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RAPID CLONAL MULTIPLICATION OF SUGARCANE (*SACCHARUM OFFICINARUM*) THROUGH CALLOGENESIS AND ORGANOGENESIS

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Abstract

The results of present investigation demonstrated that inner fresh leaves and shoot apical meristem of sugarcane were highly amenable to *In vitro* callus culture. Among the auxins, 2, 4-D @ 3.0 mg/l was more potent for callus induction and its subsequent growth. Effect of auxin-cytokinin interactions were not significant with respect to callus formation. For prolonged maintenance and curtailing the morphogenic capability, subsequent decrease in 2,4-D level was essential. Two major type of calluses developed from the cultured explants: dry nodular compact and smooth compact. Most abundantly formed callus was dry nodular and compact which was white to yellowish white in colour and was highly morphogenic. The optimum temperature for callus induction and proliferation was found to be 27 ± 1°C. A significant increase in the rate of callus initiation and proliferation was observed in cultures incubated in dark as compared with light grown cultures. Best regeneration response was obtained in hormone mediated conditions. In CP 77,400, best shoot induction response was obtained on MS medium supplemented with 1.0 mg/l BAP while in BL-4, MS medium containing 0.25 mg/l BAP + 0.25 mg/l Kinetin showed best organogenic response. Vigorous rooting of plantlets in both the varieties of sugarcane was induced after 7 days of inoculation on MS medium supplemented with 2.0 mg/l NAA.

Introduction

Sugarcane is an important cash crop and the main source of raw material for the production of sugar. It is also capable of providing renewable energy sources such as ethanol, biogas byproducts and fertilizers. It is cultivated as a commercial crop in nearly 60 countries spread over 5 continents where only 6 of them account for 65% of the world's entire sugarcane production, Brazil being the largest one (Viera, 2002). Since last few years substantial degree of decline has been noticed in sugarcane production which is not able to keep pace with the growth of sugar based industries. There are many reasons for deterioration in the productivity of sugarcane, the most important of which is attack of several pathogens like mycoplasma, bacteria, virus, fungus and pests. To keep control these rapidly advancing problems of sugarcane productivity and to meet the increasing demand of sugarcane products its continuous varietals improvement is necessary. Therefore it has become necessary to use biotechnological methods like plant tissue culture along with conventional breeding program to meet the increasing demand of sugarcane products. Realization of full potential of somatic cell genetics in higher plants is predicted on the ability to induce desired development state (Orton, 1979). The techniques of tissue culture has offered the opportunity to regenerate sugarcane plants from somatic tissues bypassing the sexual barrier. Plant tissue culture has capacity to

broaden the narrow genetic base by manipulating genes at cell level. These cells regenerate and may result in genetically stable and useful genotypes. The ability to regenerate plantlets from callus tissue of *Saccharum* species was first demonstrated by Heinz & Mee (1969). Plants regenerated through callus culture have now been obtained in large number of sugarcane species. The fascinating feature of cell culture is that one can alter one or few characters by keeping rest of the genome intact. Callus culture in sugarcane from different explants has been reported by many workers (Chen *et al.,* 1988; Rehman, *et al*., 2002 and Khan *et al*., 2004). Sorory & Hosien (2000) reported that callus production depends upon explant source and different genotypes requires different media for callus induction and its regeneration. As elucidated in literature, the response of different genotypes of sugarcane in tissue culture is different. Therefore, primarily investigation regarding genotype response are necessary and standardization of media, cultural conditions and best explant of each genotype must be accomplished first of all. The objective of the present research work was to standardize protocols for cultural conditions and explant source for callus production and plant regeneration of two genotypes of sugarcane (BL-4 and CP 77,400) for use in later study.

Material and Methods

Shoot apical meristem, spindle leaves and pith parenchyma in different sizes were used for the callus induction. 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 air flow 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 16 different concentrations of auxin and cytokinins alongwith 3% sucrose was used. pH of the medium was adjusted to 5.71±0.5. Agar was used for solidification of media. The media was autoclaved at 121 °C for 15 minutes at 15 lbs/in^2 pressure. Data was recorded on the frequency of callus formation, callus colour, type of callus compactness and smoothness of callus and fresh weight of callus. Effect of light and dark on callus induction, growth and proliferation was recorded. To standardize the medium for regeneration frequency of callus, different concentrations of growth hormones were used. To find the regeneration ability calli were first transferred to MS basal medium and then into media supplemented with different concentration of auxins and cytokinins. For *In vitro* rooting, the shoots that attained the height of 8-10 cm were separated one by one from multiplying cultures and were cultured onto ½ or full strength MS media supplemented with different concentration of different auxins. 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. Sub-culturing was carried out after every 4 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 or transparent glass beaker to provide

maximum humidity. The polythene or glass beakers were 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. Potted plants were brought out from green house into open sun light for few days. Eventually these plants were shifted into the field for further growth.

