African Journal of Biotechnology Vol. 11(42), pp. 9961-9969, 24 May, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2985
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Reproducible *in vitro* regeneration system for purifying sugarcane clones

Ghulam Mustafa[1,2] and Muhammad Sarwar Khan[1,2]*

[1]National Institute for Biotechnology and Genetic Engineering (NIBGE), P. O. Box 577, Jhang Road Faisalabad, Postcode 38000, Pakistan.
[2]Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad, Pakistan.

Accepted 11 May, 2012

Genome purification of a selected clone of sugarcane is the key to developing homogenous lines. Generally, regenerated plants after transformation are heterogeneous at genome level, and several successive rounds of selection on antibiotic-containing medium and regeneration cycles are required to purify the genome to develop a homogenous population. Sugarcane is a vegetatively propagated plant and hence it requires to be grown in the field to harvest mature cane tops carrying meristematic tissues. In the present studies, stems of *in vitro* grown plants of four indigenous genotypes namely; HSF-242, US-778, HSF-243 and HSF-240, were subjected to regeneration. Five days post incubation at various levels of 2,4-D, the segments were placed on regeneration medium containing a combination of casein hydrolysate (500 mg/L), kinetin (0.5 mg/L) and benzylaminopurine (BAP, 0.5 mg/L). Response to regeneration was varied from basal to top sections. Nevertheless, more than 137 shoots were regenerated from basal segment, suggesting that the segment consisting of meristematic tissues responded well to *in vitro* conditions. This procedure may be considered as one of the best ever published report on regeneration from *in vitro* grown plants to purify clones without subjecting the plants to field conditions and harvesting the mature cane. This technique was used to purify transgenic sugarcane plants carrying *Bacillus thuringiensis* gene.

Key words: *In vitro* explant, basal stem segment, callus induction, proficient regeneration, *Saccharum officinarum*.

INTRODUCTION

Sugarcane fulfils 70% of sugar needs worldwide (Guimarces and Sorbal, 1998) and is a major source of biofuel and biomaterials. Major impediments to traditional breeding of sugarcane are its complex genome, poor fertility, the long breeding cycle and recalcitrant nature. Since the advent of the recombinant DNA technology in 1970s, genetic manipulation of sugarcane had been attempted for the improvement of agronomic traits (Arencibia et al., 1997; Manickavasagam et al., 2004; Wu et al., 2008), production of biopharmaceuticals (Wang et al., 2005), biomaterials and engineering metabolic pathways for increased sucrose contents (Vickers et al., 2005). The application of biotechnology to sugarcane is comparatively recent. Though progress has been made in various fields but no transgenic line has yet been commercialized (Bonnett et al., 2008).

Tissue culture is a powerful tool to accelerate genetic transformation as well as breeding (Alves et al., 2011). Propagation of sugarcane under *in vitro* conditions is being carried out by harvesting cane tops of field grown plants since the advent of the tissue culture technology in 1961 (Liu, 1984). The shoot tip (Burner and Grisham, 1995) and callus cultures (Ho and Vasil, 1983; Chengalrayan and Gallo-Meagher, 2001) are two major approaches to multiply the plants. Subsequently, different

*Corresponding author. E-mail: sarwarkhan_40@hotmail.com. Tel: +92-41-2553127.

Abbreviations: 2,4-D, 2,4-Dichlorophenoxy acetic acid; **IAA,** indole-3-acetic acid; **BAP** benzylaminopurine; **IBA,** indole-3-butyric acid; **CH,** casein hydrolysate.

explants including young unfurled, mature fully expanded leaves or young dividing cells of meristematic tissues were used to improve the regeneration and multiplication process of plants (Behera and Sahoo, 2009). Somatic embryogenesis from immature inflorescence (Desai et al., 2004), young leaves (Brisibe et al., 1994) and apical meristem as well as organogenesis from young leaves (Fitch and Moore, 1990) has also been reported. Nevertheless, amongst different explants, immature leaves being highly prolific are preferred target tissues for the development of embryogenic calli (Snyman et al., 2006; Ali et al., 2010), and this was suggested to be the turning point in sugarcane biotechnology research.

Similarly, hormones in different combinations had been used to improve *in vitro* micro-propagation in sugarcane. Among these, 2,4-dichlorophenoxyacetic acid (2,4-D) had been the best choice for callogenesis, direct somatic embryogenesis (Snyman et al., 2001; Franklin et al., 2006; Behera and Sahoo, 2009) and indirect somatic embryogenesis (Guiderdoni and Demarly, 1988; Ali et al., 2007). Naphthaleneacetic acid (NAA) (Irvine et al., 1991; Lakshmanan et al., 2006) and benzylaminopurine (Ali et al., 2008; Ather et al., 2009; Biradar et al., 2009) are efficient shoot inducers, whereas indole-butyric acid (IBA) (Khatri et al., 2002; Seema et al., 2011), gibberellic acid (GA_3) (Ather et al., 2009; Dash et al., 2011) and NAA (Biradar et al., 2009; Ali, et al., 2010) has been reported for proficient rhizogenesis.

The main source of explant for sugarcane *in vitro* manipulations has been mature cane tops from field grown plants. Sugarcane is cultivated annually and it takes almost 8 to 9 months to harvest mature cane tops. In this study, *in vitro* grown plants were used to excise explant tissues for callus initiation and proliferation followed by regeneration to purify the engineered clones. Thus, developed methodology (rapid *in vitro* sugarcane propagation methodology) represents a major advance in our ability to develop homogenous lines with shortest possible time.

MATERIALS AND METHODS

Preparation and selection of plant material

Aseptic culture of four sugarcane genotypes was established for the said research work. Two months old *in vitro* grown plants developed from the young unfurled leaf tissues of four genotypes (HSF-242, US-778, HSF-243 and HSF-240) were tested for regenerability. *In vitro* grown plants having variable stem girths were used for callus induction and regeneration.

