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Research Article

Optimization of protocol for callus induction and whole plant regeneration for developing somaclonal variants in sugarcane cv. COC 671

Srilakshmi Maruprolu[1], S. Geetha[1*], R. Gnanam[2], R. Viswanathan[3], Asish K Binodh[1] and R. Sudagar[4]

[1]Center for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore -641003
[2]Center for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore -641003
[3]Division of Plant Pathology, Sugarcane Breeding Institute, Coimbatore -641003
[4]Sugarcane Research Station, Tamil Nadu Agricultural University, Melalathur- 635806,
***E-Mail**:geethagovind1@gmail.com

Abstract
The present study was carried out to optimize *in vitro* callus induction protocol and whole plant regeneration for developing red rot resistant somaclones in commercial sugarcane variety COC 671 using immature leaf sheaths as explants. In the whole experiment, the best medium for callus induction was MS medium supplemented with 2, 4-D (2.0 mg/l) recorded callus induction frequency of 100 per cent in eight days with a maximum callus fresh weight of 13.08 g/10 calli. The compact and friable embryogenic calli were regenerated in MS medium supplemented with KIN (1.0 mg/l) producing 21 shoots in 10 days with a shoot length of 8.69 cm. The well grown shoots were rooted in MS medium supplemented with NAA at 3.0 mg/L with 9 roots in 9 days which had an average root length of 3.63 cm. The regenerated plantlets were successfully acclimated under field conditions. This protocol would further be used for developing red rot resistant somaclonal variants in sugarcane.

Keywords: Sugarcane, red rot, somaclonal variants, callus induction, regeneration

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a cross pollinated and highly heterozygous plant with a variable chromosomal number (2n= 80 to 205) and is a tropical, monocotyledonous perennial grass. It is the major source of sugar in many developing countries with respect to an export product that accounts for more than 60 per cent of the world's sugar production (Guimarces and Sobral, 1998). In India, it is the second important industrial crop contributing significantly to the country's economy. It is being grown in 3.0 per cent of the total cultivable area and contributing 7.5 of per cent gross value of agricultural production in India. The main economic product is sucrose and recent studies revealed the importance of sugarcane in biofuel industry as important renewable biodiesel for the production of ethanol (Da costa *et al*., 2011). Modern cultivars of sugarcane are the result of interspecific crosses between *Saccharum officinarum, S. barieri, S. sinensis* and wild species *S. spontaneum*. The creation of variability in sugarcane through conventional breeding is highly difficult due to its high heterozygous genetic architecture, rare flowering, low fertility status, large genome size, long breeding cycle and high environmental interaction hence *in vitro* techniques are used for sugarcane improvement. Though many

in vitro propagation protocols for sugarcane has been developed by many researchers, each genotype requires a specific protocol for rapid and potential propagation. Various factors like genotype (Roy and Kabir, 2007), explants type, the position of explant, age, plant growth regulator interactions *etc.* (Cheema and Hussain, 2004 and Khan *et al.*, 2006) limited the universal application of the protocols. A number of researchers have attempted for *in vitro* genetic manipulation of sugarcane to increase yield, sugar content and tolerance to biotic and abiotic stresses using young leaf whorl explants (Vickers *et al.,* 2005, Gilbert *et al.,* 2005 and Gilbert *et al.,* 2009). Kaur *et al*. (2001) worked on three sugarcane cultivars *viz.,* COJ 64, CoJ 83 and COJ 86 using spindle explants. Baksha *et al.* (2002) worked on sugarcane cv. Isd 28 with shoot tip culture and reported that MS medium supplemented with BAP (2.0 mg/L) and IBA (0.5 mg/L) is optimal for multiple shoot induction. Lal (2003) worked on sugarcane var. BO 91 and developed a protocol for producing high frequency plant regeneration from callus by using leaf roll explants on MS medium containing 2, 4-D (5.0 mg/L) and reported high shoot differentiation (70 %) in basal MS medium. Variety COC 671 (Vasant-1) was developed by crossing Q 63 and CO 775, a high sugar yielding and early maturing variety (9-10 months). It was particularly popular among Maharasthra and Tamil Nadu farmers, due to its high quality cane production and jaggery making qualities. It was suitable for ratooning, and the ratoon production was higher than that of the *suru* crop. The yield potential was estimated to be at 265 t/ha. Though COC 671 is superior in performance, it has been vulnerable to red rot disease caused by *Colletotrichum falcatum Went*. This variety could be used to choose red rot resistant lines through plant cell and tissue culture techniques. The objective of this study was to standardize the protocol for callus induction and whole plant regeneration of sugarcane *cv*. COC 671 using immature leaf whorls as explants, which in turn would be used for developing somaclones for red rot resistance.