Results

Callus could be obtained from explants of almost any part of the original plant. However, callus formation was greatly influenced by type of explant, culture medium, photoperiod and temperature. In the present study three explants i.e., leaf, shoot apical meristem and pith segments were used for callogenesis. Explant from leaf had highest potential for callus formation and proliferation while explant from shoot apical meristem had relatively less potential for callus formation. Among various types of leaves, young and newly formed leaves resulted in better callus formation. When mature leaves were used mostly rooting callus was formed, which did not show further proliferation or regeneration response. On the other hand, explant from pith proved less efficient for callus formation and growth. Among the various concentrations of auxins used for callus induction, the concentration of 2, 4-D at 3.0 mg/l (Medium C_3) proved best for maximum callus induction and proliferation in all kinds of explants of both the varieties (Table 1). At this concentration the rate of callus formation in CP 77,400 was 100% in leaf, 90% in meristem and 60% in pith while in BL-4 it was 100, 90 and 70% in leaf, meristem and pith, respectively. Increase or decrease in the concentration of 2, 4–D adversely affected the rate of callus formation and growth. (Table 1). Eight different auxin-cytokinin combinations were also tested (media C_5 to C_{12}, Table 1). The combination of 2,4- D with BAP did not show good results in variety BL-4 while in CP 77,400 this combination proved effective (Table 1). A combination of 2, 4-D and Kinetin was promotive for BL-4, but the results were not good for CP 77,400 (Table 1). In dark regimes, callus induction was 2 days earlier than in light (Table 2a and b). Moreover, the frequency of callus formation and proliferation was also high in dark as compared to light conditions. The callus formed in dark was white to yellowish white in colour. On the other hand callus induced in light was golden brown in colour. In case of dark conditions mostly dry nodular and compact callus was formed which was morphogenic in nature (Fig. 2a). In light conditions two type of calluses were formed: Dry nodular and compact which was morphogenic and smooth compact which was non morphogenic in nature (Fig. 2b).

The proliferation response of callus varied with respect to sub-culturing. In main culture, callus induced from different explants was less prolific as compared to subsequent sub-cultures. Proliferation response increased with sub-culturing and highest proliferation response was obtained in 3rd subculturing. From the data, it is concluded that for both the varieties the selected medium regarding callus induction and proliferation was C_3 i.e. 2,4- D at the concentration of 3.0 mg/l. Excellent callus formation and proliferation was obtained at 27°C ± 1°C. Maximum morphogenic response of callus was noticed in 3rd sub-culture.

Table 1. Effect of different hormones on callus growth and development.

Treatment No.	Media concentration (mg/l)	% Age of callus induction						Proliferation*					
		Leaf		SAM		Pith		Leaf		SAM		Pith	
		CP 77,400	BL-4	CP 77,400	BL-4	CP 77,400	BL-4	CP 77,400	BL-4	CP 77,400	BL-4	CP 77,400	BL-4
C_1	MS + 2,4-D1.0	60	50	50	50	30	20	++	++	++	++	+	+
C_2	MS + 2,4-D 2.0	80	80	70	70	40	50	+++	+++	+++	+++	++	++
C_3	MS + 2,4-D3.0	100	100	90	90	60	70	++++	++++	++++	++++	++++	++++
C_4	MS + 2,4-D4.0	80	80	60	70	40	50	+++	+++	+++	+++	++	+++
C_5	MS + 2,4D 2.0 + BAP 0.5	40	30	30	30	20	20	++	++	+	++	+	+
C_6	MS + 2,4-D 3.0 + BAP 0.5	60	50	50	40	30	20	++	++	++	++	+	+
C_7	MS + 2,4-D 3.0 + BAP 0.25	90	70	80	50	60	40	+++	+++	+++	+++	+++	+++
C_8	MS + 2,4-D 4.0 + BAP 0.25	80	60	60	50	30	30	+++	+++	+++	+++	+	++
C_9	MS + 2,4-D 2.0 + Kin 0.25	30	40	20	40	20	20	+	+	+	+	+	+
C_{10}	MS + 2,4-D 3.0 + Kin 0.25	60	70	40	60	20	40	++	++	+	++	+	++
C_{11}	MS + 2,4-D 3.0 + Kin 0.5	70	90	60	60	40	50	+++	+++	+++	+++	++	++
C_{12}	MS + 2,4-D 4.0 + Kin 0.5	60	80	50	70	30	40	++	++	++	++	+	+
C_{13}	MS + BAP 2.0 + NAA 0.5	50	50	30	40	20	20	+	+	+	+	+	+
C_{14}	MS + BAP 3.0 + NAA 0.5	60	50	50	50	30	30	++	+	++	+	+	+
C_{15}	MS + BAP 3.0 + NAA0.25	70	70	60	50	40	40	++	+++	++	+++	++	++
C_{16}	MS + BAP 4.0 + NAA0.25	60	60	50	50	30	30	++	++	++	++	+	+

