RESEARCH ARTICLE

IN VITRO SCREENING OF SUGARCANE CULTIVAR Co 86032 FOR SALINITY TOLERANCE

Lamture Avinash, Narwade Prashant* and A.A. Bharose

Abstract

Salt tolerant sugarcane mutants were isolated using in vitro UV mutagenesis-selection and regeneration under NaCl salt stress. Embryogenic callus cultures of popular sugarcane cultivar Co 86032 were established from young meristematic leaf whorls on MS basal medium supplemented with 4 mg l^{-1} containing 2% sucrose and 0.7% agar. When different concentrations of NaCl were used for assessing its lethal dosage for selection, calli failed to grow beyond 50mM NaCl concentration. Besides, callus culture exposed to UV radiation exhibited lower decline in survival and calli growth and differentiation up to 125mM NaCl (3.054 mg RGR) concentration was observed. From observations, three NaCl concentrations (75, 100, 125mM) were considered for studies on in vitro *mutagenesis-selection. The sugarcane calli exposed to 75mM NaCl accumulated higher proline (5.5265 µmol) than that in the control. Total soluble protein content also was higher at 125mM NaCl concentration (0.637 mg ml^{-1} of enzyme extract from stressed calli). The genetic variability among the selected variants was analyzed using Random Amplified Polymorphic DNA (RAPD) technique. RAPD analysis resolved 77 polymorphic bands with 63.11% total polymorphism. The primer RC-15 produced four polymorphic bands of total 19 bands. The primer amplified the salt tolerant samples of 25mM to 150mM, but failed to amplify the sample of 175mM. The primer RC 16 amplified a 250bp band in all the salt tolerant samples but produced two unique bands at approximately 400 and 550 bp in 75mM and 125mM salt stressed lines. The primer OPS 09 produced eight amplicons at the highest salt concentration (175mM) probably indicating the amplification of lethal gene responsible for callus mortality. The results highlighted the need for proper assessment of induced variability by salinity stress to provide a better understanding of sugarcane cultivation under such stress conditions.*

Keywords : *Sugarcane, in vitro screening, NaCl, UV radiation, salinity tolerance*

Lamture Avinash,Narwade Prashant and A.A. Bharose
College of Agricultural Biotechnology
Marathwada Krishi Vidyapeeth
Latur, Maharashtra- 413 512, India
email: nprashanta@yahoo.com

Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the most important agro-industrial crops in both tropical and subtropical regions of the world and sugar is a major export product in many developing countries. World's 70% sugar comes from sugarcane, the principal raw material for the sugar industries (Anonymous, 2000). India is the largest producer of sugar including traditional cane sugar sweeteners, *khandsari* and *gur* with cropping area about 4.4 million hectares with yield of 64.48 tonnes per hectare of cane (http://www.faostat.fao.org). Economically sugarcane is an important industrial raw material for sugar and allied industries producing alcohol, acetic acid, butanol, paper, plywood, industrial enzymes, animal feed, etc. (Arencibia et al, 1998). Increased soil salinity is considered a noxious factor for most of the glycophytes and being a typical glycophyte sugarcane exhibits stunted growth or no growth under salinity, with its yield falling to 50% or less than its true potential. Besides, salinity in root zone of sugarcane decreases sucrose yield through its effect on both biomass and juice quality. Soil salinity reduces plant growth by perturbing different biochemical / physiological processes (Zeng and Shannon, 2000). The influence

of soil salinity on sugarcane yield and quality may be due to physiological drought and ion toxicity leading to metabolic toxicity, membrane disorganization and generation of reactive oxygen species-ROS (Hasegawa et al., 2000).

Studies conducted on different crops suggested that salt tolerance is a multigenic complex trait (Niknam and McComb, 2000), which has seriously limited the efforts to develop tolerant crop varieties through conventional breeding practices. Somaclonal variation, in combination with *in vitro* mutagenesis and selection, has been applied for screening of agronomically important mutants (Zhambrano et al., 2003). Moreover, unlike whole plant, large number of lines can be screened at a time for a desired trait and many improved mutant varieties have been released for commercial cultivation in different crop species. The novel tissue culture techniques like *in vitro* improvement of vegetatively propagated crops combined with mutation have been tried to develop salt resistant sugarcane lines (Suprasanna and Bapat, 2006).