MATERIALS AND METHODS

The current research work was carried out in the tissue culture laboratory of the Department of Plant Biotechnology, Centre for Plant Molecular Biology & Biotechnology (CPMB & B) Tamil Nadu Agricultural University Coimbatore, during 2019-21. MS Powder 4.1g (Himedia) along with different concentrations of 2, 4-D, BAP, Kin, NAA, and IBA (Sigma Grade) were used for callus induction and plant regeneration studies (**Table 1**). The pH of the media was adjusted to 5.8 with 0.1N NaOH and 0.1 N HCl. Agar agar type I (GR M666-500 G -Himedia) 8 g/L was added for solidification and the medium was then autoclaved at 121°C for 20 min.

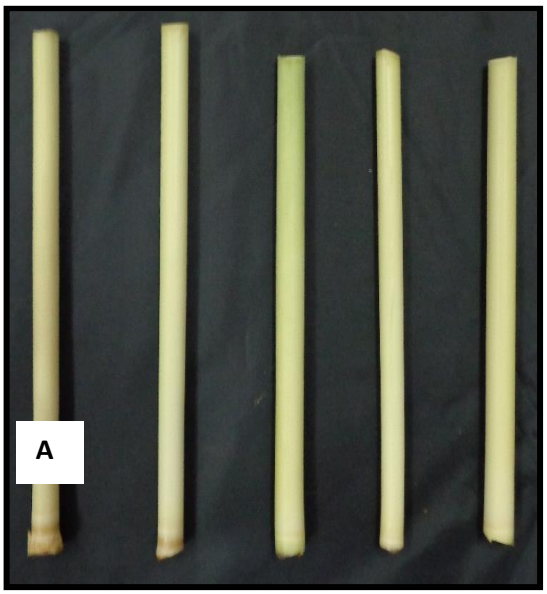
Six months old top shoots of high sucrose and high yielding highly red rot susceptible cultivar COC 671 were collected from Sugarcane Breeding Institute, Coimbatore (**Fig. 1A).** Immature inner most leaf sheaths were used as explants for induction of callus. The explants were rinsed with Bavistin (1%) along with two drops of Tween 20 for 10 min. followed by three rinses with sterile distilled water. The explants were then surface sterilized in a laminar air flow using mercuric chloride (0.1 %) for 3 min. and then thoroughly washed three times with sterile distilled water to get rid of the sterilants. Then the leaf rolls were placed on sterile filter paper for absorption of excess water on the surface and cut into 2 mm^2 and placed on an MS medium with different plant growth regulator combinations for callus induction. Ten explants were inoculated in each petriplate and cultures were kept in the dark at 25± 1°C. After four weeks of inoculation, the best treatment was adjudged based on days taken to first callus initiation, callus induction frequency after thirty days and callus fresh weight after sixty days. The sub culturing to a fresh medium was carried out after every second week.

Eight week old embryogenic calli were transferred to culture bottles (300 ml) containing regeneration medium (MS modified). Eight different combinations of BAP and Kinetin were supplemented to the MS salt and Sucrose (30 g/l) in constant amounts for regeneration experiments. Cultures were placed in the culture room at 25± 1°C under 16 h light (3000 lux) and 8 hours dark. The data recorded during the studies were days taken for shoot initiation, the number of shoots regenerated per calli and mean shoot length after four weeks the of culture period. Regenerated plantlets were then transferred to a root induction medium containing different combinations of NAA and IBA (**Table 1**). The number of days taken for root initiation, the number of roots per shoot and mean root length recorded after four weeks. Well rooted plantlets were transferred to disposable cups filled with coco peat, sand, clay (1:1:1), under high humidity (> 90%). Plants were kept in the greenhouse at 25± 1°C under 16 h light for hardening. Well grown plants with 15 cm in height were planted under field conditions for field establishment.

Experiments were set up in a Completely Randomized Design (CRD) with three replications and 10 explants per replication. The treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5% probability level according to Gomez and Gomez (1976).

