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IDEAL *IN-VITRO* CULTURE AND SELECTION CONDITIONS FOR SUGARCANE GENETIC TRANSFORMATION

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The study was aimed to identify the best combination of auxins, cytokinins, amino acids concentrations for callus induction, somatic embryogenesis, regeneration and rooting response of elite sugarcane (*Saccharum officinarum* L.) clones. Furthermore, optimal geneticin (G-418) dose has also been standardized, to select the genetically transformed sugarcane plantlets. Five different media for callus induction, embryogenesis, regeneration and rooting were tested to screen, seven elite sugarcane clones (HSF-240, SPF-213, CPF-237, CPF-245, CPF-246, CSSG-668 and CSSG-676). Different levels of selection marker (geneticin) concentrations (0, 30, 40, 50, 60 and 70 mg L^{-1}) were used in sugarcane regeneration and rooting medium. Significant variations in callus induction (49.3-86 %), somatic embryogenesis (33.3-73.3 %), regeneration (48-87 %) and rooting (46-92 %) response were observed in different media and sugarcane clones. Sugarcane clones CSSG-668, HSF-240 and CPF-237 were found to be the best for sugarcane tissue culture. Furthermore, 60 and 40 mg L^{-1} geneticin was found to be optimum concentration to select the transformed sugarcane plantlets at regeneration and rooting stage, respectively. The findings of the study could be used in sugarcane genetic transformation, micro-propagation and for the development of somaclones.

Keywords: Sugarcane, growth regulators, callus induction, regeneration, geneticin

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.), ranks amongst the world's top ten food crops and contributes 60-70% of annual sugar production in the world. More recently, it has emerged as an important bio-energy crop (Waclawovsky *et al*., 2010). Pakistan has a prominent position in the sugar producing countries in the world. It ranks 5th in sugarcane growing area, cane production and holds 15th position in global sugar production (Bashir *et al*., 2012). Its contribution in value addition to agriculture and GDP are 3.2 and 0.7 percent, respectively (Economic Survey, 2012-13). With the efforts of conventional, modern breeding and advanced biotechnological approaches, sugarcane has become the ideal crop for the future that will be helpful in fulfilling the food, feed, fiber and biofuel requirements of rapidly growing population. To meet the sugar need of the increasing population of our country and to make the sugarcane a profitable crop for the farmers, it is much needed to improve the quantity and quality of sugarcane and sugar per unit area, because despite of all efforts sugar and sugarcane production in Pakistan is still much lower than most of advanced cane growing countries of the world (Qureshi and Afghan, 2005; Arian *et al*., 2011). Sugarcane improvement through breeding is only possible in the countries where sugarcane flowers profusely and produces viable cane fuzz (Barbosa *et al*., 2012). Profuse and viable cane fuzz is produced in a decreasing day length of 12.0 hours to 11.5 hours, in the locations near to equator and it is affected badly in the subtropical and temperate areas, due to unfavorable night temperature (Junejo *et al*., 2012). Improvement of sugarcane through conventional breeding is difficult in Pakistan because the climatic conditions are not favourable for viable cane fuzz production (Shahid *et al*., 2011). In Pakistan, sugarcane improvement is dependent only on selection breeding of the imported cane fuzz (Khan *et al*., 2004). So, there is a dire need for the applications of modern biotechnological approaches such as the development of transgenic plants and exploitation of somaclonal variation to improve sugarcane clones for desirable traits. For the applications of these approaches, optimization of ideal tissue culture conditions is preliminary requirement. Tissue culture technique is being widely used in sugarcane for micro-propagation, rapid in-vitro multiplication of the elite sugarcane clones as well as for the improvements through somaclonal variation (Siddiqui *et al*., 1994; Leal *et al*., 1996; Bahera *et al*., 2009). Different amino acids and other hormonal concentrations have been optimized to achieve good somatic embryogenesis from callus (Nieves *et al*., 2008). The present research work was planned to find the best medium composition to screen out locally grown elite sugarcane clones for good tissue culture response. The clones could be further utilized for genetic transformation and for the exploitation of somaclonal variation. Another objective of this study was to determine the optimal concentration of selection agent (geneticin) to differentiate non-transformed calli form the transformed one, which is very important step in genetic transformation. Efficient

tissue culture and selection system will ensure successful genetic transformation for the development of the desired qualitative and quantitative agronomic traits in the local elite sugarcane clones.