The successful use of somaclonal variation is very much dependent on characterization of genetic stability in subsequent generations. Molecular variation in tissue culture derived plants has been characterized at DNA and protein level. Evaluation and characterization of the spontaneous and induced variants against salinity may prove to be a highly fruitful venture for their successful cultivation in stress conditions. Therefore, analysis of the induced and spontaneous genetic variation in the regenerated plants is necessary for exploiting these variants for crop improvement. Among the molecular techniques, Random Amplified Polymorphic DNA (RAPD) analysis (Williams et al. 1990) is simple, quick, easy to perform, requires small amount of DNA for analysis and no prior sequence information is required. Herein, we have presented results of *in vitro* screening for salt tolerance in callus culture of sugarcane and the effect on relative growth rate and biochemical assays on proline and proteins accumulation after UV radiations-salt stress.

Materials and methods

Embryogenic callus induction : Embryogenic callus cultures of popular sugarcane cultivar Co 86032 were established from young meristematic leaf whorls on MS basal medium (Murashige and Skoog, 1962) supplemented with 4 mg l^{-1} containing 2% sucrose and 0.7% agar. The cultures were incubated in darkness at 25 ± 2^0C and subcultured every three-week interval.

Assessment of NaCl tolerance and UV irradiation: The calli were cut in to small pieces of 5 mm size and re-inoculated on callus induction medium (CIM) at nine levels of NaCl, i.e. 0.00mM, 25mM, 50mM, 75mM, 100mM, 125mM, 150mM, 175mM and 200mM concentrations separately. Two subcultures were done at 10 days interval and maintained calli were transferred to regeneration medium, i.e. MS basal medium supplemented with 2 mg/l BAP, 0.5 mg/l NAA, and 0.5 mg/l IBA. The rate of callus growth, survival and differentiation were assessed for the determination of NaCl tolerance.

In another experiment, embryogenic calli were subjected to UV radiation for one hour using UV light (260 nm) as a source of UV radiation. Irradiated calli were transferred to callus induction medium consisting NaCl of 0.00mM, 25mM, 50mM, 75mM, 100mM, 125mM, 150mM, 175mM and 200mM concentrations separately. The cultures were regularly subcultured and maintained on CIM. The growth (mg) of both treated and non-treated calli was determined in terms of relative growth rate (RGR) after 30 days of culture on salt containing growth medium.

RGR = (Final fresh weight – Initial fresh weight) / Initial fresh weight

Estimation of free proline accumulation : Free proline content of sugarcane calli was determined as per the procedure of Bates et al (1973). Calli weighing 500 mg exposed to UV light and treated with different levels of salt were used for the study on proline accumulation.

Estimation of protein content : Determination of protein content of sugarcane calli exposed to UV light and treated with different concentrations of NaCl was determined by using Bradford protein assay procedure.

In vitro selection for salinity tolerance : Sugarcane calli exposed to UV light were cultured on regeneration medium supplemented with different levels of salt-NaCl (0.00mM, 25mM, 50mM, 75mM, 100mM, 125mM, 150mM, 175mM and 200mM). Plants regenerated from the treated calli were shifted on half MS medium supplemented with 2.5 mg l^{-1} IBA for rooting. Well-rooted plants were transferred for hardening in green house.

RAPD analysis : Genomic DNA was extracted from selected calli using CTAB (Saghai-Maroof et al., 1984) and quality checked on 0.8% agarose gels. RAPD analysis was carried out on the calli subjected to *in vitro* selection studies for salinity tolerance. Among the screened primers, the best six primers, *viz*. RC-11, RC-14, RC-15, RC-16, RC-20 and OPS-09, that showed distinct banding pattern were selected for analysis. PCR amplification reaction conditions included an initial denaturation at 94°C for five minutes, followed by 45 cycles each consisting of denaturation at 94°C for 45 seconds, primer annealing at 37°C for 1 minute and extension at 72°C for two minutes and a final extension step at 72°C for one minute.

