

Full Length Research Paper

The response of sugarcane (*Saccharum officinarum* L) genotypes to callus induction, regeneration and different concentrations of the selective agent (geneticin -418)

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Two commercial cultivars (CPF-245 and CPF-237) and three advanced lines (CSSG-668, S-2003US633, S-2003US114) of sugarcane (*Saccharum officinarum*) grown in Punjab, Pakistan were evaluated for their potential to induce callus, embryogenic callus and regeneration. Cultivar CSSG-668 was found to be the best genotype yielding maximum embryogenic callus and regeneration whereas cultivar CPF-245 exhibited lowest callus induction frequency. Five different concentrations (0, 20, 40, 60, and 80 mg/L) of the selective agent (geneticin-418) were used to optimize selection conditions with non-transformed embryogenic calli. The geneticin concentration 60 mg/L was found to be the optimal dose to select the embryogenic calli of genotypes CSSG-668, CPF-245 and S-2003US63, while 35 mg/L geneticin was found to be the best concentration for S-2003US-114. Similarly, 60 mg/L geneticin was optimum dose to select regenerated plantlets of the cultivars CSSG-668 and CPF-245 while it was 40, 25 mg/L for the cultivars S-2003US-114 and S-2003US-633, respectively. It is concluded from the present study that geneticin concentration in the range of 25 to 60 mg/L can be effectively used for the selection of transformed embryogenic calli and regenerants of different sugarcane cultivars.

Key words: Callus induction, embryogenic callus, regeneration, *Saccharum officinarum* L., selection, geneticin.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a major industrial cash crop and is widely cultivated in tropical and subtropical countries of the world for sugar and bio-ethanol production. It accounts for approximately 80% of the world's sugar production (FAO, 2009). Sugarcane is the second major cash crop in Pakistan and it is grown

over one million hectares (Economic Survey of Pakistan, 2008; 2009). Despite of all efforts, sugarcane production in Pakistan is still much lower than most of the sugarcane growing countries of the world. The low cane and sugar yields are attributed to many factors in which drought; salinity, insect pests and diseases are major constraints (Nasir et al., 2000; Khaliq et al., 2005). High ploidy, low fertility, a large genome, complex environmental interactions, slow breeding advances and back-crossing for the introduction of specific genes make conventional breeding difficult for this crop. In Pakistan, sugarcane flowers only in lower Sindh coastal areas, Jabban valley in Malakand agency, Khyber Pakhtoon Khawa and Murrree hills, but viability is still a problem due to unfavorable climatic conditions. Thus, lack of viable fuzzi production makes it difficult to improve sugarcane through conventional breeding in Pakistan. New varieties

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; BAP, benzylaminopurine; KIN, kinetin; NAA, α -naphthaleneacetic acid; GUS, β -glucuronidase; nptII, neomycin phosphotransferase II; X-Gluc, 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid; cv., cultivar.

Table 1. Composition of callus induction (CIM), callus selection (CSM) regeneration (RM) and regeneration selection medium (RSM).

Media composition	CIM (Quantity per liter)	CSM (Quantity per liter)	RM (Quantity per liter)	RSM (Quantity per liter)
MS medium	4.43 g	4.43 g	4.43 g	4.43 g
Cassein Hydrolysate	0.50 g	0.50 g	0.00l	0.00
Myo-inositol	1.00 g	1.00 g	1.00 g	1.00 g
2,4-D	4.00 mg	4.00 mg	0.50 mg	0.50 mg
Thiamine HCl	4.00 mg	4.00 mg	0.00	0.00l
Arginine	4.00 mg	4.00 mg	0.00	0.00
Sucrose	3%	3%	3%	3%
BAP	0.00	0.00	2.00 mg	2.00 mg
NAA	0.00	0.00	1.00 mg	1.00 mg
Kin	0.00	0.00	0.50 mg	0.50 mg
Geneticin	0.00	0, 20, 40, 60, 80	0.00	0, 20, 40, 60, 80

are evolved mostly through import of cane fuzz and the selection breeding (Khan et al., 2004). As genetic manipulation of the crops has emerged as a new tool for the crop improvement, therefore, genetic transformation of the desired genes in sugarcane may be helpful to cope with the sugarcane problems.

Establishment of tissue culture system is a prerequisite for genetic transformation. The first successful plant regeneration system in sugarcane was established about 40 years ago (Barba and Nickel, 1969), however, a persuasive evidence through somatic embryogenesis was reported later (Ahloowalia and Maretzki, 1983). Moreover, successful somatic embryogenesis and regeneration was further studied in sugarcane using different explants and medium composition (Falco et al., 1996; Brisibe et al., 1993; Khatri et al., 2002; Khan et al., 2004; Asad et al., 2009). The development of somatic embryogenesis (SE) was a turning point in sugarcane biotechnology (Lakshmanan et al., 2005; Ming et al., 2006). As, the callus induction, embryogenic callus production and regeneration ability in sugarcane is highly genotype dependent (Gandonou et al., 2005; Burner, 1992), therefore, the present study was aimed to study the ability of different sugarcane genotypes for embryogenic callus induction and regeneration efficiency.

