J. Crop Sci. Biotech. 2012 (June) 15 (2) : 131 ~ 136
DOI No. 10.1007/s12892-011-0111-6

RESEARCH ARTICLE

Regeneration in Sugarcane via Somatic Embryogenesis and Genomic Instability in Regenerated Plants

Saboohi Raza[1,2]*, Syeda Qamarunisa[1], Mushtaq Hussain[3], Ishrat Jamil[1], Sadia Anjum[1], Abid Azhar[1], Javed Ahmed Qureshi[1]*

[1]*The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, Pakistan*
[2]*Nuclear Institute of Agriculture (NIA), Tandojam, Pakistan*
[3]*Department of Molecular Pathology, Dow University of Health Sciences, Karachi, Pakistan*

Received: November 24, 2011 / Revised: March 14, 2012 / Accepted: April 3, 2012

Abstract

In the present study, embryogenic calli of sugarcane variety BL4 were induced using 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin in different concentrations and combinations. In contrast to earlier studies, embryogenic callus sectors were identified and isolated microscopically within 1 - 2 weeks. Subsequently, 51 media formulations were used for regeneration of proliferated embryogenic callus, using MS medium supplemented with three different cytokinins [kinetin, 6-Benzylamino purine (BAP), and thidiazuron (TDZ)] and auxins (IAA/NAA and IBA) in different combination and concentrations. After acclimatization, the genomic DNA of regenerated plants was studied to explore the insertion polymorphism of transposable elements in order to ascertain the variation among somaclones. Though low concentration of kinetin with 2,4-D was found supportive to embryogenic callus development, the highest number of regenerated plantlets was observed using BAP (1 µM), however the plantlets had very low fresh weight (2.2 g). Conversely, TDZ alone supported a significant increase in the number of plantlets regenerated (38 - 40) with higher fresh weight. The somaclones generated during this study showed considerable positional polymorphism of activator-like transposable elements possibly due to the stress associated with *in vitro* culture. This study provides a procedure to produce regenerated sugarcane plants from embryogenic callus in a relatively short time.

Key words: Activator (Ac)-like elements, insertion polymorphism of TEs, regeneration, somatic embryogenesis, sugarcane, thidiazuron (TDZ)

Introduction

Sugarcane biotechnology has gained increased attention during recent years primarily for its usage in ethanol production as an important renewable biofuel source (Cunff et al. 2008) rather than for the production of sugar. Since the 1960s, plant tissue culture has been regarded as a promising technique for improvement of genetically complex plants like sugarcane (Cunff et al. 2008; Nickell 1964). Tissue culture techniques have been applied extensively for the propagation of elite cultivars, mutation breeding, and somaclonal variations in sugarcane. Recently, sugarcane biotechnological research has gained focus on genetic engineering where the crop has been successfully transformed using microprojectile DNA bombardment, electroporation, and *Agrobacterium* for a range of new characteristics such as herbicide resistance, virus resistance, insect resistance, and altered sucrose enzyme regulation (Hotta et al. 2010; Snyman et al. 2006). Genetic transformation efforts in sugarcane rely heavily on the availability of an efficient system for *in vitro* propagation of sugarcane. The recognition of somatic embryogenesis was a turning point in sugarcane biotechnology (Lakshmanan 2006). Somatic embryogenesis offers an efficient and high volume regeneration system for the production of a large number of plants within a short period (Shah et al. 2009). For monocot species, Ho and Vasil (1983) first reported the evidence of embryogenic callus development along with two other types of

Saboohi Raza (✉)
E-mail: saboohi.raza@kibge.edu.pk
Tel: +92-213013559827 / Fax: +92-2134823887

calli at the cut end of explants cultured on Murashige and Skoog (MS) (1962) medium containing 2,4-D. Ahloowalia and Maretzki (1983) have shown the complete development of somatic embryos (SE) and their subsequent germination into whole plants. At present, a number of simple and reproducible protocols are available for SE, for instance direct somatic embryogenesis and regeneration from SE developed from immature leaf and immature inflorescence segment of sugarcane (Desai et al. 2004; Khan and Khatri 2006; Shah et al. 2009; Snyman et al. 2006).