The amplified products were resolved through agarose gel electrophoresis on 1.8% agarose gels applying 50 volt for three hours and banding pattern analyzed on gel documentation system. The amplified products were scored for presence (1), absence (0); missing or doubtful cases were scored as 9 for analysis. Band sizes were determined by comparison with 1 kb DNA ladder.

Results and discussion

Effect of NaCl stress and UV irradiation on growth and survival of sugarcane calli :

Eight concentrations of NaCl were used for assessing the lethal dosage of NaCl for selection, as crops exhibit variation in their salt tolerance potential. Calli showed tolerance up to 50mM NaCl concentration and when exposed to increasing concentration of NaCl, i.e. 75mM and above, reduction in the growth rate of sugarcane embryogenic calli was observed. These results are in agreement with an earlier report in sugarcane where a significant decline in callus growth rate occurred with 150mM NaCl (Errabii et al. 2006). A decline in callus growth upon salt stress may be due to the nutritional imbalance arising from interference of salt ions, such as Na+ and Cl- with essential nutrients involved in the uptake and translocation processes. The survival of callus culture decreased in response to 75mM NaCl and higher concentrations. This finding is consistent with earlier reports in sugarcane where relative growth rate of calli decreased as salt concentration increased (Patade et al 2008).

In vitro studies for salinity tolerance showed good response of UV irradiated calli towards growth and differentiation after one month of culture inoculation on different salt supplemented media (Fig. 1). Treatment with 25mM NaCl exhibited stimulatory response in case of callus growth (RGR 6.195 mg) which was greater than that in control (RGR 4.084 mg). Interestingly treatment of 125mM showed significant growth in callus as compared to calli stressed without UV treatment. Beyond 150mM NaCl concentration calli failed to show regeneration, may be due to necrotic growth. Patade et al (2009) found matching results as irradiated calli exhibited lower decline in callus growth with increasing salt concentrations as compared to non-irradiated calli on salt selection medium.

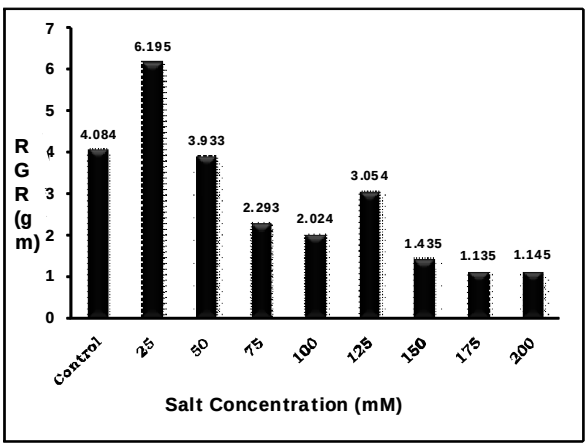

Fig.1. RGR in UV Irradiated callus culture at different NaCl concentrations

Proline accumulation in UV irradiated-salt treated callus culture

Salt stressed callus cultures exhibited higher levels of free proline accumulation as compared to the control (Fig. 2). Similarly, Gandonou et al., (2006) reported higher accumulation of proline in salt-

tolerant than in non-selected calli of sugarcane. In the present study the highest free proline content (5.5265 µmol) was recorded in 75mM NaCl concentration and calli grown at 100mM NaCl and higher concentrations showed decreasing trend of proline content. The calli stressed with 125mM NaCl concentration exhibited lower proline content (4.5440 µmol) than that stressed with 75mM NaCl (5.5265 µmol) and showed good response towards growth, probably due to the effect of UV irradiation. These findings are comparable to reports in gamma irradiated sugarcane callus culture treated with different salt concentrations where the stressed cultures with 128.3mM and higher concentrations showed decrease in proline content (Patade et al., 2009). Non-salinized irradiated calli revealed successive increase in proline content and combining the effects of gamma rays and salinity stress showed progressive increase in proline content. Proline was found to reduce the toxic effect of NaCl on helical destabilization at DNA replication (Rajendrakumar et al., 1997). As proline is osmotically active and contributes to membrane stability and mitigates the effect of NaCl on cell membrane disruption (Mansour, 1998), the increase in proline accumulation can be considered significant towards salt tolerance.

