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EFFECT OF DIFFERENT EXPLANTS AND MEDIA COMPOSITIONS FOR EFFICIENT SOMATIC EMBRYOGENESIS IN SUGARCANE (*SACCAHARUM OFFICINARUM*)

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Abstract

An efficient embryogenic response (both direct and indirect) of two different varieties of sugarcane (CP 77,400 and BL-4) was obtained in this study**.** The results demonstrates that fresh inner whorl of leaves and shoot apical meristem of sugarcane were highly amenable to *in vitro* somatic embryogenesis (both direct and indirect). A combination of 3.0 mg/l 2,4-D with 0.25 mg/l BAP was more potent for direct embryo induction and growth in both the varieties of sugarcane. For indirect somatic embryogenesis the combination of 1.0 mg/l 2,4-D with 0.25 mg/l BAP showed best results in CP 77,400 while in BL-4 it was 1.0 mg/l 2,4-D with 0.5 mg/l BAP. The optimum temperature for callus induction and proliferation was found to be 27 ± 1°C. In CP 77,400, best shoot regeneration response was obtained on MS medium supplemented with 1.0 mg/l BAP. while in BL-4, MS medium containing 1.0 mg/l BAP + 0.5 mg/l Kinetin showed best organogenic response.

Introduction

Sugarcane ranks among the worlds top 10 food crops and annually provides 70% of the sugar produced worldwide. It is cultivated as commercial crop in nearly 60 countries spread over five continents. It is an important industrial raw material for sugar, alcohol, biogas and other allied group of byproduct industries. The highest yield of sugarcane in the world is of Egypt which is about 110.8 tons per hectare while the average yield of the world is only 64.4 tonns per hectare (Anon., 2004). To meet the increasing demands of sugarcane products, its continuous varietal improvement is necessary. Sugarcane cultivar grown today are highly heterozygous and complex polyploidy produced through inter specific hybridization involving three or four species of S*accharum*. Consequently a considerable time is required to improve sugarcane varieties through conventional breeding program. Therefore, recent strategies based on biotechnological methods like Plant Tissue Culture are gaining importance. The plant tissue culture technology has become commercially viable biotechnological approach in crop improvement. In tissue culture one of the reliable and efficient methods for plant regeneration is by the formation of somatic embryos. This somatic embryogenesis is now a day best known as a pathway to induce regeneration from *In vitro* tissue cultures (Jimenez, 2001). The plants raised from somatic embryos are of single cell origin and embryogenic cultures can be synchronized and purified to obtain pure cultures of homogenous material which is more suitable for breeding and genetic analysis. Somatic embryogenesis provides huge number of individual embryos which could increase the probability of mutation many folds. In recent years the success in inducing dormancy and the accomplishment of long term storage, together with the achievement of encapsulation of somatic embryo has opened up the possibility for their use in synthetic seed technology (Litz & Gray, 1995). The

somatic embryos also appears to be more sensitive to the application of exogenous chemicals and therefore can be used for *In vitro* screening to identify plant genotype resistant to toxins or other factors like drought or salt. Development of an efficient embryogenic system is critical for the application of transgenic technology (Lakshmanan, 2005). By keeping this all background information in view, the present research work was undertaken to standardize protocols for efficient somatic embryogenesis (both direct and indirect) and plant regeneration from them in sugarcane.

Material and Methods

Shoot apical meristem, spindle leaves and pith parenchyma of different sizes were used for both direct and indirect somatic embryogenesis. Explants were obtained from field grown plants. They were washed thoroughly with tap water and then with house hold detergent to remove all the traces of dust particles. The explants were then immersed in 7.5% aqueous solution of Sodium hypochlorite for 15 minutes and were thoroughly rinsed. Sodium hypochlorite solution was decanted and apical shoots were rinsed three times with autoclaved distilled water to remove all the traces of sterilent. Inoculation was carried out in laminar airflow cabinet. It was cleaned by scrubbing with 70% ethanol solution and was irradiated with UV irradiations for 25 minutes before use.

MS medium (Murashiage & Skoog, 1962) supplemented with different concentrations of auxin and cytokinins along with 3% sucrose was used. pH of the medium was adjusted to 5.74±0.1. Agar was used for solidification of media. The media was autoclaved at 121°C for 15 minutes at 15lbs/in^2 pressure. Data was recorded on the frequency of embryo induction and proliferation. To standardize the medium for regeneration frequency of somatic embryos, different concentrations of growth hormones were used. To find the regeneration ability well developed somatic embryos were transferred to MS medium supplemented with different concentration of auxins and cytokinins. All the cultures were maintained under light intensity of 2500- 3000 lux having temperature of 27±1.0°C and photoperiod was 16 hour with 8 hours dark period in every 24 hour cycle. Total ten cultures were inoculated for each treatment. First sub-culturing was carried out after every 4 week and rest all sub-culturings were carried out after two week interval.

After establishing of complete *In vitro* plant, it was gradually acclimatized and hardened. Plantlets were thoroughly washed with running tap water to remove all the traces of agar medium. Plantlets were first transferred into pure sand inside the green house. Plants were covered with polythene bags to provide maximum humidity. The polythene was removed gradually whenever it became foggy. Plantlets were watered with Hoagland solution to fulfill its nutritive demands. After sufficient growth, the plantlets were shifted into the mixture of soil and organic manure (1:1). After 30 days the upper portion of older leaves of the plants were trimmed off. This made the plants more strong. Eventually these plants were shifted into the field for further growth.