Despite the importance of the SE in sugarcane biotechnology for the improvement of this crop, limitations in available protocols include the time required to achieve a single regenerated plant, extensive labour involved for maintenance, sub-culturing, and regeneration (Pandey et al. 2011; Snyman et al. 2006). The present study focused on finding a rapid, time-saving, and reproducible protocol for SE production and regeneration from SE with minimum use of growth regulators. For this purpose, embryogenic callus from the initial stage of callus development from immature leaf was identified, isolated, and proliferated on the same medium, and the effect of different growth regulators was studied on the regeneration potential of this embryogenic callus. Additionally, we have also demonstrated the genomic variations among the somaclones using the polymorphism as found in inter-transposable elements stationed differently among different regenerated somaclones.

Materials and Methods

Callus induction

Immature leaf rolls from 6 - 8 month old, field-grown cultivars of sugarcane variety BL4 were used for callus induction. Surface contaminants were removed using 70% ethanol for 1 min and sodium hypochlorite for 20 min. The outer leaf sheaths were aseptically removed and the inner leaves were sliced transversely into sections approximately 1 - 2 mm thick. After rinsing 2 - 3 times with sterilized distilled water, these pieces were cultured on Murashige and Skoog (1962) medium augmented with different concentrations of 2,4-D (0, 2.2, 4.5, 9, and 18 µM) and Kinetin (0, 0.46, 2.2, 4.5, 9, and 18 µM) alone and combinations of these two growth regulators. Cultures were incubated at 25 ± 2°C in the dark. Weight gain in callus and the types of calli produced were recorded.

Isolation and proliferation of somatic embryos

Whitish yellow, compact, nodular tissue clumps were picked using a binocular dissecting microscope with 4X magnification. These clumps were allowed to proliferate at different concentrations of 2,4-D (0, 2.2, 4.5, and 9 µM) at 25 ± 2°C in the dark.

Regeneration

Fifty-one different media formulations were prepared using Murashige and Skoog (1962) basal salts (MS) augmented with three different cytokinins [kinetin, 6-benzyl amino purine (BAP) and thidiazuron (TDZ)] in five different concentrations (0, 1, 4, 8, and 12 µM) with and without auxins [Indole-3-acetic acid (IAA)/ naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA)]. For each replicated trial, callus pieces weighing 50 mg each were cultured on all formulated media and incubated at 25 ± 2°C under a photoperiod of 16 h light and 8 h dark. Germinated shoots (≥ 0.5 cm), fresh weight, and shoot lengths were recorded after 30 days.

DNA extraction

DNA from the young leaves of sugarcane somaclones was extracted by CTAB method reported by Puchooa (2004).

Detection of insertional polymorphism of transposable elements (TEs)

A simple, efficient, and PCR-based technique was used to locate insertional positions of activator like TEs in the genome (Teo et al. 2005). Single primers from the internal sequences of transposable elements were used to detect the polymorphism among the somaclones. Primer was designed from the conserved region of ESTs for Activator (Ac)-like TEs from the GeneBank accessions No. gi76261907, gi76261897, and gi76261882 using CLUSTALX, GeneDoc, and primer3 software. Primer sequence used for inter-activator amplification polymorphism was CGGACTTTCATGTTCTGTGC. PCR reaction was carried out in 10 µL of reaction mixture containing 20 ng of template (genomic DNA), 3.5 mM $MgCl_2$, 0.3 mM of each dNTPs, 2.5 U of *Taq* polymerase, and 1 µM of primer in a 1x PCR reaction buffer. The amplification reaction was performed in the Eppendorf Master cycler with an initial denaturation for 4 min at 94°C, then 35 cycles: 30 s denaturation at 94°C; 35 s annealing at 55°C; 30 s extension at 72°C. Final extension was carried out at 72°C for 7 min. Amplified products were analyzed through electrophoresis on 2% agarose gel containing 1X TBE (Tris Borate EDTA) at 60 V for 1.5 h, photograph was taken under UV light using gel documentation system.

Statistical analysis

Experiments were organized as randomized complete block designs (RCBD). For callus induction, proliferation, and regeneration, each treatment had 10 replicates. Analysis of variance (*F* test) for single factor and Duncan's Multiple Range Test (DMRT) were performed using MSTAT version 1.42.