SAM = Shoot Apical Meristem

+ = Poor; ++ = Fair; +++ = Good; ++++ = Excellent

Table 2a. Effect of light and dark on callus induction in CP 77,400.

Condition	Explant	No. of explants cultured	Days for callus initiation	% Age of callus formation	Colour of callus	Type of callus
Light	Leaf	10	09 ± 0.28	90	Golden brown	Nodular/smooth compact
	SAM*	10	12 ± 0.44	80	Golden brown	Nodular/smooth compact
	Pith	10	13 ± 0.60	50	Golden brown	Nodular/smooth compact
Dark	Leaf	10	08 ± 0.24	100	White to yellowish white	Nodular compact
	SAM*	10	11 ± 0.40	90	White to yellowish white	Nodular compact
	Pith	10	11 ± 0.49	50	White to yellowish white	Nodular compact

• Shoot Apical Meristem

Table 2b. Effect of light and dark on callus induction in BL-4.

Condition	Explant	No. of explants cultured	Days for callus initiation	% Age of callus formation	Colour of callus	Type of callus
Light	Leaf	10	09 ± 0.346	90	Golden brown	Nodular/smooth compact
	SAM*	10	12 ± 0.374	80	Golden brown	Nodular/smooth compact
	Pith	10	13 ± 0.566	50	Golden brown	Nodular/smooth compact
Dark	Leaf	10	08 ± 0.283	100	White to yellowish white	Nodular compact
	SAM*	10	10 ± 0.316	90	White to yellowish white	Nodular compact
	Pith	10	12 ± 0.529	50	White to yellowish white	Nodular compact

• Shoot Apical Meristem

Organogenesis: Two types of callus i.e., morphogenic and non morphogenic, obtained from third sub-culture after eight week of inoculation was further sub-cultured either on growth regulators free or supplemented MS medium for efficient organ induction. When the morpghogenic callus of both the varieties was transferred to growth regulators free MS medium (M_1), the rate of regeneration was almost same in both the varieties, which was 60, 40 and 20% in CP 77,400 and 60%, 50% and 20% in BL-4 in leaf, shoot apical meristem and pith, respectively (Table 4). To further accelerate the rate of regeneration, MS medium was supplemented with different concentrations and combinations of BAP and kinetin.

In case of variety CP 77,400, maximum shoot differentiation was obtained in fifth sub-culture in M_2 Medium (MS medium supplemented with 1.0 mg/l BAP) (Fig. 4a, 5a and 6). At this combination 70% shoot induction was observed in callus obtained both from leaf and shoot apical meristem and 40% in callus from pith explant. In case of BL-4, best organogenic response was obtained in M_{10} medium (MS medium supplemented with 0.25 mg/l BAP and 0.25 mg/l Kinetin). At this concentration 70, 60 and 40% shoot induction was observed from callus of leaf, shoot apical meristem and pith, respectively (Fig. 4.b and 5b). Among the callus obtained from different explants, the callus derived from leaf showed better results for organ (shoot and root) induction, followed by shoot apical meristem and pith explants respectively (Table 4). Maximum organogenic response from morphogenic callus was noticed after ten week of inoculation in fifth sub-culture. By increasing the time of incubation for more than 13 week i.e., 6^{th} sub-culture, rate of organogenesis was decreased and rate of necrosis was increased. After 15 weeks i.e., in seventh sub-culture, 60% callus became dead with very poor organogenic response.