Genetic engineering deals with introduction of foreign genes into plant genome through cells, protoplasts or tissues for the production of transgenic plants that exhibit normal physiological and biological functions (Jenes et al., 1993). For successful genetic transformation, efficient selection system is essentially required. This can be achieved through negative selection using suitable selective agent (Park et al., 1998). The most important and widely used selectable marker is *nptII* (neomycin phosphotransferase) gene conferring resistance to phytotoxic amino-glycoside antibiotics, kanamycin and geneticin (Bower and Birch, 1992; Fitch et al., 1995). Geneticin

was used as selective agent for sugarcane embryogenic calli by Bower and Birch (1992). Before embarking on transformation, it is necessary to establish the effective selection system by knowing the minimal inhibitory concentration of selective agent. Until now there is no report regarding the selection system for local cultivars of sugarcane grown in Pakistan. In the present study different commercial cultivars and advanced lines of sugarcane grown in central Punjab, Pakistan were evaluated for the callus induction, embryogenesis and regeneration. Moreover, these cultivars were optimized for the selective agent geneticin (G-418) concentration so that successful transformation may be complemented with the desired genes and putative transgenic plants could be selected precisely.

MATERIALS AND METHODS

Plant materials

Plant material was collected from two sugarcane research institutes of central Punjab, Pakistan. The commercial cultivars CPF-237 and CPF-245 and advanced lines, S-2003US633, S-2003US114 were collected from the Sugarcane Research Institute (SRI), Faisalabad and one advanced line CSSG-668 from Shakarganj Sugarcane Research Institute (SSRI), Jhang. These cultivars were selected on the basis of better agronomic performance.

Callus induction and regeneration

Apical portions of healthy shoots were stripped to the terminal bud and sterilized with 70 % ethanol. Leaf rolls were peeled, under sterile conditions inside a laminar air flow hood, to cylindrical pieces approximately 3-5 mm in diameter size. Fifteen slices were cultured in three replicates on an optimized callus induction medium (Table 1). The culture plates were placed in a controlled temperature room at 26 ± 2 °C under dark conditions. The cultures were transferred on to fresh callus induction medium (CIM) every 2-3 week for long-term maintenance. Data on callus induction was recorded after 8

weeks of culture and percentage was calculated. Similarly, embryogenic callus induction percentage was also calculated as the explants producing embryogenic callus over total number of induced calli multiplied by 100. The embryogenic calli were transferred to regeneration medium (RM; Table 1) and after 12 weeks, regeneration frequency in percentage was determined.

Optimization of inhibitory concentration of the selective agent (G-418)

Stock solution (80 mg/L) of geneticin (G-418, Phyto- Technology Laboratories®, USA) was prepared, filter sterilized and stored at 4°C. Callus induction medium was autoclaved and cooled to 45 - 50°C before the addition of the selective agent (geneticin) at final concentration of 0, 20, 40, 60 and 80 mg/L. Three replicates were used for each treatment as well as for the control (without geneticin). Embryogenic calli of cv. CSSG-668, CPF-245, S-2003US114 and S-2003US633 were divided into pieces of approximately 2 - 4 mm diameter and then cultured on callus selection medium (CSM) and regeneration selection medium (RSM) for one month on both media. Actively proliferating calli were sub-cultured after two weeks to fresh media. The data on survival, dead calli and regeneration were recorded after one month treatment on selection medium and the percentages for each of these parameters were calculated. The treatment which showed 10% callus survival and regeneration, were considered as optimum media for callus selection and regeneration. Details of medium composition are shown in Table 1.

Biolistic mediated transformation to verify the optimized selection media protocol

The optimized selective agent (geneticin) concentration showing 10% calli survival was further used to observe the effectiveness for the selection of transformed embryogenic calli of cv. CSSG-668. Genetic transformation of sugarcane embryogenic calli was done with Biolistic method. A plasmid, pGreen0029 having *nptII* selection marker and *GUS* as a reporter gene was used with some modifications as described by Asad et al. (2008). The concentration and purity of the plasmid was determined with the spectrophotometer.

Preparation of gold particles and bombardment of the callus

The plasmid DNA (1 g/L) was precipitated onto 1.0 µm gold particles and bombarded using particle delivery system (PDS-1000/He, Bio-Rad Laboratories, USA) following manufacturer's instructions, in three replicates. After three days, bombarded and un-bombarded (control) embryogenic calli were transferred to callus selection medium. After two weeks the cultured calli were shifted to fresh medium. Survival percentage of bombarded and un-bombarded embryogenic calli was noted after four weeks. Histochemical *GUS* assay was done by transferring the bombarded calli to X-Gluc staining solution, followed by overnight incubation at 37 °C, as described by Jefferson et al. (1987). The number of calli showing *GUS* activity was recorded and percentage was calculated.

Statistical analyses

Statistical analysis was performed using generalized linear model-SAS (GENMOD with binomial distribution and logit link) to know about the significance and interaction of observed parameters.

RESULTS

Response of different genotypes to callus induction, embryogenesis and regeneration

Callus induction, embryogenesis and regeneration were observed in the all selected five genotypes. Different stages of tissue culture that is callus induction and subsequent plantlet regeneration are shown in Fig. 1A-F. It was observed that the callus induced in the genotypes CSSG-668, CPF-237 and S-2003US114 was compact, nodular and embryogenic, while in genotypes CPF-245 and S-2003US633, it was shiny, brownish, semi-translucent and mostly non-embryogenic. These physical characteristics were used to distinguish embryogenic and non-embryogenic calli (Fig. 1G and H). Callus induction rate varied from 77 to 91 % in different cultivars and advanced lines. Maximum callus induction (91%) was observed in cv. CPF-245 followed by cv. CSSG-668 (85%) while minimum callus induction (77 %) was observed in cv. CPF-237 and S-2003US-633 (Figure 2). Embryogenic calli induction ranged from 28 to 75% in all evaluated genotypes. Maximum embryogenic calli production (75%) was observed in cv. CSSG-668 followed by S-2003US114 (69 %) while the cv. CPF-245 produced only 28 % embryogenic callus. Similarly, maximum regeneration was observed in cv. CSSG-668 (77%) followed by cv. S-2003US114 (73 %) and minimum regeneration (35 %) was observed in cv. CPF-245 (Figure 2).