Result and Discussion

Among the various concentrations and combinations of growth regulators, 2,4–D (2.2 - 18 µM) alone and in combination with 0.46 and 2.2 µM kinetin was found to be effective in producing good quality callus. Kinetin alone did not support any callus growth, and a high concentration of kinetin (9 and 18 µM) in combination with 2,4-D was inhibitory to callus induction as also reported by Wan et al. (1988). Maximum amount of callus was proliferated on MS medium containing 4.5, 9, and 18 µM of 2,4-D. However, the differences observed in callus formation were not statistically significant ($P < 0.01$) (data not shown).

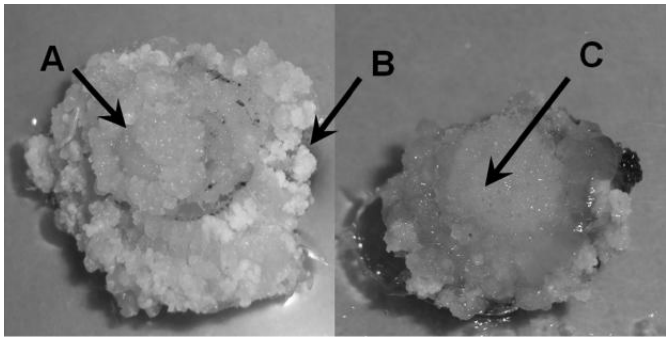

Fig. 1. Callus Induction in sugarcane: Callus produced in sugarcane variety BL4 under different concentrations of 2,4-D and kinetin. Three forms of calli were observed namely soft callus (A), compact callus (B), and mucilaginous callus (C).

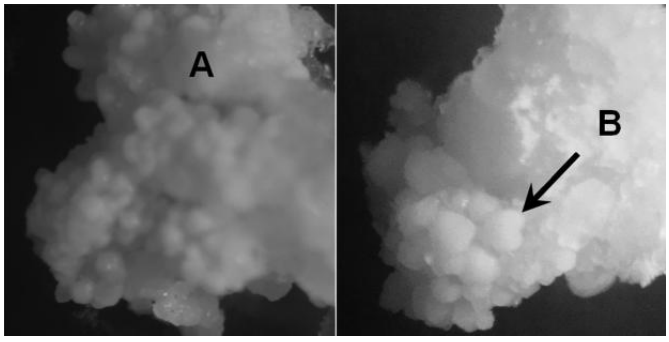

Fig. 2. Callus Proliferation: Embryogenic callus (A), 4X magnification of embryogenic callus (B); arrow indicates heart-shaped somatic embryo.

Morphologically, three types of calli (compact, soft, and muscilaginous) were produced on callus induction medium, as described by Ho and Vasil (1983; Fig. 1). In this study, the earliest embryogenic calli (Fig. 1A) isolated within 1 - 2 weeks were proliferated. No significant difference ($P < 0.01$) was found among the calli proliferated on MS medium containing different concentrations of 2,4-D (0, 2.2, 4.5, and 9 µM) (data not shown). In the proliferation medium, the embryogenic calli mass was increased by 3-fold to the initially plated tissue within 5 days and 10-fold in 25 days (Fig. 2). It is noteworthy that compact embryogenic callus was obtained within 30 - 55 days by Ho and Vasil (1983), in 10 weeks by Ahloowalia and Maretzki (1983), after 3 - 6 weeks by Desai et al. (2004), and after 2 weeks by Snyman et al. (2006). It is clear that our method seems superior to others as we obtained embryogenic callus much sooner.

Somatic cells are theoretically totipotent. However, chemical stimulus is required in most cases for making them embryogenic (Namasivayam 2007). 2,4-D is the most common synthetic auxin used to induce somatic embryogenesis from a range of plant species (Pasternak et al. 2002; Raghavan 2004) and was found to activate the expression of certain genes like Somatic Embryogenesis Receptor Kinase (SERK1) genes and Arabidopsis leafy cotyledon (LEC) genes in *Arabidopsis*, Baby Boom (BBM) gene in *Brassica napus* essential for embryogenic competence (Boutilier et al. 2002; Harada 2001; Hecht et al. 2001), conversely, inclusion of cytokinin is essential for cell division and hence it contributes towards the initial increased in the number of cells in somatic embryos (Karami et al. 2009).