In general, non morphogenic callus showed poor organogenesis. The maximum organogenic response obtained was 40% from leaf explant in N_2 medium in both the varieties (Table 5). Of the two varieties, BL-4 showed better organogenic response from leaf and shoot apical meristem as compared to CP 77,400 for the same explants. The shoots produced both from morphogenic and non-morphogenic calluses were subjected to half (½) as well as full strength of MS medium both in basal form as well as in combination with different auxins for rooting (Table 6). Only 20% rooting was obtained from shoot grown both at half as well as full strength MS basal medium within 14 days of inoculation. However, when full strength MS medium was supplemented with 2.0 mg/l NAA (R_9 medium), 90% rooting was obtained, within 7 days of incubation in CP 77,400 and 6 days of inoculation in BL-4 (Table 6). Number of roots was also high at this concentration. It was four roots per plantlet in CP 77400 and 3.4 roots per plantlet in BL-4. An increase or decrease in the concentration of NAA, not only rate of root induction and number of roots per plant was decreased but days for root formation were also increased. MS medium supplemented with a combination of 1.0 mg/l NAA and 2.0 mg/l IBA also gave good rooting response under this treatment, 80% rooting was obtained in both the varieties, within 7 days of inoculation. By increasing the concentration of either IBA or NAA, rate of root induction was decreased. Among both the varieties BL-4 showed better rooting as compared to CP 77,400 (Table 6).

Fig. 1a. Well developed dry nodular compact and highly morphogenic callus formed from leaf explant after 56 days of inoculation in 3rd subculture in MS medium containing 3.0 mg/l 2,4-D Var. CP 77,400 (2.5X).

Fig. 1b. Well developed dry nodular compact and highly morphogenic callus formed from leaf explant after 56 days of inoculation in 3rd subculture in MS medium containing 3.0 mg/l 2,4-D. Var. BL-4 (3.0X).

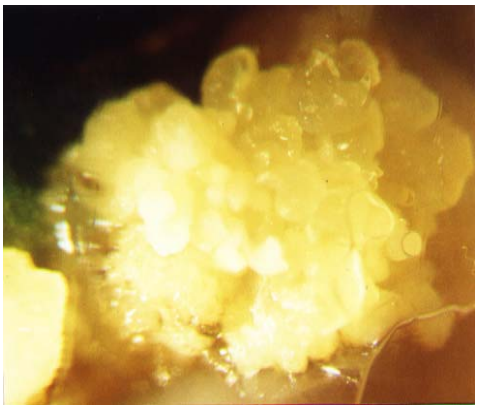

Fig. 2a. Dry nodular compact callus formed from leaf explant after 56 days of inoculation in 3rd sub- culture on MS medium containing 3.0 mg/l 2,4-D (20X).

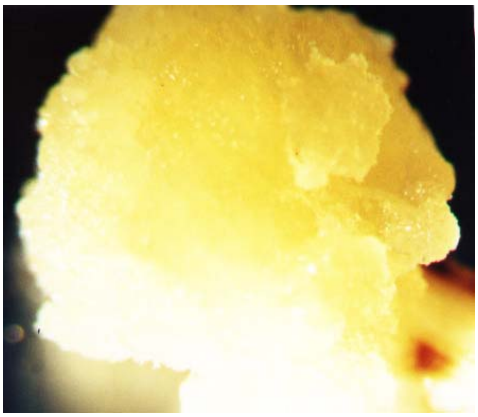

Fig. 2b. Smooth and compact callus formed from leaf explant after 56 days of inoculation in 3rd sub- culture on MS medium containing 3.0 mg/l 2,4-D (20X).

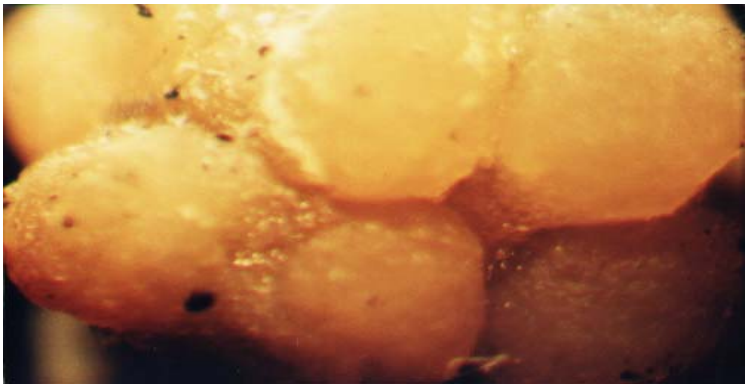

Fig. 3. A magnified view of nodular compact and morphogenic callus formed from leaf explant after 56 days of inoculation in 3rd subculture in MS medium containing 3.0 mg/l 2,4-D (20X).

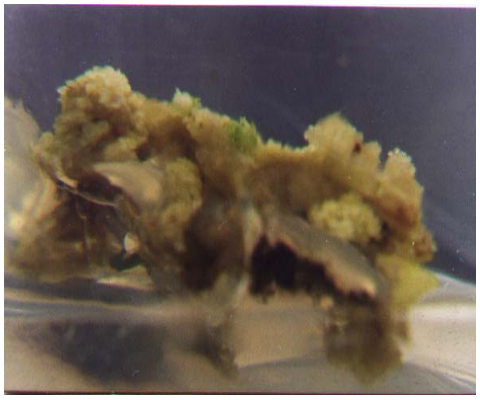

Fig. 4a. Proliferated morphogenic callus obtained from leaf explant with differentiated green patches after 9 week of inoculation in 4th sub-culture on MS medium containing 1.0 mg/l BAP Var. CP 77,400 (4X).