Callus development after short time induction in dark and light

Several concentrations and combinations of 2,4-D, kinetin, BAP, NAA, IAA, IBA and CH were used. Out of several combinations, three namely IP, IPC1 and IPC2, were used in majority of the repeat experiments. IP = MS salts (4.33 g/L), sucrose (30 g/L), myoinositol (100 mg/L), MS vitamins, casein hydrolysate (500 mg/L), kinetin (0.5 mg/L), BAP (0.5 mg/L) with different levels of 2,4-D (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/L). IPC1 = MS salts (4.33 g/L), sucrose (15 g/L), myoinositol (100 mg/L), MS vitamins, casein hydrolysate (500 mg/L), kinetin (0.5 mg/L), BAP (0.5 mg/L), 5% coconut water with different levels of 2,4-D (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/L). IPC2 = MS salts (4.33 g/L), sucrose (30 g/L), myoinositol (100 mg/L), MS vitamins, casein hydrolysate (500 mg/L), kinetin (0.5 mg/L), BAP (0.5 mg/L), 5% coconut water with different levels of 2,4-D (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/L). The media were solidified using Phytagel (2.6 g/L) and the pH was adjusted to 5.8 using KOH/HCl prior to autoclave. The stems of *in vitro* grown plants were cut into 1.5 to 2.0 mm slices from stem base to top, cultured onto IP, IPC1 and IPC2 media and incubated under dark as well as light regime for 5 days at 26 ± 1°C.

Regeneration and plant maintenance medium

After 5 days of incubation on callus induction media, calli were sub-cultured onto IP, IPC1 and IPC2 media at 26 ± 1°C with 16 h photoperiod, 2000-2500 lux day intensity for *in vitro* regeneration and multiplication. After five weeks, multiple shoots emerging from green calli were shifted to MSV medium-containing Magenta culture vessels (Phytotechnology, USA) for shoot multiplication and proliferation. Rooting and leaf development in all regenerated plants were normal. All shoots developed roots on MSV medium having 4 mg/L IBA. The developed plants were shifted to clay pots having soil and peat moss (1:3) as potting media. Plants were hardened for seven to eight days by covering with polyethylene bags. MSV medium contained: MS salt thiamine HCl 1.0 mg/L, nicotinic acid 0.5 mg/L, pyridoxine HCl 0.5 mg/L, glycine 2 mg/L, sucrose 30 g/L, pH 5.8 and phytagel 2.6 g/L.

Stereomicroscopy to evaluate homogeneity in regenerants

Genetically analyzed *Bacillus thuringiensis* (*Bt*) transformed sugarcane clones (Khan et al., 2010) were subjected to rapid *in vitro* plant propagation methodology for purification. The relevant expression of *gfp* in regenerants was tracked under stereomicroscope (SZX-10; Olympus Co., Ltd.) equipped with GFP detection system and a digital camera (DP-20; Olympus Co., Ltd.). The images produced by GFP and chlorophyll fluorescence were viewed on a computer screen attached to the microscope and processed using Adobe Photoshop software (Adobe Systems Inc.). All of the plant growth phases, including callus induction, somatic embryogenesis and organogenesis were also viewed under stereomicroscope (SZX-10; Olympus Co., Ltd.).

Data collection and analyses

A completely randomized design (CRD) with three replications per treatment was used in these studies. Total number of shoots emerged per segment were calculated after seven weeks and efficiency of different types of media and genotypes was evaluated. The experiment was repeated four times in order to evaluate its reproducibility. The data were analysed using analysis of variance (ANOVA) and Duncan's multiple range (DMR) tests (Steel and Torrie, 1986; Damon and Harvey, 1987).