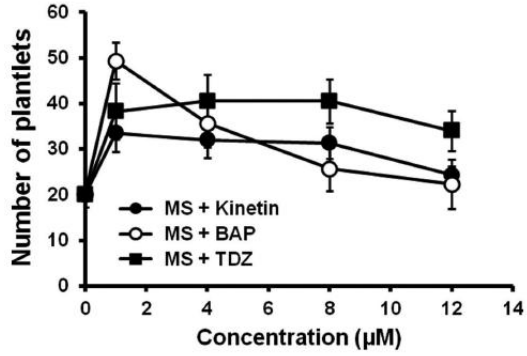

Fig. 3. Number of regenerated plantlets in MS medium with different concentrations of cytokinins alone. 50 mg of somatic embryos were planted and plantlets were counted after 30 days.

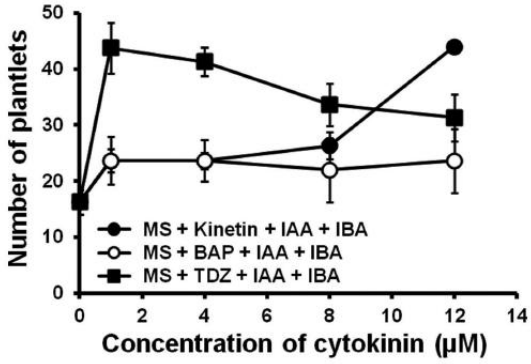

Fig. 4. Number of regenerated plantlets in MS medium with different concentrations of cytokinins + IAA (10 µM) and IBA (10 µM). 50 mg of somatic embryos were planted and plantlets were counted after 30 days.

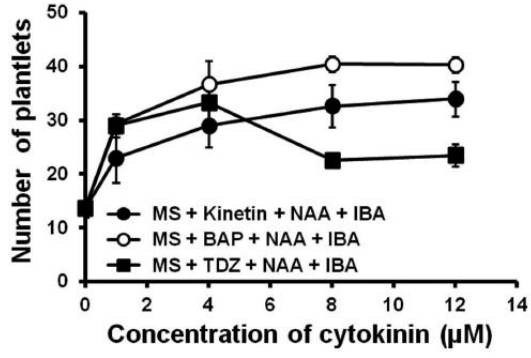

Fig. 5. Number of regenerated plantlets in MS medium with different concentrations of cytokinins + NAA (10 µM) and IBA (10 µM). 50 mg of somatic embryos were planted and plantlets were counted after 30 days.

Among all the media formulations employed for the regeneration, the performance of the regenerated plants in the media was assessed mainly by two attributes, namely number of plantlets per 50 mg of callus and fresh weight of regenerated plants. It was found that the addition of 1 µM of 6-benzylaminopurine (BAP) in the medium had a very positive influence on regeneration from the embryogenic callus (50 plantlets per 50 mg callus; Fig. 3). Seven more formulations including TDZ 4 and 8 µM alone (Fig. 3) and with IAA and IBA (Fig. 4); kinetin 12 µM with IAA + IBA (Fig. 4) and BAP 8 and 12 µM with NAA + IBA (Fig. 5) produced good regeneration ($P < 0.01$ with LSD 19.5).

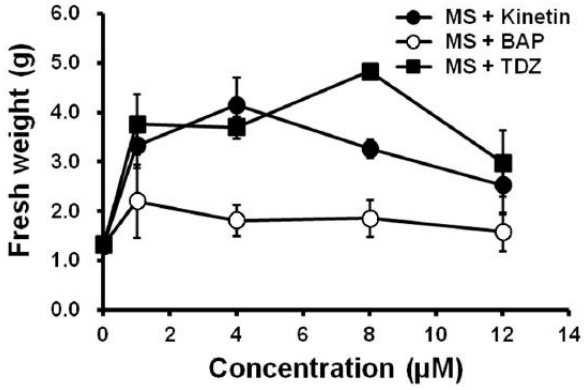

Fig. 6. Fresh weight of regenerated plantlets in MS medium with different concentrations of cytokinins alone. 50 mg of somatic embryos were planted and fresh weight were counted after 30 days.