Results

Direct somatic embryogrenesis: Initiation of direct embryo formation was observed after 14-18 days of incubation on cut edges of explant. Maximum embryogenesis for both varieties was obtained in D_7 medium i.e. MS medium supplemented with 3.0 mg/l of 2,4-D and 0.25 mg/l BAP after seven week of incubation in 3rd sub-culture. Embryo formation was 90% from leaf explant, 70% from shoot apical meristem and 40% from pith explant

(i.e. an average of 9, 7, and 5 cultures out of total 10 in each category showed direct somatic embryogenesis) in CP 77,400 while it was 90, 70 and 30% from respectively (i.e. 9, 7 and 3 cultures out of 10) in BL-4 (Table 1a & b). Initially the explant produced nodular outgrowth on the cut edges, which enlarged into pro-embryoids with in four weeks of incubation (Fig. 1a and b). These pro-embryoids developed into well developed embryos after seven week of incubation (Fig. 1b). The embryo development was dependent on concentration of 2,4-D and BAP and on the light and dark conditions. Both by increasing/decreasing the concentration of 2,4–D, decline in direct embryogenesis was observed (Table 1a & b). Similarly when concentration of 2,4–D was lowered to 2.0 mg/l (Medium D_5 and D_6) the rate of somatic embryogenesis was decreased in both the varieties of sugarcane (Table 1a & b). Direct somatic embryogenesis was normally observed in 16 hour light with 3000 lux intensity. In dark cultures direct embryogenesis was not observed. These well developed embryos germinate into root and shoot after 12 week of incubation (Fig. 2a & b).

Indirect somatic embryogenesis: Indirect embryogenesis (i.e. embryogenesis via intervening callus) was induced in explants by keeping them first in dark for four weeks and then in light. After 14 days of incubation in dark, the explant induced callus at cut edges. The callus proliferated into friable mass of yellowish brown colour, which became yellowish green when shifted from dark to light conditions (16 hour) after 4 week of inoculation in second sub-culture (Fig. 3a and b). The well developed eight week old nodular, compact and yellowish white callus (obtained after eight week of incubation on MS medium containing 3.0 mg/l 2,4-D) was shifted to MS medium supplemented with different hormones for induction of somatic embryogenesis (Table 2a & b). The embryogenic callus consisted of brownish coloured pro-embyroides on callus surface. After 11 week of incubation the pro-embryoides transferred into complete bipolar embryos. Although the process of indirect embryogenesis took more time but, number of regenerated plants, from single explant were higher due to expanded surface area of callus (Fig. 6).

Effect of auxin, cytokinin and auxin-cytokinin interaction on somatic embryogenesis: MS medium supplemented with 20 different combinations of auxins and cytokinins either alone or in combination with each other were used. Of different concentrations of 2,4-D used, 1.0 mg/l i.e. EI_1 medium provided good results for embryo induction in both the varieties. At this concentration of 2,4-D in CP 77,400, an average of 8, 7 and 4 calluses obtained from leaf, shoot apical meristem and pith respectively showed indirect somatic embryogenesis (the rate of embryo induction was 80, 70 and 40% respectively), while in case of BL-4 an average of 8,7 and 6 callus cultures (80, 70 and 60%) obtained from leaf, shoot apical meristem and pith explants respectively showed embryogenic response. Concentration of 2,4-D higher than 3.0 mg/l did not show good results (Tables 2a & b).

Of the different combinations of 2,4-D and BAP used, EI_5 medium i.e., MS medium containing 1.0 mg/l of 2,4-D with 0.25 mg/l BAP provided best results for embryogenesis in CP 77,400. In variety BL-4, the best results for indirect embryo induction were obtained in EI_6 Medium i.e., MS medium containing 1.0 mg/l 2,4-D with 0.5 mg/l BAP (Table 2a & b). When the combination of BAP and Kinetin were used (Media EI_{17} to EI_{20}), it was observed that in variety BL-4, medium EI_{17} containing 0.25 mg/l of BAP with 0.25 mg/l of kinetin provided good results for indirect embryogenesis, but in CP 77,400 the same combination was not satisfactory. All other combinations provided poor results (less than 50%) for somatic embryo formation (Tables 2a & b).

Table 1a. Direct somatic embryogenesis in CP 77,400.

SAM= Shoot apical meristem Age of culture = 7 week

Treatment No.	Media	Conc. (mg/l)	No. of explants cultured	Cultures showing embryo induction		
				Leaf	SAM	Pith
D_1	MS + 2,4- D	1	10	3.0 ± 0.282^{f}	2.0 ± 0.632^{e}	1.8 ± 0.178^{a}
D_2		2	10	6.2 ± 0.334^{bcd}	4.0 ± 0.748^{cd}	1.6 ± 0.219^{a}
D_3		3	10	7.0 ± 0.774^{bc}	5.2 ± 0.178^{bc}	4.4 ± 0.456^{b}
D_4		4	10	4.8 ± 0.715^{de}	3.8 ± 0.334^{cde}	4.4 ± 0.357^{b}
D_5	MS+2,4-D+ BAP	2.0 + 0.5	10	4.4 ± 0.21^{ef}	2.8 ± 0.912^{de}	3.0 ± 0.4^{bc}
D_6		3.0 + 0.5	10	7.2 ± 0.593^{bc}	6.0 ± 0.565^{ab}	3.0 ± 0.565^{bc}
D_7		3.0 + 0.25	10	9.0 ± 0.282^{a}	7.0 ± 0.632^{a}	4.0 ± 0.489^{b}
D_8		4.0 + 0.25	10	5.8 ± 0.334^{cde}	3.8 ± 0.438^{cde}	3.0 ± 0.565^{bc}
D_9	MS+2,4-D +Kin	1.0 + 0.5	10	3.0 ± 0.282^{f}	3.2 ± 0.334^{de}	2.0 ± 0.489^{c}
D_{10}		2.0 + 0.5	10	5.2 ± 0.334^{de}	3.0 ± 0.282^{de}	1.8 ± 0.593^{c}
D_{11}		3.0 + 0.5	10	6.0 ± 0.282^{bcd}	4.0 ± 0.282^{cd}	3.0 ± 0.8^{bc}
D_{12}		3.0 + 1.0	10	7.4 ± 0.456^{b}	4.0 ± 0.489^{cd}	4.2 ± 0.593^{b}
LSD				**1.354**	**1.682**	**1.60**

Means followed by different letters in the same column differ significantly at p = 0.05 according to Duncan's new multiple range test.

Table 1b. Direct somatic embryogenesis in BL-4.

