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Plant Regeneration through Somatic Embryogenesis from Leaf Sheath Derived Callus of Sugarcane (*Saccharum officinarum* L.) var. Isd -16

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Abstract

Leaf sheath explants of an indigenous variety Isd-16 of sugarcane (*Saccharum officinarum* L.) produced light yellow friable callus after culturing on to MS with 2,4-D (2 - 4 mg/l) and NAA (3 - 5 mg/l) singly. Callus formation was the maximum on MS + 3 mg/l 2,4-D. Callus underwent embryogenesis producing huge number of somatic embryos when subcultured on MS with 15 - 30 mg/l L-proline, 3 mg/l 2,4-D + 5 - 10% coconut water (v/v) and 3 mg/l 2,4-D + 10% CW (v/v) + 300 - 500 mg/l CH. L-proline significantly enhanced somatic embryogenesis and 25 mg/l L-proline in MS was the best culture medium formulation. Most of the somatic embryos germinated and developed plantlets after 1 - 2 weeks of incubation in proline-supplimented medium. On the other hand, maturation and germination of embryos were achieved on half-strength MS with or without 0.25 - 1.0 mg/l L-proline, and 5% coconut water (v/v). Somatic embryos derived plantlets were then successfully transferred to natural condition through successive phages of acclimation.

Introduction

Sugarcane is an important industrial crop and it is the only source of sugar production in Bangladesh. Due to lack of breeding facilities, biotechnological approach using gene transfer technology is successfully used for genetic improvement of sugarcane during past few years. Successful genetic transformation attempts have mostly employed embryogenic callus or cell cultures as the target tissue in several crop plants (Nayak et al. 1997, Newell et al. 1995) including sugarcane (Aftab and Iqbal 1999, Khalil 2001). Somatic embryogenesis is considered an efficient and high volume propagation system

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for the production of large number of plantlets in fewer steps, with a concomitant reduction in labour, time and cost (Ammirato 1987). It offers a great potentiality in crop improvement by cell selection, genetic transformation, somatic hybrid and polyploid plant production. In recent years somatic embryos are used for developing synthetic seeds, shortening the breeding cycle and transformation. Induction of somatic embryogenesis from young leaves of sugarcane was reported by Ahloowalia and Maretzki (1983), Chen et al. (1988), Marcano et al. (2000).

In Bangladesh research in this area of tissue culture has been conducted on efficient *in vitro* plant regeneration (Islam et al. 1982, Begum et al. 1995, Samad and Begum 2000, Karim et al. 2002). So far, recent years no work has been done on the improvement of protocol for induction of embryogenic culture, which helps in transgenesis. The present study was undertaken to develop efficient and stable plant regeneration through somatic embryogenesis for a popular sugarcane var. Isd -16 of Bangladesh.

Materials and Methods

Immature leaf sheath from 6 months old field-grown plants were used as explant. Apical portion of healthy shoots were stripped to the terminal bud and attached immature leaf rolls were surface sterilized with 0.1% $HgCl_2$ for 9 min. Approximately 3 slices (5 × 3 mm) were taken from each cylindrical leaf roll and cultured on MS supplemented with 2 - 4 mg/l 2,4-D and 3 - 5 mg/l NAA alone and incubated in dark for callus induction. Sub-cultures were done at every 3 weeks after incubation. In order to induce somatic embryogenesis, the proliferated friable callus was transferred to MS containing 15 - 30 mg/l L-proline, 15-30 mg/l L-glutamine, 3 mg/l 2,4-D + 5 - 10% (v/v) coconut water (CW) and 3 mg/l 2,4-D + 10% (v/v) CW + 300 - 500 mg/l casein hydrolysate (CH) with 3% (w/v) sucrose (BDH, England). The pH of the medium was adjusted to 5.6 and gelled with 0.7% (w/v) agar. The cultures were also incubated in a growth chamber at 25 ± 1ºC in dark. Number of somatic embryos was counted by the help of Stereo Microscope from about 150 mg embryogenic callus.

For germination, the embryogenic calli with developing somatic embryos were transferred to MS with or without L-proline (0.25 - 1.00 mg/l) and 5% CW (v/v) and incubated in 16/8h light/dark condition. Regenerated plantlets with well-developed root systems were transferred to pots containing soil : manure : sand (1 : 2 : 1) mixture through successive phases of acclimation. Data collected on different quantitative characters were analyzed for mean and standard error. Each experiment was performed thrice using 15 replicates. Analysis of variance was carried out and the test of significance was done by DMRT. The data in

percentage were transformed using arc-sine/square root transformation before analysis and were transformed back to percentage for presentation. All statistical analyses were conducted using MSTAT-C software.

