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Gamma Irradiation of Embryogenic Callus Cultures and *In vitro* Selection for Salt Tolerance in Sugarcane (*Saccharum officinarum* L.)

Vikas Y Patade, P Suprasanna and V A Bapat

Plant Cell Culture Technology Section, Nuclear Agriculture & Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400085, India

Abstract

Radiation induced mutagenesis followed by *in vitro* selection was employed for salt tolerance in popular Indian sugarcane (*Saccharum officinarum* L.) *cv.* CoC-671. Embryogenic calli were gamma irradiated and exposed to different levels of NaCl (42.8, 85.6, 128.3, 171.1, 213.9, 256.7, 299.5, or 342.2 mM). The relative growth rate (RGR) decreased progressively with increasing salt stress and was the least with a salt stress of 256.7 mM (0.25 ± 0.009), almost 10 fold lesser than the control. The RGR was significantly lower in 85.6 mM and higher salt stressed calli than the control. The survival percent also decreased, with an increase in NaCl concentration. In case of 10 and 20 Gy irradiated calli, regeneration was observed up to 85.6 mM NaCl selection, medium, whereas, higher treatments (128.3 mM and beyond) exhibited browning initially. However, in the subsequent subcultures, regeneration was obtained in the case of 10 and 20 Gy irradiated calli on 128.3 and 171.1 mM NaCl selections. Higher dose of gamma irradiation (40 Gy) also showed regeneration, but only with 85.6 mM NaCl selection. The unirradiated calli regenerated the highest number of plantlets followed by 10 and 20 Gy irradiated calli on salt selection. A total of 147 plantlets were selected from different salt levels. The salt selected plants are being tested for their field performance.

Key words: sugarcane, *in vitro* mutagenesis, *in vitro* selection, salt tolerance

INTRODUCTION

Sugarcane (*Saccharum* spp.) is one of the most important agro-industrial crops in the world, cultivated on more than 20 million hectares. Plant growth and productivity is severely affected by salt stress conditions (Altman 2003). Being a typical glycophyte, salinity in the root zone of sugarcane decreases the sucrose yield, through its effect on both biomass and juice quality (Lingle and Weigand 1996). The complexity and polygenic nature of salinity tolerance has seriously limited the efforts to develop the tolerant crop variety through conventional breeding practices. Somaclonal variation in combination with *in vitro* mutagenesis and selection has been applied for the isolation of agronomically useful mutants (Jain 2000; Zhambrano *et al.* 2003). Many examples related to different vegetatively propagated species, show that the combination of *in vitro* culture with selection is relatively inexpensive, simple, and efficient (Ahloowalia 1998). Chemical and/or radiation mediated *in vitro* mutagenesis and selection has been successfully used to improve agronomic traits such as salinity and drought tolerance in different crop plants (Foster 2001; Biswas *et al.* 2002; Predieri and Gatti 2004; Zhu *et al.* 2004), advocating that tissue culture selection is useful to select stress-tolerant clones.

Although studies related to the salt selection are abun-

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Correspondence P Suprasanna, E-mail: prasanna@magnum.barc.gov.in

dantly available in diverse plant species, limited research has been conducted in sugarcane (Saif-Ur-Rasheed *et al.* 2001; Gandonou *et al.* 2006). Gandonou *et al.* (2006) has studied salt stress effects by exposing the callus to a single level of 68 mM NaCl and has found that the physiological and biochemical indicators play a crucial role in salt tolerance. In these studies, it has been the authors' endeavor to employ radiation-induced mutagenesis combined with *in vitro* selection to obtain salt tolerance. Herein, results are presented on the selection for salt stress tolerance in gamma-irradiated sugarcane calli and regeneration of tolerant plants.

MATERIALS AND METHODS

Embryogenic callus cultures of popular sugarcane *cv.* CoC-671 were established from young leaf explants (Desai *et al.* 2005) and maintained through regular subcultures. The calli induced were cultured for multiplication on callus induction medium (CIM), MS (Murashige and Skoog 1962) basal salts supplemented with 100 mg L^{-1} malt extract, 100 mg L^{-1} L-glutamine, 1 000 mg L^{-1} casein hydrolysate, 5% coconut water, 2 mg L^{-1} 2,4-D, and 3% sucrose, and gelled with 0.2% Gel rite. The cultures were incubated in darkness at (25±2)°C and subcultured onto CIM at every three-week interval.