MATERIALS AND METHODS

***Plant material*:** Six to eight months old sugarcane tops of five commercial (HSF-240, SPF-213, CPF-237, CPF-245, CPF-246) and two promising clones (CSSG-668, CSSG-676) were taken from Sugarcane Research Institute (SRI), Ayub Agricultural Research Institute (AARI), Faisalabad and Shakarganj Sugar Research Institute (SSRI) Jhang, respectively.

***Preparation of explants*:** The tops were washed with sterile water followed by cleaning with autoclaved cotton dipped in ethanol to remove any external contamination. The cleaned tops were taken into laminar air flow and external whorls of the leaf were removed with sterile scalpel and blade. The peeled leaf sheath were again sterilized with 70 % ethanol for 30 seconds and thoroughly washed with sterile distilled water. Finally, leaf sheaths were removed carefully around the meristem until the cylindrical portion of 5 mm diameter were obtained with primordial and developing leaves adjacent to meristem.

***Callus initiation and somatic embryogenesis*:** The cylindrical portion was sliced into 1-3 mm thick sections. Explants were cultured on five different types of callus induction media SC-1-SC-5 in three replicates (Table 1). The cultured plates were kept in the controlled temperature room under dark conditions at 26 ± 2°C and sub-culturing was done on fresh medium every 15 days to ensure continuous supply of the nutrients. Callus formation was observed after 4-6 weeks in different clones. Final observation for callus formation was taken after eight weeks and callus induction percentage was calculated from the explants used for the experiment. Then induced calli were shifted to different embryogenic callus induction medium SEC-1 –SEC-5 (Table 1) and plates were placed in the same culture conditions as for the callus induction. Embryogenic calli formation data was collected after 6 weeks of culture and efficiency was calculated on percentage basis for different clones at different media composition.

***Shoots regeneration and rooting*:** Twenty five embryogenic calli (5-7 mm) of each sugarcane clone were cultured in three replicates on different sugarcane regeneration media, SR-1 to SR-5 (Table 1) with same culture conditions as for callus induction except putting in light (2500-3000 lux). After eight weeks, regeneration frequency was calculated by dividing the regenerated calli with total number of used explants (calli) multiplied by hundred. Eight regenerated shoots (2-3 cm long) of each clone were cultured in three replicates on five different rooting media SRM-1 to SRM-5 (Table 1) and kept in the same culture conditions as mentioned for regeneration. After four weeks, root initiation percentage was calculated from all the evaluated sugarcane clones.

***Optimization of geneticin concentration at regeneration and rooting stage*:** Geneticin (G-418, Phyto Technology Laboratories®, USA) was used as selection marker. For selection media optimization, 6-8 week old twenty five non-transformed embryogenic calli and ten regenerated shoots (2-3 cm long) were cultured in three replicates on six different regeneration and rooting selection media (SRS-1 to SRS-6 and SRSM-1 to SRSM-6), respectively. These media contained variable geneticin concentrations (Table 2). After 4 weeks, survival percentage was calculated and the concentration with 10 % survival rate was selected as

Table 1. Composition of different media used for callus induction, embryogenesis, regeneration and rooting in sugarcane

