African Journal of Biotechnology Vol. 11(15), pp. 3704-3708, 21 February, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1620
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Efficient plant regeneration through somatic embryogenesis in sugarcane (*Saccharum officinarum* L.) cultivar CPF-237

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Accepted 6 February, 2012

In the present experiment, a number of cultures were established for optimization of normal plant regeneration in sugarcane (*Saccharum officinarum* L.) cv., CPF-237. Well callus induction as well as its proliferation was observed on Murashige and Skoog (MS) medium supplemented with 3.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.0% sucrose. Almost seven-weeks old proliferated calluses were sub-cultured on MS_3 [MS, 0.4 mg L^{-1} kinetin (kin), 0.5 mg L^{-1} benzyleaminopurine (BAP), 0.3 g L^{-1} casein hydrolysate, 3% sucrose] medium for somatic embryogenesis under dark condition for four-weeks. Greenish plantlets were regenerated (8.0 plantlets per callus) on MS_{5a} (MS, 0.2 mg L^{-1} kin, 0.3 mg L^{-1} BAP, 3.0% glucose) medium in six-weeks under light conditions. Regenerated plantlets were not variant morphologically and rooted on MS_7 (MS, 0.1 mg L^{-1} indole-3-butyric acid (IBA) medium in 1½ weeks.

Key words: *Saccharum officinarum* L., shoot tips culture, callusing, somatic embryogenesis, glucose, normal plantlets, rooting.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a worldwide economic sugar crop, which contributes about 74% of world's sugar (Lichat, 2006). It is an inter-specific aneuploid hybrid (*Saccharum spontaneum* x *S. officinarum*) with high levels of polyploidy (2n:80-205). It has been presented with a very low incremental improvement rate through conventional breeding (Sreenivasan et al., 1987; Silvarola and Aguiar-Perecin, 1994; Janno et al., 1999). Meanwhile, growth rate of sugarcane is decreasing due to a number of external environmental factors. Today, innovative cellular and molecular approaches like genetic transformation are based on efficient plant regeneration through somatic embryogenesis from calluses. In this regard, *in vitro* plant regeneration of sugarcane is the most important step for insertion of new genes to its genome as well as screening of beneficial genetically-homozygous forms (Larkin and Scowroft, 1983).

Carbohydrates play vital role in cells by acting as a substrate of metabolism and precursor of various carbon build blocks of certain macromolecules. Additionally, they regulate many developmental processes among the cells (Smeekens, 2000). According to various arguments, its available resources (sugar, glucose, sorbitol or maltose) and their concentrations in initial nutrient medium (callusing culture) may be correlated with somatic embryo induction (Kamada et al., 1989; Lou et al., 1996). However, clear somatic embryogenesis among the calluses cultures of sugarcane still remain limited.

Before improving any sugarcane genotype through modern biological techniques, it is essential to establish efficient plant regeneration through somatic embryogenesis. This study also presented optimal protocols for callusing, somatic embryogenesis and then plant regeneration in sugarcane, which could be beneficial for sugarcane crop improvement through genetic engineering in future.

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Abbreviations: MS, Murashige and Skoog medium; **BAP,** benzyleaminopurine; **IBA,** indole-3-butyric acid.

Table 1. Callus proliferation and somatic embryogenesis on different plant nutrient cultures of sugarcane (*Saccharum officinarum* L.) cv., CPF-237 under aseptic conditions

S/N	MS plant nutrient culture	Callus proliferation (%)	Water content (%)	No. of somatic embryos callus^{-1}
1	MS_1	70.09±2.57	85.76±2.35	-
2	MS_2	60.97±2.92	58.59±3.84	-
3	MS_3	20.11±3.25	73.07±2.56	21.62±1.54
4	MS_4	37.18±3.01	77.12±2.43	16.42±0.97
5	MS_5	-	-	11.25±1.51
6	MS_6	-	-	7.78±0.95

MATERIALS AND METHODS

Almost six months old plants of CPF-237 commercial sugarcane local cultivar were collected from open fields. Tops of plants were dissected with fine cutter and outer leaves were peeled off to excise immature leaf tips. These were used as an explant of sugarcane for aseptic studies. They were washed in Laminar Airflow Cabinet with 90% ethanol for 1 min and also washed with sterile distilled water (dH_2O) for three times. These explants were sterilized with 30% commercial Robin Bleach® (5.25% v/v NaOCl) by stirring with magnet stirrer for 30 min. They were cultured on different MS [Murashige and Skoog, (1962) basal salts; B_5 vitamins complex (Gamborg et al., 1968); 2% sucrose and 0.8% agar)] derived nutrient cultures by supplying various concentrations and types of hormones and carbon sources for inducing acquired plant developmental.