Effect of different concentrations of geneticin on selection of callus and subsequent regeneration

In the development of transgenic plants, optimum concentration of selective agent is very critical to select the true transgenic events. In this study, effect of different concentrations of selective agent (geneticin-418) was observed on callus survival and regeneration from calli. Callus survival percentage was the same for all tested genotypes at 0 mg/L because all genotypes had the same intercepts. The effect of different concentrations of selective agent on callus survival for genotypes (CSSG-668, CPF-245 and S-2003US633) was similar as indicated in Figure 3, showing the same slope for these cultivars. Sixty milligram geneticin was found to be the suitable dose to select the calli of genotypes CSSG-668, CPF-245 and S-2003US633. In contrast, S-2003US114 showed different response to geneticin concentrations and 35 mg/L was found to be suitable dose to select the calli of this cultivar and the slope of this cultivar was also different with respect to other cultivars (Table 2; Figure 3). Interactions between cultivars and doses were also significant for callus survival (Table 4).

The different genotypes showed significant variation in regeneration efficiency on medium without geneticin (control) as indicated by different intercepts (Figure 4). In

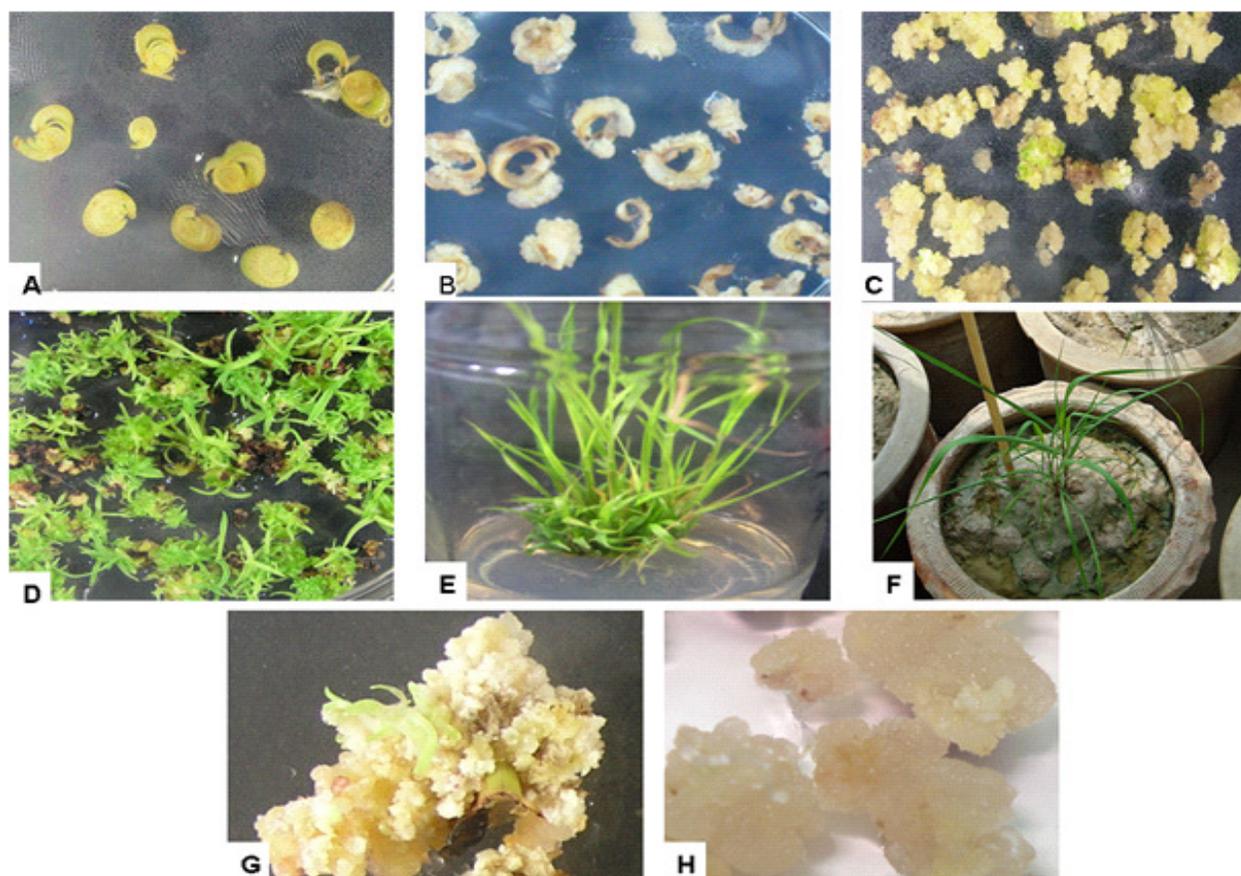


Figure 1. Tissue culture stages in sugarcane; (A) Apical discs explants on callus induction medium ; (B) Callus induction from explants; (C) callus multiplication; (D) regeneration; (E) regenerated plants on rooting medium; (F) regenerated plants in soil; (G) embryogenic calli and (H) non-embryogenic calli.

contrast, genotypes, CPF-245 and S-2003US633 had shown similar regeneration efficiency and intercepts. The genotypes (CPF-245 and S-2003US114) responded to the increasing dose of selective agent similarly as the slope of regression line was constant between these genotypes but CSSG-668 and S-2003US-633 had shown different patterns. Geneticin (60 mg/L) was found to be optimum for the 10 % survival of regenerated plantlets in the cultivars CSSG-668 and CPF-245 while for the cultivars S-2003US114 and S-2003US633) it was 40, 25 mg/L, respectively (Table 3; Figure 4). Interactions between cultivars and doses were also significant for regeneration efficiency (Table 4).

