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AN EFFICIENT, SHORT AND COST-EFFECTIVE REGENERATION SYSTEM FOR TRANSFORMATION STUDIES OF SUGARCANE (*SACCHARUM OFFICINARUM* L.)

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Abstract

Sugarcane genetic transformation efforts are seriously hampered by the lack of an efficient and reproducible regeneration system. An efficient, short and cost-effective regeneration system, through direct embryogenesis, was developed for local cultivars and elite lines of sugarcane. Using 1-2 mm thick meristematic young leaf whirls, direct embryogenesis was achieved in Murashige & Skoog (MS) medium supplemented with 2, 4-dichlorophenoxyacetic acid (2, 4-D) under cool white fluorescent light for 16 hour/day at 25 ± 2 ℃ within three weeks. Of the various concentrations of 2, 4-D tested, 3 mg/L induced the highest frequency of embryogenic callus (60 %). The embryos germinated in the fourth week on the same medium. Shoot proliferation and multiplication was carried out in liquid MS medium containing benzyl aminopurine (BAP) at a concentration of 1 mg/L. The improved regeneration system will particularly be useful in our ongoing genetic transformation studies.

Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the economically important crops widely cultivated in the tropics to subtropics, and annually provides around 60 to 70 % of the world's sugar. Sugarcane is used as raw material for the production of sugar, alcohol, plywood and other pharmaceutical products. Considering its importance in the agricultural industry, concerted efforts are being made for its improvement using biotechnological approaches particularly genetic transformation.

Tissue culture is employed in almost all current practical transformation systems to achieve a workable efficiency of gene transfer, selection and regeneration of transformants. Lack of a robust regeneration system to regenerate transformed plants at a satisfactory frequency is still the key factor which seriously limits the improvement of crops through genetic transformation (Popelka & Altpeter, 2003). In transformation studies a tissue culture system which helps regenerate large number of cells accessible to gene transfer, retain the capacity of cells for regeneration for the duration of the target preparation, cell proliferation and selection treatments is highly desirable. The direct introduction of genes for desired traits into elite crops particularly vegetatively propagated like sugarcane, the need to avoid somaclonal variation becomes overriding in the choice of regeneration systems (Birch, 1997).

Somatic embryogenesis offers an efficient and high volume regeneration system for the production of large number of plants within a short period. The plants derived from direct somatic embryogenesis usually are unicellular in origin and hence genetically uniform. Somatic embryogenesis in sugarcane has been reported using young leaves (Chen *et al*., 1988; Fitch & Moore, 1990; Brisibe *et al*., 1994), immature inflorescences (Blanco *et al*., 1997; Desai *et al*., 2004) and apical meristems (Ahloowalia & Maretzki, 1983). More recently a protocol for direct somatic embryogenesis has been reported using MS medium supplemented with NAA and kinetin (Desai *et al*., 2004).

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Successful genetic transformation attempts have mostly employed embryogenic callus/cell cultures as the target tissue. Previously, embryogenic callus was induced in the presence of a combination of an auxin and a cytokinin (Fitch & Moore, 1990; Santosa *et al*., 2004) and regeneration was obtained by reducing the concentration of the auxin or deleting it from the medium (Ahloowalia & Maretzki, 1983; Desai *et al*., 2004), or by media supplementation with thidiazuron (TDZ) (Chengalrayan & Gallo-Meagher, 2001). A major limitation of this callus system is the repeated subculture to select embryogenic callus portions among highly proliferating non-embryogenic tissue. This process is not highly reproducible and furthermore increases the chances of somaclonal variation (Karp, 1991). As these limitations have become unavoidable, strategies to improve plant regeneration must necessarily include manipulation of the explant material to embark upon new morphogenetic pathways.

The objective of the present study was to develop a robust and cost-effective regeneration system for sugarcane through direct embryogenesis without an intervening callus phase which can be used in studies aimed at genetic transformation of our elite sugarcane germplasm.

Materials and Methods

Direct embryogenesis: Four sugarcane genotypes (HSF-240, CSSG-668, HSF-242 and SPSG-26) provided by Sugarcane Research Institute, Jhang, Pakistan and maintained at the experimental fields of School of Biological Sciences, University of the Punjab, Lahore were used as source of explants in this study. Apical portions of healthy shoots were stripped to the terminal bud and detached from field grown plants. The fresh material was brought to tissue culture laboratory and attached immature leaf rolls from apical portions were peeled under sterile conditions without sterilization to cylinders of approximately 5 mm diameter. Six to eight serial slices (1-2 mm thick) were removed from each cylinder immediately above the apical meristem and cultured in MS medium (Murashige & Skoog, 1962) supplemented with 3 % sucrose and various concentrations of 2, 4-D ranging from 2 to 5 mg/L.