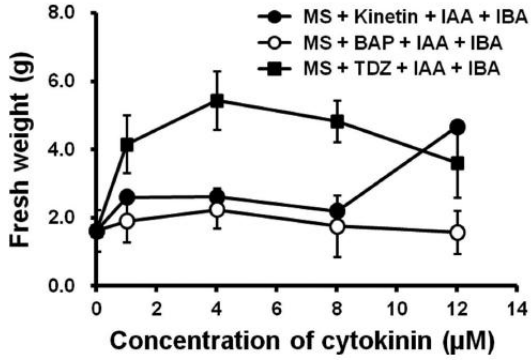

Fig. 7. Fresh weight of regenerated plantlets in MS medium with different concentrations of cytokinins + IAA (10 µM) and IBA (10 µM). 50 mg of somatic embryos were planted and fresh weight was counted after 30 days.

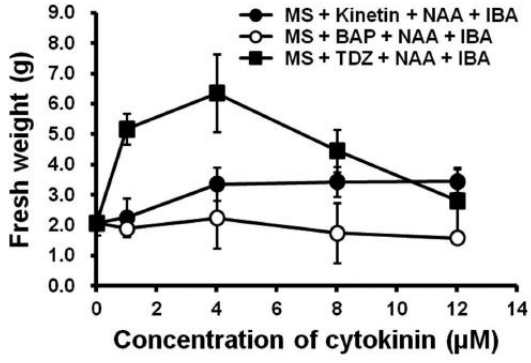

Fig. 8. Fresh weight of regenerated plantlets in MS medium with different concentrations of cytokinins + NAA (10 µM) and IBA (10 µM). 50 mg of somatic embryos were planted and fresh weight was counted after 30 days.

Comparison among the fresh weight of the regenerated plantlets in all media showed that TDZ at 1, 4, and 8 µM alone and in combination with IAA/NAA + IBA produced healthier plantlets compared to other formulations ($P < 0.01$ with LSD 2.32) (Figs. 6, 7, and 8) (Chengalrayan and Gallo-Meagher 2001). Medium containing kinetin alone (4 µM) substantially increases the weight, but it was due to vigorous growth of somatic embryos (Wan et al. 1988), rather than the regenerated shoots. However, the medium containing kinetin (12 µM) in combination with IAA

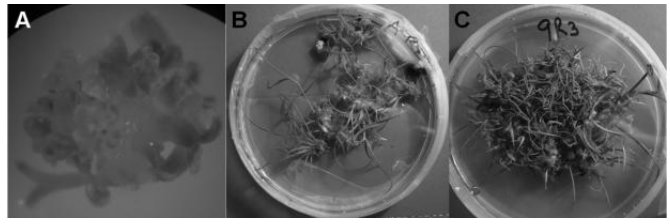

Fig. 9. Regeneration of somatic embryos observed using binocular microscope under 4X magnification (A). Further growth of regenerated embryos from somatic embryos are shown in MS medium (B) and in thidiazuron (TDZ) (4 µM) (C).

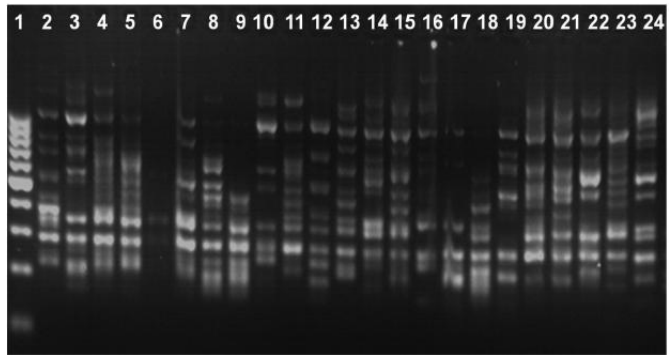

Fig. 10. Inter-transposons amplified polymorphism (ITAP): Lane 1, 100 bp molecular weight marker; Lane 2-23, ITAP profile of somaclones; Lane 24, ITAP profile of BL4 (mother plant).

+ IBA proved to be a good medium for increased number of plantlets produced (44 ± 5.0) and the gain in fresh weight (4.66 ± 0.65 g) (Figs. 4 and 7) (Khan and Khatri 2006).