Effect of optimized geneticin (G-418) concentration on transformed sugarcane calli and *GUS* expression

Biolistic mediated transformation method was successfully used to transform the sugarcane genotype CSSG-668 with *nptII* selectable marker and *GUS* reporter gene. Successful transformation can be seen by the

presence of difference in physical effects of geneticin on transformed and non-transformed embryogenic calli of cv. CSSG-668 and the presence of *GUS* expression in transformed embryogenic calli. Growth of non-transformed embryogenic calli was completely inhibited at 60 mg/L G-418 and the transformed embryogenic calli showed the optimum or normal growth at this concentration (Figure 5). Seventy seven percent of bombarded embryogenic calli were killed at 60 mg/L geneticin, while none of the non-transformed calli survived at this concentration. Ninety three percent of the geneticin resistant calli showed *GUS* expression when subjected to histochemical *GUS* assay (Figure 6).

DISCUSSION

Availability of reproducible and efficient somatic embryogenesis system strengthens the transgenic technology in sugarcane because embryogenic calli is the most suitable target tissue for genetic transformation (Snyman et al., 1996). We have optimized a suitable

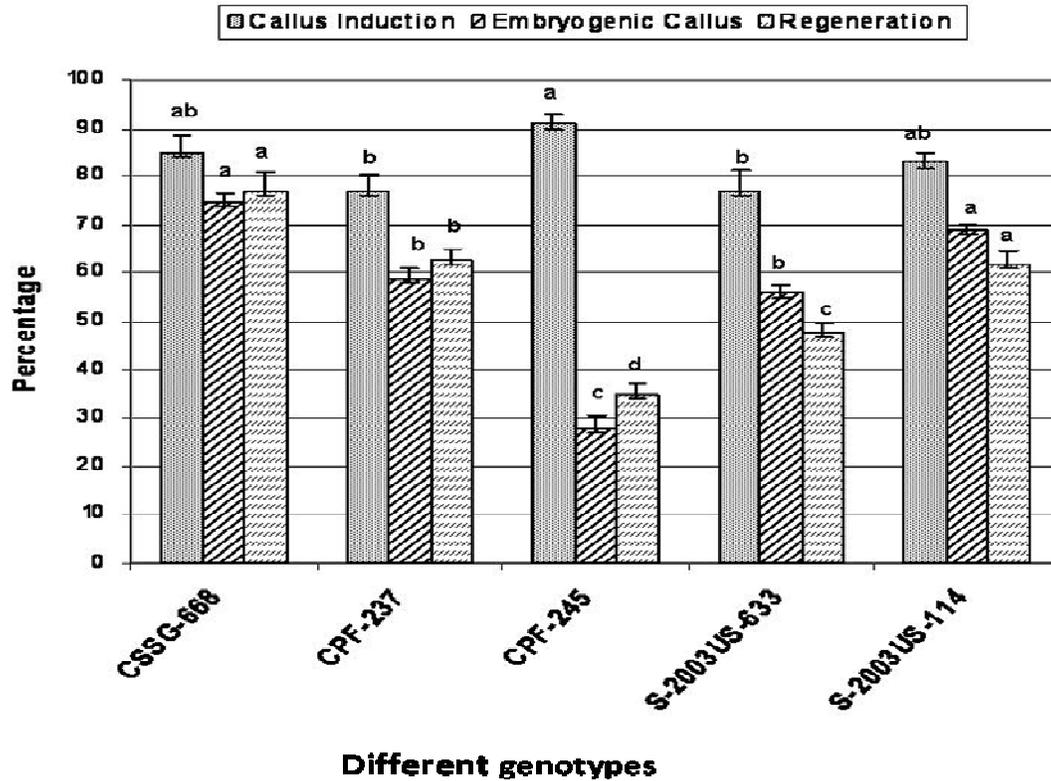


Figure 2. Callus induction, embryogenic callus and regeneration percentage of five different sugarcane genotypes.