RESULTS

Callus induction

Two-months old *in vitro* grown plants were sacrificed to

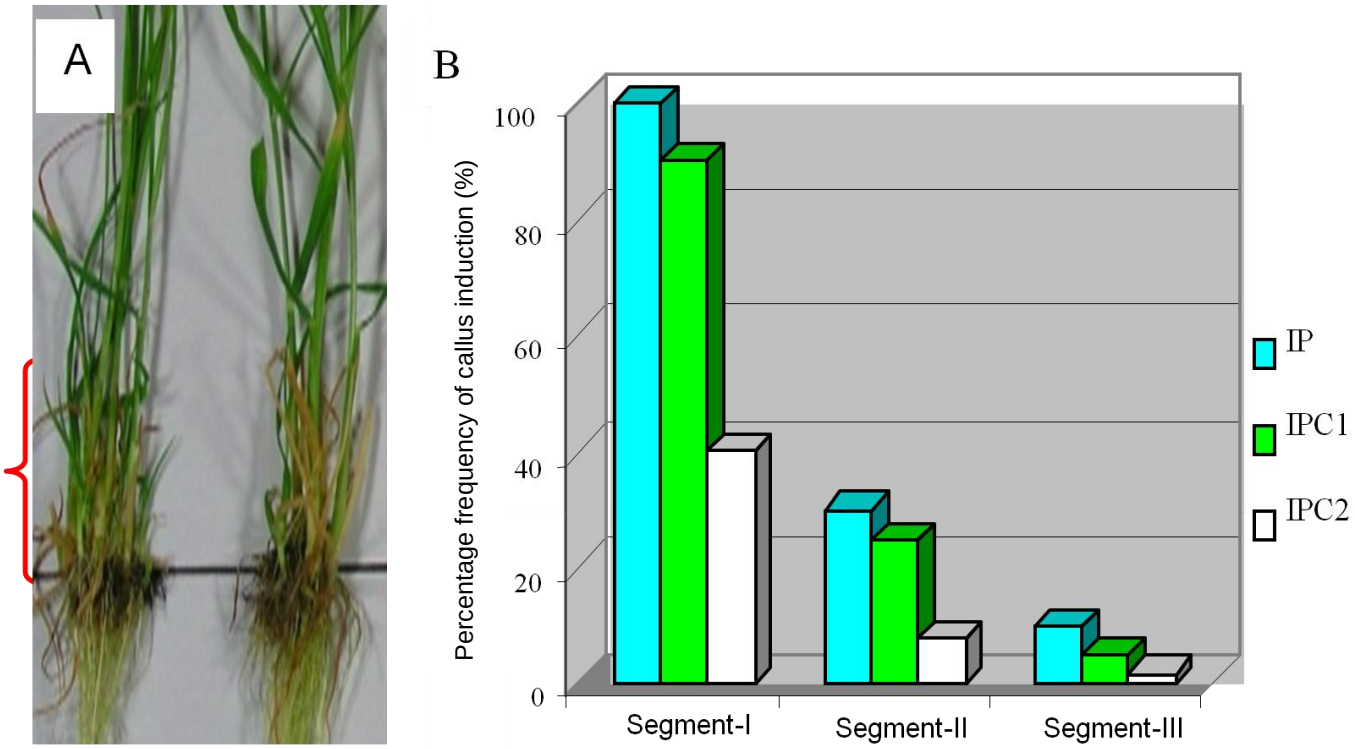

Figure 1. Graph showing comparison of % age frequency of callus induction on three different media, and the first three basal segments. A, Explant which was excised from *in vitro* grown plants and was sliced into pieces of 1.5 - 2.0 mm. B, IP medium was evaluated to be the best as compared with IPC1 and IPC2. Callus induction and proliferation was maximum on segment-I as compared with segment-II and segment-III.

prepare stem sections (Figure 1A). The stem was cut into thin sections and placed on MS medium containing different combinations of growth regulators for callus induction. All stem segments from base to top were evaluated. Callus induction percentage varied from basal to top sections where maximum callus was induced from first basal slice (Figure 1B). No callus induction was observed on fourth to onward sections. Nevertheless, very minute callus was witnessed on second and third slices. As far as optimal combination of growth regulators for callus induction is concerned, IP medium was rated the best medium because all genotypes responded well to this combination of hormones. The response of callus induction to various levels of 2,4-D was found to be genotype dependent, as HSF-243, HSF-240 and US-778 responded well at 1.5 mg/L, whereas HSF-242 showed encouraging response at 1.0 mg/L of 2,4-D. Stem segments cultured on IPC1 and IPC2 media responded to callus induction, but calli were mucilaginous and gummy in appearance. The effect of culture regime (light/dark) on callus induction was also observed. Overall, complete dark regime proved to be more favourable for callus induction as compared to light/dark (16:8 h) regime.

Callus induction and regeneration from basal stem segment-I and II, respectively was carried out and representative plates showed the callus induction and regeneration on segments cultured on IP medium (Figure 2A and B). Amongst stem segments of variable thickness, the slices of 1.5 to 2.0 mm appeared to be the most responsive to callus induction and proliferation. The shoots that were proliferated on plates (Figure 2C and D) were shifted to boxes for rooting (Figure 2E). Finally, rooted plants were acclimatized in pots containing mixture of soil + peat moss at 1:3 (Figure 2F).

Regeneration

Synergistic effect of auxin and cytokinin on shoot regeneration

After five days incubation in the dark, explants were cultured onto MS medium containing growth regulators in various concentrations and combinations. IP medium containing casein hydrolysate (500 mg/L), kinetin (0.5 mg/L) and BAP (0.5 mg/L) with 0.25 to 0.5 mg/L 2,4-D appeared to be the best combination revealing their synergistic effect on regeneration. Thus, IP medium was evaluated to be the best as compared to IPC1 or IPC2. The IPC1 and IPC2 containing 5% coconut water with full strength MS medium (IPC1) or half strength MS medium (IPC2) showed poor response to regeneration. As far as genotype response to regeneration is concerned,