Treatments	Callus induction media					Embryogenic callus media					Regeneration media					Rooting media				
	SC-1	SC-2	SC-3	SC-4	SC-5	SEC-1	SEC-2	SEC-3	SEC-4	SEC-5	SR-1	SR-2	SR-3	SR-4	SR-5	SRM-1	SRM-2	SRM-3	SRM-4	SRM-5
Casein Hydrolysate (gL^{-1})	0	0.25	0.50	0.75	1.0	0	0.25	0.50	0.75	1.0	-	-	-	-	-	-	-	-	-	-
2-4-D ($mg\,L^{-1}$)	1.5	2.5	3.5	4.0	4.5	1.0	1.25	1.50	1.75	2.0	0	0.12	0.25	0.50	0.75	-	-	-	-	--
Arginine ($mg\,L^{-1}$)	0	1.5	2.5	3.5	4.5	0	1.5	2.5	3.5	4.5	0	1.50	2.50	3.50	4.50	0.5	0.5	0.5	0.5	0.5
Glycine ($mg\,L^{-1}$)	0	1.5	2.5	3.5	4.5	0	1.5	2.5	3.5	4.5	0	1.50	2.50	3.50	4.50	1.5	1.5	1.5	1.5	1.5
Cystine ($mg\,L^{-1}$)	0	1.5	2.5	3.5	4.5	0	1.5	2.5	3.5	4.5	0	1.50	2.50	3.50	4.50	2.5	2.5	2.5	2.5	2.5
Coconut water (V/V) %	0	2.5	5.0	7.5	10.0	0	2.5	5.0	7.5	10.0	-	-	-	-	-	-	-	-	-	-
BAP ($mg\,L^{-1}$)	-	-	-	-	-	-	-	-	-	-	0.5	1.00	1.50	2.00	2.50	3.5	3.0	2.5	2.0	1.5
Kinetin ($mg\,L^{-1}$)	-	-	-	-	-	-	-	-	-	-	0	0.00	0.25	0.50	0.75	-	-	-	-	-
NAA ($mg\,L^{-1}$)	-	-	-	-	-	-	-	-	-	-	0	0.25	0.50	0.75	1.00	-	-	-	-	-
IBA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.5	4.0	3.5	3.0	2.5

SC-1 to SC-5 and SEC-1 to SEC-5 supplemented with 4.43 $g\,L^{-1}$ MS salts (Phyto Technology Laboratories), 1 $g\,L^{-1}$ Myo-inositol, 30 $g\,L^{-1}$ Sucrose, 3.6 $g\,L^{-1}$ Phytagar, pH 5.7-5.8.

SR-1 to SR-5 and SRM-1 to SRM-5 supplemented with 2.20 $g\,L^{-1}$ MS salts with vitamins + 1 $g\,L^{-1}$ Myo-inositol, 30 $g\,L^{-1}$ Sucrose, 3.60 $g\,L^{-1}$ Phyt agar, pH 5.7-5.8.

Each media was sterilized by autoclaving at 121°C for 20 min and cooled to 45^{0}C. Then, poured (~35 mL) into 15 x 100 mm petri plates except rooting media which was poured into glass jars.

Table 2. Composition of different sugarcane regeneration and rooting selection media

Treatments	Regeneration selection media						Rooting selection media					
	SRS-1	SRS-2	SRS-3	SRS-4	SRS-5	SRS-6	SRSM-1	SRSM-2	SRSM-3	SRSM-4	SRSM-5	SRSM-6
Geneticin (mg.L^{-1})	0	30	40	50	60	70	0	30	40	50	60	70

SRS-1 to SRS-5 supplemented with MS salts with vitamins 4.43 g L^{-1}, Myo-inositol 01 g L^{-1}, 2,4-D 0.25 mg L^{-1}, Casein Hydrolysate (CH) 0.5 g L^{-1}, NAA 0.50 mg L^{-1}, Arginine 2.5 mg L-1, Sucrose 30 g L^{-1}, Glycine 2.5 mg L^{-1}, Cystine 2.5 mg L^{-1}, BAP 1.5 mg L^{-1}, Kinetin 0.25 mg L^{-1}, Phytagar 3.60 g L^{-1} and pH between 5.7 to 5.8.