SAM= Shoot Apical meristem Age of Culture = 7 week

Treatment No.	Media	Conc. (mg/l)	No. of explants cultured	Cultures showing embryo induction		
				Leaf	SAM	Pith
D_1	MS + 2,4- D	1	10	4.2 ± 0.438^{cd}	2.2 ± 0.593^{de}	2.0 ± 0.692^{b}
D_2		2	10	5.2 ± 0.521^{bc}	3.0 ± 0.4^{cde}	2.0 ± 0.282^{ab}
D_3		3	10	6.0 ± 0.632^{b}	5.0 ± 0.632^{bc}	4.0 ± 0.748^{ab}
D_4		4	10	5.0 ± 0.282^{bc}	4.2 ± 0.521^{bcd}	4.0 ± 0.748^{a}
D_5	MS+2,4-D+ BAP	2.0 + 0.5	10	4.0 ± 0.489^{cd}	3.0 ± 0.632^{cde}	2.2 ± 0.438^{ab}
D_6		3.0 + 0.5	10	6.0 ± 0.748^{b}	5.0 ± 0.4^{bc}	4.0 ± 0.8^{a}
D_7		3.0 + 0.25	10	9.0 ± 0.282^{a}	7.2 ± 0.657^{a}	3.0 ± 0.8^{ab}
D_8		4.0 + 0.25	10	4.0 ± 0.565^{cd}	4.0 ± 0.565^{bcde}	3.0 ± 0.4^{ab}
D_9	MS+2,4-D +Kin	1.0 + 0.5	10	3.0 ± 0.489^{d}	2.0 ± 0.484^{e}	2.0 ± 0.489^{ab}
D_{10}		2.0 + 0.5	10	5.2 ± 0.178^{bc}	4.0 ± 0.565^{bcde}	2.0 ± 0.282^{ab}
D_{11}		3.0 + 0.5	10	5.0 ± 0.638^{bc}	6.0 ± 0.489^{ab}	3.0 ± 0.632^{ab}
D_{12}		3.0 + 1.0	10	5.0 ± 0.296^{bc}	5.0 ± 0.632^{bc}	3.0 ± 0.489^{ab}
LSD				**1.549**	**1.875**	**1.90**

Means followed by different letters in the same column differ significantly at p = 0.05 according to Duncan's new multiple range test.

Table 2a. Effect of different hormones on indirect embryo induction in CP 77,400.

Treatment No.	Media	Conc. (mg/l)	No. of callus transferred	Cultures showing Indirect embryo induction		
				Leaf	*SAM	Pith
EI_1	MS+2,4-D	1	10	8.0 ± 0.565^{abc}	7.0 ± 0.748^{b}	4.0 ± 0.4^{bc}
EI_2		2	10	7.2 ± 0.438^{bcd}	6.0 ± 0.282^{b}	5.0 ± 0.4^{b}
EI_3		3	10	6.0 ± 0.565^{cde}	5.0 ± 0.489^{bcd}	4.0 ± 0.489^{bc}
EI_4		4	10	4.0 ± 0.8^{efgh}	3.0 ± 0.282^{de}	3.0 ± 0.632^{cd}
EI_5	MS+BAP+2,4-D	0.25 + 1.0	10	9.8 ± 0.178^{a}	9.0 ± 0.282^{a}	7.0 ± 0.632^{a}
EI_6		0.50 + 1.0	10	9.0 ± 0.282^{ab}	7.0 ± 0.8^{b}	5.0 ± 0.282^{b}
EI_7		1.0 + 1.0	10	7.0 ± 0.632^{bcd}	6.0 ± 0.4^{bc}	4.0 ± 0.489^{bc}
EI_8		1.5 + 2.0	10	3.0 ± 0.489^{fgh}	3.2 ± 0.657^{de}	2.0 ± 0.632^{de}
EI_9	MS+Kin+2,4-D	0.25 + 1.0	10	6.0 ± 0.848^{cde}	6.0 ± 0.282^{bc}	5.0 ± 0.489^{b}
EI_{10}		0.50 + 1.0	10	5.0 ± 0.632^{def}	5.0 ± 0.282^{bcd}	3.0 ± 0.565^{cd}
EI_{11}		1.0 + 1.0	10	2.8 ± 0.593^{gh}	3.0 ± 0.748^{de}	1.0 ± 0.282^{e}
EI_{12}		1.5 + 2.0	10	2.0 ± 0.571^{h}	2.0 ± 0.489^{e}	1.0 ± 0.4^{e}
EI_{13}	MS+BAP	1	10	6.0 ± 0.632^{cde}	5.0 ± 0.489^{bcd}	3.0 ± 0.748^{cd}
EI_{14}		2	10	6.2 ± 0.593^{cde}	5.0 ± 0.632^{bcd}	4.0 ± 0.4^{bc}
EI_{15}		3	10	6.2 ± 0.867^{efg}	4.0 ± 0.632^{cd}	3.0 ± 0.632^{cd}
EI_{16}		4	10	4.2 ± 0.521^{efg}	3.0 ± 0.282^{de}	1.0 ± 0.282^{e}
EI_{17}	MS+BAP+Kin	0.25 + 0.25	10	6.0 ± 0.632^{cde}	5.0 ± 0.489^{bcd}	5.0 ± 0.4^{b}
EI_{18}		0.5 + 0.25	10	5.0 ± 0.4^{def}	5.0 ± 0.282^{bcd}	5.0 ± 0.489^{b}
EI_{19}		1.0 + 0.25	10	6.0 ± 0.489^{cde}	6.0 ± 0.632^{bc}	5.0 ± 0.565^{b}
EI_{20}		1.0 + 0.5	10	7.0 ± 0.748^{bcd}	7.0 ± 0.8^{b}	4.0 ± 0.632^{bc}
LSD				**1.86**	**1.70**	**1.604**

Means followed by different letters in the same column differ significantly at P = 0.05 according to Duncan's new multiple range test.