Among all cytokinins used for plant regeneration of somatic embryos, BAP and TDZ without addition of any other auxin allowed for a significant increase in the number of regenerated plantlets at low concentration (1 and 4 µm, respectively). However, the plantlets produced in the presence of BAP had very low fresh weight as compared to any other treatment, hence very low survival rate when transferred to simple MS medium for acclimatization. In contrast to BAP, TDZ showed highly significant increased number of regenerated plantlets with maximum fresh weight (Chengalrayan and Gallo-Meagher 2001; Fig. 9).

Thidiazuron (TDZ: N-phenyl- N'-[(1,2,3-thidiazol-5-yl)urea]) a synthetic herbicide, has been found to be a potent regulator of plant growth and development under *in vitro* condition (Murch and Saxena 2001; Qureshi and Saxena 1992). TDZ has also been reported to replace auxin and cytokinin requirement for somatic embryogenesis in plant tissue (Visser et al. 1992). Unlike other cytokines commonly in use that are adenine based, TDZ is a urea-based cytokinin and therefore non-degradable by cytokinin-oxidase in plant tissues. This quality ensures cost effectiveness of TDZ, persistent in tissues transforming them from cytokinin dependence to cytokinin autonomy (Makara et al. 2010). The role of TDZ in the induction of plant morphogenesis involves the stimulation of individual plant growth regulators processes and the maintenance of the physiological stasis of the plant tissue during the culture process (Murch and Saxena 2001).

The genomic analysis by the inter-transposon amplified polymorphism showed considerable positional polymorphism of activator like transposable elements in the regenerated plantlets

(Fig. 10). It has been suggested that the genome-wide dispersed repetitive sequences that remain dormant during the developmental phase are then activated under stress conditions like wounding, pathogen attack, and cell culture (Grandbastien 1998; Wessler 1996). It is conceivable that the dedifferentiation (callus induction) and re-differentiation may provide a genomic stress induced by the *in vitro* culture conditions that ensued the activation of transposable elements (TEs). Indeed activation of TEs during *in vitro* culturing has been observed in maize and potato (Momose et al. 2010; Rhee et al. 2009). TEs are regarding as one of the major cause of the genomic instability primarily because of their ability to move within the genome and their intervention in the epigenetic regulation of the genes (Lippman et al. 2004). In light of above mentioned studies, it is plausible to say that somaclone variants may be the function of differential localization and/or change in copies of repeats, which in addition to provide varying characteristics in the somaclone but also facilitate to delineate them genetically.

References

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

Boutilier K, Offringa R, Sharma VK, Kieft H, Ouellet T et al. 2002. Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. Plant Cell 14: 1737-1749

Chengalrayan K, Gallo-Meagher M. 2001. Effect of various growth regulators on shoot regeneration of sugarcane. In Vitro Cell. Dev.-Plant 37: 434-439

Cunff LL, Garsmeur O, Raboin LM, Pauquet J, Telismart H et al. 2008. Diploid/polyploid syntenic shuttle mapping and haplotype-specific chromosome walking toward a rust resistance gene (*Bru1*) in highly polyploid sugarcane (2n~12x~115). Genetics 180: 649-660

Desai NS, Suprasanna P, Bapat VA. 2004. Simple and reproducible protocol for direct somatic embryogenesis from cultured immature inflorescence segments of sugarcane (*Saccharum* spp.). Curr. Sci. 87: 764-768

Grandbastien MA .1998. Activation of plant retrotransposons under stress conditions. Trends Plant Sci. 3: 181-187

Harada JJ. 2001. Role of *Arabidopsis LEAFY COTYLEDON* genes in seed development. J. Plant Physiol. 158: 405-409

Hecht V, Vielle-Calzada J, Hartog MV, Schmidt EDL, Boutilier K, Grossniklaus U, de Vries SC. 2001. The Arabidopsis *somatic embryogenesis receptor kinase 1* gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol. 127: 803-816

Ho WJ, Vasil IK.1983. Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.) I. The morphology and physiology of callus formation and ontogeny of somatic embryos. Protoplasma 118: 169-180

Hotta CT, Lembke CG, Domingues DS, Ochoa EA, Cruz GMQ et al. 2010. The biotechnology roadmap for sugarcane omprovement. Trop. Plant Biol. 3: 75-87

Karami O, Aghavaisi B, Pour AM. 2009. Molecular aspects of somatic-to-embryogenic transition in plants. J. Chem. Biol. 2: 177-190