Shoot proliferation and multiplication: The embryogenic calli with induced shoot meristem were transferred to MS liquid medium supplemented with various concentrations of BAP (0-2 mg/L) for shoot proliferation and multiplication. All cultures were transferred to fresh medium every 2-3 weeks. Total number of shoots and percentage of shoots, greater than 1 cm in length, were determined 8 weeks after culture initiation.

Culture conditions: The pH of all media was adjusted to 5.7 and autoclaved at 15 psi for 15 minutes at 121°C. Initial cultures i.e. serial slices were cultured on agar (8 g/L) solidified medium in test tubes. After shoot meristem induction the explants were transferred to MS medium in jars containing sterile surgical cotton or agar as supporting agent. All cultures were incubated in a culture room at 25 ± 2°C under cool white fluorescent light (2.5-30 k Lux) with a 16-hour photoperiod. Sub-culturing was carried out every fortnightly.

Results and Discussion

Direct embryogenesis: Young sugarcane leaves are known to be good explant source for callus production (Brisibe *et al*., 1994; Chengalrayan & Gallo-Meagher 2001) and 2, 4-D has been used to induce callus from various sugarcane explants (Oropeza and Garcia, 1996; Gallo-Meagher *et al*., 2000). Therefore, young leaves of sugarcane elite genotypes

were used as starting material. Another advantage of using young apical portions is that the inner 5 mm thick cylinder is highly sterile and thus sterilization process can be avoided. Swelling of the explants and initiation of embryo-like structures were observed in the first week of the culture. In the next three weeks, well developed embryos were observed all over the cultured explants (Fig. 1A). The presence of embryos within 2-3 weeks of culture indicated direct embryogenesis. The highest frequency of embryogenesis (60%) from the 1 mm long young meristematic leaf whirl explants was observed in MS medium supplemented with 3 mg/L 2,4-D (Fig. 2).

Although use of immature inflorescence (Chengalrayan & Gallo-Meagher, 2001, Desai *et al*., 2004) for high number of plants per explant (185–200) through direct embryogenesis has been reported, areas where sugarcane flowering is a problem because of humidity, temperature and latitude, immature leaves are the best source of explants. The apical portions are abundantly available and are free of disease which can be directly inoculated into the media under sterile conditions thus saving time and resources.

Direct embryogenesis in sugarcane has been reported using a combination of auxins and cytokinins with three to six weeks of dark treatment and then 12 hour photo period for another four weeks (Desai *et al*., 2004). However, in the present study we obtained shoot meristem induction from embryogenic callus in the same medium containing 2, 4-D (3 mg/L) and maintaining the cultures under cool fluorescent light for 16 hours without dark treatment. We found no change in the shoot meristem induction frequency by using naphthaleneacetic acid (NAA), in combination with kinetin or TDZ (data not shown). The culture time was reduced by 3 to 6 weeks. The protocol developed in this study is now being routinely employed to 4 elite genotypes of sugarcane. The embryogenic response of cultures was previously attributed to the presence of a suitable combination of growth regulators (Desai *et al*., 2004). The present study suggests that the protocol for direct embryogenesis in sugarcane can further be simplified and shortened by using 2, 4-D alone instead of a combination of auxins and cytokinins.

Shoot proliferation and multiplication: The effect of various concentrations of BAP on shoot regeneration was evaluated after 4 weeks using this embryogenic callus with induced shoot meristem as starting material (Fig. 1). Callus with well-developed embryos was transferred to MS medium containing various concentrations of BAP. The highest shoot regeneration frequency (20 per explant) was achieved when BAP was used at a concentration of 1 mg/L in liquid MS medium (Fig. 3). In the present study addition of NAA or kinetin in the medium did not alter the number of shoots produced and BAP alone remained the most effective treatment for shoot regeneration and proliferation.

Results on the use of different gelling/supporting media for shoot proliferation and multiplication are given in Fig. 4. The average number of shoots regenerated in media solidified with 0.8% agar or liquid media containing cotton as supporting agent were 20 and 18, respectively. Using cotton bed instead of agar as supporting agent was not only cost effective but also enhanced the number of shoots regenerated, possibly due to efficient nutrient uptake (Fig. 4).

