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Partial Desiccation of Embryogenic Calli Improves Plant Regeneration in Sugarcane (*Saccharum* Spp.)

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Abstract

Partial desiccation of embryogenic calli cultures or somatic embryos leads to different physiological changes and maturation of somatic embryos, leading to improved plant regeneration. Embryogenic calli was induced from immature inflorescence segments and young leaf rolls of sugarcane (*Saccharum officinarum* hybrids CoC-671) on Murashige and Skoog's basal medium enriched with different concentrations of 2,4-D (1-4 mg/l), L-glutamine (100mg/l), malt extract (100mg/l), casein hydrolysate (1000 mg/l) and coconut milk (5%) and solidified with 0.2% gelrite. The embryogenic calli were subjected to desiccation for 1-8 h. Desiccation of the calli for 6-7 h resulted in enhancement of plant regeneration frequency (83-96%) as compared to control (12%). Plantlets exhibited vigorous growth to maturity in the greenhouse. Partial desiccation of embryogenic calli offers as a simple method for improving plant regeneration frequency in sugarcane.

Key words: Saccharum spp., embryogenic calli, desiccation, plant regeneration

Introduction

Sugarcane is an important commercial crop in many developing / developed countries. Considering its importance in the agricultural industry, concerted efforts are being made for its improvement using conventional and biotechnological techniques. Sugarcane micropropagation from apical meristems has been commercially useful because of the time it saves in multiplying the promising varieties and clones, facilitating the acquisition of large volumes of material besides useful in eliminating pathogens. Tissue culture techniques are now widely used in sugarcane improvement programs (Guiderdoni *et al.*, 1995) and plant regeneration has been obtained from young leaf rolls (Ho and Vasil 1983; Chen *et al.*, 1988) and immature inflorescences (Liu, 1993). Somatic embryogenesis in cell and callus cultures has also become the choice for high volume propagation system and setting up such a large-scale delivery system would be desirable in multiplying new sugarcane varieties. The success of genetic manipulation in sugarcane is dependent on highly embryogenic calli and efficient plant regeneration system (Bower and Birch, 1992, Arancibia et al., 2000). Effect of different growth regulators on sugarcane regeneration has been studied and thiadiazuron was found as the most suitable, over other growth regulators (Chengalrayan and Gallo-Meagher, 2001).

In many plant species, the conversion of embryos is related to poor quality of embryos and lack of maturation and desiccation tolerance (Bapat and Rao, 1996). Desiccation probably completes the developmental process and initiates biochemical events, which prepare embryos for germination (Kermode and Bewley, 1985). Partial desiccation has been reported to enhance somatic embryogenesis and plant development in spruce (Roberts 1991), wheat (Carman, 1988), Grape (Gray, 1989), soybean (Hammat and Davey, 1987) and rice (Rance *et al.*, 1994). In the present investigation, we report that partial desiccation of embryogenic calli enhanced plant regeneration frequency in sugarcane, which may offer as a rapid regeneration system in case of mutagenized or genetically transformed sugarcane cultures.

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Materials and Methods

Establishment of cultures

The fresh plant material for isolating young leaf bases and immature inflorescence segments of sugarcane variety CoC-671 was collected from Sugarcane and Gur Research Centre, Kolhapur, India. The old leaf base coverings were removed carefully without damaging the internal young and delicate tissue, followed by the immersion of portion of inflorescence and apical shoots in absolute alcohol for 5 min for surface sterilization. The outer leaf base coverings were then removed using sterile forceps. Young and immature segments (3-6 mm) of inflorescence and young leaf bases (3-6mm) were inoculated on Murashige and Skoog's (1962) media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 1-3 mg/L), L- glutamine (100 g/L), malt extract (ME; 100 mg/L), casein hydrolysate (CH; 1 g/L), coconut water (CW; 5%) and sucrose (3-4%). The pH of the medium was adjusted to 5.8 and the cultures were maintained in dark initially for 3-6 weeks and thereafter incubated in the culture room at 27±1℃ under white cool, fluorescent light (1000 lux) for 10-12 h/day, with relative humidity of 70-80%.