SRSM-1 to SRSM-5 supplemented with ½ MS (2.2 g L^{-1}), Myo-inositol 01 g L^{-1}, 2,4-D 0.25 mg L^{-1}, CH 0.5 g L^{-1}, NAA 0.50 mg L^{-1}, Arginine 2.5 mg L^{-1},Glycine 2.5 mg L^{-1}, Cystine 2.5 mg L^{-1}, BAP 0.25 mg L^{-1}, IBA 1.5 mg L^{-1} , Phytagar 3.60 g L^{-1}, Sucrose 60 g L^{-1} and pH 5.7-5.8.

Each media was sterilized by autoclaving at 121^{0}C for 20 min and cooled till 45^{0}C. Then, poured (~ 35 mL) into 15 x 100 mm petri plates except rooting media which was poured into glass jars.

optimal dose.

***Statistical analysis*:** The data were analyzed by using the statistics 8.1 software and treatments were compared by using least significant difference (LSD) at 5% probability level.

RESULTS AND DISCUSSION

***Optimal growth hormones, amino acids for in vitro sugarcane regeneration*:** An efficient protocol supporting good callusing and regeneration is essential for successful genetic transformation of the commercial clones with minimal somaclonal variation (Asad *et al*., 2009). In the present study, sugarcane plantlets were regenerated successfully from all the tested clones. Ideal growth regulators and amino acids combination were identified for the sugarcane callus induction, somatic embryogenesis, regeneration and rooting. Amino acids are generally used in tissue culture media as a source of organic nitrogen and as a requirement for embryo specific genes (Grewal *et al*., 2006). Significantly, variable callus induction (49.3-86%), somatic embryogenesis (33.3-73.3%), regeneration (48-87%) and rooting (46-92%) were observed in all tested media and clones (Fig. 1 a, b, c, d). Variation in regeneration ability was observed among the seven clones; however some clones showed similar results for regeneration. Maximum callus induction (86%) was observed in CSSG-668 at SC-4, while minimum 52 and 49.3% was observed in CPF-245 and CPF-246 at SC-1 and SC-5 media compositions (Table 1). Out of the five tested combinations, SC-4 showed the best response for callus induction in all the cultured clones. However, non-significant difference was observed for callus production at SC-3 and SC-4 media for the clones HSF-240, SPF-213 and CPF-246 (Fig. 1a). Findings in this study indicated that 4 mg