Khan IA, Khatri A. 2006. Plant regeneration via organogenesis or somatic embryogenesis in sugarcane; histological studies. Pak. J. Bot. 38: 631-636

Lakshmanan P. 2006. Invited review addendum: Somatic embryogenesis in sugarcane- an addendum to the invited review 'In Vitro Cell. Dev. Biol. Plant 41: 345-363; 2005. In Vitro Cell. Dev.-Plant 42: 201-205

Lippman Z, Gendrel A, Black M, Vaughn MW, Dedhia N. 2004. Role of transposable elements in heterochromatin and epigenetic control. Nature 430: 471-476

Makara AM, Rubaihayo PR, Magambo MJS. 2010. Carry-over effect of Thidiazuron on banana *in vitro* proliferation at different culture cycles and light incubation conditions. Afr. J. Biotechnol. 9: 3079-3085

Momose M, Abe Y, Ozeki Y. 2010. Miniature inverted-repeat transposable elements of stowaway are active in potato. Genetics 186: 59-66

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

Murch SJ, Saxena PK. 2001. Molecular fate of thidiazuron and its effect on auxin transport in hypocotyls tissues of *Pelargonium x hortorum* Bailey. Plant Growth Regul. 35: 269-275

Namasivayam P. 2007. Acquisition of embryogenic competence during somatic embryogenesis. Plant Cell. Tiss. Org. Cult. 90: 1-8

Nickell LG. 1964. Tissue and cell culture of sugarcane: Another research tool. Hawaii Plant Rec. 57: 223-229

Pandey RN, Rastogi J, Sharma ML, Singh RK. 2011. Technologies for cost reduction in sugarcane micropropagation. Afr. J. Biotechnol. 10: 7805-7813

Pasternak PT, Prinsen E, Ayaydin F, Miskolczi P, Potters G, Asard H, Onckelen HAV, Dudits D, Feher A. 2002. The role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. Plant Physiol. 129: 1807-1819

Puchooa D. 2004. A simple, rapid and efficient method for the extraction of genomic DNA from lychee (*Litchi chinensis* Sonn.) Afr. J. Biotechnol. 3: 253-255

Qureshi JA, Saxena PK. 1992. Adventitious shoot induction and somatic embryogenesis in several varieties of hybrid seed geranium (*Pelargonium x hortorum* Bailey). Plant Cell Rep. 11: 443-448

Raghavan V. 2004. Role of 2,4-dichlorophenoxyacetic acid (2,4-D) in somatic embryogenesis on cultured zygotic embryos of Arabidopsis: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2,4-D. Am. J. Bot. 91: 1743-1756

Rhee Y, Lin H, Buell R, Childs K, Kaeppler S. 2009. A c2 allele of maize identified in regenerant-derived progeny from tissue culture results from insertion of a novel transposon. Maydica 54: 429-437

Shah AH, Rashid N, Haider MS, Saleem F, Tahir M, Iqbal J. 2009. An efficient, short and cost-effective regeneration system for transformation studies of sugarcane (*Saccharum officinarum* L.). Pak. J. Bot. 41: 609-614

Snyman SJ, Meyer GM, Richards JM, Haricharan N, Ramgareeb S, Huckett BI. 2006. Refining the application of direct embryogenesis in sugarcane: effect of the developmental phase of leaf disc explants and the timing of DNA transfer on transformation efficiency. Plant Cell Rep. 25: 1016-1023

Teo CH, Tan SH, Ho CH, Faridah QZ, Othman YR, Heslop-Harrison JS, Kalendar R, Schulman AH. 2005. Genome constitution and classification using retrotransposon based marker in the orphan crop banana. J. Plant Biol. 48: 96-105

Visser C, Qureshi JA, Saxena PK. 1992. Morphoregulatory role of thidiazuron: Substitution of auxin and cytokinin requirement for the induction of somatic embryogenesis in geranium hypocotyl cultures. Plant Physiol. 99: 1704-1707

Wan Y, Sorensen EL, Liang GH. 1988. The effects of kinetin on callus characters in alfalfa (*Medicago sativa* L.). Euphytica 39: 249-254

Wessler SR. 1996. Plant retrotransposons: Turn on by stress. Curr. Biol. 8: 959-961