The embryogenic calli were subjected to gamma radiation using Gamma Cell 220 at dose rate of 9.6 Gy min^{-1}. The irradiation doses were 10, 20, 30, 40, or 50 Gy. Radiation-treated calli were immediately cultured on CIM to eliminate the radiolysis hazards and subcultured at least thrice, at monthly intervals, on the same medium (CIM) before using for further studies. Survival percent of the calli was recorded in terms of white proliferating clumps (WPCs).

Gamma irradiated calli (200 mg) were cultured on CIM supplemented with different levels of salt-NaCl (42.8, 85.6, 128.3, 171.1, 213.9, 256.7, 299.5, or 342.2 mM). Callus growth was determined in terms of relative growth rate (RGR) after four weeks of culture on a salt selection medium by using formula, RGR = (Final fresh weight - Initial fresh weight)/Initial fresh weight. The putatively tolerant calli were exposed to salt selection in subsequent cycles.

Plantlets were regenerated after two to three weeks of transfer of salt selected calli on regeneration medium, that is, CIM without 2,4-D. About 5 cm long individual shoots were transferred on half MS medium with 2 mg L^{-1} NAA for rooting. The regeneration efficiency was expressed in terms of number of plantlets regenerated in a particular treatment of gamma irradiation and salt stress. The rooted plantlets were hardened in the green house. Each treatment consisted of 15 calli (five per each 9.5 cm diameter culture plate) and the values were given in the form of mean ± standard error. Experiments consisting of treatments and control were replicated thrice and percent values were Arcsine transformed, and analysis of variance (ANOVA) was carried out using the IRRISTAT program (IRRI 2003).

RESULTS AND DISCUSSION

Survival percent of the gamma irradiated calli, in terms of WPCs, decreased progressively with an increase in the irradiation dose. The highest survival was observed in the control cultures (85.7%), whereas, the lowest survival was noted in 50 Gy irradiated cultures (Fig.1). The extent of browning showed an increasing trend with an increase in irradiation dose (Fig.2). Cultures exposed to 20 Gy irradiation, exhibited almost 50% survival as compared to the nonirradiated control cultures. Hence it was considered as the LD_{50} dose for sugarcane embryogenic cultures. Lower doses (10 and 20 Gy) and the control regenerated higher number of plantlets and regeneration response decreased beyond 20 Gy irradiation. Doses of 30 Gy and higher did not

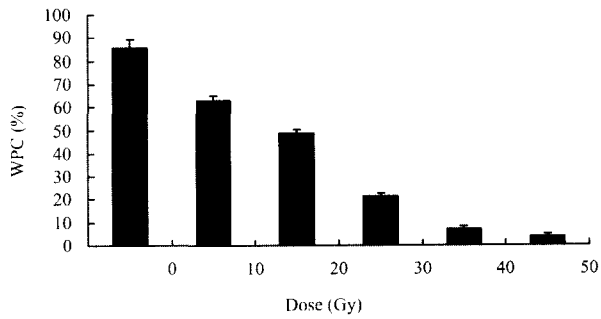

Fig. 1 Effect of gamma irradiation on survival percentage of embryogenic cultures in sugarcane following 4 weeks of post-irradiation culture. Data are mean values ± SE.

show any sign of regeneration in the first subculture, however, continued subculture of these cultures on regeneration medium resulted in plant regeneration.

In case of salinity tolerance, observations after one month of culture showed decreasing trend of fresh weight gain with the increase in NaCl concentration. The RGR decreased with increasing salt stress (Fig.3) and was the least with salt stress of 256.7 mM (0.25 ± 0.009), almost 10 fold less than the control. The RGR in case of salt selection with 85.6 mM NaCl was significantly lower than the control, however, the differences between RGR of control and that of 42.8 mM treatment were not significant. The survival percent also decreased with increase in NaCl concentration. Selection medium with more than 171.1 mM NaCl exhibited no survival or regeneration (Figs.4 and 5). Calli turned completely brown in these treatments.