RESULTS AND DISCUSSION

Six months old top shoots of COC 671 were used for the collection of immature leaves as explants. Surface sterilized leaf bits inoculated on MS medium with different concentrations of 2,4-D alone (0.5-3.5 mg/l) or 2,4-D (1- 4 mg/l) in combination with BAP (0.5 or 1mg/l). Days to callus induction in all the tested medium combinations were effective at $P < 0.05$ (**Fig. 1(B), Table 2**). The number of days taken for the first initiation of the callus ranged between 8 – 21 days. Among the fifteen combinations tried, the earliest callus induction was observed in MS medium supplemented with 2,4-D (2 mg/l) in minimum duration of 8 days, while it took a maximum of 21 days for the



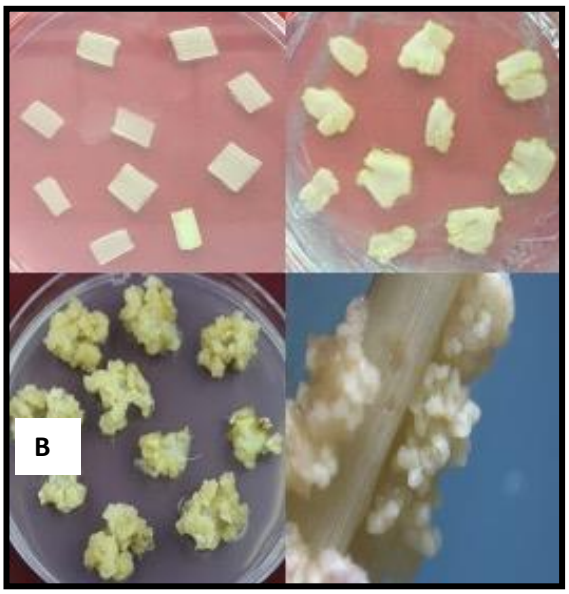


Fig. 1 (A) Leaf *Explant* **(B) Bulging of explants, Callus induction from margins and proliferation**

Table 1. Different callus induction and regeneration media composition

S.No	Callus induction media				S.No	Regeneration media				S.No	Rooting media			
	MS salt (g/L)	Sucrose (g/L)	2,4-D (mg/L)	BAP (mg/L)		MS salt (g/L)	Sucrose (g/L)	BAP (mg/L)	Kin (mg/L)		MS salt (g/L)	Sucrose (g/L)	NAA (mg/L)	IBA (mg/L)
T1	4.10	30.00	0.50	0.00	M1	4.10	30.00	1.00	0.00	R1	4.10	30.00	0.50	0.00
T2	4.10	30.00	1.00	0.00	M2	4.10	30.00	2.00	0.00	R2	4.10	30.00	1.00	0.00
T3	4.10	30.00	1.50	0.00	M3	4.10	30.00	3.00	0.00	R3	4.10	30.00	2.00	0.00
T4	4.10	30.00	2.00	0.00	M4	4.10	30.00	4.00	0.00	R4	4.10	30.00	3.00	0.00
T5	4.10	30.00	2.50	0.00	M5	4.10	30.00	0.00	1.00	R5	4.10	30.00	0.00	0.50
T6	4.10	30.00	3.00	0.00	M6	4.10	30.00	0.00	2.00	R6	4.10	30.00	0.00	1.00
T7	4.10	30.00	3.50	0.00	M7	4.10	30.00	0.00	3.00	R7	4.10	30.00	0.00	2.00
T8	4.10	30.00	1.00	0.50	M8	4.10	30.00	0.00	4.00	R8	4.10	30.00	0.00	3.00
T9	4.10	30.00	2.00	0.50										
T10	4.10	30.00	3.00	0.50										
T11	4.10	30.00	4.00	0.50										
T12	4.10	30.00	1.00	1.00										
T13	4.10	30.00	2.00	1.00										
T14	4.10	30.00	3.00	1.00										
T15	4.10	30.00	4.00	1.00										