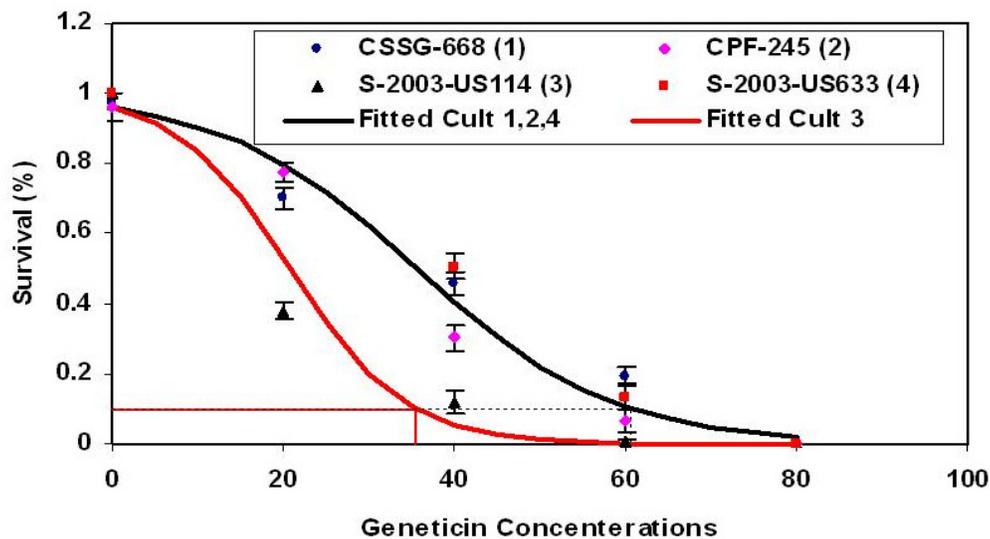


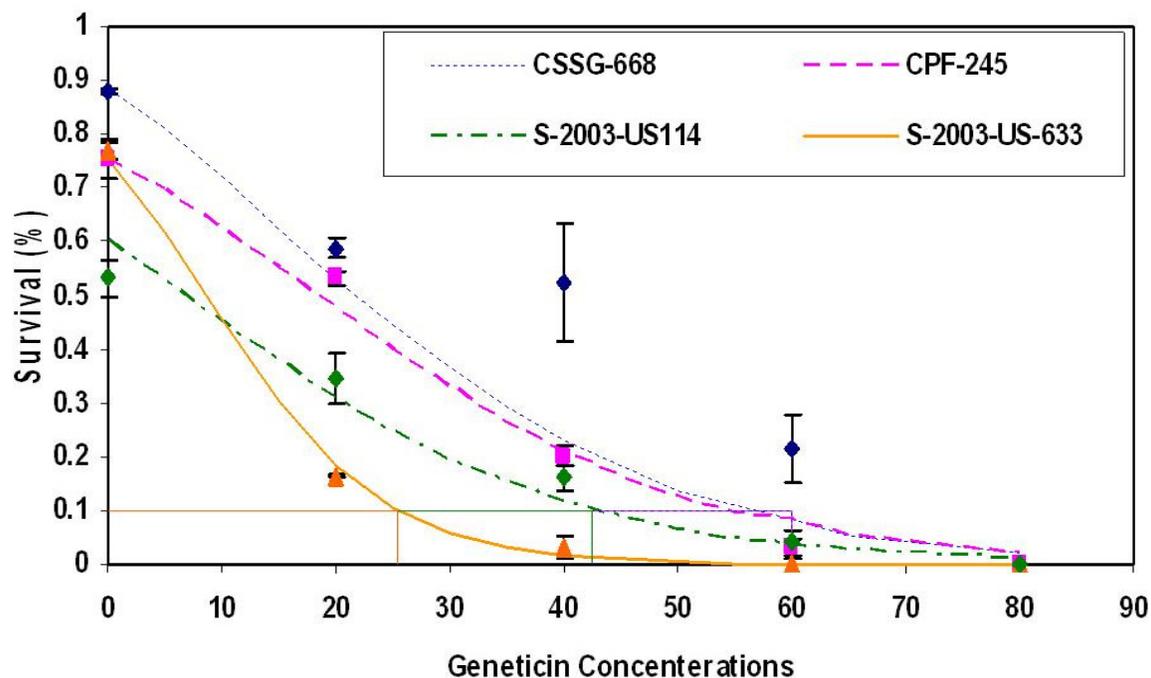
Figure 3. Effect of different concentrations of geneticin on callus survival of different cultivars.

media for production of embryogenic calli and regeneration in sugarcane (Unpublished data). These optimized media were used to observe the response of different sugarcane genotypes to callus induction,

embryogenic callus production and regeneration. The results showed that callus induction, embryogenic callus production and regeneration varied among the evaluated genotypes which might be due to genotypic variability

Table 2. Analyses of parameters estimates for effect of geneticin dose on callus survival in different genotypes.

Parameter	Estimate	SE	Pr > chisq	Geneticin doze for 10% survival (mg/L)
Intercept (1,2,3,4)	3.1310	0.1708	<0.001	
Slope				
Cultivar (1,2,4)	-0.1508 + 0.0627	0.0084	< 0.001	60
Cultivar (3)	- 0.1508	0.1000	< 0.001	35

**Figure 4.** Effect of different concentrations of geneticin on regeneration of different cultivars.**Table 3.** Analyses of parameters estimates for effect of geneticin dose on regeneration in different genotypes.

Parameter	Estimate	SE	Pr> chisq	Cultivars	Geneticin Doze for 10% survival (mg/L)
Intercept					
Cultivar (CPF-245, S-2003-US114)	1.1281	0.3596	<0.001	CSSG-668	60
Cultivar (CSSG-668)	2.0408	0.3733	<0.001	CPF-245	60
Cultivar (S-2003-US633)	0.4153	0.168	<0.001		
Slope					
Cultivar (CSSG-668, CPF-245, S-2003 US633)2,4,)	-0.1305 + 0.0689	0.0244	<0.001	S-2003-US633	45
Cultivar (S-2003-US114)	- 0.1305	0.0124	<0.001	S-2003-US114	25

(Figure 2). These results are in agreement with the findings of Gandonou et al. (2005) in sugarcane. They observed that callus induction rate varied from 69.23 to 95.87 % which indicated that callus induction ability is greatly influenced by the genotypes. Moreover, Badawy

et al. (2008) and Burner (1992) had also reported that the callus induction capacity in sugarcane is genotype dependent.

