Improved method of *in vitro* culture in hybrids of sugarcane

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Abstract: *The callusing and regeneration potential of intergeneric and interspecific hybrids of Saccharum was assessed. The hybrids used for the study were S.officinarum x Erianthus, S. officinarum x S. spontaneum, S. officinarum xSclerostachya and commercial sugarcane variety x S. officinarum. Cytological analysis has been conducted and it confirmed the hybridity of these clones. Data on culture efficiency and regeneration efficiency revealed that callusing and regeneration potential of these hybrids greatly depend on genotype. The culture efficiency of S. officinarum x Sclerostachya was less (40%) when compared to other hybrids (80-100%). Production of non embryogenic calli was more in this hybrid. A differential influence of parental genome on cell proliferation and morphogenesis was observed. In order to increase callus regeneration, the embryogenic calli were subjected to partial desiccation. Calli desiccated for five hours resulted in 40% weight loss and these calli showed enhanced plant regeneration response invariably in all hybrids over other desiccation treatments and control. The desiccation treatment showed improvement in conversion of somatic embryos into healthy plants as compared to plants obtained from the non-desiccated embryogenic calli. The method described is simple, inexpensive and does not require advanced technical workers or equipment. Since it is applicable to all the hybrids studied, the method can be useful to increase the rate of in vitro regeneration especially in in vitro recalcitrant materials. Partial desiccation can also be employed to stimulate regeneration of embryogenic callus cultures undergoing physical and chemical mutagenesis in sugarcane and other crops.*

Key words: Sugarcane, *in vitro* culture, desiccation,

INTRODUCTION

Sugarcane (*Saccharum*) is one of the most important field crops grown in the tropics and sub-tropics. It has a very complex genome. Sugarcane is a suitable candidate for the application of plant biotechnology and genetic engineering tools, as limitation exists because of its complex polyploidaneuploid
genome, narrow genetic base, poor fertility, susceptibility to various diseases and pests and the long duration required to breed elite cultivars. There has been continuous effort towards the refinement of protocols for efficient sugarcane somatic embryogenesis *in vitro*. Effective utilization of biotechnological approaches depends on efficient and reliable plant regeneration systems (Guiderdoni *et al*., 1995; Lakshmanan *et al*., 2005; Lakshmanan, 2006; Suprasanna and Bapat, 2005). Innovative approaches of cell and tissue culture possess a significant promise for the development of new genetic variability for the improvement of the crop. Other than this the embryogenic cultures have found their place in wide variety of applications, from obtaining virus resistant plants through somaclonal variation (Oropeza *et al*., 1995), to mutagenesis and *in vitro* selection (Suprasanna *et al*., 2006; Patade *et al*., 2006) and to developing transgenic plants (Bower *et al*., 1996; Arencibia *et al*., 1998).There has been a continuous effort towards refinement of protocols for efficient sugarcane morphogenesis *in vitro* as demonstrated by studies conducted in the past 8-10 years (Snyman *et al*., 2000, 2001; Geijskes *et al*., 2003: Desai *et al*., 2004a, b; Suprasanna and Bapat, 2005; Patel *et al*., 2007). These efforts are driven with the intention to improve the efficiency and speed of the tissue culture system used. In most of the cases frequency of plant regeneration from callus cultures depends directly on the frequency of somatic embryogenesis. A number of factors like genotype, medium constituents, auxins, sugar, amino acids and growth regulators influence callus induction and regeneration. Among these factors the genotype appears to be an important factor influencing the efficiency of *in vitro* culture. In sugarcane little is known about the importance of genotype of *in vitro* culture ability. In this crop two earlier reports (Burner, 1992; Gandonou *et al*., 2005) revealed that genotype affected callusing response. In a previous study using different sugarcane species, hybrids and varieties, it has been reported that callus production ability in sugarcane is genotype dependent (Sobhakumari *et al*., 2009). In this *in vitro* culture study with different intergeneric and interspecific hybrids, differential influence of parental genome on cell proliferation and morphogenesis was observed. Some of the hybrids, especially those evolved from wild species or related genera showed poor regeneration from callus. The hybrids with high amount of phenolic compounds were also recalcitrant to *in vitro* culture. Much research has been focused on modifying culture conditions and regeneration procedures to get high quality callus and increase shoot regeneration frequency. Partial desiccation treatments have been reported to be beneficial for embryogenesis and plant regeneration in several plant species like Soybean (Hammat and Davey, 1987), rice (Vinod Saharan *et al*., 2004), banana (Srinivas *et al*., 2006) and sugarcane (Suprasanna, 2008).

The purpose of this study was to test the ability of two intergeneric and two interspecific hybrids of *Saccharum* to callus induction, embryogenic callus production and plant regeneration using young leaf bits as explant in order to determine genotypic influences with *in vitro* response in sugarcane and identify the hybrids with low regeneration capacity to improve its regeneration *via* partial desiccation of callus.