50 ml $CaCl_2$(prepared by adding 4.4 g of anhydrous$CaCl_2$ in 500 ml of distilled water) and 8 g Agar Agar type I also added in above media pH 5.8

young leaf to respond to callus induction when inoculated on MS basal medium. When 2,4-D was tried alone, at lower concentrations, the callus induction was noticed in 8-9 days but as the concentration was increased, it had been extended to 12-16 days. The addition of BAP (0.5 or 1.0 mg/l) to lower or higher concentrations of 2, 4-D did not bring down the days taken for callus induction and it took 9- 15 days. According to Grattaapaglia and Machado (1998), 2, 4-D tended to stimulate callus formation, even at low concentrations. The growth

regulator showed an effect on the RNA metabolism by inducing the transcription of messenger RNA capable of coding proteins required for the growth and hence, promoting chaotic cell proliferation, *i.e*., callus formation (George, 1996). Optimal callus induction could be obtained through manipulating 2,4-D concentrations and the duration of its presence in the induction medium (Zheng and Konzak, 1999) as different genotypes responded differently to varying 2,4-D levels (Arzani and Mirodjagh, 1999). According to Ozias-Akins and Vasil (1982) increased levels of 2,4-D beyond 2 mg/l inhibits cell division. Rashid *et al*. (2009) reported good callus initiation at 2 mg/l 2,4-D in sugarcane cultivar HSF-240. Though, Gopitha *et al.* (2010) worked on the same variety of COC 671, reported callus initiation at 15 days after inoculation of explant on MS medium supplemented with 3.0 mg/l 2,4-D and 10% coconut milk. This study showed early callus initiation at a lower 2,4-D concentration of 2.0 mg/l and it also reduced the culture duration for callus initiation. This is attributed to the age of explants or the used cultural conditions.

Callus induction was obtained in all the treatments, regardless of the concentrations of the hormones used. The highest callus induction frequency (100%) was observed in MS medium fortified with 2 mg/l of 2, 4-D (T4) alone and the lowest callus induction frequency (23.33%) was observed in basal MS medium. At higher concentrations of 2,4-D, more than 2 mg/l, there was a reduction in callus induction frequency as well as with the addition of BAP at all concentrations of 2,4-D. More than 90 per cent callus induction frequency was recorded in (MS+2,4-D (3.5 mg/l) and (MS+2,4-D (1.0 mg/l)+BAP(1.0 mg/l) (**Fig. 2 and Table 2**). Jamil *et al.* (2017) obtained 95 per cent callus induction on culture media supplemented with 2,4-D @ 2.0 mg/l. However, Karim *et al.* (2002) and Gopitha *et al.* (2010) reported a high frequency of callus induction (90-100 %) at 3 mg/l of 2, 4-D. Highly significant ($p<0.05$) values (**Table 2**) were seen for callus fresh weight for all the treatments. There was a wide variation recorded in the experiments for the callus fresh weight and it ranged from 2.03 g to 13.08 g per 10 calli. The callus fresh weight obtained was the highest in (2,4-D, 2 mg/l). The optimum plant growth regulator for callus fresh weight was from 2,-4-D either alone @ 2 and 3 mg/l or 2,4-D @ 2.0 mg/l with 0.5 BAP and 2,4-D @ 2 and 3 mg/l with BAP 1.0 mg/l recorded an average fresh weight of 8.5-9.5 mg/l(**Fig. 3A, 3B and Table 2).**

Considering the three parameters studied during callus induction, *viz*., days to first callus initiation, callus induction frequency and callus fresh weigh**t**, the best medium combination was found to be MS medium fortified with 2,4-D (2.0 mg/l), which could record 100 per cent callus induction in 8 days with a maximum fresh weight of 13.08 g/10 calli. Similarly, 2 4-D @ 3 mg/l alone or 2,4-D 3 mg/l with BAP 1.0 mg/l could record a fresh weight of 9.30 and 9.527 even though the callus induction frequency was 56.66 and 76.66 per cent, respectively. Basal MS medium showed the least response for callus induction (**Table 2**). The callus cultures were maintained through frequent sub culturing (up to 3 sub cultures) after every 10-15 days interval on to fresh medium for further proliferation. The physical appearance, morphology and nature of the calli were also documented during the experiments**.** Based on morphological and physical appearance, there were two types of calli observed, (i) compact whitish cream colour (**Fig 2A**) (ii) friable whitish cream colour callus (**Fig 2B**) and both of them were embryogenic after three sub culturing, the proliferated calli were transferred to shoot regeneration medium (**Fig. 3A and 3B**).

The proliferated callus was sub cultured to a regeneration medium (MS) supplemented with BAP or Kinetin in different concentrations (1, 2, 3 and 4.0 mg/l). During the regeneration studies, days to shoot initiation, the number of shoots per calli and average shoot length were recorded to adjudge the best treatment.