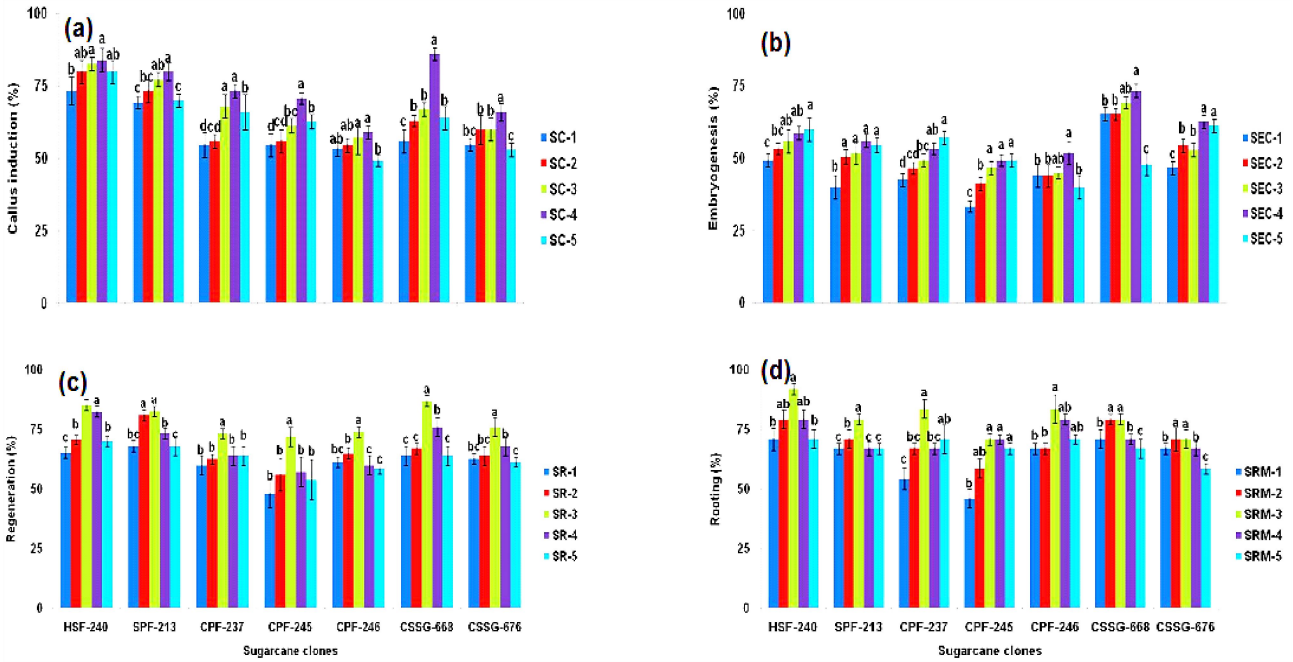

Figure 1. Percentage response of seven sugarcane cultivars on different media combinations for; (a) callus induction, (b) embryogenesis, (c) regeneration, (d) rooting initiation. Error bars indicate the standard deviation of mean (n=3). Graph bars with the same letter are not significantly different at $P<0.05$.

L^{-1} 2,4-D, was the best for callus induction which is in line with previous reports (Kureel *et al*., 2006). Furthermore, addition of amino acids also helped in enhancement of callus induction and optimum concentration was 3.5 mg L^{-1} for all tested amino acids. This study also showed that callus development was dependent on genotype which is in accordance with the findings of Badawy *et al*., (2008). Callus texture and colour was also noted and it was observed that the callus at SC-3 and SC-4 medium was mostly white, yellow brown, granular and compact having maximum capability for embryogenesis and regeneration. While at SC-1, SC-2 and SC-5, most of the produced calli were loose granular, watery, watery loose, watery compact to compact texture. The results support the findings of Badawy *et al*. (2008) who also found similar type of callus induction in sugarcane. These physical properties have shown the impact on embryogenesis and regeneration of the clones. Maximum somatic embryogenesis (73.3%) was observed in the clone CSSG-668 at SEC-4 combination, while minimum (33.3%) in CPF-245 at SEC-1 (Fig. 1b). The results showed that decreased 2,4-D concentration and presence of amino acids were important for better embryogenesis. Optimum concentration of 2,4-D and of all tested amino acids was 1.75 mg L^{-1}, 3.5 mg L^{-1}, respectively. It was also evaluated that the capacity of clones for embryogenic callus induction was variable in different clones at different media. However, SEC-4 media combination was found to be the best for embryogenesis in all the tested clones (Fig. 1b). Previously similar response for callus production in different sugarcane clones has been reported (Bower and Birch, 1992). Regeneration was also observed in all the cultivars and tested combinations (Fig. 2). Maximum regeneration (87%) was observed in the clone, CSSG-668 followed by HSF-240 (85.3%) at SR-3 media combination while minimum regeneration (48%) was observed in CPF-245 at SR-1. All the clones exhibited better regeneration ability at SR-3 media. However, SPF-213 showed similar response for regeneration at SR-2 media (Fig. 1c). Rooting response was different at different concentrations of auxin and cytokinin, and different clones used (Fig. 3). Among the tested concentrations, 3.5 mg L^{-1} IBA and 2.5 mg L^{-1} BAP were found most optimum for rooting. Among the tested clones maximum rooting (92%) was observed in HSF-240 within 15 days of shoots culturing. On the other hand, lowest rooting response (46%) was observed in the clones CPF-245 at SR-1 media combination with higher concentration of IBA (4.5 mg L^{-1}), BAP (3.5 mg L^{-1}), and without amino acids (Fig. 1d). This study clearly indicated that single hormone was not enough for efficient regeneration. Therefore, the results suggest that combination of cytokinins, auxins and amino acids are necessary for efficient sugarcane regeneration. Positive effects of growth hormones