For callus induction and its proliferation, they were cultured on MS nutrient medium supplemented with 3.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) for callus induction under dark conditions (MS_1). Almost three-weeks old callus culture was sub-cultured on four MS nutrient media: MS_2 (MS, 0.2 mg L^{-1} benzyleaminopurine (BAP), 0.1 mg L^{-1} kinetin, 2% sucrose), MS_3 (MS, 0.4 mg L^{-1} kin, 0.5 mg L^{-1} BAP, 0.3 g L^{-1} casein hydrolysate, 3% sucrose), MS_4 (MS, 1.0 mg L^{-1} BAP, 1.0 mg L^{-1} naphthaleneacetic acid (NAA), 2% sucrose) and including MS_1 (MS, 3.0 mg L^{-1} 2,4-D, 2% sucrose) to determine best medium for efficient callus proliferation. After four-weeks of culture, callus proliferation rate was calculated by applying an equation as given below:

$$\text{Callus proliferation (\%)} = \frac{Callus\,Final\,Wt - Callus\,Initial\,Wt}{Callus\,Final\,Wt} x100$$

Three weeks old callus culture on MS_1 was sub-cultured on four different medium (MS_1, MS_2, MS_3 and MS_4). When culture was four-weeks old, water contents were determined. It was calculated by an equation as given below:

$$\text{Relative water content (RWC \%)} = \frac{Callus\ or\ plantlets\ F\,Wt - D\ Wt}{Callus\ or\ plantlets\ F\,Wt} x100$$

The fresh weight (F Wt) of the proliferating callus from each callus culture was measured immediately on excision. Dry weight (D Wt) was taken after oven drying at 80°C for 48 h. Undifferentiated multiplied callus were subjected to induce differentiation or somatic embryogenesis. Almost seven-weeks old well proliferated callus from MS_1 was sub-cultured on four different nutrient media: MS_3 (MS, 0.4 mg L^{-1} kin, 0.5 mg L^{-1} BAP, 0.3 g L^{-1} casin hydrolysate, 3% sucrose), MS_4 (MS, 1.0 mg L^{-1} BAP, 1.0 mg L^{-1} NAA, 3% sucrose), MS_5 (MS, 0.2 mg L^{-1} kin, 0.3 mg L^{-1} BAP, 3% sucrose) and MS_6 (MS, 1.0 mg L^{-1} NAA, 0.5 mg L^{-1} 2,4-D, 3% sucrose). These cultures were incubated in dark-room for four-weeks.

Somatic embryo induced calluses from MS_3 and MS_4 were sub-cultured on MS_5 (MS, 0.2 mg L^{-1} kin, 0.3 mg L^{-1} BAP, 3% sucrose), MS_{5a} (MS, 0.2 mg L^{-1} kin, 0.3 mg L^{-1} BAP, 3% glucose), MS_6 (MS, 1.0 mg L^{-1} NAA, 0.5 mg L^{-1} 2,4-D, 3% sucrose) and MS_{6a} (MS, 1.0 mg L^{-1} NAA, 0.5 mg L^{-1} 2,4-D, 3% glucose) media. These cultures were incubated in light conditions for almost six-weeks or until plant regeneration was observed. Plant regeneration and normal plant regeneration efficiency were calculated by applying formulas as given below:

$$\text{Plant regeneration efficiency} = \frac{\#\ of\ \text{Re}\ generated\ Plantlets}{Total\ \#\ of\ somatic\ embryos} x100$$

$$\text{Normal plant regeneration efficiency} = \frac{\#\ of\ \text{Re}\ generated\ Normal\ Plantlets}{Total\ \#\ of\ \text{Re}\ generated\ Plantlets} x100$$

Regenerated normal plants were rooted on MS_7 (MS, 0.1 mg L^{-1} IBA) medium in growth room under light conditions for 1½ weeks.

The pH of each medium was adjusted to 5.7 to 5.8 with 0.1 N HCl or NaOH solutions and solidified with 0.8% agar. They were autoclaved at 121°C and 15 lbs inch^{-2} pressure for 15 min. The cultures were maintained at 25 ± 1°C in low light (0.13 µmol m^{-2} s^{-1}, 2 h) and dark conditions.