Table 2b. Effect of different hormones on indirect embryo induction in BL-4.
***SAM = Shoot apical meristem Age of culture = 11 week**

Treatment No.	Media	Conc. (mg/l)	No. of callus transferred	Cultures showing Indirect embryo induction		
				Leaf	*SAM	Pith
EI_1	MS+2,4-D	1	10	8.0 ± 0.565^{abc}	7.0 ± 0.748^{ab}	6.0 ± 0.748^{a}
EI_2		2	10	8.0 ± 0.692^{abc}	6.0 ± 0.489^{abc}	5.0 ± 0.632^{ab}
EI_3		3	10	6.0 ± 0.489^{cd}	5.0 ± 0.282^{bcd}	5.0 ± 0.489^{ab}
EI_4		4	10	4.0 ± 0.632^{ef}	4.2 ± 0.521^{cd}	3.0 ± 0.632^{bc}
EI_5	MS+BAP+2,4-D	0.25 + 1.0	10	8.2 ± 0.334^{ab}	8.0 ± 0.4^{a}	5.0 ± 0.489^{ab}
EI_6		0.50 + 1.0	10	9.8 ± 0.178^{a}	8.0 ± 0.692^{a}	6.0 ± 0.565^{a}
EI_7		1.0 + 1.0	10	7.2 ± 0.657^{bc}	6.0 ± 0.565^{abc}	4.0 ± 0.632^{abc}
EI_8		1.5 + 2.0	10	7.0 ± 0.632^{bc}	3.0 ± 0.489^{de}	2.0 ± 0.489^{c}
EI_9	MS+Kin+2,4-D	0.25 + 1.0	10	7.0 ± 0.489^{bc}	6.0 ± 0.748^{abc}	5.0 ± 0.894^{ab}
EI_{10}		0.50 + 1.0	10	5.0 ± 0.4^{de}	5.0 ± 0.4^{bcd}	4.0 ± 0.632^{abc}
EI_{11}		1.0 + 1.0	10	4.0 ± 0.489^{ef}	4.0 ± 0.632^{cd}	2.0 ± 0.282^{c}
EI_{12}		1.5 + 2.0	10	3.0 ± 0.565^{f}	2.0 ± 0.489^{e}	2.0 ± 0.489^{bc}
EI_{13}	MS+BAP	1	10	8.0 ± 0.489^{abc}	6.0 ± 0.8^{abc}	6.0 ± 0.748^{a}
EI_{14}		2	10	8.0 ± 0.489^{abc}	6.0 ± 0.565^{bc}	5.0 ± 0.4^{ab}
EI_{15}		3	10	6.2 ± 0.593^{bcd}	5.8 ± 0.593^{de}	4.0 ± 0.489^{abc}
EI_{16}		4	10	4.0 ± 0.84^{ef}	3.0 ± 0.632^{de}	3.2 ± 0.657^{bc}
EI_{17}	MS+BAP+Kin	0.25 + 0.25	10	8.0 ± 0.565^{abc}	7.0 ± 0.565^{ab}	6.0 ± 0.748^{a}
EI_{18}		0.5 + 0.25	10	6.0 ± 0.632^{cd}	6.0 ± 0.489^{abc}	5.0 ± 0.4^{ab}
EI_{19}		1.0 + 0.25	10	5.0 ± 0.748^{de}	5.0 ± 0.692^{bcd}	4.0 ± 0.489^{abc}
EI_{20}		1.0 + 0.5	10	5.0 ± 0.282^{de}	5.0 ± 0.489^{bcd}	5.0 ± 0.489^{ab}
LSD				**1.707**	**1.821**	**2.006**

Means followed by different letters in the same column differ significantly at P = 0.05 according to Duncan's new multiple range test.

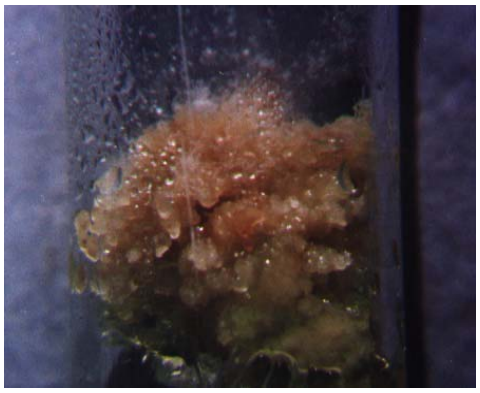

Fig.1a Embryogenic callus derived from immature leaf after 7 week of inoculation on MS medium containing 3.0 mg/l 2,4-D and 0.25 mg/l BAP (4X).

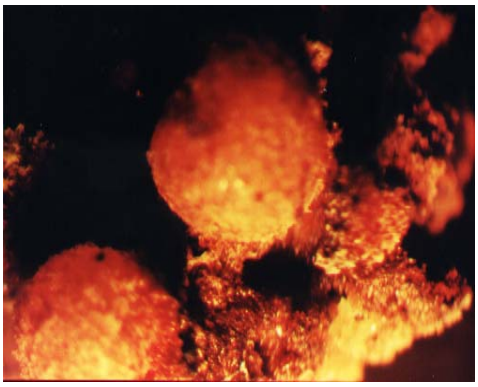

Fig. 1b A magnified view of well developed embryos derived from leaf explant after seven week of inoculation on MS medium containing 3.0 mg/l 2,4-D +v0.25 mg/l BAP (20X).

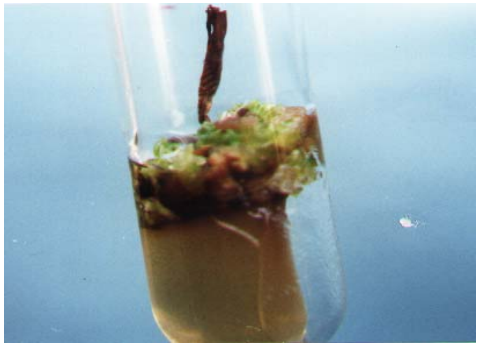

Fig. 2a. Direct embryogenesis and its germination into root and shoot after 12 week of incubation on MS medium containing 3.0 mg/l 2,4-D+0.25 mg/l BAP. Var. CP 77,400 (2X).