Ability of different genotypes to produce the embryogenic calli is the most critical parameters (Bower

Table 4. Chi-Square means of geneticin dose, cultivar and dose-cultivar interaction for callus survival and regeneration.

Type 3 analysis for callus survival				Type 3 analysis for regeneration			
Source	DF	Chi-Square	Pr > ChiSq	Source	DF	Chi-Square	Pr > ChiSq
Cult	3	10.72	0.0134	Cult	2	70.52	<.0001
Dose	1	1168.11	<.0001	Dose	1	678.79	<.0001
Dose*Cult	3	21.17	<.0001	Dose*Cult	1	53.53	<.0001

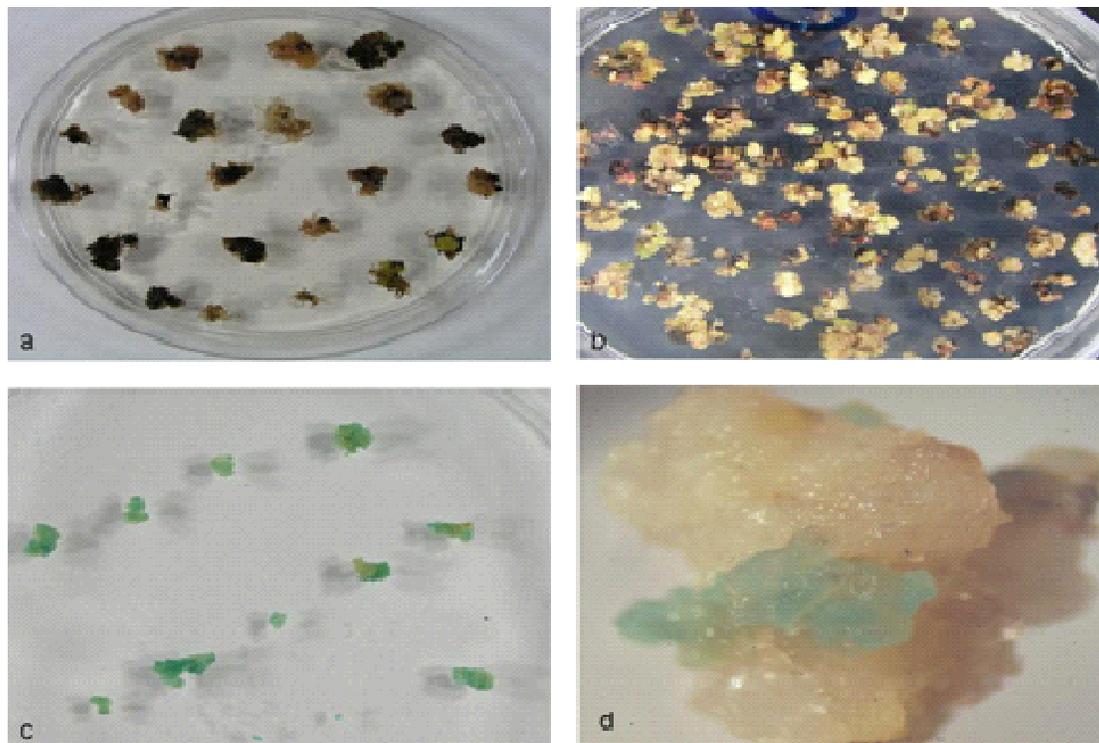


Figure 5. Physical effects of geneticin on (A) non-transformed and transformed (B) embryogenic callus; (C) *GUS* activity of survived embryogenic callus and (D) microscopic view of calli showing *GUS* activity.

and Birch, 1992). It reveals the capacity of calli to regenerate plantlets from one cell or few numbers of cells. Our results showed the presence of significant variation (28-75 %) in different genotypes for the production of embryogenic calli. This variation in callus production of different genotypes was also reported by other authors who were able to produce embryogenic callus in sugarcane (Liu, 1993; McCallum et al., 1998; Gandonou et al., 2005). On the contrary, Badawy et al. (2008) reported that all three genotypes of sugarcane in their experiment, showed high embryogenic callus percentages (about 95%) but non-significant difference were observed among them. Significant variations in regeneration of all the tested genotypes in the present study were observed that may have resulted due to the genetic variability in the genotypes which showed different behavior for callus induction and

regeneration, these results are in line with the results of Gill et al. (2004) in sugarcane. They concluded that two varieties showed significant difference in their response for percent shoot regeneration. Similar results were also reported by Gandonou et al. (2005) in sugarcane. On the contrary, Khan et al. (2009) observed non-significant difference in shoot induction from three different sugarcane cultivars.