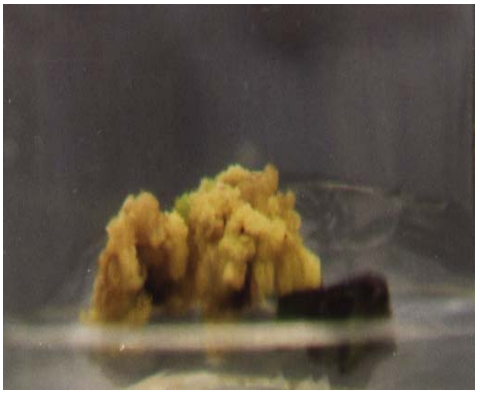

Fig. 4b. Proliferated morphogenic callus obtained from leaf explant with differentiated green patches after 9 week of inoculation in 4th sub-culture on MS medium containing 0.25 mg/l BAP + 0.25 mg/l Kin. Var. BL-4 (4X).

Fig. 5a. Direct regeneration from callus obtained from leaf explant after 10 week of inoculation in 5th subculture on MS medium containing 1.0 mg/l of BAP Var. CP 77,400 (3.5X).

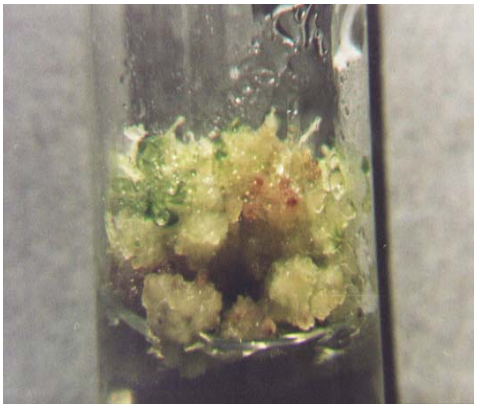

Fig. 5b. Direct regeneration from callus obtainedfrom leaf explant after 10 week of inoculation in 5th subculture on MS medium containing 0.25 mg/l BAP+ 0.25 mg/l Kin. Var. BL-4 (4X).

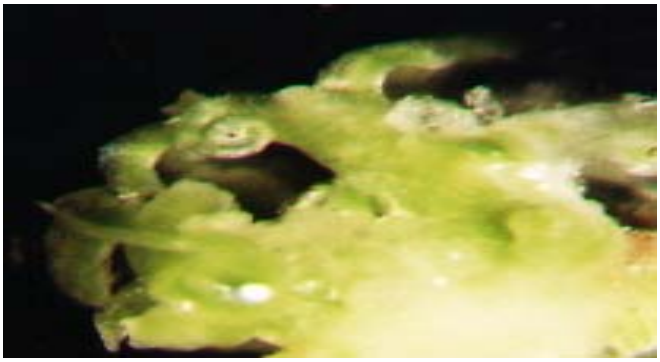

Fig. 6. Magnified view of plant regeneration form callus obtained from leaf explant after 10 week of inoculation in 5th subculture (20X).

Table 3. Effect of sub-cultures on callus growth.

Variety	CP 77,400			BL-4		
Explant	**Leaf**	**SAM***	**Pith**	**Leaf**	**SAM***	**Pith**
Main culture	1.45 ± 0.017^{d}	1.42 ± 0.010^{e}	1.32 ± 0.005^{d}	1.32 ± 0.019^{d}	1.05 ± 0.0288^{d}	1.06 ± 0.024^{d}
1st subculture	1.68 ± 0.019^{c}	1.64 ± 0.015^{d}	1.52 ± 0.015^{c}	1.51 ± 0.015^{c}	1.19 ± 0.0125^{c}	1.18 ± 0.017^{c}
2nd subculture	1.82 ± 0.0075^{b}	1.79 ± 0.015^{b}	1.78 ± 0.008^{b}	1.77 ± 0.01^{b}	1.32 ± 0.017^{b}	1.30 ± 0.0141^{b}
3rd subculture	1.98 ± 0.014^{a}	1.94 ± 0.017^{a}	1.95 ± 0.015^{a}	1.84 ± 0.017^{a}	1.45 ± 0.0075^{a}	1.45 ± 0.0216^{a}
4th subculture	1.78 ± 0.003b	1.72 ± 0.0125^{c}	1.55 ± 0.012^{c}	1.56 ± 0.015^{c}	1.09 ± 0.0262^{d}	1.07 ± 0.019^{d}
5th subculture	1.40 ± 0.0125^{d}	1.39 ± 0.0141^{e}	1.29 ± 0.010^{d}	1.32 ± 0.014^{d}	0.84 ± 0.0141^{e}	0.80 ± 0.0125^{e}
6th subculture	1.30 ± 0.0216^{e}	1.28 ± 0.010^{f}	1.20 ± 0.016^{e}	1.20 ± 0.018^{e}	0.69 ± 0.0145^{f}	0.70 ± 0.0082^{f}
7th subculture	1.22 ± 0.017^{f}	1.21 ± 0.008^{g}	1.13 ± 0.008^{f}	1.13 ± 0.019^{f}	0.62 ± 0.018^{g}	0.65 ± 0.010^{f}
L.S.D.	**00.055**	**0.0479**	**0.0443**	**0.058**	**0.068**	**0.061**