MATERIALS AND METHOD

The four hybrids used for the study were obtained from the experimental field of Sugarcane Breeding Institute, Coimbatore, India. The hybrids were (1) *S. officinarum* x *Erianthus*, (2) *S.officinarum* x *S.spontaneum* (3) Co 7201 x *S.officinarum* (4) *S.officinarum* x *Sclerostachya*. Six month old clones were selected to collect the healthy shoots for explant preparation. The outer leaves were removed and surface of shoots were wiped with alcohol. Two or three outer whorls were removed and the apical portion of the stem
(10cm) was cut and taken into laminar air flow chamber. The developing leaves encircling the growing point were dissected out and bits of about 0.5cm x 0.5cm were cut with the help of sterile forceps and scalpel. The excised explants (10/ culture bottle) were immediately inoculated onto MS medium (Murashige and Skoog, 1962) supplemented with 3mg /l 2,4 Dichlorophenoxyacetic acid and 20g/l sucrose. The pH was adjusted to 5.8 with 1.0 N NaOH and all media were solidified with 8.0g/l agar before autoclaving for 20min at 120° C. Subculturing was done at 15-20 days interval. Observations were made on callus induction time, callus weight, callus induction percentage, callus morphology and embryogenic callus production. To study the effect of partial desiccation, about 5g embryogenic calli after 2months on the callus maintenance medium were transferred to sterile Petri dishes containing sterile Whatman filter paper disks on sterile silica gel. The dishes were sealed with para film and kept at 25±1° C in dark for 8h. Moister content was determined as per the method described by Desai *et al*. (2004). Initial weight of the calli was noted and after each hour (1-8h), weight was taken and moisture content calculated as follows:

$$MC = \frac{\text{Initial weight of the calli} - \text{Final weight}}{\text{Initial weight}} \times 100.$$

After desiccation the callus was transferred to regeneration medium (MS+1.0 mg/l kinetin+0.25 mg/l BAP +0.5 mg/l GA3). Observations were recorded on fresh weight of callus, survival of callus and number of plantlets regenerated. Experiments consisting of treatments and control were replicated thrice.

RESULTS AND DISCUSSION

Clones exhibited considerable variation in time taken for callus induction and subsequent growth and colour of the callus. Among the four hybrids selected for the study, three of them were having *S. officinarum* as female parent and one having it as male parent. The hybrid in which *Erianthus* as the male parent showed callus induction response within two weeks whereas the hybrid with *S. spontaneum* as the male parent took three weeks to respond. The hybrid Co 7201 x *S. officinarum* took 22 days for callus induction. These results indicated that callus induction ability was greatly influenced by the genotype and in this study especially by the parental genome. Callus induction rate varied from 60% - 100%. These high callus induction percentages revealed the high capacity of the sugarcane cultivars tested to induce callus from leaf explants. Both V1 (*S. officinarum* x *Erianthus*) and V3 (Co 7201 x *S. officinarum*) showed 100% callus induction, whereas the hybrids V2 (*S. officinarum* x *S. spontaneum*) and V4 (*S. officinarum* x *Sclerostachya*) showed 80% and 60% callus induction respectively. These results indicate that callus induction ability is greatly influenced by the genotype and this finding is in agreement with those already reported in sugarcane (Gandonou *et al*., 2005), *Oryza sativa* (Abe and Futsuhara,1986; Mikami and Kinoshita, 1988; Hoque and Mansfield, 2004), *Primula* sp. (Schween *et al*., 2003) and *Triticum* (Zale *et al*., 2004). Embyogenic characteristic of the callus is a very important parameter. It reveals the capacity of the callus to regenerate a plant from one cell or a few numbers of cells. Distinction between embryogenic and non embryogenic callus was determined on the basis of the external appearance of the callus. The culture efficiency of the hybrids was calculated in terms of the number of explants that produced friable embryogenic calli. For the variety V1the culture efficiency was 100% because all the explants that were inoculated in the callus medium showed embryogenic callus induction in this hybrid. For culture efficiency
hybrid V2 and V4 were weak (60% and 40% respectively), where as hybrid V3 was intermediate with 80% efficiency.

The capacity to produce embryogenic callus depends on genotype. In *in vitro* studies the choice of the potential genotype that could be improved depends mainly on their capacity to plant regeneration. The embryogenic calli were transferred to differentiation medium and kept in light. The time taken for the initiation of green shoot