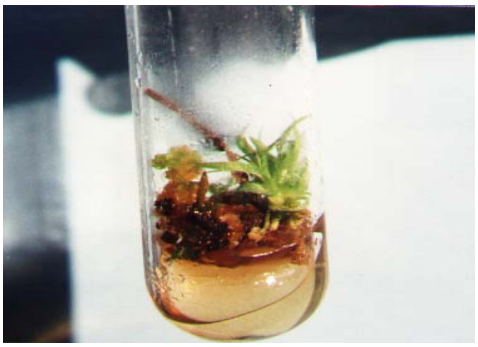

Fig. 2b. Direct embryogenesis and its germination into root and shoot after 12 week of incubation on MS medium containing 3.0 mg/l 2,4-D+0.25 mg/l BAP. Var. BL-4 (2X).

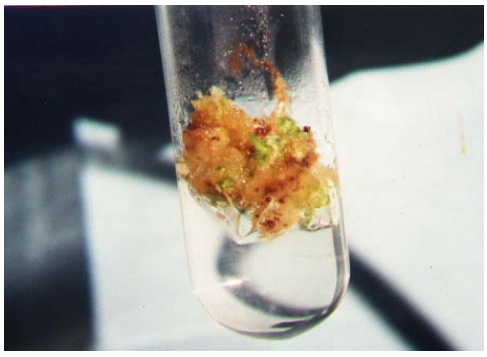

Fig. 3a. Indirect embryo induction from leaf explant after 12 week of incubation on MS medium containing 0.25 mg/l BAP + 1.0 mg/l 2,4-D Var. CP 77,400 (2X).

Fig. 3b. Indirect embryo induction from leaf explant after 12 week of incubation on MS medium containing 0.5 mg/l BAP + 1.0 mg/l 2,4-D Var. BL-4 (1X).

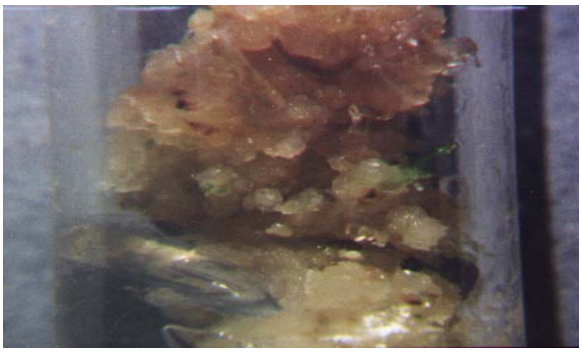

Fig. 4 Different stages of plant regeneration through embryos via intervening callus after 11 week of incubation on MS medium containing 0.25 mg/l BAP+1.0 mg/l 2,4-D (4X).
(a) Callus (b) Embryogenesis (c) Shoot Regeneration

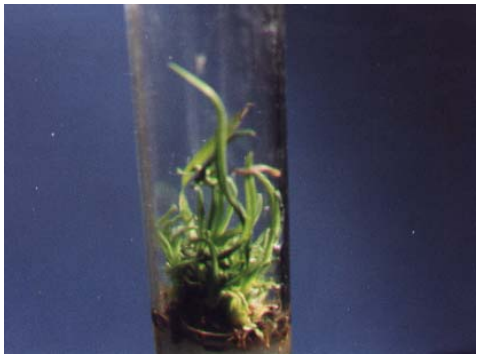

Fig. 5a. Differentiation from bipolar embryo obtained from leaf explant after 14 week of incubation on MS medium containing 1.0 mg/l BAP Var. CP 77,400 (1.5X).

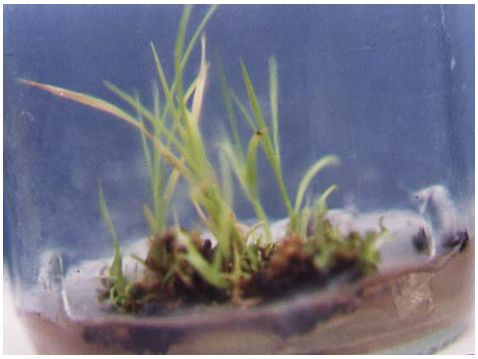

Fig. 5b. Differentiation from bipolar embryo obtained from leaf explant after 14 week of incubation on MS medium containing1.0 mg/l BAP+0.25 mg/l Kin Var. BL-4 (1.5X).

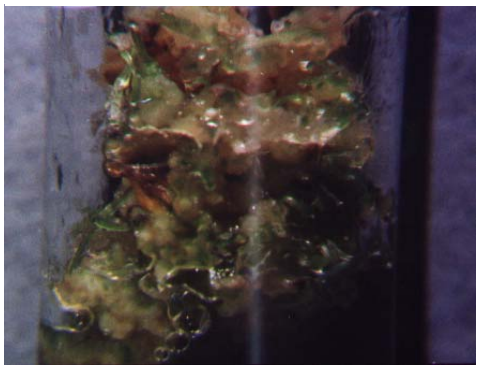

Fig. 6 Shoot regeneration form somatic embryos obtained from leaf explant after 13 week of incubation (4X).

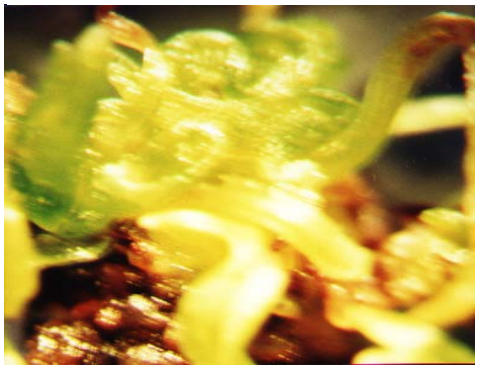

Fig. 7. Magnified view of shoot regeneration form somatic embryos obtained from leaf explant after 13 week of incubation (20X).