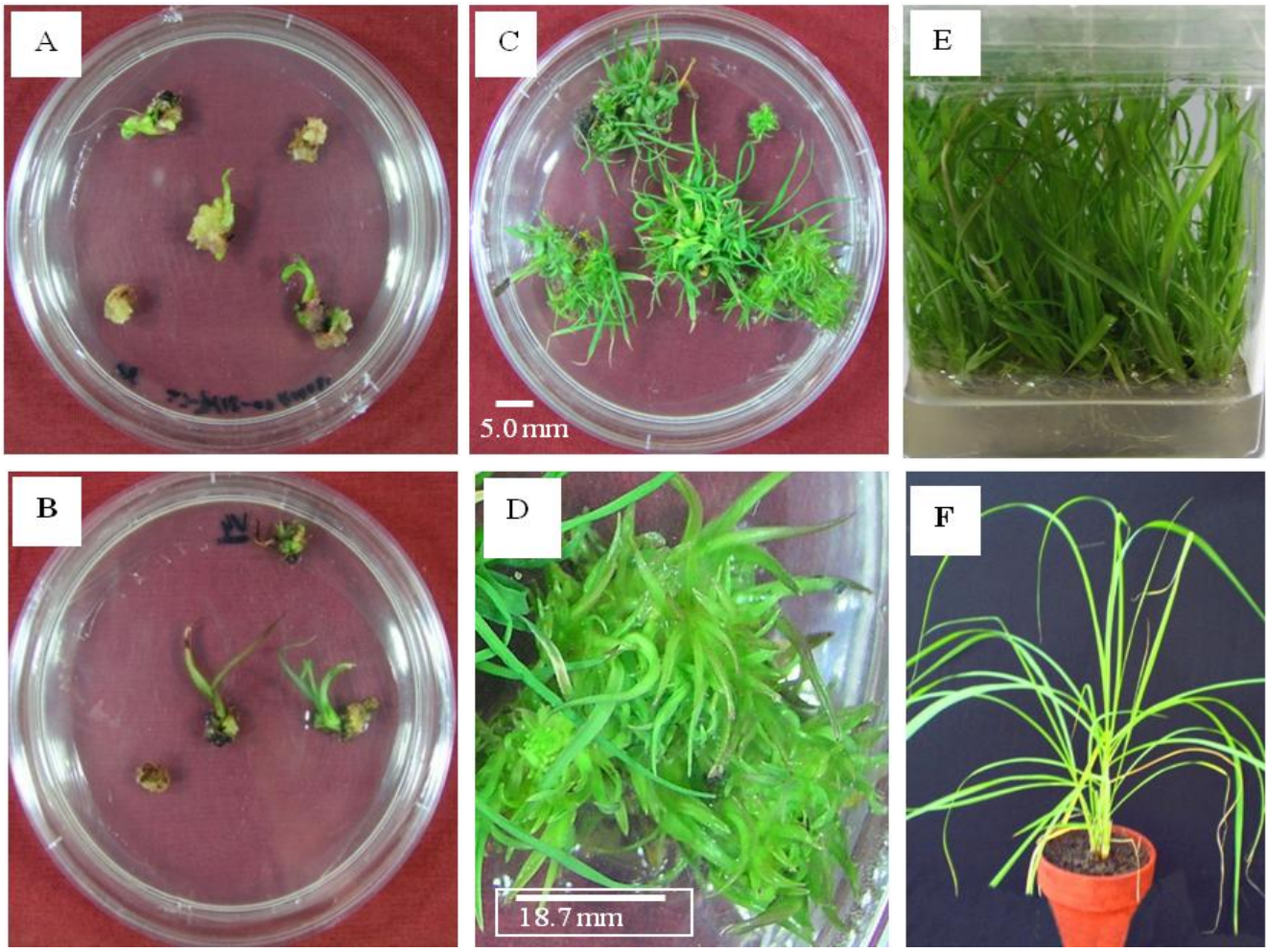

Figure 2. Callogenesis and regeneration from basal stem segments of *in vitro* grown plants. A and B, Callus induction and regeneration from basal stem segment-I and II, respectively. C, Plate B after six to seven weeks of culture on IP medium. D, Close up of shoots from plate C. E, *In vitro* plants proliferating in boxes. F, Hardening of plants raised through rapid *in vitro* plant regeneration methodology in a mixture of soil + peat moss at 1:3.

genotype HSF-243 resulted in maximum shoot induction and more than 137 shoots per segment were counted. Other genotypes also responded well to regeneration as 117, 81 and 95 shoots per segment were counted in genotypes HSF-242, US-778 and HSF-240, respectively (Figure 3). Hence, it is inferred that the developed combination of hormones is the best combination owing to be genotype independent. The resultant shoot clumps were shifted to Magenta culture vessels (Phytotechnology, USA) for further proliferation (Figure 2F).

Analysis of variance (ANOVA) showed that the effect of various media, genotypes and their interaction were highly significant ($P<0.01$). Amongst various genotypes, CPF-243 showed the highest mean value (89.83 shoots per stem segment) for shoot induction followed by HSF-242 which has 77.58 shoots per stem segment (Table 1). Mean values for the interaction of various media and genotypes highlighted that IP medium supplemented with 2,4-D at levels of 0.25 and 0.5 mg/L is the most suitable medium for *in vitro* regeneration from all genotypes.

Impact of stem position on regeneration

All stem segments (from base to top) were evaluated for regeneration. One of the critical parameters was the position of the callus-developing segment on the *in vitro* grown plant. Among the stem slices, 1st basal segment responded extraordinarily to regeneration in all genotypes used in the present studies. This was perhaps due to the presence of meristematic cells in that region of the stem. In case of 2nd segment, resultant shoots were not regenerant, rather, segments elongated and developed into shoots (Figure 2C). Stereomicroscopy, using dark and bright field, exhibited the development of calli (Figure 4A), which upon transfer onto the IP1 medium developed embryos (Figure 4B to E) and shoots (Figure 4F).

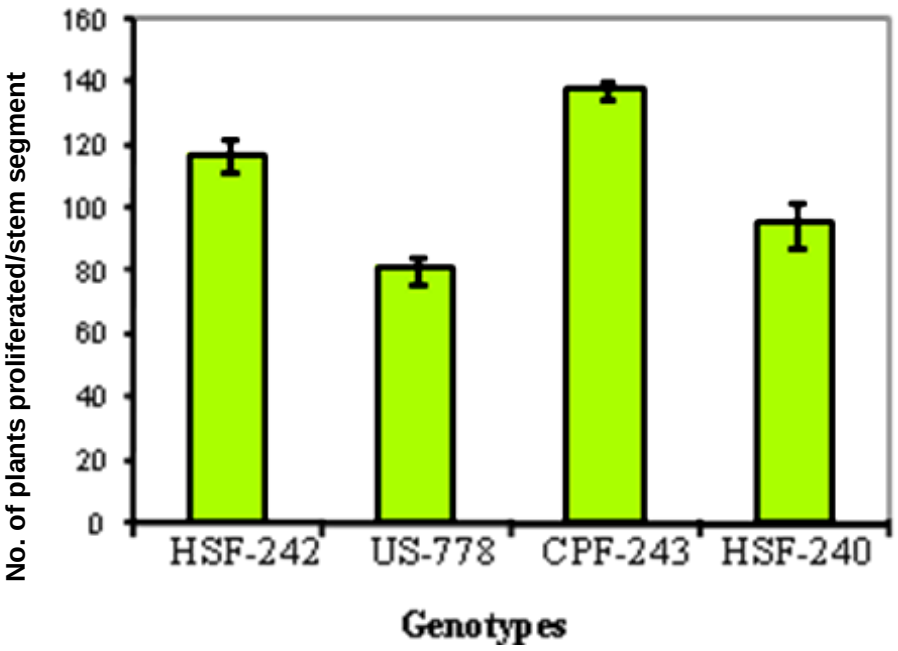

Figure 3. Graphical representation of shoot induction from four genotypes. Maximum shoot induction was observed in genotype CPF-243 followed by HSF-242, HSF-240 and US-778.

Table 1. Analysis of variance for shoot induction among the four genotypes using *in vitro* plant.

K value	Source	Degree of freedom	Sum of squares	Mean square	F value	Significance	Probability
2	Media	3	84561.562	28187.187	471.0951	***	0.0000
4	genotype	3	8979.729	2993.243	50.0263	***	0.0000
6	Media × Genotype	9	3133.021	348.113	5.8181	***	0.0001
-7	Error	32	1914.667	59.833			
Total		47	98588.979				

In genotype CPF-243 maximum number of shoots was observed, followed by HSF-242, HSF-240 and US-778.

Rooting and acclimatization

The developed independent shoots were shifted onto MSV medium for proliferation and root development. Almost all regenerated shoots developed roots on MSV medium however, copious rooting was observed when MSV medium was supplemented with IBA at a concentration of 4 mg/L. The entire process to develop plants with copious roots took 8 to 10 weeks. The established plants with copious roots were shifted to pots for acclimatization (Figure 2F). Best hardening response was obtained in a mixture of soil and peat moss (1:3).