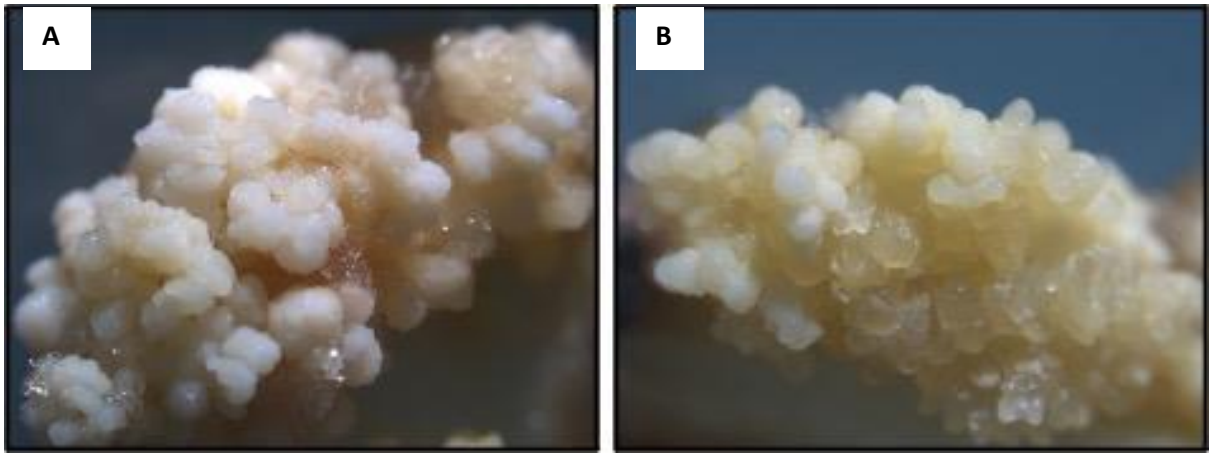


Fig. 2 (A) Compact and (B) Friable Callus

Table 2. Effects of 2,4-D alone or 2,4-D in combination with BAP on callus induction from immature leaves of*cv* CoC 671

Code	Medium with plant growth regulators (mg/L)	Days to callus initiation	Callus induction frequency (%)	Callus fresh weight (g/ 10 calli)
T0	Control (T0)	21.33±0.66^{h}	23.33±3.33^{h}	2.03±0.00^{h}
T1	MS+2,4-D (0.5)	9.66±0.33^{b}	76.66±3.33^{def}	3.05± 0.08^{hg}
T2	MS+2,4-D(1.0)	9.33±0.33^{b}	73.33±3.33^{f}	3.90±0.20^{gf}
T3	MS+2,4-D(1.5)	9.33±0.33^{b}	70.66±3.33^{bc}	5.10±0.35^{fe}
T4	MS+2,4-D(2.0)	**8.00±0.88^{a}**	**100.00±0.00^{a}**	**13.08±0.63^{a}**
T5	MS+ 2,4-D(2.5)	14.00±0.67^{e}	66.66±6.66^{bc}	2.94±0.09^{h}
T6	MS+2,4-D(3.0)	14.66±0.67^{e}	56.66±6.66^{b}	9.30±0.67^{b}
T7	MS+2,4-D (3.5)	16.00±0.67^{g}	90.33±3.33^{ef}	6.60±0.50^{d}
T8	MS+2,4-D (1.0)+ BAP(0.5)	13.33±0.67^{d}	70.66±6.66^{cd}	6.26±0.39^{ed}
T9	MS+2,4-D (2.0)+BAP(0.5)	15.00±0.58^{f}	80.00±10.00^{bc}	9.26±0.37^{b}
T10	MS+2,4-D (3.0)+ BAP(0.5)	12.33±0.67^{c}	80.66±3.33^{cd}	2.34±0.17^{h}
T11	MS+ 2,4-D (4.0)+BAP(0.5)	10.00±1.00^{b}	73.33±6.66^{cd}	9.26±0.37^{b}
T12	MS+2,4-D (1.0)+ BAP(1.0)	12.33±0.67^{c}	93.33±3.33^{ef}	4.82±0.25^{f}
T13	MS+2,4-D (2.0)+ BAP (1.0)	14.33±0.67^{e}	83.33±3.33^{cde}	8.56±0.33^{cb}
T14	MS+2,4-D (3.0)+ BAP (1.0)	15.66±0.67^{f}	76.66±6.66^{cd}	9.52±0.86^{b}
T15	MS+2,4-D (4.0)+ BAP(1.0)	12.79±0.67^{c}	76.66±3.33^{cd}	7.67±0.92^{dc}
	Mean	12.80	85.470	6.484
	CD	1.94	1.41	5.700
	SEm(+)	0.67	5.16	0.487
	SED	0.95	7.30	0.689
	C.V.%	9.09	10.46	11.61

