Karnataka J. Agric. Sci., 22(1) :(21-24) 2009

In vitro plant regeneration using shoot tip culture in commercial cultivar of sugarcane*

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(Received : April, 2008)

Abstract: A protocol for direct shoot regeneration without intervening callus phase was developed by using shoot tip culture on MS (Murashige and Skoog) medium for Sugarcane (*Saccharum* spp.) variety CoC-671. The study revealed that aseptically inoculated shoot tips of sugarcane in BAP (Benzylamino purine) @ 2.0 mg/l established the shoot induction and shoot multiplication was also observed at same concentration. At 2 mg/l of BAP (79.64%), the shoots were weak, tiny and non-separable, whereas at 1.0 mg/l of BAP (21.84%) rate of multiplication was low (5.46) but resulted in desirable quality, well grown, easily separable and healthy plantlets. The root induction was observed NAA (Napthalene acetic acid) @ 2 mg/l (70.00%) and complete plantlets were hardened and transferred to greenhouse for establishment with a survival rate of 72 per cent.

Key words: Shoot tip culture, sugarcane, shoot induction, root induction, *in vitro* regeneration

Introduction

Sugarcane is the most important sugar crop in the world and accounts for about 70 per cent (Anonymous, 2005) of the worlds total sugar production. In sugarcane, micropropagation is important for rapid multiplication of elite genotypes/clones and for the quick spread of new varieties (Nickell and Heinz, 1973). Tissue culture of sugar-cane has received considerable research attention because of its economic importance as a cash crop. Plant regeneration through tissue culture technique would be a viable alternative for improving the quality and production of sugar-cane. Initial attempts to regenerate plants through in vitro technique were made on sugar-cane by Nickell (1964) and Heinz and Mee (1969). In sugarcane conventional propagation is through sets which is slow, usually one to ten in a period of one year. Moreover, pathogens keep on accumulating generation after generation which reduces the yield and quality of sugarcane.

Usually due to lack of multiplication procedures, it requires 10 - 15 years to complete the selection cycle and to get an improved variety for commercial cultivation. The time spent for this multiplication is considered a serious economic problem, mainly in view of the higher yields that would be obtained by planting the new variety earlier on a large commercial scale, therefore efficient propagation systems are required for mass multiplication. Micropropogation through tissue culture holds immense potential for mass multiplication and subsequent rejuvenation and quality production (Heinz and Mee, 1969)

Induction of callus and regeneration of plants using sugar-cane varieties of India were reported elsewhere (Shukla *et al*., 1994, Islam *et al.*, 1996, Gosal *et al*., 1998). However, reports are scarce on shoot tip culture in sugar-cane varieties of India. In callus phase there are the maximum chances of mutation and somaclonal variation when plant regenerated from somatic embryos. Multiplication and germplasm preservation of sugar-cane is possible for this purpose; shoot tip has a greater potentiality. Therefore, the present investigation has been undertaken to establish plant regeneration protocol through shoot tip culture in sugar-cane

The present communication thus deals with the studies of direct shoot regeneration from shoot tip, multiple shoots and root induction and then establishment of plants in green house.

Material and methods

Sugarcane plants, variety CoC-671, were obtained from the Agricultural Research Station, Sankeswar, University of Agricultural Sciences, Dharwad. The plants were raised and maintained under field conditions as per the recommended agronomic practices. These plants served as the source of explants for the *in vitro* studies.

Shoot tips were collected from 6-8 months old well established plants stem segments of about 5 cm, together with meristem tip, were excised in a laminar airflow chamber. They were sterilized for one minute with fungicide (carbendazim @ 0.1%) and bactericide (streptomycin 0.1%) and then with 80 per cent ethanol for 45 seconds. The segments were then washed 3 times with double distilled water under sterile conditions and then shoot tip was dissected.

Media used for shoot tip culture establishment (MC-I), multiplication of shoots (MC-II) and root induction (MC-III) was containing the ingredients as furnished in table 1. A half strength medium of (Murashige and Skoog, 1962) salts (1/2 MS) was also used mainly for growth of plant lets.

Micropropagation was conducted in a well defined conditions of the culture room and maintained at 25± 2^0C, uniform

*Part of M.Sc.(Agri.) thesis submitted by the senior author to the University of Agricultural Sciences, Dharwad-580005, India.

light (Ca. 1000 lux) provide by fluorescent tubes (7200^{0}K) over a light/dark cycles of 16/08 hours. The resulting plantlets were contacted and transplanted into polystyrene well containing peat soil and plants were transferred to greenhouse. After 20 days.