Purification of sugarcane clones by rapid *in vitro* plant propagation methodology

For visual tracking of transformed cells, the plants confirmed for transgene integration into the genome by polymerase chain reaction (PCR) were analyzed using stereomicroscope equipped with GFP detection system.

The green fluorescent protein fluoresces green under UV light whereas chlorophyll fluoresces red. It was observed that the size of fluorescent sectors was different in different leaves and in different leaf layers in heterogeneous transgenic plants.

However, when these plants were subjected to rapid *in vitro* plant propagation methodology with selection pressure, regenerants showed more uniform fluorescence sectors, depicting increased level of homogeneity (Figure 5), thus facilitating purification of transgenic clones. Exposing totipotent cells to selection pressure ensured the survival/proliferation of cells having sufficient transgene expression and elimination of untransformed from heterogeneously transformed cells, and purification of homogonous plants from heterogonous population.

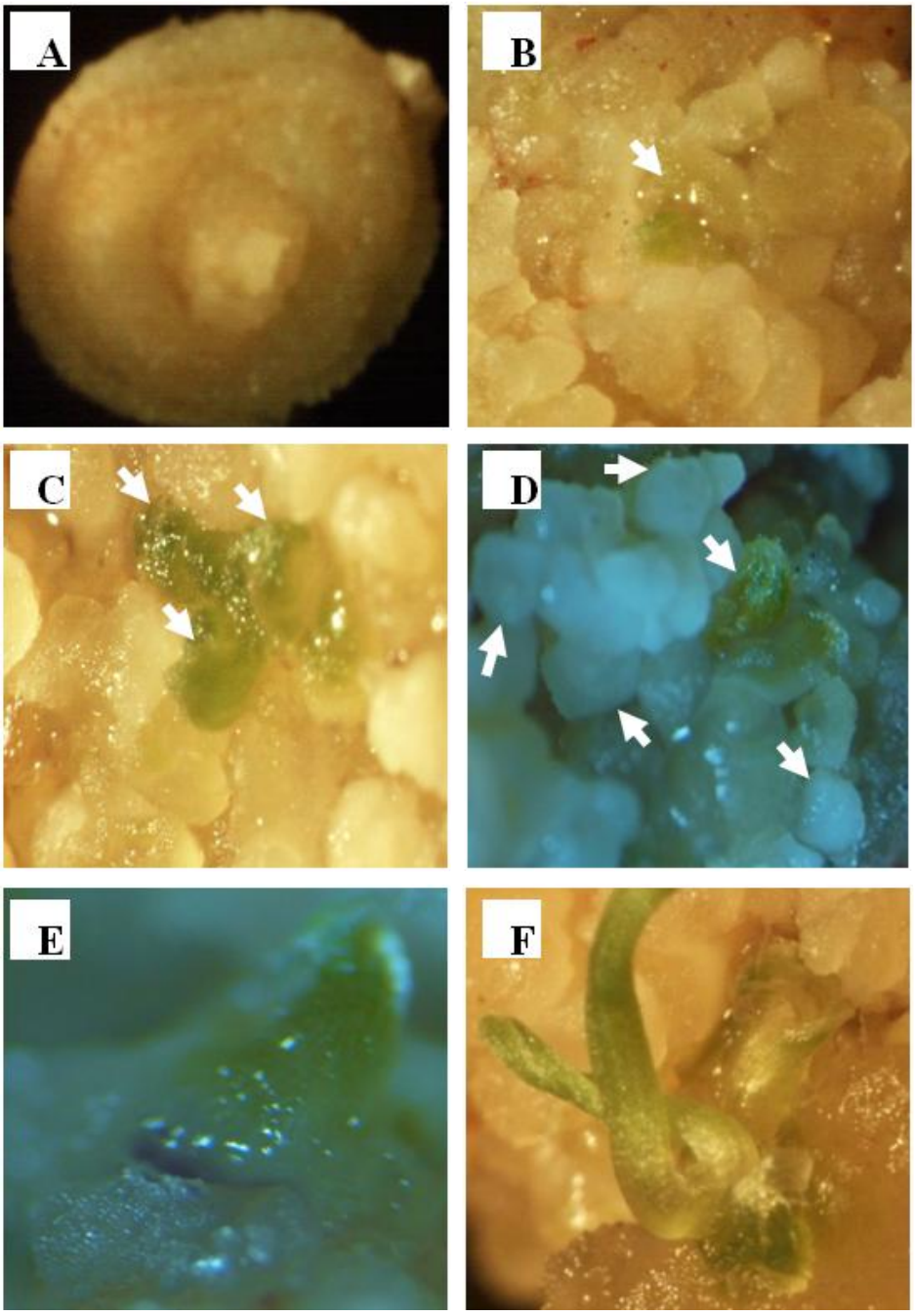

Figure 4. Stereomicroscopy for callus induction and subsequent regeneration of calli from basal segments of stem. A, Short time callus induction in the dark. B and C, Short time induced calli after incubation in light (white arrows). D, White somatic embryoids on callus (white arrows). E, Somatic embryos on calli derived from the basal stem segment. F, Somatic embryo derived from *in vitro* plants developing into shoots.