Regeneration from somatic embryos: Once the somatic embryogenesis had been induced either by direct or indirect method, further development of embryo was identical in both the cases. Embryogenic calluses were shifted first of all to hormone free medium for differentiation. These embryos germinated into root and shoot after 12 weeks of incubation (Figs. 6 and 7). Maximum regeneration was obtained after 14 week of incubation. In medium R i.e., in hormone free MS medium, in case of CP 77,400, 76% cultures obtained from leaf explant showed regeneration, Similarly somatic embryos obtained from shoot apical meristemand pith showed 60 and 56% regeneration response (i.e an average of 7.6, 6 and 5.6 cultures out of 10 in each type of explant showed regeneration), while in BL-4 regeneration potential in the same medium was 80% in cultures obtained from leaf explant, 64% in cultures from shoot apical meristem and 56% from pith explant (i.e. 8, 6.4 and 5.6 cultures respectively).

MS media supplemented with different hormones were also tested to see their effect to enhance regeneration potential of embryos. For this purpose 20 different media containing different concentration of hormones were tested (Tables 3a & b).

Two auxins, 2,4-D and NAA were supplemented in varying concentration to MS medium. In variety CP 77,400, in R_{13} medium i.e., medium containing 1.0 mg/l of 2,4-D, 7, 6.6 and 5 cultures (i.e. 70, 66 and 50%) showed regeneration. In case of BL- 4, an average of 7.4, 6.4 and 5 (74, 64 and 50%) embryogenic cultures obtained from leaf, shoot apical meristem and pith explants respectively exhibited regeneration. In both the varieties, by increasing the concentration of 2,4-D, regeneration potential was gradually decreased and dropped to zero at 4.0 mg/l of 2,4-D (R_{16} medium,). Other auxin i.e., NAA did not show good results for regeneration in both the varieties (Table 3).

Two cytokinins BAP and kinetin were also used either alone or in combination with each other for shoot regeneration. In CP 77,400, R_1 medium containing BAP at the concentration of 1.0 mg/l showed maximum regeneration which was average 9, 8 and 6.4 cultures (90, 80 and 64%) in somatic embryos obtained from leaf, shoot apical meristem and pith, respectively (Table 3a) while in BL-4, an average of 8.4, 8 and 6 embryogenic cultures (i.e. 84, 80 and 60%) obtained from leaf, shoot apical meristem and pith respectively showed regeneration (Table 3b). In both the varieties, increase in the concentration of BAP, resulted in decreased rate of regeneration. When Kinetin was used alone no good results were obtained.

In combination of BAP and kinetin, CP 77,400 did not show good results while in BL-4 excellent regeneration was obtained when 1.0 mg/l of BAP in combination with 0.5 mg/l of kinetin was used in the MS medium. At this concentration 90% regeneration was obtained in leaf, 84% in shoot apical meristem and 66% in pith. Increasing in the concentration of kinetin resulted in adverse effects i.e., regeneration potential of embryos was decreased to 44, 40 and 30% in cultures obtained from leaf, shoot apical meristem and pith, respectively. Almost similar results were obtained when concentration of BAP higher than 2.0 mg/l was used (Tables 3a & b).

Effect of incubation period on survival of embryos: Optimum period for incubation of embryos was two week in embryogenic medium after which sub-culturing was done. By increasing incubation period for more than two week in embryogenic medium percentage of survival was decreased. After 2 week of incubation 10% necrosis was noticed in direct and 16% in indirect embryogenesis. After four week of incubation there was an abrupt decline in the percentage of survival in indirect embryogenesis while in direct embryogenesis a gradual decline was observed. At this incubation period 80% embryos survived in direct and 60% in indirect embryogenesis (Table 4).

Table 3a. Effect of different hormones on regeneration potential of embryos in CP 77,400.
***SAM = Shoot apical meristem Age of culture = 14 week**

Treatment No.	Media	Conc. (mg/l)	No. of cultures examined	Average number of cultures showing regeneration		
				Leaf	*SAM	Pith
R	MS Basal	MS Basal	10	7.6 ± 0.357^{ab}	6.0 ± 0.489^{bcd}	5.6 ± 0.606^{abc}
R1	MS + BAP	1.0	10	9.0 ± 0.4^{a}	8.0 ± 0.565^{a}	6.4 ± 0.606^{a}
R2		1.5	10	7.6 ± 0.357^{ab}	7.0 ± 0.282^{ab}	6.0 ± 0.489^{ab}
R3		2.0	10	6.0 ± 0.565^{bcd}	5.0 ± 0.565^{cdef}	5.0 ± 0.4^{abcd}
R4		2.5	10	4.4 ± 0.456^{def}	3.0 ± 0.282^{ghi}	3.0 ± 0.4^{efg}
R5	MS + Kin	1.0	10	7.6 ± 0.669^{ab}	7.0 ± 0.489^{ab}	5.0 ± 0.748^{abcd}
R6		1.5	10	7.0 ± 0.8^{bc}	6.4 ± 0.726^{abc}	4.6 ± 0.632^{bcde}
R7		2.0	10	6.0 ± 0.565^{bcd}	5.4 ± 0.606^{bcde}	4.0 ± 0.282^{cdef}
R8		2.5	10	4.4 ± 0.219^{def}	4.0 ± 0.282^{efgh}	3.0 ± 0.4^{efg}
R9	MS+BAP+Kin	1.0 + 0.25	10	7.6 ± 0.669^{ab}	7.0 ± 0.632^{ab}	5.0 ± 0.632^{abcd}
R10		1.0 + 0.5	10	6.0 ± 0.8^{bc}	5.6 ± 0.606^{bcde}	4.0 ± 0.4^{cdef}
R11		2.0 + 0.25	10	5.6 ± 0.536^{bcd}	4.0 ± 0.489^{efgh}	4.4 ± 0.456^{bcde}
R12		2.0 + 0.5	10	4.6 ± 0.726^{def}	3.4 ± 0.219^{fghi}	3.0 ± 0.4^{efg}
R13	MS+2,4-D	1.0	10	7.0 ± 0.632^{bc}	6.6 ± 0.536^{abc}	5.0 ± 0.748^{abcd}
R14		2.0	10	4.4 ± 0.606^{def}	4.4 ± 0.726^{defg}	3.4 ± 0.456^{def}
R15		3.0	10	2.0 ± 0.489^{g}	2.0 ± 0.489^{i}	1.6 ± 0.606^{ghi}
R16		4.0	10	0^{h}	0^{j}	0^{i}
R17	MS+NAA	1.0	10	4.0 ± 0.489^{ef}	3.4 ± 0.669^{fghi}	2.4 ± 0.606^{fgh}
R18		2.0	10	4.0 ± 0.4^{ef}	3.0 ± 0.4^{ghi}	1.5 ± 0.219^{ghi}
R19		3.0	10	3.0 ± 0.489^{fg}	2.6 ± 0.357^{hi}	1.0 ± 0.282^{hi}
R20		4.0	10	0^{h}	0^{j}	0^{i}
LSD				**1.673**	**1.55**	**1.55**

Means followed by different letters in the same column differ significantly at P = 0.05 according to Duncan's new multiple range test.