Desiccation

About 20g of embryogenic calli derived from immature inflorescence segments was desiccated using sterilized silica gel in a sterile desiccator. Calli was desiccated for 0, 1 to 8 h and after each desiccation interval of 1 h, about 2 g of calli was transferred to regeneration medium (MS basal medium without 2,4-D) in three replications. The experiment was repeated three times and data were recorded on the embryo conversion, regeneration frequency and plantlet number.

Moisture content (MC)

About 2 g of embryogenic calli was transferred onto autoclaved dry filter paper and kept on 20 g of silica gel in a desiccator. Initial weight of embryogenic calli was noted and after every hour, weights were taken and moisture content was calculated as, MC = Initial weight of the calli-Final weight / Initial wt × 100. After desiccation, calli was transferred onto regeneration medium without 2,4-D, supplemented with 3% sucrose and gelled with 0.2% Phytagel (Sigma USA). Conversion frequency was calculated as the embryogenic calli developed with good shoots. All the experiments were repeated thrice and the data were recorded on the embryo conversion, regeneration frequency and shoot number per culture.

Plant regeneration and acclimatization

The healthy somatic embryo derived plants were cultured on MS basal media without any growth regulators for root induction. The rooted plants were transferred for hardening in small plastic pots containing autoclaved soil, covered with polythene sheets and maintained under 25-27℃ at 70% relative humidity.

Results and Discussion

Establishment of cultures

The immature inflorescence segments cultured on various combinations of MS media enriched with different nitrogenous and complex compounds (Table 1) showed swelling within four days and formation of the embryogenic calli within three weeks (Figure 1. A-C). About 90% of the inflorescence explants responded well for calli induction and calli turned highly compact, nodular within four weeks (Figure 1. A, B), while only 40% of the young leaf base explants responded and showed initiation of calli from the leaf bases as well as from the cut ends of the leaves. Calli derived from the leaf base explant was of mixed type with watery and nodular calli.

The response of immature inflorescence and young leaf base explants to the different combinations of the media is presented in Table 1. Highly embryogenic and compact nodular calli were obtained when the immature inflorescence segments were cultured on S3 media, while young leaf bases when cultured on SL1 media showed development of embryogenic and non-embryogenic calli. It was observed that media enriched with complex nitrogenous compounds like, casein hydrolysate, malt extract and coconut water was necessary for the induction of highly embryogenic calli from immature inflorescence segments or young leaf base explants. Further, it was observed that supplementation of media with higher concentrations of 2,4-D (S4, S5) induced watery type of calli, while addition of coconut water in combination with 1mg/L of 2,4-D also yielded watery type calli in both the explants.

Desiccation

The embryogenic calli of sugarcane showed rapid moisture loss upon partial desiccation for 0-8 h (Figure 2). The level of moisture loss remained constant after 7 h of desiccation of the calli. Initially within one hour, 29.7% moisture was lost and after 7 h treatment, almost 90% of the moisture was lost. Calli desiccated for different periods showed varied

Table 1. Response of inflorescence and young leaf explants of sugarcane var. CoC-671 for embryogenic calli induction on MS media supplemented with various concentrations of growth regulators

No.	2,4-D[b] (mgl^{-1})	BA (mgl^{-1})	Glu (mgl^{-1})	ME (mgl^{-1})	CH (mgl^{-1})	CW (%)	Sucrose% (w/v)	%Response	Growth	Type of calli
CoC-671: Immature inflorescence explant										
S1	1	--	--		--	5	3		+[a]	Watery
S2	2	1					3	30	+	Watery
S3	1	--	100	100	1000	5	3	90	++ +	Embryogenic
S4	3		100	100	1000	5	3	60	++ +	Watery
S5	4				500	5	3	60	++	Watery
CoC-671: Young leaf base explant										
SL1	1	--	100	100	1000	5	3	65	+	Nodular, Watery calli
SL2	3	--	100	100	1000	5	3	78	++	Watery

a: '+' - slow growth; '++' - moderate; '+++' - fast, b: 2,4-D- 2,4-dichlrophenoxyacetic acid; BA- Benzyl adenine; Glu- L-glutamine; ME- malt extract; CH- Casein hydrolysate; CW- coconut water