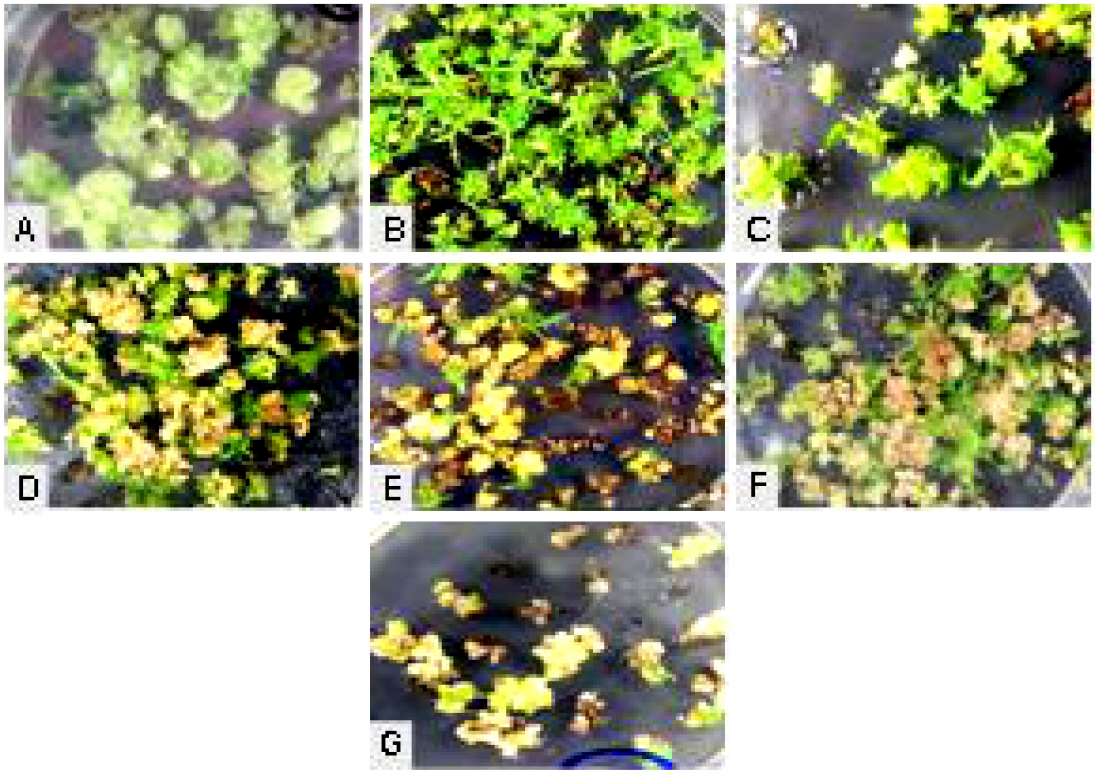

Figure 2. Regeneration in different sugarcane clones at SR-3 media; (A) HSF-240 (B) CSSG-668, (C) CPF-246 (D) SPF 213 (E) CPF-237, (F) CSSG-676 (G) CPF-245.