Table 3b. Effect of different hormones on regeneration potential of embryos in BL-4.
***SAM = Shoot apical meristem Age of culture = 14 week**

Treatment No.	Media	Conc. (mg/l)	No. of cultures examined	Average number of cultures showing regeneration		
				Leaf	*SAM	Pith
R	MS Basal	MS Basal	10	8.0 ± 0.282^{abc}	6.4 ± 0.456^{bcd}	5.6 ± 0.456^{ab}
R1	MS + BAP	1.0	10	8.4 ± 0.219^{ab}	8.0 ± 0.489^{ab}	6.0 ± 0.489^{ab}
R2		1.5	10	7.0 ± 0.282^{bcd}	7.0 ± 0.489^{abc}	5.0 ± 0.4^{bc}
R3		2.0	10	7.4 ± 0.456^{bcd}	5.6 ± 0.606^{cde}	4.6 ± 0.357^{bc}
R4		2.5	10	4.4 ± 0.456^{ef}	3.6 ± 0.456^{fg}	3.0 ± 0.565^{de}
R5	MS + Kin	1.0	10	7.0 ± 0.632^{bcd}	6.0 ± 0.489^{cd}	5.0 ± 0.565^{bc}
R6		1.5	10	6.6 ± 0.456^{cd}	5.6 ± 0.219^{cde}	4.6 ± 0.536^{bc}
R7		2.0	10	6.0 ± 0.4^{d}	5.6 ± 0.456^{cde}	4.0 ± 0.282^{cd}
R8		2.5	10	4.0 ± 0.489^{ef}	4.0 ± 0.282^{efg}	3.0 ± 0.4^{de}
R9	MS+BAP+Kin	1.0 + 0.25	10	8.0 ± 0.282^{abc}	7.0 ± 0.748^{abc}	5.4 ± 0.536^{abc}
R10		1.0 + 0.5	10	9.0 ± 0.282^{a}	8.4 ± 0.219^{a}	6.6 ± 0.357^{a}
R11		2.0 + 0.25	10	7.4 ± 0.726^{bcd}	6.0 ± 0.8^{cd}	4.0 ± 0.489^{cd}
R12		2.0 + 0.5	10	4.4 ± 0.357^{ef}	4.0 ± 0.282^{efg}	3.0 ± 0.565^{de}
R13	MS+2,4-D	1.0	10	7.4 ± 0.669^{bcd}	6.4 ± 0.726^{bcd}	5.0 ± 0.4^{bc}
R14		2.0	10	6.0 ± 0.4^{d}	5.0 ± 0.489^{def}	4.0 ± 0.282^{cd}
R15		3.0	10	4.4 ± 0.219^{ef}	4.0 ± 0.282^{efg}	3.0 ± 0.4^{de}
R16		4.0	10	0^{g}	0^{i}	0^{g}
R17	MS+NAA	1.0	10	4.6 ± 0.357^{e}	3.4 ± 0.219^{fgh}	2.4 ± 0.456^{ef}
R18		2.0	10	4.0 ± 0.282^{ef}	3.0 ± 0.282^{gh}	2.0 ± 0.282^{ef}
R19		3.0	10	3.0 ± 0.489^{efg}	2.0 ± 0.632^{h}	1.4 ± 0.357^{f}
R20		4.0	10	0^{g}	0^{i}	0^{g}
LSD				**1.293**	**1.46**	**1.319**

Means followed by different letters in the same column differ significantly at P = 0.05 according to Duncan's new multiple range test.

Table 4. Effect of incubation period on survival of embryo.

Source of Embryogenesis	Number of cultures examined	Embryogenic cultures survived (14 week old) at different incubation periods (week)						
		0	2	4	6	8	10	LSD
Direct	10	10^{a}	9 ± 0.4^{ab}	8 ± 0.282^{bc}	7.6 ± 0.456^{c}	7.4 ± 0.536^{c}	6 ± 0.4^{d}	1.261
	% Age of survival	100	90	80	76	74	60	
Indirect	10	10^{a}	8.4 ± 0.219^{b}	6 ± 0.4^{c}	4.6 ± 0.219^{d}	2.6 ± 0.357^{e}	1.6 ± 0.486^{e}	0.969
	% Age of survival	100	84	60	46	26	16	

Conclusion

Regarding regeneration potential of embryogenesis, the best selected medium for variety CP 77,400 was medium R_1 i.e. MS media containing 1.0 mg/l of BAP, while in BL-4 the combination of 1.0 mg/l BAP with 0.5 mg/l of kinetin i.e. medium R_{10} gave best results (Tables 3; Fig. 5a and b). It was observed that the mode of direct embryogenesis was rapid than the indirect one. In some explants direct embryogenesis was obtained even after four weeks of incubation. The rate of survival of direct embryos was higher than indirect embryos.

Discussion

Somatic embryogenesis is a process in which a bipolar structure, resembling a zygotic embryo, develops from a non-zygotic cell without vascular connection with the original tissue. Acquisition of embryogenic competence largely rely on dedifferentiation when the existing developmental information must be stopped or altered in order to make the cells responsive for new signals. It is generally accepted that the reactivation of cell division in somatic plant cells is required for dedifferentiation (Nagata *et al*., 1994) and the establishement of embryogenic competence (Dudits *et al*., 1995; Yeung, 1995). Feher *et al*., (2003) reported that during this somatic-to-embryogenic transition, cells have to dedifferentiate, activate their cell division cycle and reorganize their physiology, metabolism and gene expression patterns.

