



## DEVELOPMENT OF PROTOCOL FOR MASS MULTIPLICATION OF TWO ELITE VARIETIES OF SUGARCANE THROUGH MICROPROPAGATION.

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**ABSTRACT:** Sugarcane (*Saccharum officinarum* L.) also called "noble canes" by Dutch scientists, belongs to family *Poaceae* and genus *Saccharum*. Sugar cane is the main source of sugar in all tropical and subtropical countries of the world with an annual production of 40 - 41 million tons. Sugar i.e. sucrose is necessary part of daily diet and a major portion of total sugar produced is used in alcoholic beverages, soft drinks, ice-creams, chocolates, canning industries and its by-products i.e. molasses and bagasse are also utilized as raw material for manure, fiber board and paper making units and even as fuel in sugar mills. Sugar cane is a tall, perennial grasses which is propagated vegetatively by stem cuttings called 'setts' having healthy buds. The conventionally propagated sets thus use a substantial number of canes and yet do not ensure uniformity, pest free or disease free status of planting material. The *in vitro* regeneration methods instead ensure large number of uniform, disease free and vigorous planting material in relatively lesser time. In this study we were emphases on the shoot tips containing auxiliary meristem were inoculated in MS medium with a fixed concentration of BAP (1mg/lit) for shoot tip initiation and establishment. Small shoots started appearing with in 7-10 days in all cultures bottles. Maximum shoot lengths were obtained in SM-II media i.e. MS supplemented with 0.25mg/lit BAP. After 30 days of incubation the average shoot length recorded in SM-II medium was found to be 1.63cm in Co-86032 and 1.65cm in Co-94012. Similarly the regenerated shoots were used for root induction in root forming media. The response of root formation was different in all media, as highest root formation average was observed in RM-III (3 mg/lit NAA & 3 mg/lit IBA) followed by RM-II media. Average number of plants showing roots was 9.6 in Co-86032 whereas it was recorded low in Co-94012 i.e. 7.8 in RM-III.

**Key words:** Sugarcane, *In-vitro*, Axillary meristem, Shoot and root multiplication

### INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) also called "noble canes" by Dutch scientists, belongs to family *Poaceae* and genus *Saccharum*. Sugar cane is the source of sugar in all tropical and subtropical countries of the world with an annual production of 40 - 41 million tons (www.indiastat.com, 1996). Sugar i.e. sucrose is necessary part of daily diet and a major portion of total sugar produced is used in alcoholic beverages, soft drinks, ice-creams, chocolates, canning industries. The by-products of sugar industry i.e. molasses and bagasse are also utilized as raw material for manure, fiber board and paper making units and even as fuel in sugar mills. Sugar cane is a tall, perennial grass which is propagated vegetatively by stem cuttings called 'setts' having healthy buds. The cane used for planting are preferably well grown immature plants of about 10-12 months age and free from pests. The conventionally propagated setts thus use a substantial number of canes and yet do not ensure uniformity, pest free or disease free status of planting material. The *in vitro* regeneration methods instead ensure large number of uniform, disease free and vigorous planting material in relatively lesser time. Development of tissue culture method for sugarcane production was initiated almost three decades ago in late 1970s using meristem culture [1].

Several studies have reported use of MS (Murashige and Skoog's) media with 0.1 to 1.5 mg/L of BAP and 1.0 to 5.0 mg/L of IBA & 1.0 to 7.0 mg/L of NAA in different concentration and combination to be suitable for *in-vitro* regeneration. The *in-vitro* culture techniques have also been used for development of new variety of sugarcane capitalizing somaclonal variation. Due to polysomic and polyploidy genome of sugarcane several efforts have been made to develop superior sugarcane varieties using somaclonal variation tool with improved productivity and eliminating physiological defects like spines, leaf drying, and disease susceptibility. These include many of the 'Co' releases like Co 86032, Co 92007, Co 92029, Co 93005, Co 94003, Co 94012, Co 94003, Co 95016, Co 99011 and Co 99012 ([www.sugarcane.res.in](http://www.sugarcane.res.in)). Of these varieties, Co-86032 and Co 94012 were released for cultivation in tropical regions including Maharashtra, Madhya Pradesh, Chhattisgarh and Andhra Pradesh and are known to possess higher sugar recovery (16-17%). In the present study we have attempted to standardize protocol for mass multiplication of CO86032 and CO994012 varieties through *in-vitro* regeneration. Differential concentration of 6-Benzyl amino purine (6-BAP) and combinations of Indole Butyric acid (IBA) and Naphthalene acetic acid (NAA) were used for optimization of multiple shoot induction and root induction response in both the varieties [2].