In conclusion, the induction of direct somatic embryogenesis using leaf rolls as described in the present study is cost-effective and could be useful in rapid propagation of elite sugarcane varieties. The number of shoots regenerated was about 20 per explant, and assuming an average of 7 slices per young leaf cylinder, the total number of plants that can be generated is around 140 per plant in around eight weeks. The highly efficient regeneration system should allow transformation both with biological as well as physical methods to be more successful in developing transgenic cultivars of sugarcane in a short time.

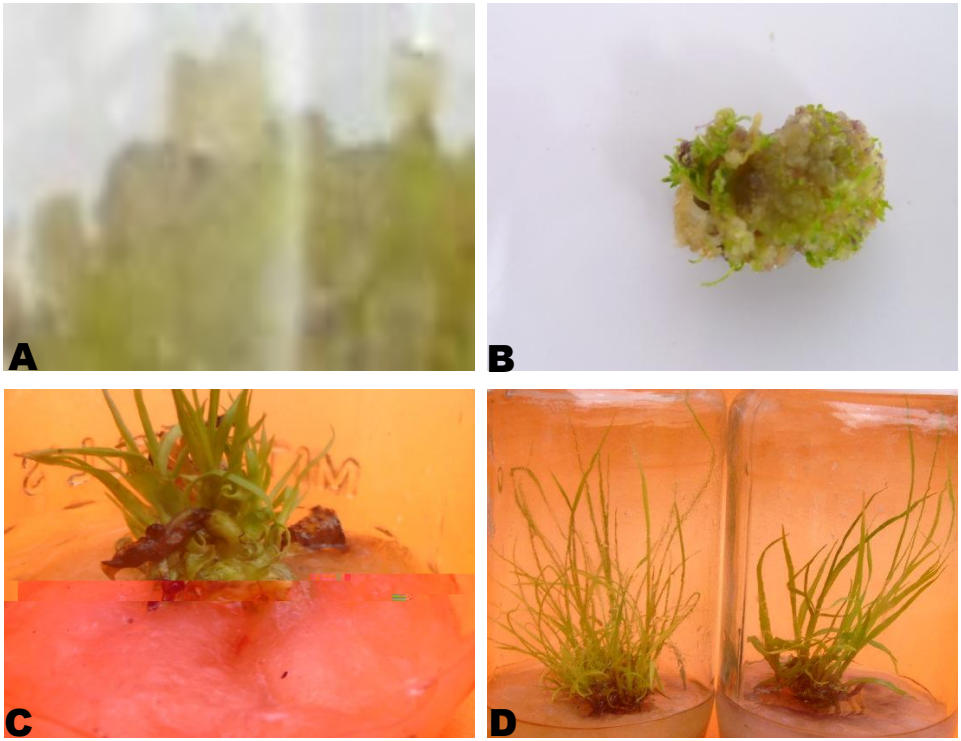
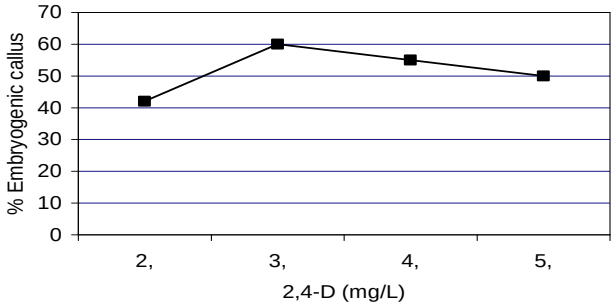


Fig. 1. Stages of direct embryogenesis and shoot regeneration in sugarcane: A): Embryogeic callus developed from 4 week of culture. B): Embryogenic callus with more than 60 % shoot meristem induction. C and D): Shoot regeneration and multiplication on cotton bed in liquid MS medium.



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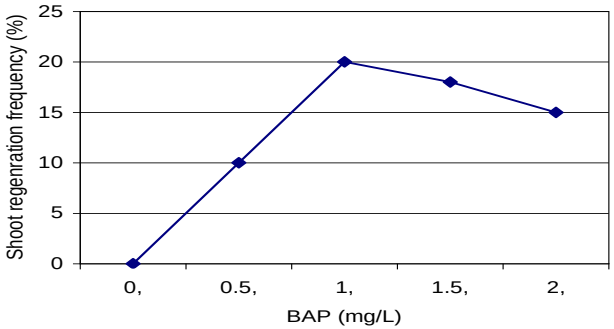

Fig. 3: Effect of different concentrations of BAP on shoot regenration from embryogenic callus