In the present study, somatic embryogenesis was achieved both by direct and indirect pathways. Embryogenic calli are characterized by cells organized into embryoid structures while cells of non-embryogenic calli formed disorganized mass. Quiroz *et al.*, (2002) reported that both direct and indirect embryos have unicellular origin.

Results of present study revealed that the induction of direct somatic embryogenesis was dependant on type of explant, supplemented hormones and light and dark conditions. Among different explants used, the leaf explant exhibited high frequency of embryo induction. Rui & Seppo (2004) reported in rye that genotype, culture medium, sucrose, gel agent and auxin influenced somatic embryogenesis of immature embryo significantly. Lakshmanan *et al*., (2006) reported the best response of young leaf explants for direct somatic embryogenesis in sugarcane. Himanshu *et al.*, (2000) and Chengalrayan & Meagher (2001) also used leaf explant for somatic embryogenesis in sugarcane.

For direct somatic embryogenesis MS medium was supplemented with either auxins, cytokinins or auxin-cytokinin combinations. Best results were obtained in medium containing auxin-cytokinin combination. Among different combinations used maximum embryogenesis was observed in medium containing 3.0mg/l 2,4-D and 0.25 mg/l BAP. Results obtained by Geeta & Padmanabhan (2001) and Mukherjee *et al.,* (2001) also support our study. Himanshu *et al*., (2000) reported that the best response of sugarcane for somatic embryogenesis can be obtained in MS medium supplemented with 4.0 mg/l 2,4-D and 0.5 mg/l kinetin. However, in to present investigation the use of 2,4-D either alone or in combination with kinetin failed to show good results for direct somatic embryogenesis.

In the present study it was observed that direct somatic embryos appeared on cut edges of leaf incubated at 16 hour light and 8 hour dark conditions. The cultures placed under complete darkness did not produce somatic embryos but when shifted to light

somatic embryos appeared. Siddiqui (1993) and Geeta & Padmanabhan (2001) reported similar findings for direct somatic embryogenesis, when they used leaf explant in 16 and 8 hours light and dark conditions. Zhang *et al*., (2005) reported the formation of direct somatic embryos from cut edges of leaf, petiole and stem explant in *golden pothos*. Histological examination of direct embryogenesis revealed that at approximately one week after the explant had been placed in culture the development of embryo began in the form of small, isodiametric and densely cytoplasmic cells that underwent a series of organized division (Quiroz *et al*., 2002).

The formation of somatic embryos *via* intervening callus is called indirect somatic embryogenesis (Emons, 1994). The results of present investigation also demonstrate that leaf explant is fairly good source for embryo induction through callus. The callus starts proliferating into friable creamy mass of well organized callus tissues. Somatic embryos arose from single cell on the surface of proliferating callus in 4 to 5 weeks. After 4 to 5 weeks of incubation in MS medium containing less amount of 2,4-D and BAP, globular structure of embryo emerged on callus surface which later formed scutellar and coleoptilar stage. Similar pattern of embryo formation was observed by Deepti *et al*., (2002) in rice.

The results of present study also demonstrate the effect of type, concentration and interaction of auxins on the induction of indirect somatic embryogenesis. Among auxins, both 2,4-D successfully induced embryo but NAA could not provide satisfactory results. Further study revealed that by decreasing the concentration of 2,4-D, rate of somatic embryogenesis was increased. Jimenez (2001) and Yang *et al*., (2003) also obtained somatic embryos by lowering the concentration of auxin 2,4-D in *Phragmites communis.* Ceccarelli (2000) reported somatic embryogenesis in *Dacus carota* even by the removal of 2,4-D. In the continued presence of auxin, a differential change in the gene expression (probably associated with increased demethylation of DNA (Lo *et al.,* 1989) in PEM (i.e. pro-embryogenic masses) occurs (Litz & Gray, 1995). Under these circumstances, the PEM within the culture synthesize all the gene products necessary to complete the globular stage of embryogenesis. At that point the PEM also contain many other mRNAs and proteins whose continued presence generally inhibits the continuation of the embryogenic programme. The removal or decrease in the concentration of auxin results in the inactivation of a number of genes so that the embryogenic programme can now proceed (Zimmerman, 1993).

Best results for indirect somatic embryogenesis in present investigation were observed when combination of auxin (2,4-D) with cytokinin was used. One of the possible targets of auxin action in this respect is the induction of the expression of the cdc2 gene coding for the key regulatory protein kinase of the cell cycle. Auxin alone can result in the accumulation of this protein in high amounts but for the activation of the kinase the presence of cytokinin is required (Pasternak *et al*., 2000). Among different combinations of auxins and cytokinins used, 1.0 mg/l of 2,4-D with 0.25 mg/l of BAP provided best results for embryo induction in CP 77,400 while in case of BL-4, concentration of BAP was increased to 0.5 mg/l with same concentration of 2,4-D i.e. 1.0 mg/l. These results also showed the different response of different genotypes on same medium. Milad *et al.,* (2001) reported that the entire *In vitro* cultivar trait are highly and significantly influenced by difference in genotype. Nuutila *et al.,* (2002) also reported that the cultivation of same specie may differ drastically in their requirement for medium components.

In the present study it was observed that during somatic embryogenesis, sustainability and further growth of germinated embryos was the real obstacle in obtaining complete plant. The rate of embryo survival declined sharply with age of culture. Most embryogenic culture lost their growth potential due to browning of tip or complete necrosis of whole culture.

During somatic embryogenic induction of cells, there is differential gene expression resulting in the synthesis of mRNA and proteins. This genetic information in turn elicits diverse cellular and physiological response that are involved in "switching over" of the developmental program of somatic cells (Archana & Paramjit, 2002). Various model systems have been investigated to understand the mechanism of gene regulation during somatic embryogenesis. However, the precise mechanism controlling plant gene expression and detailed steps by which these genes direct the plant specific process of somatic embryogenesis is still far from being clearly understood.

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