buds was observed. As in callus induction the hybrid V1 took lesser time (7 days) for induction of shoot buds. Within two weeks regeneration was initiated in all the hybrids. Regeneration efficiency in terms of regenerable calli was calculated and it was observed that in all the cases the regeneration efficiency was below 50%. Though such genotypes that show high frequency of regenerable callus are advantageous in tissue culture programmes, no correlation was observed between the available embryogenic calli and percentage of plant let regeneration. All the embryogenic calli were not showing successful morphogenesis to form plant lets. In the case of hybrid V4 (*S. officinarum* x *Sclerostachya*) only 16% morphogenesis could be obtained from the embyogenic calli. In other hybrids also the regeneration rate was not high (20 – 48%). In order to improve the regeneration efficiency of the calli obtained from these hybrids a protocol with partial desiccation was tried. In this study, the intensity of the desiccation treatment has been expressed in terms of percentage of fresh weight loss rather than in terms of moisture content in order to avoid the influence of ambient environmental factors such as temperature, humidity and seasonal effects. The 8h partial desiccation treatment resulted in a decrease in water content from the desiccated calli. When the moisture content was calculated, it was found that initially 20-25% moisture was lost within 1h of desiccation and up to 80% moisture loss was seen after 8h. Calli desiccated for 5h showed better response (80%) over other desiccation treatments and control. From the hybrid V4 (*S. officinarum* x *Sclerostachya*) an average of 6 plantlets were obtained in control where as 22 plantlets were obtained after 5h desiccation. After 5h desiccation 40-45% moisture loss was observed. Transferring matured somatic embryos to MS medium supplemented with 0.25mg/l BAP, 0.5mg/l GA3 and 1.0mg/l NAA stimulated leaves and roots whose morphology was identical to that of seedlings. Desiccation treatment showed improvement in the conversion of somatic embryos into healthy plants as compared to plants obtained from nondesiccated embryogenic calli. Partial desiccation increased the regeneration of sugarcane varieties 2-4 fold (Fig. 1).

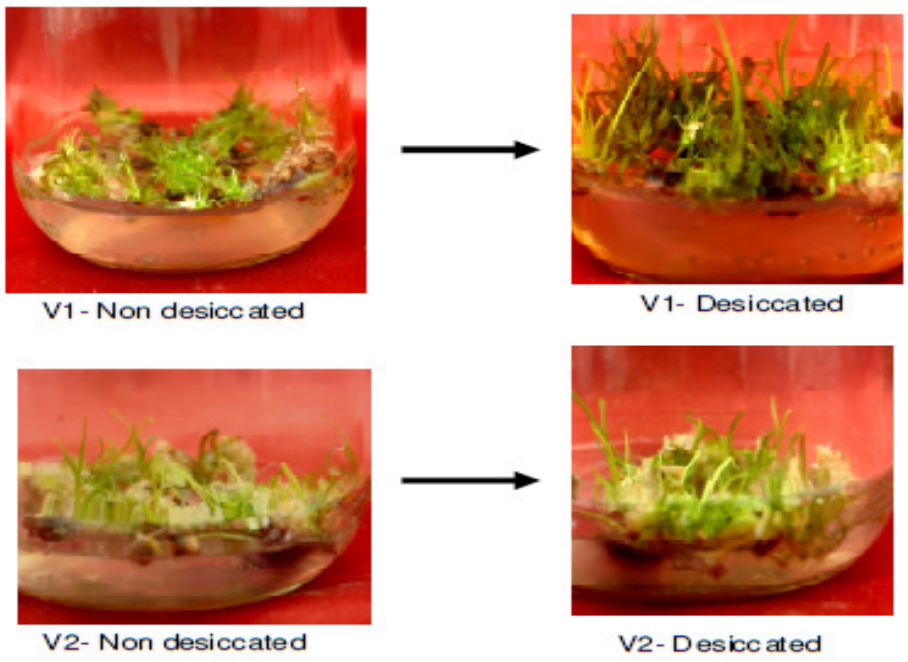

Fig. 1. Effect of desiccation on *in vitro* regeneration in sugarcane

It has been reported that slow desiccation caused stimulation of loss of trigger and substantial accumulation of starch and protein in comparison to no or rapid dehydration (Etiene *et al*., 1993). As in the case of indica rice (Chand and Sahrawat, 2001) and *Pinus kesia* (Malabadi *et al*., 2004) it was found that desiccation treatment of embryogenic calli was needed to induce somatic embryo maturation and plantlet formation from sugarcane embryogenic calli. The results suggest that a complex relationship exists between the water content of embryogenic calli and maturation of somatic embryos. Some reports say that the desiccation treatment might trigger rapid biochemical changes in the calli and under water stress specific enzymes or polipeptides probably appear in the callus culture (Rance *et al*., 1994). Attree and Fowke (1993) reported that desiccation treatment stimulated the accumulation of storage reserves and triglycerides that have a positive effect on somatic embryo maturation. The effect of partial drying may cause a breakdown of endogenous ABA (Abscisic acid) or decrease the sensitivity of embryos to ABA which could release embryos from development constrains and allow germination to proceed (Kermode *et al.*, 1989).

In conclusion, the present study suggests that partial desiccation of embryogenic callus improves the maturation process of somatic embryos of different hybrids of sugarcane and this method is feasible, reproducible and offers a tremendous potential for large scale *in vitro* regeneration especially in *in vitro* recalcitrant cultivars.

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