RESULTS AND DISCUSSION

In this experiment, a system for normal plant regeneration in sugarcane (*S. officinarum* L.) cv., CPF-237 was established. Through optimization of various spatial and temporal culture compositions, abnormalities were reduced in the developing plantlets (Webb et al., 1983; Eudes et al., 2003). Callus in CPF-237 sugarcane cultivar was induced in explants (immature leaf tips) on MS_1 plant nutrient medium under dark conditions. De-differentiation as well as callus development was observed among the cultures (Figure 2b). After three-weeks of callusing culture, calluses from MS_1 were sub-cultured on MS_2, MS_3 and MS_4 including MS_1. Maximum callus proliferation was observed on MS_1 culture (Figure 2a). The seven-weeks old well proliferated calluses were sub-cultured from MS_1 to MS_3, MS_4, MS_5 and MS_6 media for somatic embryogenesis in dark conditions (Table 1).

Explant
Immature leaf cuttings
3-4 mm

↓

Callusing
3.0 mg L^{-1} 2,4-D (MS_1)
Dark conditions
7-weeks

↓

Somatic embryogenesis
0.5 mg L^{-1} BAP + 0.4 mg L^{-1} kin +
0.3 g L^{-1} casin hydrolysate + 3% sucrose
(MS_3); Dark conditions;
4-weeks

↓

Plant regeneration
0.2 mg L^{-1} kin + 0.3 mg L^{-1} BAP + 3%
glucose, (MS_{5b})
Light conditions
6-weeks

↓

Plant rooting

$\frac{1}{2}$ MS_0 + 0.1 mg L^{-1} IBA (MS_7)
Light conditions;
$1\frac{1}{2}$-weeks

Figure 1. A schematic representation of an optimized protocol for efficient plant regeneration through somatic embryogenesis in sugarcane (S. officinarum L.) cv., CPF-237.

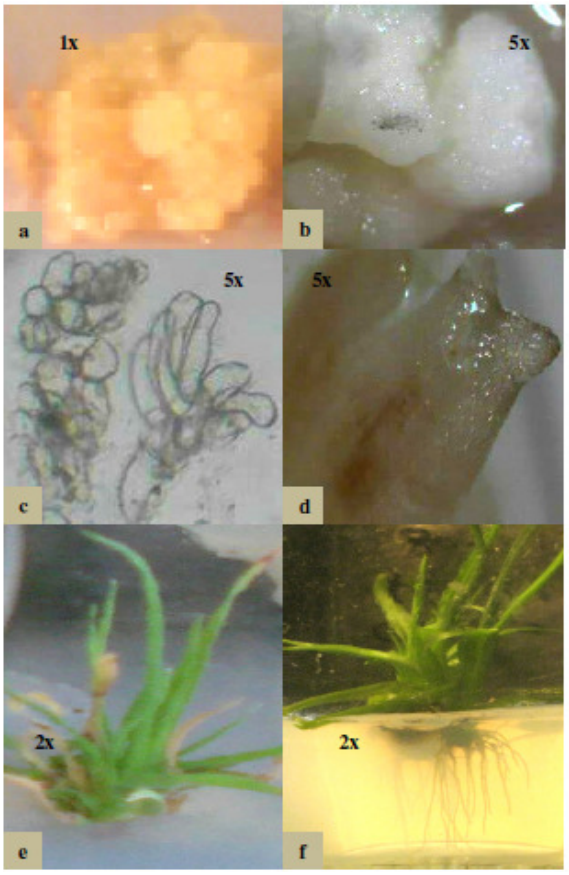

Figure 2. Different steps of optimized protocol for normal plant regeneration in sugarcane (*S. officinarum* L.) cv., CPF-237 through somatic embryogenesis. a, Callus proliferation after 4-weeks; b, calluses proliferation in somatic embryo induction medium (MS_3) after 2-weeks; c, various somatic cells increased in size after 4-weeks of culture; d, developing somatic embryos in 4-weeks old somatic embryogenesis culture; e, emerging shoots from somatic embryos after 3-weeks in plant regeneration medium (MS_{5b}); f, developing plantlets from the somatic embryogenic callus after 7-weeks of culture; g, root induction on MS_7 medium in regenerated plantlets after 1½ weeks.

Within four-weeks, somatic embryos were developed on MS_3 medium. The somatic embryo induced cultures from MS_3 and MS_4 were sub-cultured on MS_5, MS_{5a}, MS_6 and MS_{6a} media in light conditions for shoot induction. Maximum number of plantlets were regenerated in MS_{5a} (sub-cultured callus from MS_3) medium (Figure 2 and Table 2).