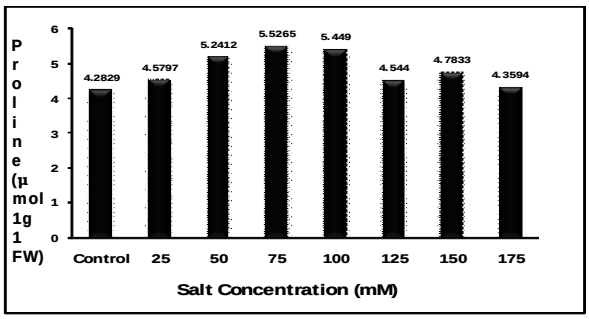

Fig.2. Poline content in UV Irradiated callus culture at different NaCl concentrations

Total protein content in UV irradiated-salt treated callus culture

The protein content was found significantly higher in 125mM NaCl stressed calli than control and other salt stress calli (Fig. 3). The callus challenged with 125mM NaCl showed approximately 2.5 times (i.e. 0.637 mg ml^{-1} of enzyme extract from stressed calli) increase in protein level as compared to the control

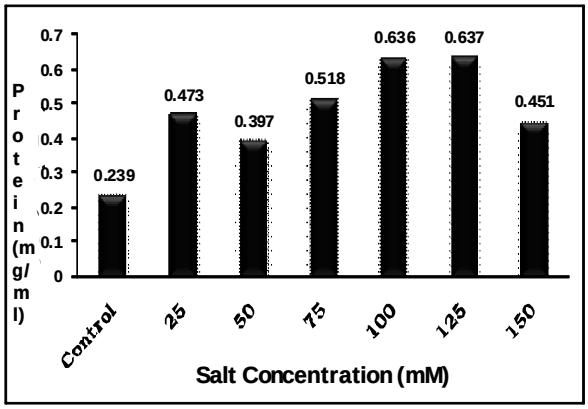

Fig.3. Protein content in UV Irradiated callus culture at different NaCl concentrations

(0.239 mg ml^{-1}) treatment. A higher content of soluble proteins has been observed in salt tolerant cultivars of barley, sunflower, finger millet and rice (Ashraf and Harris, 2004). The calli stressed with salt beyond 150mM NaCl showed decreasing trend of protein accumulation and did not show any growth due to burning during selection cycles on salt containing medium. Singh et al. (1987) reported that proteins accumulated in plants under saline conditions provided a storage form of nitrogen that was re-utilized later to play a role in osmotic adjustment.

In vitro selection

In the present study, the sugarcane embryogenic calli were exposed to different levels (0-200mM) of salt-NaCl stress for assessment of salt tolerance and the results indicated that sugarcane calli could tolerate up to 50mM NaCl. Besides, the callus exposed to UV radiation exhibited lower decline in survival and interestingly showed calli growth and differentiation up to 125-150mM NaCl (3.054 mg RGR) concentration where the RGR was greater than 75-100mM NaCl. The calli stressed with 150mM NaCl concentration failed to exhibit differentiation during further selection cycles.