*SAM = Shoot apical meristem

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. Organogenesis from morphogenic callus.

Treatment No.	Media	Concentration (mg/l)	% Age of shoot induction					
			Leaf		SAM		Pith	
			CP 77,4000	BL-4	CP 77,4000	BL-4	CP 77,4000	BL-4
M_1	MS Basal		60	60	40	50	20	20
M_2	MS + BAP	1.0	70	60	70	50	40	30
M_3		1.5	50	40	40	40	30	30
M_4		2.0	40	40	40	40	20	20
M_5		2.5	20	30	20	20	10	10
M_6	MS + Kinetin	1.0	40	40	30	40	20	20
M_7		1.5	40	40	20	30	10	10
M_8		2.0	20	20	20	20	10	10
M_9		2.5	10	10	10	10	00	10
M_{10}	MS+BAP+Kinetin	0.25+0.25	60	70	50	60	30	40
M_{11}		0.5+0.25	50	70	40	50	30	30
M_{12}		1.0+ 0.50	30	60	30	40	20	20
M_{13}		2.0+0.50	20	40	10	30	10	20
M_{14}	MS + 2,4-D	1	30	40	30	30	20	20
M_{15}		2	30	30	20	30	10	20
M_{16}		3	40	50	40	40	10	10
M_{17}		4	30	30	20	30	10	10

Photoperiod = Sixteen hours light and eight hours dark.

Number of cultures examined = 10 each and Age of culture = 11 week

Table 5. Organogenesis from non-morphogenic callus.

Treatment No.	Media	Concentration (mg/l)	% Age of shoot induction					
			Leaf		SAM		Pith	
			CP 77,4000	BL-4	CP 77,4000	BL-4	CP 77,4000	BL-4
N_1	MS Basal		30	30	30	30	10	10
N_2	MS + BAP	1.0	40	40	30	40	10	10
N_3		1.5	20	40	20	20	10	10
N_4		2.0	10	20	10	10	10	10
N_5		2.5	10	20	10	10	00	00
N_6	MS + Kinetin	0.25 + 0.25	30	40	30	40	10	10
N_7		0.5 + 0.25	30	40	20	20	10	10
N_8		1.0 + 0.50	20	30	10	20	00	00
N_9		2.0 + 0.50	10	10	10	10	00	00

Number of cultures examined = 10 each and Age of culture = 11 week

Table 6. Rooting of induced shoots.