Irradiated callus cultures on salt selection media showed a decrease in callus growth with increase in salt concentration. The survival response exhibited a decreasing trend with increasing salt concentrations. Next to the control, the 85.6 mM NaCl selection showed better callus growth, however, the least response with callus browning was observed at higher concentrations. In case of 10 and 20 Gy irradiated cultures, regeneration was observed up to 85.6 mM NaCl selection medium (Fig.4). The treatments with 128.3 mM and higher concentrations exhibited browning and did not show any sign of regeneration in the first subculture cycle. However, in a subsequent subculture, regeneration was obtained in 10 and 20 Gy irradiated calli on 128.3 and 171.1 mM NaCl selection medium. In 40 Gy irradiated calli, regeneration could be seen on 85.6 mM NaCl selection. However no regeneration was observed on salt selection in case of 30 and 50 Gy irradiated calli (Fig.4). A total of 147 plantlets were selected from different salt selection media. The unirradiated calli regenerated the highest number of plantlets followed by 10 and 20 Gy irradiated calli on salt selection.

Radiation mutagenesis combined with *in vitro* culture has proved effective in the induction of novel genetic variation, selection, and multiplication of mutant clones aimed at crop improvement (Foster 2001; Biswas *et al.* 2002; Zhu 2004). Assessment of the radiosensitivity in terms of LD_{50} is the first step of *in vitro* mutagenesis, with physical mutagens such as gamma

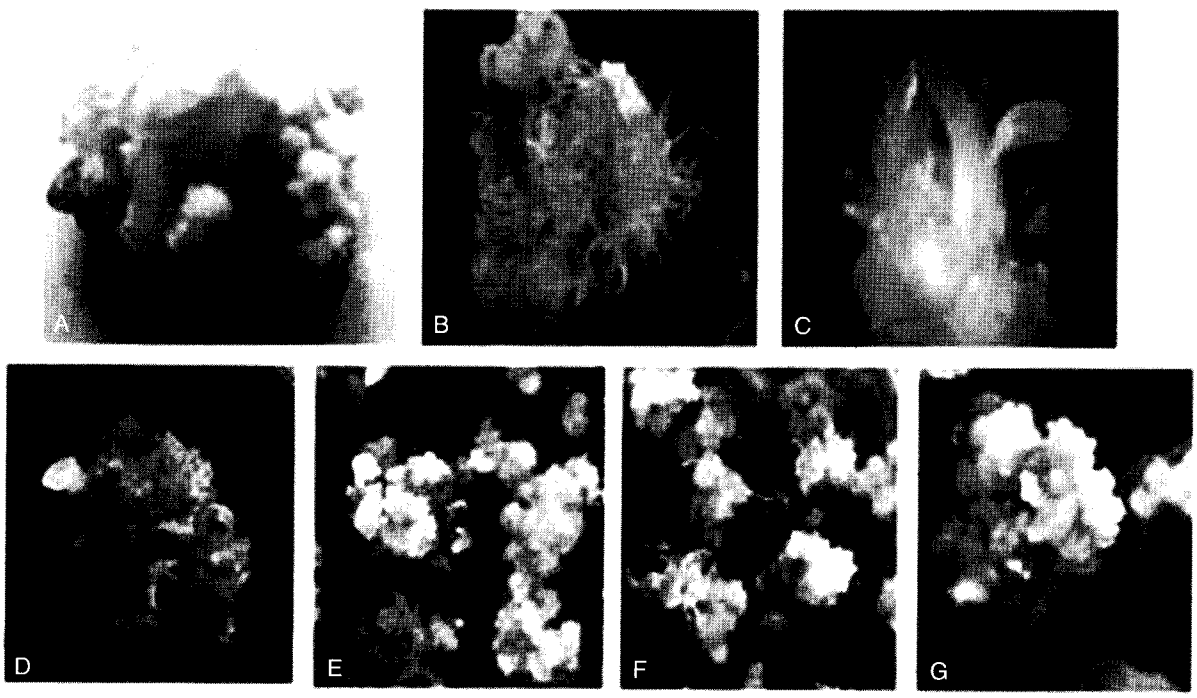

Fig. 2 Regeneration from gamma irradiated calli of sugarcane *cv.* CoC-671. A-C, regeneration from control non irradiated calli; D-G, regeneration from calli irradiated at 10, 20, 30, and 40 Gy, respectively.