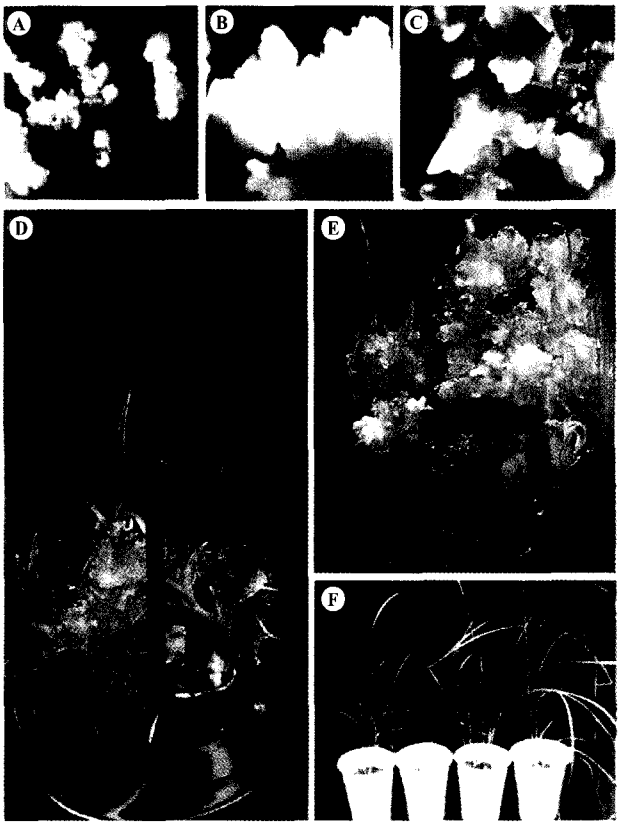

Figure 1. Plant regeneration from embryogenic calli derived from inflorescence explants of sugarcane cv. CoC-671.

A. Callus induction of inflorescence explants,
B. Highly embryogenic callus,
C. Callus showing development of embryos (arrows show developing embryos),
D. Desiccated callus showing plant regeneration.
E. Non-desiccated calli showing greening
F. Hardened regenerated plants in the greenhouse.

response of regeneration (Figure 3). Among the different desiccation periods, 6 and 7 h treatments yielded better response (83 and 96% respectively) over other treatments and control (12%). Plantlets per culture ranged from 1.4± 0.16 to 3.8±0.197 for calli desiccated for 1-6 h, whereas plantlet number increased to 9.0±1.67 with 6 h desiccation and 11.05±1.82 with 7 h desiccation (Figure 3). The desiccation treatment showed improvement in the conversion of somatic embryos into healthy plants (Figure 1.D) as compared to plants obtained from the non-desiccated embryogenic calli (Figure 1.E). The plants obtained through desiccation treatment were hardened in the green house (Figure 1.F) and all the plants were found to be morphologically normal.

Calli obtained from immature inflorescence was found to be highly embryogenic (>90%) and developed into somatic embryos after four weeks of culture and plantlets when maintained either on the same media or media devoid of growth regulators (Figure 1. B, C). In sugarcane it was earlier reported that the calli obtained from immature inflorescence was highly embryogenic, compact and nodular and plant regeneration was through atypical embryogenesis-organogenesis and the regenerants were phenotypically normal (Vasil and Vasil, 1985). Although thidiazuron was useful to stimulate regeneration frequency in embryogenic calli (Gallo- Meagher *et al.*, 2000), thidiazuron failed to induce similar response in the embryogenic cultures of CoC-671 (data not shown), which could be attributed to different genotypes used in the studies. Browning of the embryogenic tissue and poor regeneration frequency prompted us to employ partial desiccation treatment of the embryogenic calli and it was observed that the desiccation of the embryogenic calli of sugarcane for 6 and 7 h gave high regeneration fre-