## MATERIALS AND METHOD

Sugarcane varieties Co-86032 & Co-94012 (Fig.1) were obtained from Sugarcane Breeding Institute, Coimbatore and these were raised and maintained in research field of Aditya Biotech Lab & Research Pvt. Ltd. Raipur. The plants were used as mother plant for selecting suitable explants for initiation of *in-vitro* cultures.

Auxiliary meristem was selected from 8-10 month old healthy plants. Buds were taken into the lab and surface sterilized using Bavistin 0.1 % and Ampicillin 0.01 % followed by treatment with 0.2 % Mercuric chloride for 10 minutes. The sterilized explant was washed thrice with distilled water and buds were dissected to appropriate size (0.5 - 1 cm) under laminar air flow. The explant was inoculated on MS (Murashige & Skoog's) medium supplemented with fixed concentration of BAP (6 - Benzyl aminopurine), for establishment of initiation culture (Table. 1). The *in-vitro* cultures were incubated and maintained in incubation chamber having 1200-1500 lux light intensity. The incubation temperature was 22-25° C with 16 hours light and 8 hours dark period in every 24 hours cycle. First subculture was done after five weeks of inoculation and rest of the subculture was done after four weeks of incubation. MS medium supplemented with three different concentration of BAP was used to induce shoot multiplication. Well grown multiple shoots were transferred to root induction media having three different concentration and combination of BAP. Similarly for *in-vitro* root induction in individual shoots of both the varieties of sugarcane three different concentrations and combination of IBA (Indole Butyric Acid) and NAA (Naphthalene Acetic Acid) Table.1 were used. Three percent sucrose was used as common in all the media. The pH of the medium was adjusted to 5.83 with the help of 1 N solution of HCl and NaOH. Agar was used as the solidifying agent in medium at a concentration of 0.6%.

The rooted plantlets were transferred to sterile coco peat media for primary hardening under semi-controlled condition with 26-30° C temperature and 70-80% relative humidity. After 35 to 40 days primary hardened plants were transferred to shade house for secondary hardening. The secondary hardening of plants was done for a period of 45 to 60 days in poly bags filled with soil, sand and compost in the ratio of 3:3:1.



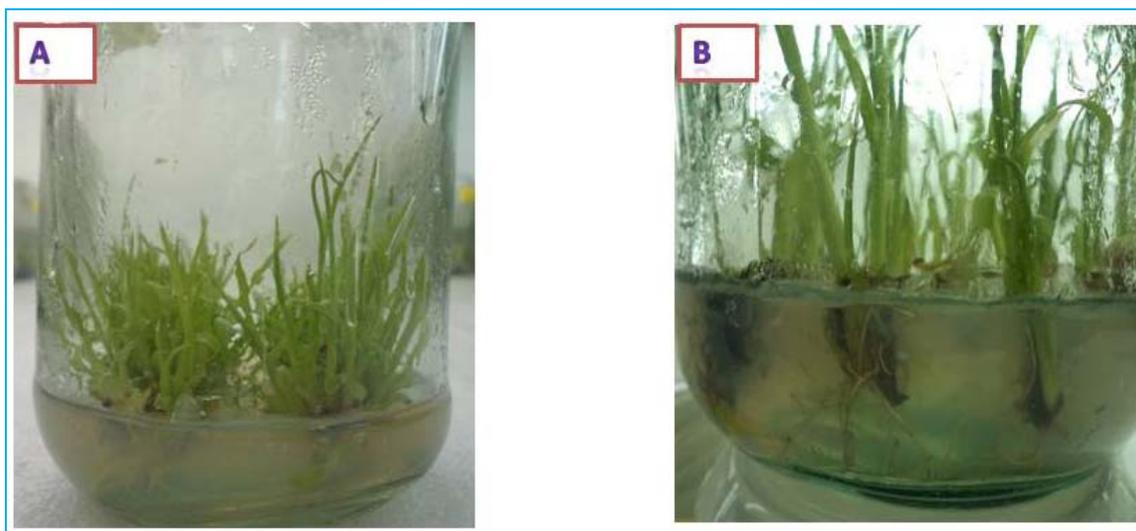
Fig: 1. Two elite Genotypes of the sugarcane A. Co-86032 and B. Co-94012