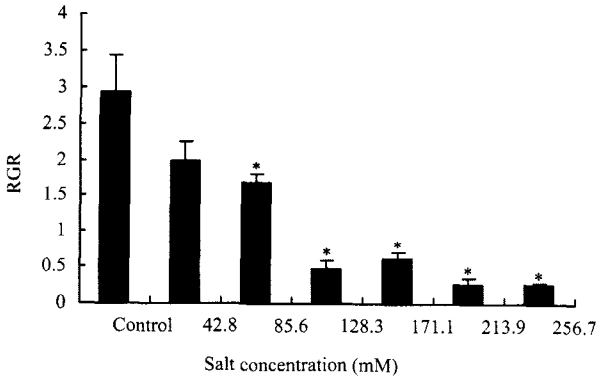

Fig. 3 Effect of increasing NaCl concentration on relative growth rate (RGR) of embryogenic cultures of sugarcane following 4 weeks of exposure to NaCl. Data are mean values ± SE. * Significantly different over control at $P=0.05$.

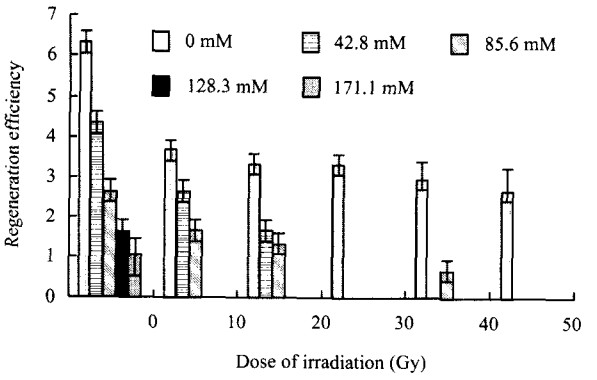

Fig. 4 Regeneration response of gamma irradiated calli on different sub-lethal doses of salt (NaCl). Regeneration efficiency is the number of plantlets regenerated. Data are mean values ± SE.