Results and Discussion

Young sugarcane leaves are known to be good explant sources for callus production (Ho and Vasil 1983, Brisibe et al**.** 1994). Therefore, young leaves of sugarcane var. Isd-16 were used as starting materials on MS containing high concentrations of 2,4-D and NAA alone for callus induction. The explants were induced to develop callus in all culture media formulations but the degree of callus proliferation varied from 13 - 91% (Fig. 1). The highest frequency (91%) of explants induced callus formation in medium having 3 mg/l 2,4-D. This result corroborates with that of Heinz and Mee (1969) and Chengalrayan and Gallo-Meagher (2001). After two subcultures at 2 - 3 weeks intervals on the same medium, these calli became light yellow, friable and embryogenic in nature.

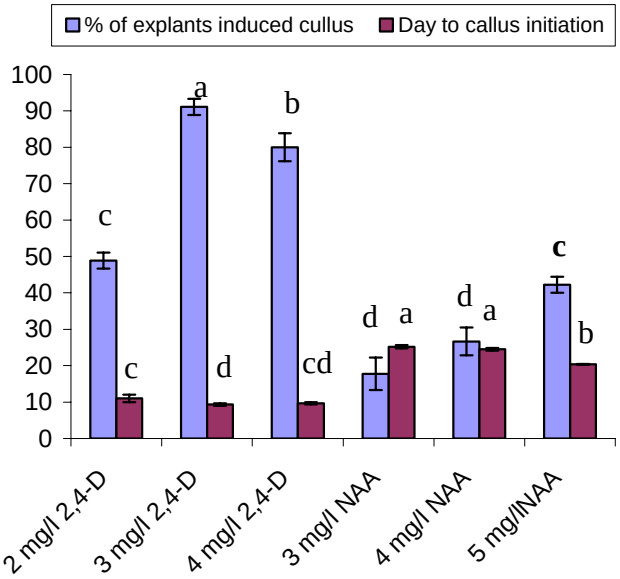

Fig. 1. Effect of different concentrations of 2,4-D and NAA on induction of callus from leaf sheath explants of sugarcane Isd-16. Bar represents Mean ± SE. Bars marked by the same letter indicate that values are not significantly different from one other at 5% level according to DMRT.

In order to induce somatic embryogenesis, the proliferated friable calli were transferred to MS with various concentrations and combinations of L-proline, L-glutamine, 2,4-D + CW, 2,4-D + CW + CH (Table 1). Amino acids such as L proline, L glutamine, glutamic acid, asparagine play a key role for induction of

somatic embryogenesis in sugarcane. The development of somatic embryo in presence of amino acids was reported in maize (Carvalho et al. 1997), in wheat (Yadava and Chawla 2002), and also in sugarcane (Sinha et al. 2000 and Desai et al. 2004). In the present investigation, L-glutamine did not show any positive

Table 1. Effect of various concentrations and combinations of L-proline, L-glutamine, 2,4-D, CW and CH on somatic embryogenesis through callus culture of sugarcane var. Isd-16.

Supplements (mg/l)	Days to embryo initiation (Mean ± SE)	% of callus induced somatic embryogenesis	No. of somatic embryos/150 mg embryogenic callus (Mean ± SE)	Remarks
L-proline				
15	12.84 ± 0.22a	51de	31.95 ± 3.72d	cal + emb
20	8.07 ± 0.30cd	80b	59.13 ± 0.82b	cal + emb + minisht
25	6.60 ± 0.14d	91a	75.58 ± 2.90a	cal + emb + ms/pt
30	8.11 ± 0.31cd	71bc	50.16 ± 2.20c	cal + emb + minisht
L- glutamine				
15 - 30	-	-	-	cal
2,4-D + CW% (v/v)				
3.0 + 5	12.85 ± 0.15a	44e	36.69 ± 5.06d	cal + emb
3.0 + 10	10.12 ± 0.18bc	71bc	54.36 ± 3.32bc	do
2,4-D + CW% (v/v) + CH				
3.0 + 10 + 300	12.98 ± 0.65a	60de	35.89 ± 1.23d	cal + emb
3.0 + 10 + 400	9.58 ± 1.43bc	80b	58.91 ± 1.11b	do
3.0 + 10 + 500	11.69 ± 1.70ab	69bcd	49.64 ± 0.37c	do

Mean values within a column having the same letter are not significantly different according to DMRT at 5% level. cal + emb = callus with embryos, cal + emb + minisht = callus + embryos with minishoots, cal + emb + ms/pt = callus + embryos with minishoots/plentlets.