Results and discussion

Shoot tip containing apical meristem of cultivar CoC-671 were inoculated with different concentrations of BAP for shoot tip initiations and establishment. Small shoots started appearing within 7-10 days in many of the culture tubes (Table 2). Initially there was problem of tissue browning (Liu, 1981 and 1984) due to release of phenols from the base of shoot tip, which slightly hindered the shoot growth. Sub culturing was done frequently (7 days) during the early stages of establishment to reduce the adverse effect of browning of tissues and the release of pigments in the medium (Gosal *et al*., 1998; Dobariya., 1994).

The per cent establishment varied between different levels of BAP. Frequency of establishment was maximum with BAP @ 2.0 mg/l (72%), on which shoots were active and healthy compared to other treatments. At this level of BAP at the end of 25th day, 10-15 shoots originated from a single shoot tip meristem

Table 1. Media used for shoot tip establishment (MC-I), multiplication of shoots (MC-II) and root induction of shoots (MC-III)

Ingredients	MC-I mg/l	MC-II mg/l	MC-III mg/l
1. Macro nutrients	MS	MS	MS
2. Micro nutrients	MS	MS	MS
3. Vitamins			
- Thiamine-Hcl	1.00	1.00	1.00
- Pyridoxine-Hcl	1.00	1.00	1.00
- Nicotin acid	1.00	1.00	1.00
- Glycine	10.00	10.00	10.00
- Myoinositol	100.00	100.00	100.00
- Biotin	-	0.50	0.50
4. Hormones			
- BAP	2.00	2.00	-
- NAA	-	-	2.00
5. Other supplements			
- Coconut water	-	10% v/v	-
6. Sucrose	30.00	30.00	30.00
7. Agar	8.00	8.00	8.00
8. pH before autoclaving	5.8	5.8	5.8

Table 2. Effect of benzylamino purine on establishment of shoots

Sl. No.	Treatment	Concentration of growth regulator (mg/l)	No. of shoot tips inoculated	No. of shoot tip established
1	MS	0.00	25	0 (0.00)
2	MS + BAP	0.50	25	2 (8.00)
3	MS + BAP	1.00	25	4 (20.00)
4	MS + BAP	2.00	25	18 (72.00)
	Mean		-	6.00
	SEm±		-	0.27
	CD (0.01)		-	1.13

→ Culture medium: Murashige and Skoog
→ BAP : Benzylamino purine
→ Volume of the medium: 16ml / culture. pH - 5.8
→ Number of replications: 5. No of cultures/replications = 5
→ Culture conditions: Temperature: 26 ± 2^{0}C. Light / Dark period: 16 / 8 Hrs
→ Culture period: 28 Days. Inoculum: 300 ± 10 mg / culture
→ ** Values outside the parenthesis are transformed, where as in parenthesis are actual values

(Table 2,). Similar results were obtained by Dhumale *et al.* (1994) with BAP @ 3 mg/l and NAA @ 1 mg/l. The study of Anon. (1985) indicated that normal shoot initiation and development was obtained in two cultivars CO-62171 and CO-7201 with BAP @ 0.05 mg/l only. These observations suggest that concentration of BAP required for shoot initiation and establishment varies with genotypes. The amount of cytokinin applied and found adequate depends on the genotype used and the micropropagation strategy employed. Most investigators prefer to secure proliferation of shoots along with normal development of shoot from the cultured bud or the meristem. In such cases higher cytokinin levels have been used, whereas, normal development of the shoot from the bud meristem might require very low levels of the growth regulators, as in case of Sreenivasan and Jalaja (1983).

The rate of multiplication is high in adventitious shoots and somatic embryogenesis but the occurrence of somaclonal variation among the plantlets probably due to incipient callus is a draw back. In order to get true to type plants, the only option presently available is to secure enhanced release of axillary buds through meristem/shoot tip culture.

Although the rate of multiplication is low in shoot tip culture but variation is nil or very less. Micropropagation coupled with apical meristem culture technique ensures the production of whole plants that are generally identical. BAP at different concentration levels have been tried in the present investigation (Table 3). BAP @ 2 mg/l, recorded the highest overall rate of multiplication (79.64%) followed by BAP @ 1.0 mg/l (21.84%). These results are in contrast to the report of Alam *et al*. (1995) where 0.5-1 mg/l BAP were used for shoot multiplication.

The average rate of multiplication (79.64) observed at 2 mg/l BAP, similar to results observed by Hendre *et al*. (1983) with respect to rate of multiplication. But shoots were weak tiny and non separable making it difficult to manage. In this there is a chance of de-novo shoots through incipient callus, which goes unnoticed (Shepard *et al*., 1980). Therefore, it was considered undesirable to use 2 mg/l BAP for sugarcane, although multiplication rate was the highest (79.64%). Here plants were tiny and in closely associated packs, indicating that atleast some of them are de-novo shoots. At this rate of multiplication genetic changes are quite possible, normally the

multiplication rate of 5-10 per cent with in 25 days is sufficient to produce large number of shoots economically in most species.