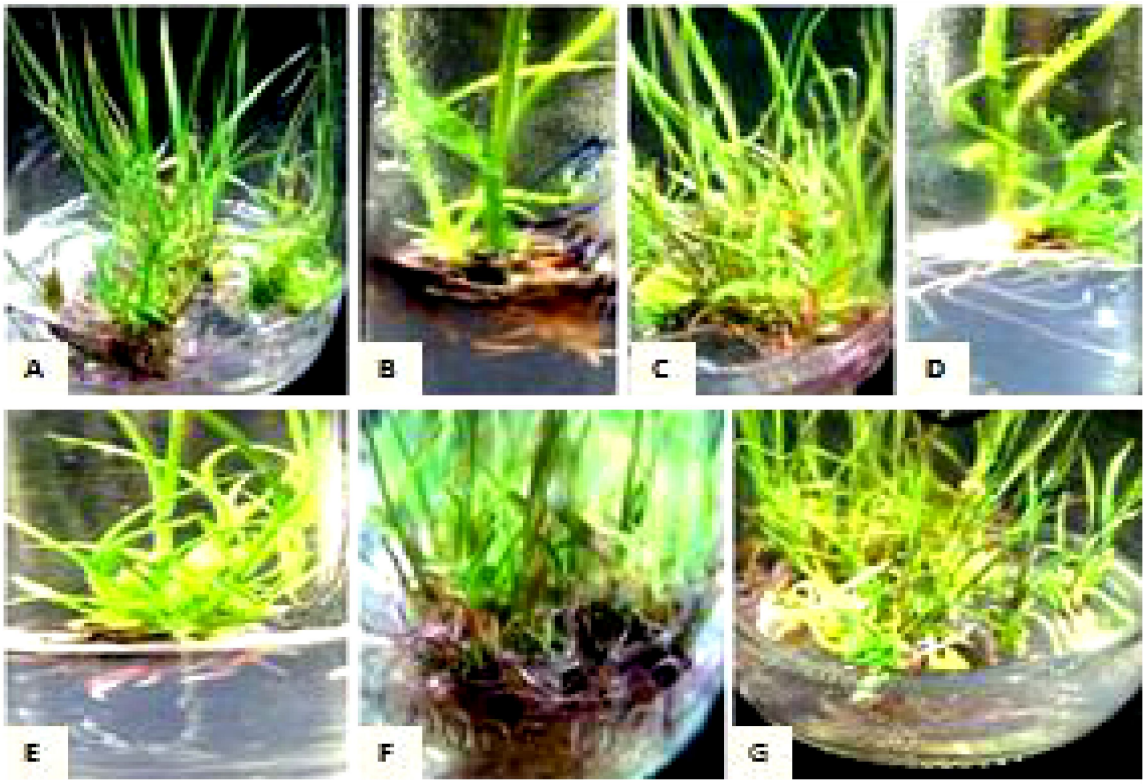

Figure 3. Rooting in different sugarcane clones at SRM-3media; (A) CPF-245, (B) SPF-213, (C) CSSG-676, (D) HSF-240, (E) CPF-237, (F) CSSG-668, (G) CPF-246.

combinations on sugarcane regeneration were also reported by previous authors (Tripathi *et al*., 2000; Roy and Kabir, 2007). In comparison to previous findings, this study resulted in identification of optimal amino acid concentrations and combinations for efficient sugarcane regeneration. Regeneration potential of different sugarcane clones have been reported to be specific and clone dependent (Khan *et al*., 2004; Gandonou *et al*., 2005).