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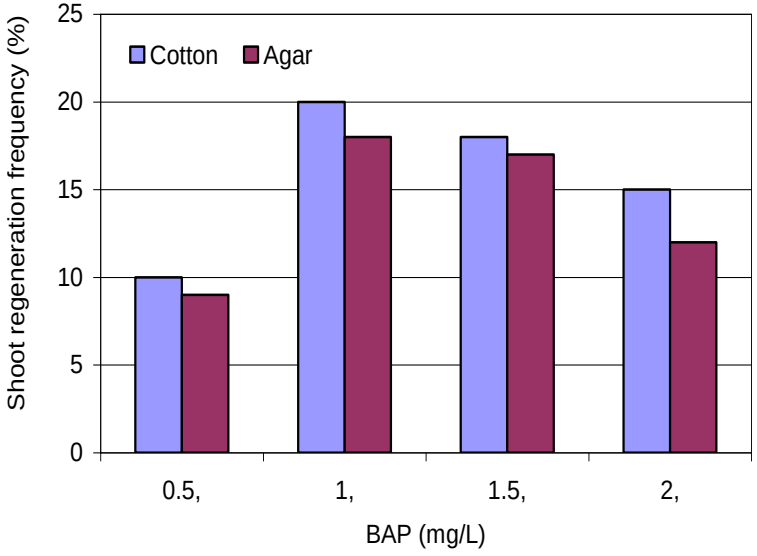

Fig. 4. The influence of explant supporting agents (cotton and agar) on shoot regeneration in sugarcane.

References

Ahloowalia, B. S. and A. Maretzki. 1983. Plant regeneration via somatic embryogenesis in sugarcane. *Plant Cell Rep*., 2: 21-25.

Birch, R.G. 1997. Plant Transformation: Problems and Strategies for Practical Application. *Annu. Rev. Plant Physiol. Plant Mol. Bio*l., 48: 297-326.

Blanco, M.D.A., N. Nieves, M. Sanchez, C.G. Borroto, R. Castillo, J.L. Gonzalez, M. Escalona, E. Baez and Z. Hernandez. 1997. Protein changes associated with plant regeneration in embryogenic calli of sugarcane (*Saccharum* sp.). *Plant Cell Tiss. Organ Cult*., 51: 153-158.

Brisibe, E.A., H. Miyake, T. Taniguchi and E. Maeda. 1994. Regulation of somatic embryogenesis in long term callus cultures of sugarcane (*Saccharum officinarum* L.). *New Phytol*., 126: 301-307.

Chengalrayan, K. and M. Gallo-Meagher. 2001. Effect of various growth regulators on shoot regeneration of sugarcane. *In Vitro Cell. Dev. Biol. Plant*, 7: 434-439.

Chen, W.H., M.R. Davey, J.B. Power and E.C. Cocking. 1988. Control and maintenance of plant-regeneration in sugarcane callus cultures. *J. Exp. Bot.*, 39: 251-261.

Desai, N.S., P. Suprasanna and V.A. Bapat. 2004. Simple and reproducible protocol for direct somatic embryogenesis from cultured immature inflorescence segments of sugarcane (*Saccharum* spp.). *Current Science*, 87: 764-768.

Fitch, M.M.M. and P.H. Moore. 1990. Comparison of 2, 4-D and picloram for selection of long-term totipotent green callus cultures of sugarcane. *Plant Cell Tiss. Organ Cult*., 20: 157-163.

Gallo-Meagher, M., R.G. English and A. Abouzid. 2000. Thidiazuron stimulates shoot regeneration of sugarcane embryogenic callus. *In Vitro Cell. Dev. Biol. Plant*, 36: 37-40.

Karp, A. 1991. On the current understanding of somaclonal variation. In *Oxford Surveys of Plant Molecular and Cell Biology*. (Ed.): H.F. Miflin. Oxford University Press, New York, pp. 1-58.

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

Oropeza, M. and E. Garcia. 1996. Somaclonal variants resistant to sugarcane mosaic virus and their agronomic characterization. *In Vitro Cell. Dev. Biol. Plant*, 32: 26-30.

Popelka J.C. and F. Altpeter. 2003. Evaluation of rye (*Secale cereale* L) inbred lines and their crosses for tissue culture response and stable genetic transformation of homozygous rye inbred line L22 by biolistic gene transfer. *Theor. Appl. Genet*., 107: 583-590.

Santosa, D.A., R. Hendroko, A. Farouk and R. Greiner. 2004. A rapid and highly efficient method for transformation of sugarcane callus. *Mol. Biotechnol.*, 28: 113 -119.

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