Van Harten, A.M. (1998). Mutation Breeding: Theory and Practical Applications. Cambridge University Press, London.

Wolf, F.T., Tilford, R.H. and Martinez, M.L. (1976). Effect of phenolic acid and their derivatives upon the growth of *Avena* coleoptiles. *Z. Pflanzenphysiol*. **80:** 243-250.

Xing, W. and Rajashekar, C. B. (2001). Glycine betaine involvement in freezing tolerance and water stress in *Arabidopsis thaliana*. *Environ. Expt. Bot*. **46:** 21-28.