Isolation and selection of transformed cells containing stably integrated gene is one of the major steps in production of transgenic plants which can be achieved by knowing the minimum concentration of selective agent that can inhibit the growth of non-transformed cells and allow transformed cells to survive. This will make the transformation process more efficient resulting in very low occurrences of chimeras. Sreeramanan et al. (2006) stated that determination of optimum concentration of

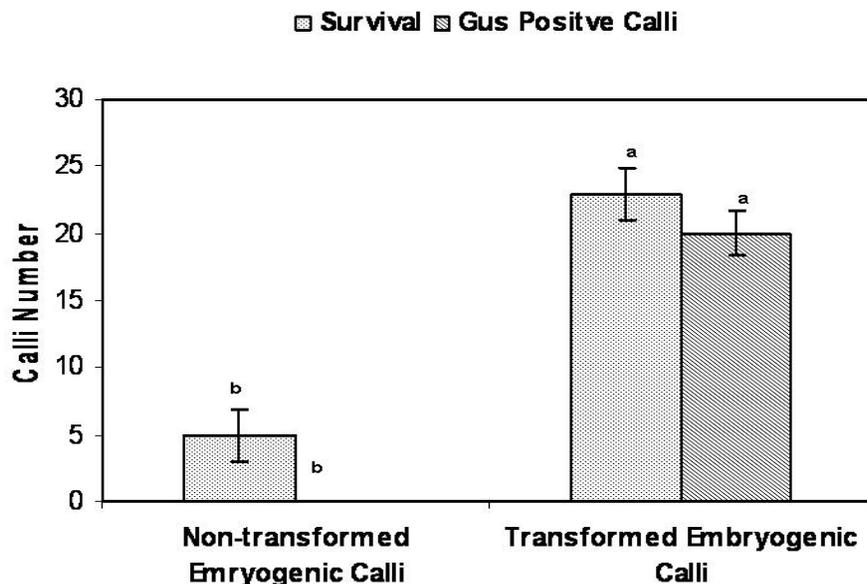


Figure 6. Survival and *GUS* positive calli number of transformed and non-transformed embryogenic callus.

selective agent is the most important step in stable transformation as this can make selection process more efficient. Since, the minimum inhibitory concentration of selective agent is tissue and species specific (Parveez et al., 1996), we have optimized the minimum inhibitory concentration of geneticin (G-418) for embryogenic calli of different sugarcane genotypes. It was observed that geneticin (G-418) is efficient selection agent for embryogenic sugarcane calli. Similarly, effectiveness of geneticin in sugarcane was reported by Elliot et al. (1999). This variation in different monocots might be due to different levels of endogenous resistance which means that endogenous resistance may be tissue or genotype specific (Parveez et al., 1996).

It was found that 60 mg/L geneticin (G-418) concentration was the most effective for cv. CSSG-668, CPF-245 and S-2003US633, while 35 mg/L was effective for S-2003US114. This variation in sensitivity of cv. S-2003-US114 to selective agent might be due to different level of endogenous resistance. Similarly, Van Boxtel et al. (1995) reported that the sensitivity to selective agents was genotype dependent. On the other hand, similar sensitivity of other three genotypes to selective agent might be due to same level of endogenous resistance. These optimum concentrations may increase effectiveness of the selection system for tested genotypes but these were contrary to the report of Bower et al., (1996) in sugarcane.

In the present study cultivar CSSG-668 proved to be very useful for efficient callus induction, embryogenic callus production and bombarded embryogenic calli gave of embryogenic calli of sugarcane was achieved through biolistic mediated transformation method. Similar findings

good transformation efficiency. The stable transformation were also achieved in sugarcane by Vickers et al., (2005); Khalil (2002), Bower and Birch, (1992), Bower et al., (1996). It was confirmed by the presence of resistance in transformed tissue to high dose of geneticin and most of the survived tissue (93 %) at this concentration showed *GUS* expression. Similarly, Christou (1996) reported that β -glucuronidase is useful for detecting the tissues receiving foreign genes and for determining the number of expressing cells. The present study might be useful to produce embryogenic calli, which can be transformed efficiently under optimum concentration (60 mg/L) of the selectable marker like geneticin. This study might also be helpful to screen other genotypes for callus induction, regeneration and transformation purposes.