As the growth of calli decreased with increasing salt concentration from previous observations, three NaCl concentrations (75, 100, 125mM) were considered for studies on *in vitro* mutagenesis-selection. Somaclonal variation in combination with *in vitro* mutagenesis offers potential for the isolation of salinity and drought tolerant lines in a short duration employing *in vitro* selection (Suprasanna and Rao, 1997; Suprasanna et al., 2006). Salt-tolerant cell lines

have been developed in several glycophytic species such as alfalfa (Croughan et al., 1978), potato (Sabbah and Tal, 1990), wheat (Piri et al., 1994), tomato (Kripkyy et al., 2001), rice (Basu et al., 2002), sunflower (Davenport et al., 2003) and *Catharanthus roseus* (Elkahoui et al., 2005) with the help of *in vitro* methods.

Plants when confronted with salinity stress, respond with a significant accumulation of compatible solutes like proline and total soluble proteins. These compatible solutes or osmoprotectants can reach high levels for enhanced stress tolerance through scavenging of free radicals and protecting enzymes (Sharma and Dubey, 2005). In the present study, sugarcane calli exposed to 75mM NaCl accumulated higher proline (5.5265 µmol) than control. A change in proline content has been correlated with its capacity to tolerate and adapt to salinity conditions (Gandonou et al., 2006). Total soluble protein content was also higher at 125mM NaCl concentration (0.637 mg ml^{-1} of enzyme extract). Several salt-induced proteins have been identified in plant species and have been classified into two distinct groups: salt stress proteins (Pareek et al., 1997) which accumulate only due to salt stress; and stress associated proteins (Ali et al., 1999) which accumulate in response to heat, cold, drought, water logging, and high and low mineral nutrients, besides salt stress. Proteins may be synthesized de novo in response to salt stress or may be present constitutively at low concentration and increase when plants are exposed to salt stress (Pareek et al., 1997).

The plantlets selected from salt stressed condition were hardened initially on sand and soil rite and then the hardened plantlets were transferred to polybags filled with potting mixture and maintained under saline condition in green house.

RAPD analysis of UV irradiated-salt stressed sugarcane calli

The variability among the control (without UV radiation-salt stress) and UV irradiated-salt stressed calli (i. e. 25, 75, 125, 175 mM) was analyzed using RAPD molecular marker technique. Of the total screened primers for sugarcane genome, the best suited six (RC-11, RC-14, RC-15, RC-16, RC-20 and OPS-09) that showed distinct banding pattern were selected for analysis. All the primers were able to generate 122 amplicons with an average of 20.23 amplicons per primer, of which 77 (63.11%) amplicons were polymorphic, accounting for an average of 12.83 polymorphic bands per primer.

The primer RC-15 produced four polymorphic bands among 19 bands observed and amplified salt tolerant samples (25mM to 150mM) with four bands each (Fig. 4). However, it failed to amplify the sample of 175mM. The primer RC 16 amplified a 250bp band in all the salt tolerant samples and produced two unique bands of 400 and 550 bp in 75mM and 125mM salt stressed lines, respectively. That might be due to good callusing response, high RGA and high proline content in such samples. The primer OPS 09 produced eight amplicons probably leading to the amplification of lethal gene responsible for callus mortality at the highest salt concentration of 175mM. The RAPD profile revealed polymorphism among the selected tolerant lines from the control treatment.