DISCUSSION

Availability of reproducible, efficient and facile regeneration system is a fundamental pre-requisite for the genetic transformation as well as micropropagation of sugarcane (Snyman et al., 1996). Sugarcane *de novo* regeneration has been well explored (Brumbley et al., 2008), but most of the studies are reported on field grown explants, except Tiel et al. (2006) and Garcia et al. (2007). Tiel et al. (2006) used *in vitro* grown plants as explant source and reported maximum regeneration frequency of 16.95 shoot per explant. Similarly Garcia et

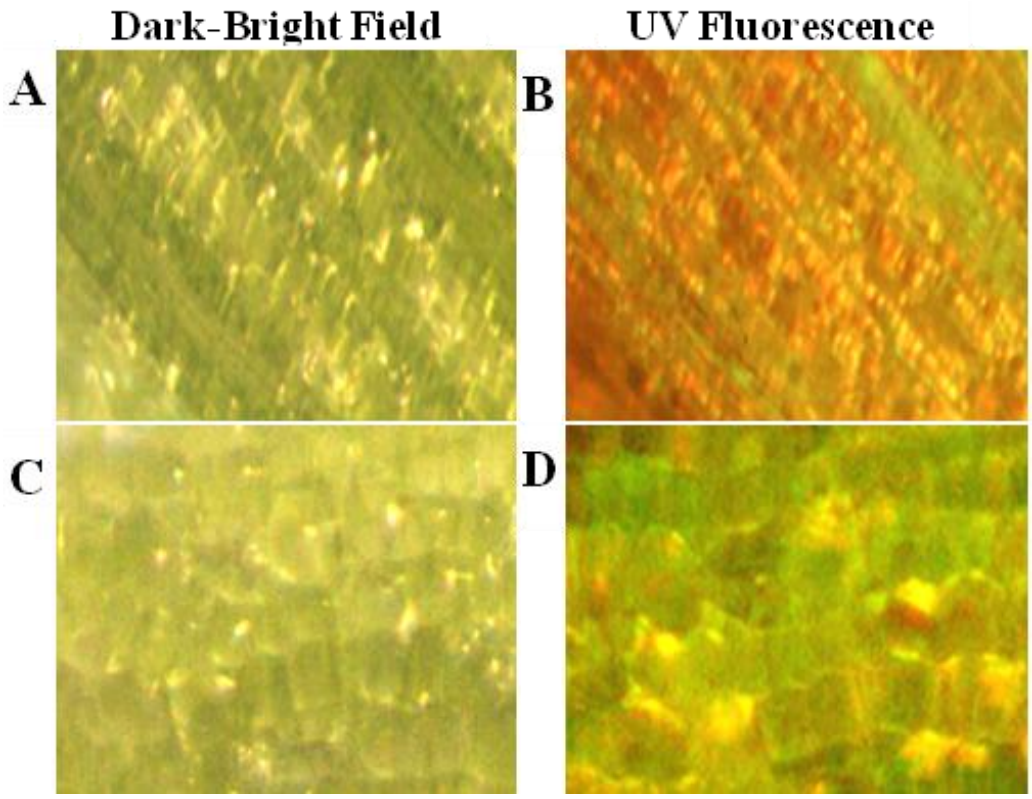

Figure 5. Determination of homogeneity in transgenic shoots using stereomicroscope. A and C, Leaf sections under dark-bright field; B and D, The same leaf sections under UV light. GFP fluoresced green under UV light, whereas chlorophyll fluoresced red.

al. (2007) used *in vitro* grown plants as explants but focused on the effects of growth regulators and light on the morphogenic patterns as well as the cellular origin of regeneration processes in certain genotypes. Our studies resulted in one of the best ever published report on regeneration (Figure 2D and E) from *in vitro* grown plants. Further, it would facilitate purification of clones without subjecting the plants to field conditions. The protocol is proficient and reproducible with regeneration frequency of more than 137 shoots/stem segment, almost 7 to 8 times higher than that earlier reported.

Moreover, after short time callus induction in the dark, explants were subsequently transferred to light on IP medium for regeneration as proposed by Franklin et al. (2006). We used auxin (2,4-D) and cytokinins (BAP and kinetin) in combination with casein hydrolysate to improve *in vitro* plant regeneration since auxins applied extrinsically are capable of erasing the genetically programmed physiology of plant tissues, by reverting them to dedifferentiated state (callus induction). Induced calli may contain meristematic nodules which never differentiate further, unless suitable conditions are provided. Wounding also acts as a stimulus for dedifferentiation and callus formation. These wound responses involve auxins and cytokinins and seem to be the biological trigger for plant regeneration (Potrykus, 1989). Growth-promoting concentrations of auxin have been found to stimulate the synthesis of both DNA and RNA within the nuclei. This in turn controls various metabolic pathways (Dash et al., 2011). Auxin causes changes in DNA methylation, leading to the reprogramming of differentiated cells. It is also reported to regulate both division and expansion of plant cells mediated by a putative auxin binding protein ABP1 (Chen et al., 2001).

In this study, it was observed that dark regime proved better for short time callus induction as absence of light creates a stress environment and helps in dedifferentiation of organized tissues to form unorganized callus tissues. Direction of differentiation could be influenced by the ratio of exogenously supplied auxin and cytokinin. These two groups of growth regulators play an important role in unlocking totipotent expression. We evaluated that lower levels of auxin speed up organogenesis and cytokinin also supports it synergistically if applied in lower concentration (0.5 mg/L). It is inferred from these studies that continuous exposure of the ex-plant tissues to reduced level of 2,4-D (0.25 to 0.5 mg/L) supplemented with casein hydrolysate (500 mg/L), kinetin (0.5 mg/L) and BAP (0.5 mg/L) in the presence of light, resulted in excellent shoot induction. Hence, auxin (2,4-D) is not only a significant determinant of callus induction, but also of *in vitro* organogenesis in sugarcane. Sugars promote

osmotic potential and increased respiration results in increased synthesis of cell wall material leading to cell elongation.

BAP is also a considerable stimulator of shoot induction and had been used in most of the recent regeneration studies (Ather et al., 2009; Behera and Sahoo, 2009; Dibax et al., 2011). In sugarcane, rooting is induced either by the addition of indole-3-butyric acid (Cheema and Hussain, 2004; Ali et al., 2008) or by depriving the medium of growth regulators (Lakshmanan et al., 2006) or even by supplementation of the medium with high sucrose content (Singh et al., 2006). In our studies, regenerants developed roots on growth regulator free medium but copious rooting was achieved in the medium augmented with IBA (4 mg/L). Further, IBA was found to play a key role in root developmental regulation as root number increased with increase in IBA concentration but root length decreased with increase in IBA concentration (Mustafa and Khan, unpublished). As far as the impact of stem position on regeneration is concerned all of the stem segments, except 1st basal segment, did not respond to hormones. The morphogenic fate of a cell is determined to a great extent by its neighbouring cells/ tissues (Lyndon and Francis, 1992). If excised, cells are released from the control of neighbouring cells and under appropriate culture conditions, express their morphogenic potential. Further, these basal stem segment tissues contain meristematic cells as a result, more responsive to dedifferentiation and differentiation.