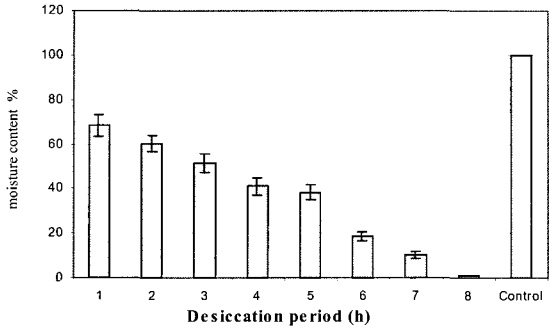

Figure 2. Change in moisture content in embryogenic callus of sugarcane during desiccation.

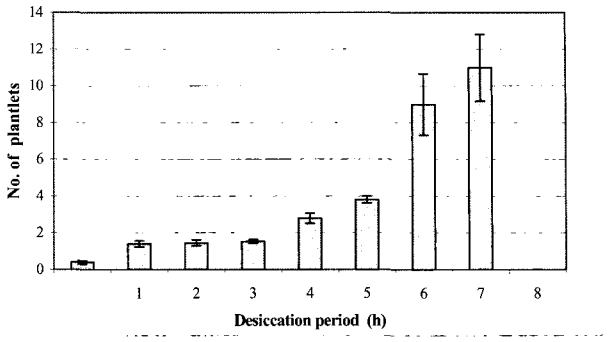

Figure 3. Effect of desiccation on regeneration of plantlets from embryogenic callus of sugarcane

quency. Partial desiccation of embryogenic calli has been useful for enhancement in somatic embryo differentiation and plant regeneration in some economically important crops like soybean (Hammat and Davey 1987, wheat (Carman 1988) and rice (Rance *et al.*, 1994). In banana, we have earlier observed desiccation mediated enhancement (66%) of embryo conversion compared to 56% in control (Srinivas 2002). Partial desiccation of the mature somatic embryos, corresponding to a decrease in water content from 90% to 75%, significantly improved germination rates (from 25% to 80%) in date palm (Fki *et al.*, 2003). Desiccation treatments given to somatic embryos of *Hevea*, also resulted in improved germinability and conversion of embryos into plants (Etienne *et al.*, 1993) and in the maturation and germination of horse chess nut somatic embryos (Capuana and Debergh, 1997). ABA plays a major role in conferring desiccation tolerance (Iida *et al.*, 1992, Attree *et al.*, 1990), either through the induction of the expression of maturation genes or suppression of proteins related to germination (Bapat and Rao, 1996).

Though the embryogenic calli of sugarcane possess very good potential for plantlet regeneration, most of the time it exhibits precocious germination of the embryos and shoot development with crowded nature and abnormal plants like albino, xantha were also observed. (Fitch and Moore 1990). The precocious germination generally occurs in cultured embryos when the maturation has been 'short circuited' and the maturation is transitory, frequently indispensable stage between embryo development and embryo germination phases (Quatrano, 1987). The desiccation of embryogenic calli of sugarcane showed good development of embryos with maturation and conversion into healthy plants with roots. We have routinely used this protocol and regenerated more than 150 plants that exhibited maximum survival (95%) during hardening in the greenhouse. Upon transfer to the field, these plants exhibited more vigor and high tillering compared to control plants (unpublished results).

Several micropropagation systems have been developed for sugarcane that include stages of shoot formation, elongation and rooting followed by acclimatization. Prospective use of these is often constrained by lower multiplication rates and genotype dependence and hence there is a need to develop more efficient regeneration systems. The results obtained in this study suggest that it is possible to obtain high frequency plant regeneration in sugarcane using simple and inexpensive method of desiccation. Such an improved regeneration should facilitate generating plants after *in vitro* mutagenesis or genetic transformation. We have already employed this technique for improving plant regeneration from irradiated embryogenic cultures in sugarcane (unpublished results).

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