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REFERENCES

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

- Anonymous (2008-2009). Pakistan Economic Survey, Finance Division, Government of Pakistan, (www.finance.gov.pk).
- Asad S, Arshad M, Mansoor S, Zafar Y (2009). Effect of various amino acids on shoot regeneration of sugarcane (*Saccharum officinarum* L.). *Afr. J. Biotechnol.* 8(7): 1214-1218.
- Asad S, Mukhtar Z, Nazir F, Hashmi JA, Mansoor S, Zafar Y, Arshad M (2008). Silicon Carbide Whisker-Mediated Embryogenic Callus Transformation of Cotton (*Gossypium hirsutum* L.) and Regeneration of Salt Tolerant Plants. *Mol. Biotechnol.* 40: 161-169.
- Badawy OM, Nasr MI, Alhendawi RA (2008). Response of sugarcane (*Saccharum* species hybrid) genotypes to embryogenic callus induction and *in vitro* salt stress. *Sugar Tech.* 10(3): 243-247.
- Barba R, Nickell LG (1969). Nutrition and organ differentiation in tissue culture of sugarcane—a monocotyledon. *Planta*, 89: 299-302.
- Bower R, Birch RG (1992). Transgenic sugarcane plants via micro-projectile bombardment. *Plant J.* 2: 409-416.
- Bower R, Elliott AR, Potier BAM, Birch RG (1996). High-efficiency, micro-projectile mediated co-transformation of sugarcane, using visible or selectable markers. *Mol. Breed.* 2(3): 39-249.
- Brisibe EA, Nishioka D, Miyake H, Taniguchi T, Maeda E (1993). Developmental electron microscopy and histochemistry of somatic embryo differentiation in sugarcane. *Plant Sci.* 89: 85-92.
- Burner MD (1992). Regeneration and phenotypic variability of plants cultured *in vitro* from mature sugarcane caryopses. *J. Am. Soc. Sugar Cane Technol.* 12: 82-90.
- Christou P (1996). Transformation technology. *Trends Plant Sci.* 1: 423-431.
- Economic Survey of Pakistan (2008-09). Finance Division, Government of Pakistan, (www.finance.gov.pk).
- Elliott AR, Cambell JA, Dugdale B, Brettell RIS, Grof CPL (1999). Green fluorescent protein facilitates rapid in-vivo detection of genetically transformed plant cells. *Plant Cell Rep.* 20: 157-162.
- Food and Agriculture organization (FAO) (2009). Food outlook June.
- Falco MC, Mendes BMJ, Neto AT, Da-Gloria BA (1996). Histological characterization of *in-vitro* regeneration of *Saccharum* sp. R. *Bras. Fisiol. Veg.* 8(2): 93-97.
- Fitch M, De La Cruz A, Moore P (1995) Effectiveness of different selection markers for sugarcane transformation. *Plant Genome II; Int Plant Genome Conf.* p. 57.
- Gandonou CH, Errabii T, Abrinii J, Idaomar M, Chibi F, Skali Senhaji N (2005). Effect of genotype on callus induction and plant regeneration from leaf explants of sugarcane. *Afr. J. Biotechnol.* 4(11): 1250-1255.
- Gill NK, Gill R, Gosal SS (2004). Factors enhancing somatic embryogenesis and plant regeneration in sugarcane (*Saccharum officinarum* L.). *Indian J. Biotechnol.* 3: 119-123.
- Jefferson RA, Kavanagh TA, Bevan MW (1987). *GUS* fusions: β -glucuronidase as a sensitive and versatile gene fusion in higher plants. *EMBO J.* 6: 3901–3907.
- Jenes B, Moore H, Cao J, Zhang W, Wu R (1993). Techniques for gene transfer. In Kung S, Wu R (eds). *Transgenic Plants*, San Diego: Academic Press Inc. 1: 125-146.
- Khalil SM (2002). Regeneration via somatic embryogenesis and micro-projectile mediated co-transformation of sugarcane. *Arab. J. Biotechnol.* 5(1): 19-32.
- Khalid A, Ashfaq M, Akram W, Choi JK, Lee J (2005). Effect of plant factors, sugar contents, and control methods on the Top Borer (*Scirpophaga nivella* F.) Infestation in selected varieties of sugarcane. *Entomological Res.* 35: 153-160.
- Khan IA, Dahot MU, Seema N, Yasmeen S, Bibi S, Raza G, Khatri A, Naqvi MH (2009). Direct regeneration of sugarcane plantlets: a tool to unravel genetic heterogeneity. *Pak. J. Bot.* 41(2): 797-814.
- Khan IA, Khatri A, Nizamani GS, Siddiqui MA, Khanzada, MH, Dahar NA, Seema N, Naqvi MH (2004). *In vitro* studies in sugarcane. *Pak. J. Biotech.* 1: 6-10.
- 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(1): 41-43.
- Lakshmanan P, Geijskes RJ, Aitken KS, Grof CPL, Bonnett GD, Smith GR (2005). Sugarcane biotechnology: the challenges and opportunities. *In-Vitro Cell. Dev. Biol. Plant*, 41: 345-363.
- Liu MC (1993). Factors affecting induction, somatic embryogenesis and plant regeneration of callus from cultured immature inflorescences of sugarcane. *J. Plant Physiol.* 141: 714-720.
- McCallum J, Snyman SJ, Meyer GM, Botha FC (1998). Comparison of selected commercial sugarcane varieties for use in a stable transformation programme. *Proc S. Afr. Sug. Technol. Ass.* p. 72.
- Ming R, Moore PH, Woo KK, D'Hont A, Glaszmann JC, Tew TL, Mirkov TE, Silva JD, Jifon J, Rai M, Schnell RJ, Brumbley SM, Lakshmanan P, Comstock JC, Paterson AH (2006). Sugarcane improvement through breeding and biotechnology. *Plant Breed. Rev.* 27: 17-117.
- Nasir NM, Qureshi RH, Aslam M (2000). Effect of salinity on emergence of sugarcane lines. *Pak. Sugar J.* 15: 12-14.
- Park S, Rose S, Zapata C, Srivatanakul M, Smith R (1998). Cross protection and selective marker genes in plant transformation. *In-Vitro Cell Dev. Biol. Plant.* 34: 117-121.
- Parveez GKA, Chowdhury MKU, Saleh NM (1996). Determination of minimal inhibitory concentration of selection agents for oil palm (*Elaeis guineensis* Jacq.) transformation. *Asia Pacific J. Mol. Biol. Biotech.* 4: 219-228.
- Parveez GKA (1998). Optimization of parameters involved in the transformation of oil palm using the biolistic method. Ph.D. Thesis, Universiti Putra, Malaysia.
- 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.
- Sreeramanan M, Maziah M, Abdullah MP, Rosli NM, Xavier R (2006). Potential selectable marker for genetic transformation in Banana. *Biotech.* 5(2): 189-197.
- Van Boxtel J, Eskes A, Berthouly M (1995). Glufosinate as an efficient inhibitor of callus proliferation in coffee tissue. *In-Vitro Cell. Dev. Biol. Plant*, 33: 6-12.
- 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. *Aust J. Agric. Res.* 56(1): 57-68.