Another important aspect of the present study was to harvest several advantages of using *in vitro* grown plants as explant source compared to field grown plants such as; A) currently, the only realistic means for the production of disease-free seed canes of newly developed varieties is tissue culture (Lorenzo et al., 2001). This can further be accelerated by adopting rapid *in vitro* sugarcane propagation methodology that would cut short the time for selection and mass multiplication of new lines. B) Development of stable transgenic crops, particularly transplastomic lines require purification of primary transformants to a state of homoplasmy through repeated cycles of selection and regeneration (Khan and Maliga, 1999). In monocots, the absence of leaf based regeneration system is a major bottleneck in the purification of transplastomic lines as the rice transplastomic lines remained heteroplasmic in the absence of a protocol for carrying out repeated cycles of selection and regeneration (Lee et al., 2006). We used this technique for the purification of nuclear transformed (*Bt*) sugarcane plants (Khan et al., 2010). Heterogeneous transformants, when posed to 2nd round of selection and regeneration using the said methodology, their level of homogeneity was predominantly increased as revealed by GFP fluorescence under stereomicroscope (Figure 5). The totipotent tissues when cultured on selective regeneration media each cell was exposed to selection pressure, resulting in survival of only the cells having ability to tolerate the selection pressure. Thus, escapees and heterogeneously transformed cells were eliminated. The methodology devised may further be extended to genetically engineer chloroplast genome of sugarcane as callogenesis and morphogenesis responsive tissues (cortical chlorenchyma) are structurally and functionally similar to the leaf mesophyll containing mature chloroplasts (Yiotis et al., 2006). Hence, may facilitate repeated cycles of selection and regeneration for the purification of transplastomic clones. C) High contamination levels and absence of physiological uniformity among the field grown plants are other major problems of using field grown plants as ex-plant source, but rapid *in vitro* sugarcane propagation methodology provides physiologically uniform material and requires no disinfestations. D) Another limitation of using field grown plants is the availability of plants. They are only available for very strict period of time affecting experimentation, but *in vitro* grown plants are available throughout the year whenever required for experimentation.

The protocol presented here is very efficient as number of shoots developed is very high compared to the numbers reported in the literature. Hence, in addition to supporting genetic engineering programmes, it would also help in the betterment of existing sugarcane multiplication system.

ACKNOWLEDGEMENTS

The authors would like to thank the Ministry of Science and Technology for funds to Muhammad Sarwar Khan (MSK) and the Higher Education Commission of Pakistan for supporting the student in completing the research for PhD degree, and Sugarcane Research Institute, AARI, Faisalabad, Pakistan, for providing the plant material.

REFERENCES

Ali A, Naz S, Iqbal J (2007). Effect of different explants and media compositions for efficient somatic embryogenesis in sugarcane (*Saccharum officinarum*). Pak. J. Bot., 39: 1961-1977.

Ali A, Naz S, Siddiqui FA, Iqbal J (2008). An efficient protocol for large scale production of sugarcane through micropropagation. Pak. J. Bot., 40: 139-149.

Ali S, Iqbal J, Khan MS (2010). Genotype independent *in vitro* regeneration system in elite varieties of sugarcane. Pak. J. Bot., 42: 3783-3790.

Alves SAO, Lemos OFde, Santos Filho BG, da Silva ALL (2011). *In vitro* embryo rescue of interspecific hybrids of oil palm (*Elaeis oleifera* x *Elaeis guineensis*). J. Biotechnol. Biodiv., 2: 1-6.

Arencibia A, Vazquez RI, Prieto D, Tellez P, Carmona ER, Coego A, Hernandez L, De la Riva GA, Selman HG (1997). Transgenic sugarcane plants resistant to stem borer attack. Mol. Breed., 3: 247-255.

Ather A, Khan S, Rehman A, Nazir M (2009). Optimization of the protocols for callus induction, regeneration and acclimatization of sugarcane cv. Thatta-10. Pak. J. Bot., 41: 815-820.

Behera KK, Sahoo S (2009). Rapid *in vitro* micro propagation of sugarcane (*Saccharum officinarum* L. cv-Nayana) through callus culture. Nat. Sci., 7: 1-10.

Biradar S, Biradar DP, Patil VC, Patil SS, Kambar NS (2009). *In vitro* plant regeneration using shoot tip culture in commercial cultivar of sugarcane. Karnataka J. Agric. Sci., 22: 21-24.

Bonnett GD, Nowak E, Olivares-Villegas JJ, Berding N, Morgan T, Aitken KS (2008). Identifying the risks of transgene escape from sugarcane crops to related species, with particular reference to *Saccharum spontaneum* in Australia. Trop. Plant Biol., 1: 58-71.

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

Brumbley SM, Snyman SJ, Gnanasambandam A, Joyce P, Hermann SR, da Silva JAG, McQualter RB, Wang ML, Egan B, Patterson AH, Albert HH, Moore PH (2008) Sugarcane. In: Kole C, Hall TC (eds). A compendium of transgenic crop plants, vol 7: sugar, tuber and fiber crops. Blackwell, Oxford, pp. 1-58.

Burner DM, Grisham MP (1995). Induction and stability of phenotypic variation in sugarcane as affected by propagation procedure. Crop Sci., 35: 875-880.

Cheema KL, Hussain M (2004). Micropropataion of sugarcane through apical bud and axillary bud. Int. J. Agric. Biol. ,6: 257-259.

Chen JG, Shimomura S, Sitbon F, Sandberg G, Jones AM (2001). Role of auxin-binding protein 1 in leaf cell growth. Plant J., 28: 607-617.

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

Damon RA, Harvey WR (1987). Experimental design, ANOVA and regression. Harper and Row Publishers, New York, p. 165.

Dash M, Mishra PK, Mohapatra D (2011). Mass propagation via shoot tip culture and detection of genetic variability of *Saccharum officinarum* clones using biochemical markers. Asian J. Biotechnol., 3: 378-387.

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: 764-8.