**Table: 1 Different medium used for shoot multiplication and root induction.**

S.No.	Ingredients	SM-I (mg/L)	SM-II (mg/L)	SM-III (mg/L)	RM-I (mg/L)	RM-II (mg/L)	RM-III (mg/L)
1.	Macro nutrients	MS	MS	MS	½ MS	½ MS	½ MS
2.	Micro nutrients	MS	MS	MS	½ MS	½ MS	½ MS
3.	Thiamine-Hcl	1.00	1.00	1.00	1.00	1.00	1.00
4.	Pyridoxine-Hcl	1.00	1.00	1.00	1.00	1.00	1.00
5.	Nicotin acid	1.00	1.00	1.00	1.00	1.00	1.00
6.	Glycine	2.00	2.00	2.00	2.00	2.00	2.00
7.	Myoinositol	100	100	100	100	100	100
8.	BAP	0.1	0.25	0.50	-	-	-
9.	NAA	-	-	-	1.00	3.00	3.00
10.	IBA	-	-	-	1.00	1.00	3.00
11.	Sucrose	30000	30000	30000	30000	30000	30000
12.	Agar	6000	6000	6000	6000	6000	6000
13.	pH	5.83	5.83	5.83	5.83	5.83	5.83

## RESULT AND DISCUSSION

The present study was carried out to optimize rooting and shooting medium for *in-vitro* propagation of sugarcane at large scale. Sugarcane is an importance cash crop of India and forms the backbone of many agro-industries. The large number of mother plants might differ in their morphological, agronomic and economically important traits which are a major reason of yield loss. Production of large number of uniform sugarcane plants having desirable characteristics would significantly improve sugarcane production and would also facilitate proper crop management practices [3]. Shoot tip containing auxiliary meristem of cultivar Co-86032 Co-94012 were used as explants for *in-vitro* regeneration. The shoot tips containing auxiliary meristem were inoculated in MS medium with a fixed concentration of BAP (1mg/lit) for shoot tip initiation and establishment (Fig.2). Small shoots started appearing with in 7-10 days in all cultures bottles. Similar results have been reported by Dhumale *et al* (1994) for initiation where BAP @ 3mg/lit and NAA 1mg/lit were used. Biradar *et al* (2008) [4] also reported that auxiliary bud is the most suitable explant for initiation with MS medium containing BAP @ 2 mg/L concentration.



**Fig: 2. Shoot and Root Multiplication A. Shoot Multiplication in MS +BAP (1mg/lit),  
B. Root Multiplication in MS+3 mg/lit NAA & 3 mg/lit IBA**

Ten random culture bottles of each variety were studied for observing shoot length, multiple shoots and survival percentage of the shooting cultures during cycles of multiplication. Maximum shoot lengths were obtained in SM-II media i.e. MS supplemented with 0.25mg/lit BAP (Table 2.).

After 30 days of incubation the average shoot length recorded in SM-II medium was found to be 1.63cm in Co-86032 and 1.65cm in Co-94012, whereas minimum shoot length of 1.52cm in Co-86032 and 1.55 cm in Co-94012 was observed in SM-III i.e. MS supplemented with 0.1mg/lit BAP [5]. It was also observed that shoots in SM-I were as elongated as SM-II but the shoot were found succulent and weak. The shoots obtained in SM-III and SM-I was not appropriate for further sub culturing and root regeneration. The three different media showed differential response for multiple shoot formation. After 4-5 week of shoot growth, actively growing shoots were transferred to fresh medium in jars for further growth and proliferation. It was observed that highest number of shoots were observed in SM-II and SM-III media (Table. 2) and least number of shoots were recorded in SM-I medium (Table. 2) in third cycle of multiplication. Proliferation of shoot started & during secondary proliferation stage, lateral shoots developed from base of newly initiated shoot. As a result a dense mass of shoot (25-30) was developed in culture. The best shoot multiplication response for sugarcane varieties were obtained in SM-II medium i.e. MS Medium supplemented with 0.25mg/lit BAP (Table-2). The medium showed about 21.09 in Co-86032 and 20.86 in Co-94012 shoots in an average after 1 month of incubation. Though SM-3 medium i.e. MS medium supplemented with 0.5mg/lit. BAP also showed high numbers of multiple shoots but the shoots were succulent and curly in nature that made them highly unsuitable for rooting [6].