effect on somatic embryogenesis. This observation is in agreement with the results obtained by Ho and Vasil (1983). On the other hand, L-proline was found to be effective for enhancing the frequency of embryogenesis. Among the different concentrations, 25 mg/l L-proline was proved to be the most effective formulation for somatic embryogenesis from leaf sheath derived calli. A large number of somatic embryos with different developmental stages such as, globular, oblong, heart shaped, scutellar, and coleoptilar stages appeared over the surface of the embryogenic callus. Moreover, embryo induction, maturation and germination simultaneously occurred in mass of the same callus (Fig. 2a-c). The addition of L-proline to the medium facilitated the increase in reduced nitrogen in the medium and became available to the cells that might enhance the development of embryo and subsequent germination of somatic embryos to complete plantlets. It was observed that the media containing 3 mg/l 2,4-D + 10%

CW and 3 mg/l 2,4-D + 10% CW + 400 - 500 mg/l CH also showed positive effect of somatic embryogenesis. Development of embryogenic callus subjected to high contents of 2,4-D and CH in combination with CW was also reported in sugarcane (Khail 2001). Somatic embryo germination was achieved on half-strength MS with or without low concentrations of L-proline and 5% CW but the best germination (84.44%) was recorded in half-strength MS0 (Table 2, Fig. d) which corroborated with the result of Ho and Vasil (1983).

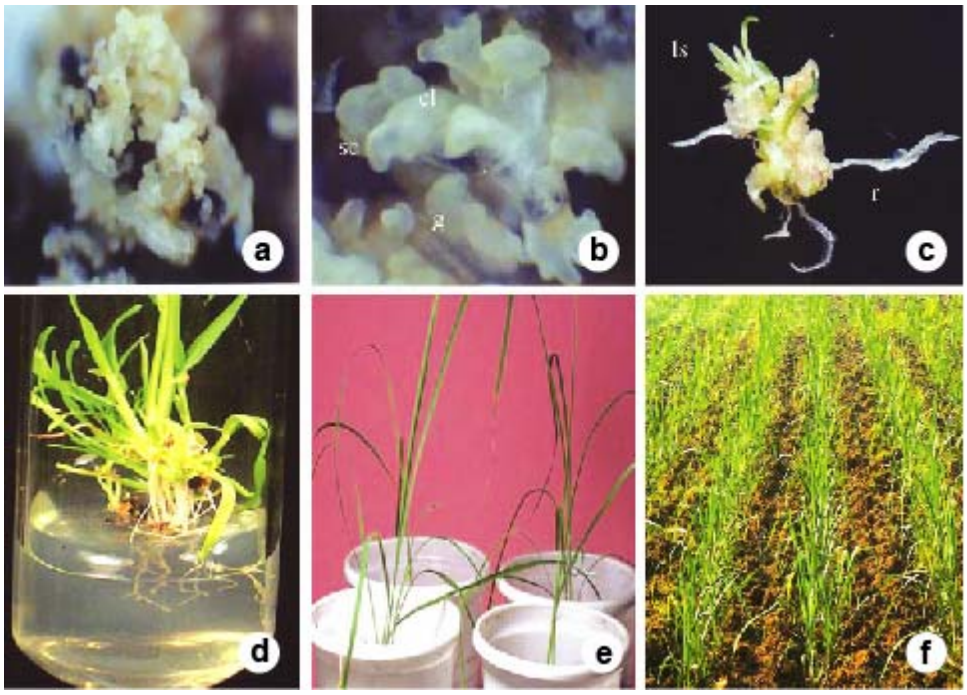

Fig. 2. Somatic embryogenesis in sugarcane: (a) White cluster of somatic embryos with various developmental stages on 25 mg/l L-proline containing MS. (b) Globular (g) and coleoptiler (cl) embryos with scutellum (sc). (c) Germinating somatic embryos with leafy scutellum (ls) and roots (r). (d). Cluster of somatic embryos germinated on half-strength MS0. (e) Acclimatized plantlets in plastic pot. (f) Plantlets growing in the field.

The age of the callus was an important factor for somatic embryo induction. Only 6 - 10 weeks old callus induced somatic embryogenesis. According to Smith and Street (1974) changes in ploidy of the cultured cells may lead to loss of embryogenic potential in long-term culture. Somatic embryogenesis occurs in short time culture and their ability decreases with increasing duration. The plantlets were successfully transferred to field through successive phases of acclimatization (Fig. 2e-f).

Table 2. Effect of half-strength MS with or without different concentrations of L-proline and CW on germination of somatic embryos of sugarcane var. Isd-16.

Supplements	Germination (%)
Half strength MS	84.44a
Half strength MS + proline mg/l	
0.25	71.12ab
0.5	66.66bc
1.0	51.11c
Half strength MS + 5% CW (v/v)	53.33c

Mean values within a column having the same letter are not significantly different according to DMRT at 5% level.

Present study elucidated an efficient protocol for plant regeneration *via* embryogenesis of sugarcane (*Saccharum officinarum* L.) var. Isd-16 and this will be helpful in conducting genetic transformation.

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