Dibax R, Alcantara GB de, Filho JCB, Machado MP, de Oliveira Y, da Silva ALL (2011). Plant regeneration of sugarcane cv. RB931003 and RB98710 from somatic embryos and acclimatization. J. Biotechnol. Biodiv., 2: 32-37.

Fitch MMM, Moore PH (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.

Franklin G, Arvinth S, Sheeba CJ, Kanchana M, Subramonian N (2006). Auxin pretreatment promotes regeneration of sugarcane (*Saccharum* spp. hybrids) midrib segment explants. Plant Growth Regul., 50: 111-119.

Garcia R, Cidade D, Castellar A, Lips A, Magioli C, Callado C, Mansur E (2007). *In vitro* morphogenesis patterns from shoot apices of sugarcane are determined by light and type of growth regulator. Plant Cell Tiss. Organ Cult., 90: 181-190.

Guiderdoni E, Demarly Y (1988). Histology of somatic embryogenesis in cultured leaf segments of sugarcane plantlets. Plant Cell Tiss. Organ Cult. 14: 71-88.

Guimarces CT, Sobral WS (1998). The *Saccharum* complex: relation to other *andropogoneae*. Plant Breed. Rev., 16: 269-288.

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

Irvine JE, Benda GTA, Legendre BL, Machado GR (1991). The frequency of marker changes in sugarcane plants regenerated from callus culture. II Evidence for vegetative and genetic transmission, epigenetic effects and chimeral disruption. Plant Cell Tiss. Organ Cult. 26: 115-125.

Khan MS, Ali S, Iqbal J (2010). Developmental and photosynthetic regulation of δ-endotoxin reveals that engineered sugarcane conferring resistance to 'dead heart' contains no toxins in cane juice. Mol. Biol. Rep., 38: 2359-2369.

Khan MS, Maliga P (1999). Fluorescent antibiotic resistance marker for tracking plastid transformation in higher plants. Nat. Biotechnol., 17: 910-915.

Khatri A, Khan IA, Javed MA, Siddiqui MA, Khan MKR, Khanzada MH, Dahar NA, Khan R (2002). Studies on callusing and regeneration potential of indigenous and exotic sugarcane clones. Asian J. Plant Sci., 1: 41-43.

Lakshmanan P, Geijskes RJ, Wang L, Elliott A, Grof CPL, Berding N, Smith GR (2006). Developmental and hormonal regulation of direct shoot organogenesis and somatic embryogenesis in sugarcane (*Saccharum* spp. interspecific hybrids) leaf culture. Plant Cell Rep. 25: 1007-1015.

Lee SM, Kang K, Chung H, Yoo SH, Xu XM, Lee SB, Cheong JJ, Daniell H, Kim M (2006). Plastid transformation in the monocotyledonous cereal crop, rice (*Oryza sativa*) and transmission of transgenes to their progeny. Mol. Cells, 21: 401-410.

Liu MC (1984). Sugarcane In: Sharp W H, Evans DA, Ammirato PV, Yamada, Y., eds. Handbook of plant cell culture, New York: Macmillan, 2: 572-605.

Lorenzo JC, Ojeda E, Espinosa A, Borroto C (2001). Field performance of temporary immersion bioreactor derived sugarcane plants. *In Vitro* Cell. Devel. Biol. Plant, 37: 803-806.

Lyndon RF, Francis D (1992). Plant and organ development. Plant Mol. Biol., 19: 51-68.

Manickavasagam M, Ganapathi A, Anbazhagan VR, Sudhakar B, Selvaraj N, Vasudevan A, Kasthurirengan S (2004). Agrobacterium mediated genetic transformation and development of herbicide resistant sugarcane (*Saccharum* species hybrids) using axillary buds. Plant Cell Rep., 23: 134-143.

Potrykus I (1989). Gene transfer to cereals: An assessment. Trends Biotechnol., 7: 269-273.

Seema S, Oad FC, Khan IA, Tunio S, Siddiqui MA, Yasmeen S, Khatri A, Bibi S (2011). Influence of phytohormone on the organogenesis of sugarcane. Pak. J. Bot. 43: 1531-1534.

Singh N, Kumar A, Garg GK (2006). Genotype dependent influence of phytohormone combination and subculturing on micropropagation of sugarcane varieties. Ind. J. Biotechnol., 5: 99-106.

Snyman SJ, Huckett BI, Botha FC, Watt MPA (2001). comparison of direct and indirect somatic morphogenesis for the production of transgenic sugarcane (*Saccharum* spp. hybrids). Acta Hortic., 56: 105-108.

Snyman SJ, Meyer GM, Carson DL, Botha FC (1996). Establishment of embryogenic callus and transient gene expression in selected sugarcane varieties. S. Afr. J. Bot., 62: 29-37.

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.

Steel RGD, Torrie JH (1986). Regression lineal. In Bioestadi stica: Principios Y. procedimientos. Madrid: McGraw Hill, pp. 231-261.

Tiel K, Enriquez GA, Ceballo Y, Soto N, Fuentes AD, Ferreira A, Coll Y, Pujol M (2006). Development of a system for rapid plant regeneration from *in vitro* sugarcane (S*accharum officinarum* L) meristematic tissue. Biotechnol. Appl. 23: 22-24.

Vickers JE, Grof CPL, Bonnett GD, Jackson PA, Morgan TE (2005). Effects of tissue culture, biolistic transformation, and introduction of *PPO* and *SPS* gene constructs on performance of sugarcane clones in the field. Aus. J. Agric. Res., 56: 57-68.

Wang ML, Goldstein C, Su W, Moore PH, Albert HH (2005). Production of biologically active GM-CSF in sugarcane: a secure biofactory. Transgenic Res., 14: 167-178.

Wu Y, Zhou H, Que YX, Chen RK, Zhang MQ (2008). Cloning and identification of promoter *Prd29A* and its application in sugarcane drought resistance. Sugar Tech., 10: 36-41.

Yiotis C, Manetas Y, Psaras GK (2006). Leaf and green stem anatomy of the drought deciduous Mediterranean shrub *Calicotome villosa* (Poiret) Link. (Leguminosae). Flora, 201: 102-107.