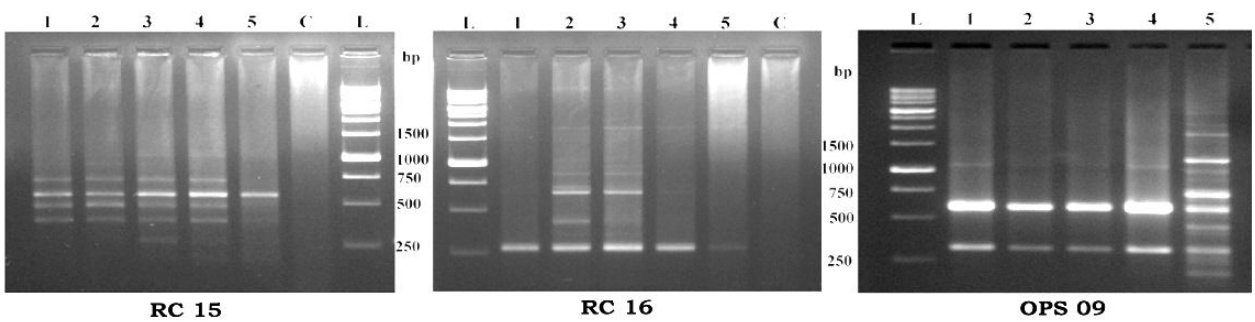

Fig. 4. Lane 1- 25mM; 2-75mM; 3- 100mM; 4- 125mM; 5- 175mM; C-Control; L- Ladder

The application of molecular markers technique will prove helpful to establish efficient *in vitro* system for selection of abiotic stress tolerant clones through *in vitro* mutagenesis. Mutagenesis through radiation in combination with tissue culture technique seems suitable for the improvement of vegetatively propagated crops (Maluszynski., 1995; Lee et al., 2002). The study of Patade et al. (2006) on RAPD analysis of genetic variation among the *in vitro* calli selected for salinity tolerance from irradiated and non-irradiated cultures suggest that the variation can be detected at the stage of regeneration even before hardening in the green house. The proper assessment of induced variability adjacent to salinity and other abiotic stresses may prove highly productive scheme for its economic cultivation under the stress conditions.

Acknowledgements

We would like to thank the Department of Biotechnology, GOI, New Delhi; Marathawada Krishi Vidyapeeth, Parbhani and College of Agricultural Biotechnology, Latur for financial support for the completion of this work.

References

Ali G, Srivastava PS, Iqbal M (1999). Proline accumulation, protein pattern and photosynthesis in regenerants grown under NaCl stress. Biol Pl 42 89–95

Anonymous (2000). Sugarcane Breeding Institute. Quarterly (November).

Arencibia AE, Carmona P, Tellez M T, Chan S M, Yu L, Trujillo, Oramas P. (1998). An efficient protocol for sugarcane (*Saccharum* spp.) transformation mediated by Agrobacterium tumefaciens. Transgenic Res 7: 213–222

Ashraf M, Harris PJC (2004) Potential biochemical indicators of salinity tolerance in plants. J Pl Sci 166: 3–16

Basu S, Gangopadhyay G, Mukherjee BB (2002) Salt tolerance in rice in vitro: implication of accumulation of Na+, K+ and proline. Pl Cell Tissue Organ Cult 69:55–64

Bates L S (1973) Rapid determination of free proline for water stress studies. Pl & Soil 39:205-207

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Croughan TP, Stavarek SJ, Rains DW (1978) Selection of a NaCl-tolerant line of cultivated alfalfa cells. Crop Sci 18:959–963

Davenport BS, Gallego MS, Benavides PM, Tomaro LM (2003) Behaviour of antioxidant defense system in the adaptive response to salt stress in Helianthus annuus L. cells. Pl Growth Regul 40:81–88

Elkahoui S, Herna´ndez AJ, Abdelly C, Ghrir R, Limam FB (2005) Effects of salt on lipid peroxidation and antioxidant enzyme activities of Catharanthus roseus suspension cells. Pl Sci 168(3):607–613

Errabii T, Gandonou C, Essalmani H, Abrini J, Idaomar M, Skali-Senhaji N (2006) Growth, proline and ion accumulation in sugarcane callus cultures under drought-induced osmotic stress and its subsequent relief. African J Biotech 5(16):1488-1493

Gandonou CH, Errabii T, Abrini J, Idaomar M, Senhaji NS (2006) Selection of callus cultures of sugarcane (Saccharum sp.) tolerant to NaCl and their response to salt stress. Pl Cell Tissue Organ Cult 87:9–16

Hasegawa PH, Bressan RA, Zhu JK, Bohnert H J (2000) Plant cellular and molecular responses to high salinity. Annu Rev Pl Physiol Pl Mol Biol 51:463–499

http://www.faostat.fao.org

Kripkyy O, Kerkeb L, Molina A, Belver A, Rodrigues-Rosales P, Donaire PJ (2001) Effects of salt-adaptation and salt-stress on extracellular acidification and microsome phosphohydrolase activities in tomato cell suspensions. Pl Cell Tissue Organ Cult 66:41–47