***Optimal geneticin concentration to select transformed sugarcane plantlets*:** Precise selection of genetically transformed plant cells is a critical step for the development of transgenic plants. This can be done by identifying the optimal concentration of selection agent where only genetically transformed cells having resistance to antibiotic can grow. It can reduce the possibility of chimeras and ultimately results in efficient genetic transformation process. Identification of optimum selective agent concentration is the most important step for stable genetic transformation. It has also been observed that the minimum inhibitory concentration of selective agent is tissue or species specific (Parveez *et al*., 2007; Raza *et al*., 2010). Therefore, in this study, the optimal geneticin concentration was identified, which can be used to select transformed sugarcane cells of different genotypes at regeneration and rooting stage. It was observed that 60 mg L^{-1} geneticin was the optimum level for the clones HSF-240, CPF-245 and CSSG-668 where only 10% tissues survived. While for the clones CPf-237, CPF-246 and CSSG-676 geneticin concentration 50 mg L^{-1} was found to be optimum. The clone SPF-213 has shown 10% survival at geneticin concentration 40 mg L^{-1}. Zero callus survival, and regeneration was observed at the geneticin 70 mg L^{-1}. It is evident from the data that geneticin concentration 40-60 mg L^{-1} could be used for the optimum selection of the transformed calli of different sugarcane clones (Fig. 4a). Furthermore, different geneticin concentrations (0-70 mg L^{-1}) were also checked for optimization of rooting selection media to select the transformed shoots of different clones at rooting stage. This study showed that geneticin at 50 mg L^{-1} was the best to select the transformed shoots of HSF-240, SPF-213, CPF-245, CPF-246, CSSG-668 and CSSG-676 because only 10% shoots survived at this concentration. On the other hand, cv. CPF-237 behaved differently and geneticin 40 mg L^{-1} found to be optimum for selection because only 13% shoots survived at this concentration. All the shoots at higher doses 60 and 70 mg L^{-1} of geneticin were albino and no rooting was observed. It is evident from the data that for the selection of regenerated transformed shoots of sugarcane for rooting, the optimum level of selection agent (G-418) could be 40-50 mg L^{-1} (Fig. 4b). These findings are in line with

previous reports (Raza *et al*., 2010). It was found that geneticin is very effective to select sugarcane tissues. Similar effectiveness of geneticin had been reported for the selection of genetic transformants in sugarcane (Elliot *et al*., 1999) and, other monocots like barley (Ritala *et al*., 1994) and sorghum (Tadesse *et al*., 2003).

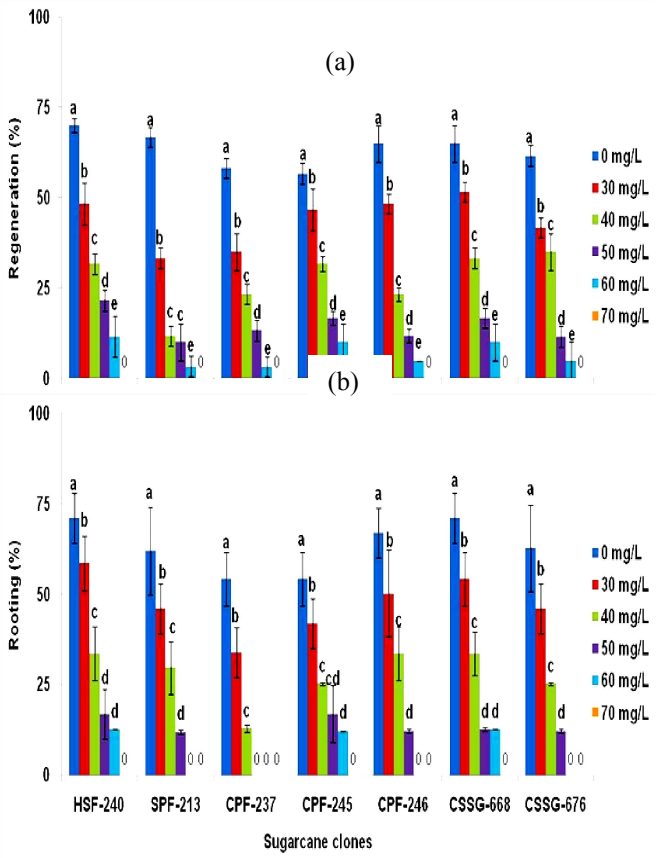

Figure 4. Response of seven sugarcane cultivars on media containing different geneticin concentrations at; (a) regeneration stage, (b) rooting stage. Error bars indicate the standard deviation of mean (*n*=3). Graph bars with the same letter are not significantly different at *P<0.05*.

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