	Media Concentration (mg/l)	% Age of root formation		Days for root induction		Number of roots/plant	
		CP 77,400	BL-4	CP 77,400	BL-4	CP 77,400	BL-4
R	½ MS	20	20	14 ± 1.0a	14.0 ± 0.5477^{a}	1.1 ± 0.330^{d}	1.0 ± 0.2^{g}
R_1	MS Basal	20	20	13 ± 0.693ab	14.0 ± 0.53^{a}	1.3 ± 0.285	1.3 ± 0.145
R_2	½ MS + IAA 1.0	20	70	12 ± 0.9592^{ab}	9.0 ± 0.5657^{c}	1.7 ± .2846^{cd}	2 ± 0.3162^{ef}
R_3	½ MS + IAA 2.0	80	60	11 ± 0.9899^{bc}	8.0 ± 0.4^{cd}	2.6 ± 0.2529^{bc}	2.3 ± 0.247^{de}
R_4	½ MS + IAA 3.0	60	40	12 ± 0.9487^{ab}	8.0 ± 0.4472^{cd}	2.4 ± 0.2898^{bc}	2.0 ± 0.2449^{ef}
R_5	½ MS + IAA 1.0	70	20	08 ± 0.2828^{d}	11.8 ± .7042^{b}	1.8 ± 0.3098c^{d}	1.4 ± 0.1549^{fg}
R_6	½ MS + IAA 2.0	60	80	09 ± 0.4^{cd}	9.0 ± 0.6164^{c}	2.0 ± 0.3741^{bcd}	2.0 ± 0.245^{ef}
R_7	½ MS + IAA 3.0	40	60	08 ± 0.3742^{d}	9.0 ± 0.3742^{c}	2.0 ± 0.2449^{bcd}	2.1 ± 0.2983^{ef}
R_8	MS + NAA 1.0	60	60	09 ± 0.8124^{cd}	8.0 ± 0.4^{cd}	3.0 ± 0.2^{ab}	2.3 ± 0.2025^{de}
R_9	MS + NAA 2.0	90	90	07 ± 0.4^{d}	6.0 ± 0.4472^{e}	4.0 ± 0.3464^{a}	3.4 ± .2898^{abc}
R_{10}	MS + NAA 3.0	80	80	08 ± 0.316^{d}	8.0 ± 0.6928^{cd}	3.0 ± 0.4243^{ab}	3.0 ±0.2828^{bcd}
R_{11}	NAA 1.0 + IBA 2.0	80	80	07 ± 0.3162^{d}	7.0 ± 0.4898^{de}	4.0 ± 0.3464^{a}	3.6 ± 0.3225^{ab}
R_{12}	NAA 2.0 + IBA 4.0	80	80	07 ± 0.3742^{d}	7.0 ± 0.3464^{de}	2.0 ± 0.3742^{bc}	3.9 ± 0.2983^{a}
R_{13}	NAA 1.5 + IBA 3.0	70	70	09 ± 0.3464^{cd}	9.0 ± 0.4898^{c}	2.0 ± 0.3162b^{cd}	2.7 ± 0.2025^{cde}
				1.91	**1.52**	**0.9457**	**0.765**

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

Discussion

Callogenesis: Callus growth follows a typical logrithmic pattern involving slow initial cell division induction period requiring auxin, a rapid cell division phase involving active synthesis of DNA, RNA and protein followed by a gradual cessation of cell division along with differentiation of callus mass (Hartmann *et al.,* 1999). Among different hormones used for callus induction and proliferation, mostly auxins are reported to be most effective. Among different auxins used, 2, 4-D is reported to have better results for callus induction and proliferation in cereals. It is reported to exhibit its vitality in almost all cereal crops (Wen *et al.*, 1991).

Present investigation demonstrated the effect of auxins and auxins-cytokinins interaction on callus induction and proliferation in sugarcane. Best results for callus induction and proliferation was obtained on MS medium containing 3.0 mg/l 2, 4-D in both the varieties of sugarcane (Table 1). Mannan & Amin (1999) and Prajapati *et al., (*2000) reported the better performance of 2, 4-D for callus induction and proliferation in sugarcane. The promotive effect of the same dose of 2, 4-D for maximum callus induction and proliferation in sugarcane was frequently reported by many other scientists (McCallum *et al.,* 1998; Somashekhar *et al.,* 2000; Javed *et al.*, 2001).

The hormone mediated callus induction and subsequent growth on *a priori* is dependent on certain factors which may trigger the complete chain of events that influence the ability of cultured cells to grow in an organized fashion. Plant tissues, therefore, must have receptors for hormones. These hormones interact with specific receptors that reside either on cell membrane or within the cytoplasm (Mockeviciute & Anisimoviene, 1999). Affinity and concentration of receptors on the surface of the target tissues determine the type of response. Specific binding site for both auxin (Kim *et al*., 2001) and cytokinin have been identified (Yamada *et al*., 2001). A class of proteins called expansins mediates the proton ability to cause cell wall loosening (McQueen *et al*., 1995). These expansins break the hydrogen bonds between the polysaccharide components of the wall (Cosgrove, 2001). Proton (H+) pumping and lowering of cytosolic pH result in an elevation of intracellular calcium level (Shishova *et al*., 1999). Both cytosolic pH and calcium ions act as second messengers in early auxin action (Zhang, 2003). Calcium ions, either themselves and or along with calcium binding proteins e.g., calmodulin activate the protein kinase cascade which in turn activates other proteins, including the transcription factors (Wagner 2001). These factors presumably interact with the auxin-response elements and regulate the expression of auxin-inducible or auxin-responsive genes and exert its effect on cell cycle and stimulate cell division (Johri & Mitra, 2001).