Lee YI, Lee IS, Lim YP (2002) Variations in sweet potato regenerates from gamma ray irradiated embryogenic callus. J Pl Biotech 4(4):163-170

Maluszynski, M, Ahloowalia BS, Sigurbjoornsson B (1995) Application of in vitro mutation techniques for crop improvement. Euphytica 85: 303-315

Mansour MMF (1998) Protection of plasma membrane of onion epidermal cells by glycinebetaine and proline against NaCl stress. Pl Physiol Biochem 36:767–772

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

Niknam SR, McComb J (2000). Salt tolerance screening of selected Australian woody species-a review. Forest Ecol Mgmt 139: 1-19

Pareek A, Singla SL, Grover A (1997) Salt responsive proteins/genes in crop plants. In: PK Jaiwal, RP Singh, A Gulati (eds), Strategies for Improving Salt Tolerance in Higher Plants, pp 365–391. Oxford and IBH Publication Co, New Delhi

Patade VY, Suprasanna P (2009) An in vitro radiation induced mutagenesis-selection system for salinity tolerance in sugarcane. Sug Tech 11 (3): 246-251

Patade VY, Suprasanna P, Bapat VA (2006) Selection for abiotic (salinity and drought) stress tolerance and molecular characterization of tolerant lines in sugarcane. BARC Newsletter. Issue no. 273.

Patade, VY, Suprasanna P, Bapat VA (2008) Effects of salt stress in relation to osmotic adjustment on sugarcane (*Saccharum officinarum* L.) callus cultures. Pl Growth Regul 55:169–173

Piri K, Anceau CS, EL Jaafari, Lepoivre P, Semal J (1994) Se´lection in vitro de plantes androge´ne´tiques de ble´ tendre re´ sistantes a‘ la salinite´. In AUPELF-UREF John Libbey Eurotext, Paris (eds), Quel avenir pour l’ame´ lioration des plantes. 311–320.

Rajendrakumar CSV, Suryanarayana T, Reddy AR (1997) DNA helix destabilization by proline and betaine: Possible role in the salinity tolerance process. FEBS Letters 410:201-205

Sabbah S, Tal M (1990) Development of callus and suspension cultures of potato resistant to NaCl and mannitol and their response to stress. Pl Cell Tissue Organ Cult 21:119–128.

Saghai Maroof MA, Solima KM, Jorgenson RA, Allard RW (1984) Ribosomal DNA spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc Nat Acad Sci USA 81:8014-8019

Sharma P, Dubey RS (2005) Modulation of nitrate reductase activity in rice seedlings under aluminium toxicity and water stress: Role of osmolytes as enzyme protectent. J Pl Physiol 162:854-864

Suprasanna P, Bapat VA (2006) Advances in the development of in vitro culture systems and transgenics in sugarcane. International Symposium on Technologies to Improve Sugar Productivity in Developing Countries, pp 629- 636, China

Suprasanna P, Rao PS (1997). Selection of mutants using plant cell and tissue culture techniques. In: Hemantaranjan A (ed) Advances in Plant Physiology, Vol.1. pp 103-122. Scientific Publishers, Jodhpur, India

Suprasanna P, Meenakshi S, Bapat VA (2006) Integrated approaches of mutagenesis and in vitro selection for crop improvement. In: Ashwani Kumar NS, Shekhawat (eds), Recent advances in Plant tissue culture and its applications. IK Int Publ, New Delhi, India (In Press)

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535.

Zambrano AY, Demey JR, Fuch M, Gonzalez V, Rea R, De Souza O, Gutierrez Z (2003) Selection of sugarcane clones resistant to SCMV. Pl Sci 165:221-225

Zeng I, Shannon MC (2000) Effect of salinity on grain yield and yield components of rice at different seedling densities. Agron J 92:418–23.