**Table: 2 Shoot Multiplication and induction response in Co-86032 & Co-94012 under different concentration of 6- Benzyl Amino purine (BAP).**

Media	Media Composition (mg/Litre)	Days to Shoot Induction		Shoot Length (in cm)		No. of Shoots/bunch		Survival %	
		Co-86032	Co-94012	Co-86032	Co-94012	Co-86032	Co-94012	Co-86032	Co-94012
SM-1	MS+0.1 BAP	10.5	10.6	1.54	1.60	20.83	20.55	86.11	85.23
SM-2	MS + 0.25 BAP	9.8	10.1	1.63	1.65	21.09	20.86	96.61	91.23
SM-3	MS + 0.50BAP	9.7	9.9	1.52	1.55	21.26	21.01	91.46	90.09

Different concentration of BAP in shoot multiplication medium was found to affect survival of shoots. The percent of survival varied between different shooting media carrying different levels of BAP, where maximum survival rate was found in medium containing BAP @ 0.25mg/lit (96.61%) in Co-86032 and 91.23 in Co-94012. The shots in SM-II medium were active and healthy compared to other two media [7]. The regenerated shoots were used for root induction in root forming media. Half strength MS medium supplemented with 3 different concentration and combinations of NAA & IBA was used. The response of root formation was different in all media, as highest root formation average was observed in RM-III (3 mg/lit NAA & 3 mg/lit IBA) followed by RM-II media (Table. 3) (Fig.2) and least root formation average was recorded in RM-I media (Table. 3).

**Table: 3 Root formation response in Co-86032 & Co-94012 with different concentration and combination of Naphthalene acetic acid (NAA) and Indole butyric acid (IBA).**

Media	Media Composition (mg/Litre)	Days to Shoot Induction		No. of Plants showing root		Average root frequency		Survival %	
		Co-86032	Co-94012	Co-86032	Co-94012	Co-86032	Co-94012	Co-86032	Co-94012
RM-1	½ MS+ 1 NAA + 1 IBA	16.7	17.15	5.6/10	5.1/10	*Low	*Low	61.23	59.33
RM-2	½ MS + 3 NAA + 1 IBA	14.66	16.05	8.5/10	6.9/10	*Medium	*Low	71.12	61.52
RM-3	½ MS + 3 IBA	10.28	14.06	9.6/10	7.8/10	*High	*Medium	93.55	74.17

\***Low**- 2-5 Roots, poor health, Blackish color, **Medium**- 5-10 Roots, Normal Health, Whitish Color, **High**- <20 Roots, Heavy Bunch, Healthy Roots, Whitish Color

Average number of plants showing roots was 9.6 in Co-86032 whereas it was recorded low in Co-94012 i.e. 7.8 in RM-III. Root formation was always recorded best in RM-III with heavy bunch, healthy roots more than 20 roots per plant and whitish in colour. It took only 10 days for initiation of roots in Co-86032 variety when inoculated in RM-III media whereas Co-94012 took almost 15 days for root initiation in same media. The two varieties showed differential root formation and developmental response. The roots observed in Co-94012 were smaller, less dense and dark brown in colour. The poor root formation and growth in Co-94012 was recorded in all the three rooting media (RM-I, RM-II and RM-III) under this study. Thus suggesting influence of genotypic variation in *in-vitro* root regeneration of Co-94012 variety rendering it less successful under *in-vitro* based mass propagation. Further optimization of root induction and growth related parameters of Co-94012 is required and are under progress.

## CONCLUSIONS

The shoot tips containing axillary meristem were inoculated in MS medium with a fixed concentration of BAP (1mg/lit) for shoot tip initiation and establishment. Small shoots started appearing with in 7-10 days in all cultures bottles. Maximum shoot lengths were obtained in SM-II media i.e. MS supplemented with 0.25mg/lit BAP. After 30 days of incubation the average shoot length recorded in SM-II medium was found to be 1.63cm in Co-86032 and 1.65cm in Co-94012. Similarly the regenerated shoots were used for root induction in root forming media. The response of root formation was different in all media, as highest root formation average was observed in RM-III (3 mg/lit NAA & 3 mg/lit IBA) followed by RM-II media. Average number of plants showing roots was 9.6 in Co-86032 whereas it was recorded low in Co-94012 i.e. 7.8 in RM-III.

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