The cultured explant in this study comprised of shoot apical meristem, newly formed leaves around meristem and pith parenchyma. As these different explants consisting of callus are triggered by some hormone, the higher the meristematic region within the callus mass, the greater will be the probability of differentiation (Fitch & Moore, 1993). The results of our findings also reaffirm same results. So the callus formed from shoot apical meristem and newly formed whorl of leaves around meristem exhibited maximum morphogenic potential due to their greater meristematic nature, while callus originating from pith parenchyma showed least morphogenic response. Hoque & Mansfield (2004) also confirmed that younger explants were more efficient in callus induction. Cultures incubated at 27 ± 1ºC produced maximum callus induction and proliferation. However, Khan (1998) reported 28 ± 1ºC as the optimum temperature for callus formation. The

discrepancy may depend on the cultivar used. The results of present study has also shown that continuous dark period has more promotive effect for callus induction and proliferation as compared to continuous and interrupted light.

Organogenesis: Organogenesis *In vitro* consists of many aspects such as phytohormone perception, dedifferentiation of differentiated cells to acquire organogenic competence (Lo *et al.*, 1997), re-entry of quiescent cells into cell cycle, and organization of cell division to form specific organ primordia and meristems (Burritt & Leung, 1996). The present study also highlights the differentiated response of morphogenic and non-morphogenic callus into shoot or root on hormone free or selected regeneration medium. The results of present study revealed that morphogenic callus (dry nodular and compact) exhibited more potential for shoot regeneration even on hormone free medium. The callus subjected to hormone free medium may contain a population of cells which were the part of original meristematic tissues. Meristematic cells of callus retain relatively higher totipotency and swiftly induce the organ formation even on the basal medium (Siddiqui, 1993). Part of nodular and smooth callus which did not differentiate in hormone free medium, when shifted into the medium supplemented cytokinins, underwent organogenesis or somatic embyogenesis. Initiation of differentiation begins with differentiation of group of parenchyma cells to produce centre of meristematic activity called meristemoids which leads to organ formation. The fate and shape of differentiated plant cells can be determined by the size of their undifferentiated precursors.

In the present study, some cultures showed differentiation even in the presence of same concentration of 2, 4-D, which was needed for callus formation. This can be explained on the fact that the uptake of 2, 4-D is dependant on available receptor sites. Higher the receptor sites greater will be the metabolism of 2, 4-D. When 2, 4-D molecules interact with 2, 4-D receptors, the level of 2, 4-D in medium drops which facilitate the growth of cells that restored their totipotency during these metabolic activities.

Results of present study also revealed that non-morphogenic callus also underwent differentiation on MS medium supplemented with cytokinins but its organogenic potential was very poor. This can be explained on the fact that certain factors may be required which promote the ability of cells in *In vitro* cultures to grow in an organized fashion which leads to the modification of subsequent growth. Peros *et al.,* (1990) also reported an increase in morphogenic response of callus after cytokinin treatment. Cytokinin plays a role in organogenesis by stimulating cell division both *In vivo* and *In vitro.* Immunocytochemistry and direct measurement of cytokinin both reveal high cytokinin levels in mitotically active areas, such as the root and shoot meristems and very low levels are found in tissues where the cell cycle is arrested (Corbesier *et al*., 2003; Rashotte, 2005). Application of exogenous cytokinin to some organs that normally lack this hormone has been shown to induce cell division (Agostino & Joseph, 1999). The ability of cytokinins to initiate shoots from callus in tissue culture and the initiation of ectopic meristems in cytokinin overproducing plants suggest a role for cytokinins in SAM (Shoot apical meristem) development. One possible mechanism by which cytokinins influence SAM development is by regulating gene expression. The knotted1 (kn1) homeobox family of genes is expressed exclusively in the SAM and is involved in its development and maintenance (Kerstetter, 1997). Rupp *et al.*, (1999) examined the expression of KNAT1 and STM (Shoot meristemless gene) (*Arabidopsis* homologs of

kn1) in transgenic *Arabidopsis* expressing it under the control of a heat shock promoter. The steady-state mRNA levels of both KNAT1 and STM were elevated following heat shock and were correlated to elevated cytokinin levels. Elevated KNAT1 and STM transcript levels were also observed in untreated amp1 plants, implying that endogenous cytokinin can also induce expression of these homeobox genes. These results suggest that cytokinins may act upstream of KNAT1 and STM in regulating SAM development.

Present study further highlighted the effect of different explants on morphogenic potential of callus. Explants from meristem and from inner roll of leaves covering the meristem showed higher differentiation potential. This may be due to their greater meristematic nature. Shaheeen & Mirza (1989) also reported that leaf was better explant source in sugarcane. Similar results were reported by Shahid *et al.,* (1990).

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