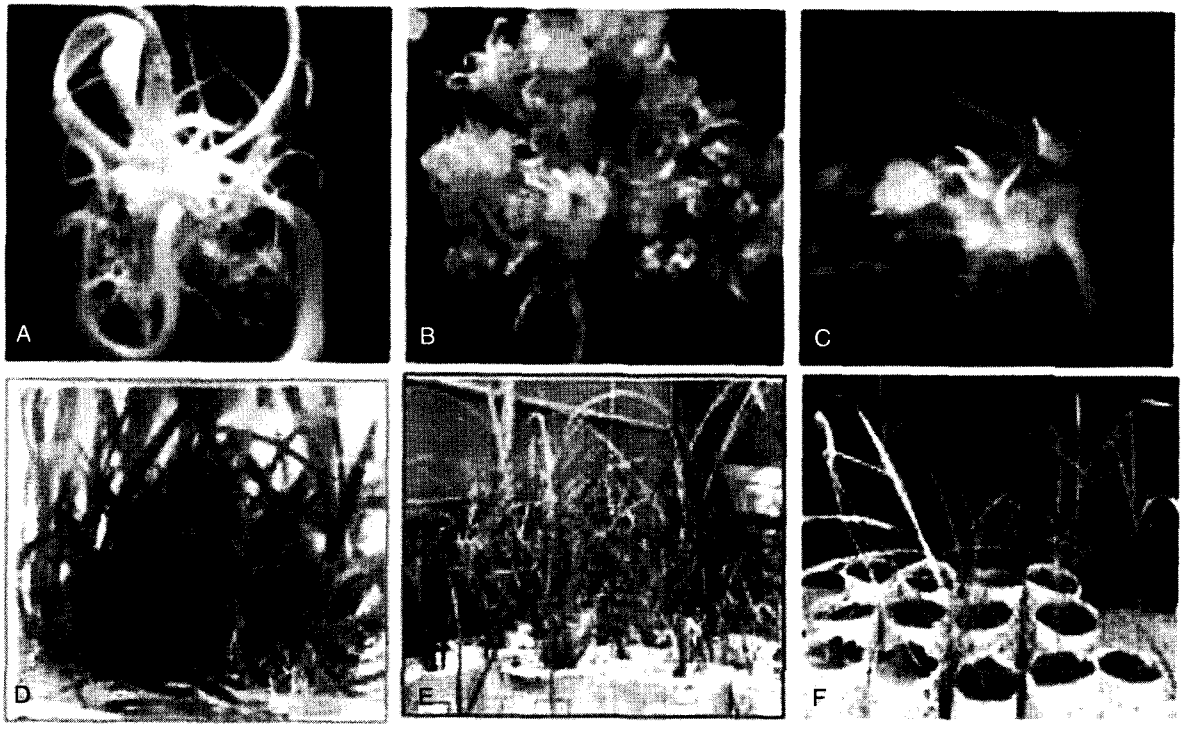

Fig. 5 Regeneration of gamma irradiated calli at different sub lethal doses of salt. A-C, regeneration on control, 42.8, and 171.1 mM salt selection media, respectively; D, rooting of 171.1 mM salt selected plantlets; E and F, hardened salt selected plants.

radiation, to optimize the suitable dose for mutagenesis. In the present study, the sugarcane embryogenic callus cultures were exposed to different doses of gamma irradiation, and post irradiation survival response was evaluated in terms of WPCs. The percentage of WPC exhibited a decreasing trend with an increase in radiation dose, suggesting an inverse correlation of the radiation dose and proliferation capacity. In the present study, 20 Gy was considered as LD_{50} after four weeks of the post irradiation culture. Taras *et al.* (1999) characterized the radio-sensitivity of cells using LD50 as the criteria. However, LD_{20} or LD_{30} had also been used as the optimum dose, as these levels of mutagens were not highly toxic for the tissues (Colijin *et al.* 1979). In

the present study, irradiated calli in the post irradiation phase were immediately subcultured onto the fresh medium as radiolysis produced toxic compounds in the medium. Saif-Ur-Rasheed *et al.* (2001) recorded 20 Gy as a LD_{50} dose for a young leaf derived sugarcane callus, and a drastic decrease in regeneration frequency with an increased dose of irradiation. Four to five percent regeneration was observed in the 60 Gy treatment. Also in the present study higher number of plantlets was regenerated from lower doses (10 and 20 Gy) as compared to higher doses. In most cases of *in vitro* selection for salinity, selection was either applied to explants or cell/callus culture by inclusion of growth inhibitory levels of salt in the selection medium (Suprasanna and Rao 1997). To study the salt inhibitory level, the nonirradiated callus was exposed to different NaCl concentrations and the results indicated regeneration of only up to 171.1 mM selections. The survival response and the growth of calli also showed a decreasing trend with an increasing concentration of salt. Therefore in studies on *in vitro* mutagenesis and selection, the media were designed accordingly. The irradiated callus cultures on salt selection media showed a decline in callus growth with increase in salt concentration. Also the survival response in all gamma irradiated cultures exhibited a decreasing trend with increasing salt concentration. These findings were consistent with the earlier reports (Ehsanpour and Fatahian 2003) wherein; the effect of salt stress in Medicago sativa callus cultures was seen up to 120 mM NaCl with a decline in callus growth. Wheatley *et al.* (2003), when studying the development of salt adoption in greater yam, observed no growth on 100 mM salt selection. Decline in growth of callus upon salt stress could be on account of dehydration of cells through low water potential or nutritional imbalance because of interference of saline ions (Na^+, Cl^-) with essential nutrients in both uptake and translocation process. Gandonou *et al.* (2006) studied the salt stress effects by exposing the callus to a single level of 68 mM NaCl and found that the physiological and biochemical indicators played a crucial role in salt tolerance.

It is plausible that inorganic ions and proline amass adequately to permit osmotic adjustment of sugarcane under a salt stress regime. Screening regenerated plants under field conditions is underway, to further confirm stress-tolerance of the selected lines. Regeneration of plants exhibiting an enhanced tolerance to abiotic stress is one of the important goals for the biotechnological improvement of crop plants. Enhanced salinity tolerance may prove beneficial to improve the competitiveness of the popular sugarcane cultivars and their commercial cultivation in saline areas.

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