Almost four-weeks old normal growing plantlets either regenerated through direct or indirect methods were rooted on MS_7 medium under light conditions. Profuse-rooting was observed in each cultivar within 1½ weeks (Figure 2). Somewhat wilting was observed among the cultures when rooted shoot clumps were transferred from rooting medium to soil containing polythene bags for plant hardening. They were grown in growth room conditions for 10-days then shifted to open field conditions.

Although, regenerated plantlets were normal in this study, however fluctuations in timing as well as culture conditions induced structural abnormalities in developing plantlets, for instance, when calluses were younger than six-weeks sub-culture on somatic embryogenesis medium. Less or no plant regeneration was observed. Similarly, 10-weeks old calluses also influenced the abnormal characters in developing plantlets. Meanwhile, efficient and normal plant regeneration was seen when somatic embryogenesis was induced in seven-weeks old calluses in the presence of sucrose (3%) instead of glucose (3%) in MS_3 medium. This culture presented good regeneration ability when sub-cultured on MS_{5a} plant regeneration medium (Table 2). Similarly, somatic embryogenesis carried out by culturing same calluses on MS_4 and MS_5 showed the development of abnormal plant clumping on MS_6 and MS_{6a} plant regeneration cultures (Figure 1).

Plant regeneration responses are based on appropriate initial explant selection and a number of factors including callusing have been considered to affect somatic embryos induction, their maintenance and then regeneration into plantlets (Liu, 1993; Thorpe, 1995; Mozdhorst et al., 1997). During somatic embryogenesis,

Table 2. Plant regeneration efficiency in sugarcane (*S. officinarum* L.) cv., CPF-237 from somatic embryos on different plant nutrient culture under aseptic conditions.

Cultivar name	Somatic embryo induction culture	MS plant nutrient culture			
		MS_{5a}	MS_{5b}	MS_{6a}	MS_{6b}
Plantlets callus^{-1} regenerated through somatic embryogenesis					
CPF-237	MS_3	16.42±0.95	18.92±1.01	7.99±0.99	6.44±0.29
	MS_4	5.32±0.05	5.91±0.01	3.99±3.41	3.41±0.09
Normal plant regeneration (%)					
CPF-237	MS_3	75.95±5.04	87.51±4.02	36.96±4.05	29.79±3.12
	MS_4	32.40±3.02	35.94±3.21	24.30±2.97	20.77±2.78
Respective heights of the regenerated plantlets					
CPF-237	MS_3	2.56±0.10	3.44±0.14	2.24±0.28	2.29±0.15
	MS_4	2.68±0.15	3.23±0.12	2.53±0.17	2.55±0.09

both medium composition as well as time and its incubation conditions are very important aspects for deciding the ability, form and number of developing shoots from them. Importance of carbon-source has also been measured during different stages of plant development. Callus growth and shoot initiation are slow in the presence of sucrose, while the phenomenon is reversed because of the presence of glucose in plant regeneration medium (Table 2). Sucrose is found to stimulate somewhat dedifferentiation, enhance callus formation and also somatic embryogenesis in case of sugarcane rather than shoot formation (Strickland et al., 1987; Kunitake et al., 1997; Javed and Ikram, 2008). Meanwhile, plant regeneration in sugarcane from variably old callus cultures is possible (Heinz and Mee, 1969; Ahloowalia and Meretzki, 1983) to develop soma-clonal variation (Kaur et al., 2002). Such variations are undesirable phenomena for *in vitro* germplasm conservation as well as mass cloning of new developing varieties or plant improvement through genetic transformation of commercial varieties. Somatic embryogenesis among callusing cultures has been considered as an important aspect for plant regeneration of transgenic cells identified in different sugarcane cultivars (Fitch and Moore, 1993; Aftab and Iqbal, 1999).

Normal somatic embryogenesis as well as shoot induction has been optimized through specification of special and temporal conditions of the culture. Here, efficient and normal plant regeneration is the reflection of suitable somatic embryo induction. Established protocols (direct and indirect) are very efficient and economical. These systems may be useful for developing transgenic plants through *Agrobacterium*-mediated method in future.

ACKNOWLEDGEMENTS

This research was financially supported by Pakistan Agriculture Research Council (PARC), Islamabad, Pakistan, under Agricultural Linkage Programme (ALP). Authors are heartily thankful to them.

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