Among the other treatments, BAP @ 1 mg/l recorded better overall rate of multiplication (21.84%) and another with 0.5 mg/l (8.04%). Overall rate of multiplication at these levels of BAP resulted in desirable quality well grown easily separable and healthy plant lets (Table 3). At this rate of multiplication one can produce enormous number (2 x 10^8) of plants from a single shoot tip in a year over a 4-5 weeks micropropagation cycles.

Rooting can be achieved either by transferring the shoots to medium lacking cytokinin with or without a rooting hormone.

Table 3. Effect of benzylamino purine on regeneration of shoot tip explants

Tr. No.	Treatments	Concentration of growth regulator (mg/l)	Average number of shoots per culture (Multiple shoots) (25 DAI)
1	S1=MS + BAP	0.00	0.52 (2.08)
2	S2=MS + BAP	0.50	2.01 (8.04)
3	S3=MS + BAP	1.00	5.46 (21.84)
4	S4=MS + BAP	2.00	19.91 (79.64)
5	S5=MS + BAP+NAA	2.00 + 1.00	1.89 (7.56)
	Mean	-	5.96
	SEm+	-	0.03
	CD(0.01)	-	0.10

→ Culture medium: Murashige and Skoog

→ BAP : Benzylamino purine

→ Volume of the medium: 16ml / culture. pH - 5.8

→ Number of replications: 5. No of cultures/ replication = 5

→ Culture conditions: Temperature: 26 ± 2^{0}C. Light / Dark period: 16 / 8 Hrs

→ Culture period: 28 Days. Inoculum: 300 ± 10 mg / culture

→ ** Values outside the parenthesis are transformed, where as in parenthesis are actual values

rooting (Larkin, 1982, Shukla *et al.*, 1994, Alam *et al*., 1995, Islam *et al*., 1996) and more than 5 mg/l NAA inhibits rooting. The concentration of hormone varies with variety to variety.

Hardening of plantlets is an important step in tissue culture studies. Sreenivasan and Sreenivasan (1992) observed 90-95 per cent survival of plantlets in poly house under shade. They used potting mixture of sieved, silt and rotten press mud 1:1:1 proportion. The plantlets with good root system were taken and transplanted into potting mixture by trimming the leaves.

Rooting can be improved by lowering the concentration of macro salts to a half or less. This is analogous to the report of Mohatkar *et al*. (1993) who obtained profuse and healthy rooting of sugarcane shoots on half strength of MS medium.

Auxin is essential for root induction in many species. However, high auxin concentration has been reported to inhibit the root elongation phase (Thimann, 1977). The cultivar CoC-671 recorded 80 per cent frequency with NAA @ 2 mg/l, whereas NAA @ 3 mg/l recorded 70 per cent of rooting (Table 4). This might be due to inhibition of rooting at higher concentration of NAA because it inhibits the root induction and elongation. Many workers also reported that 5 mg/l NAA was good for

Table 4. Effect of napthalene acetic acid on root induction

Tr. No.	Treatment	Concentration of growth regulator (mg/l)	No. of healthy roots
1	MS	0.00	5 (50.00)
2	MS + NAA	0.50	6 (60.00)
3	MS + NAA	1.00	7 (70.00)
4	MS + NAA	2.00	8 (80.00))
5	MS + NAA	3.00	7 (70.00)
	Mean	-	6.6
	SEm+	-	0.32
	CD(0.01)	-	1.27

→ Culture medium: Murashige and Skoog

→ NAA: Napthalene acetic acid

→ Volume of the medium: 16ml / culture. pH - 5.8

→ Number of replications: 5. No of cultures/ replication = 5

→ Culture conditions: Temperature: 26 ± 2^{0}C. Light / Dark period: 16 / 8 Hrs

→ Culture period: 28 Days. Inoculum: 300 ± 10 mg / culture

→ Values in parenthesis are in percentage

The survival rate of micropropagated plants was 72 per cent. This is due to the variation in the method of propagation and environmental factors because temperature and humidity are the two key factors, which control survival rate of plant lets in polyhouse. The largest problem concerns the drop in relative humidity from near 100 per cent in the culture vessels to much lower values in the polyhouse and also in field. Due to absence of epicuticular waxon in-vitro shoots leads to excessive water loss. Temperature around 24-26^{0}C and relative humidity around 75-90 per cent resulted in 90 per cent survival Doobariya (1994).

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