Each value represents the mean ± SE of three replicates and 10 explants/ replication. Different letters in the same column indicated the significant differences at p ≤ 0.05.(Duncan's multiple range test)

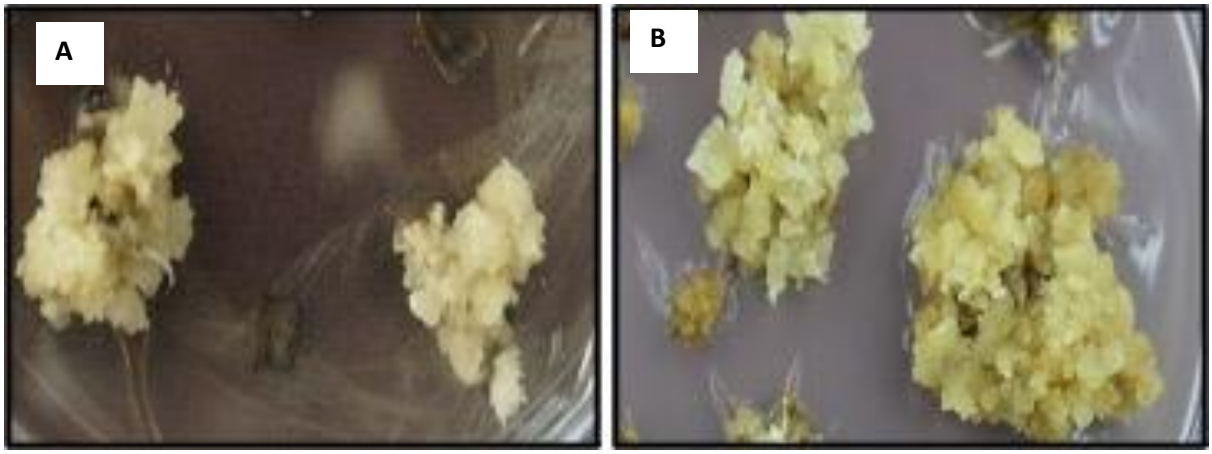


Fig. 3. (A) Poor proliferation of callus (B) Higher proliferation of callus

Eight combinations tried showed a significant difference ($p<0.05$) for shoot regeneration (**Fig. 4A, 4B and Table 3**). Shoot initiation was the earliest (10 days) when the medium was supplemented with either BAP or KIN at 1 mg/l and the duration extended to 15 and 16 days for shoot regeneration, when MS medium was fortified with higher concentrations of either BAP or Kinetin at 4.0 mg/l, respectively. The rest of the treatments took 11-14 days to regenerate shoots from both the types of the embryogenic calli (**Table 3**). Among the plant hormones, cytokinins like BAP and KIN play an active role in shoot regeneration from the callus. These results were in accordance with the studies conducted by Abu-Romman *et al.* (2015) and they reported that 1 mg/l KIN was effective for shoot induction in cucurbits. Primarily, cytokinins have a major role on plant development, such as the regulation of shoot formation, shoot multiplication and the promotion of cell division and expansion by Mok and Mok (2001). Variation in the activity of different cytokinins can be explained by their different uptake rate reported in different genomes by Blakesly (1991), varied translocation rates to meristematic regions and metabolic processes in which cytokinin may be degraded or conjugated with sugars or amino acids to form biologically inert compounds as reported by Kaminek (1992). Baday (2020), Dinesh *et al*. (2015) and Trippathy *et al*. (2020) reported that 1 mg/l BAP was optimum for shoot regeneration in sugarcane. Therefore, the present study clearly witnessed the influence of cytokinin in shoot regeneration from callus and it also gave an insight to the levels of hormones required for better shoot regeneration in sugarcane.

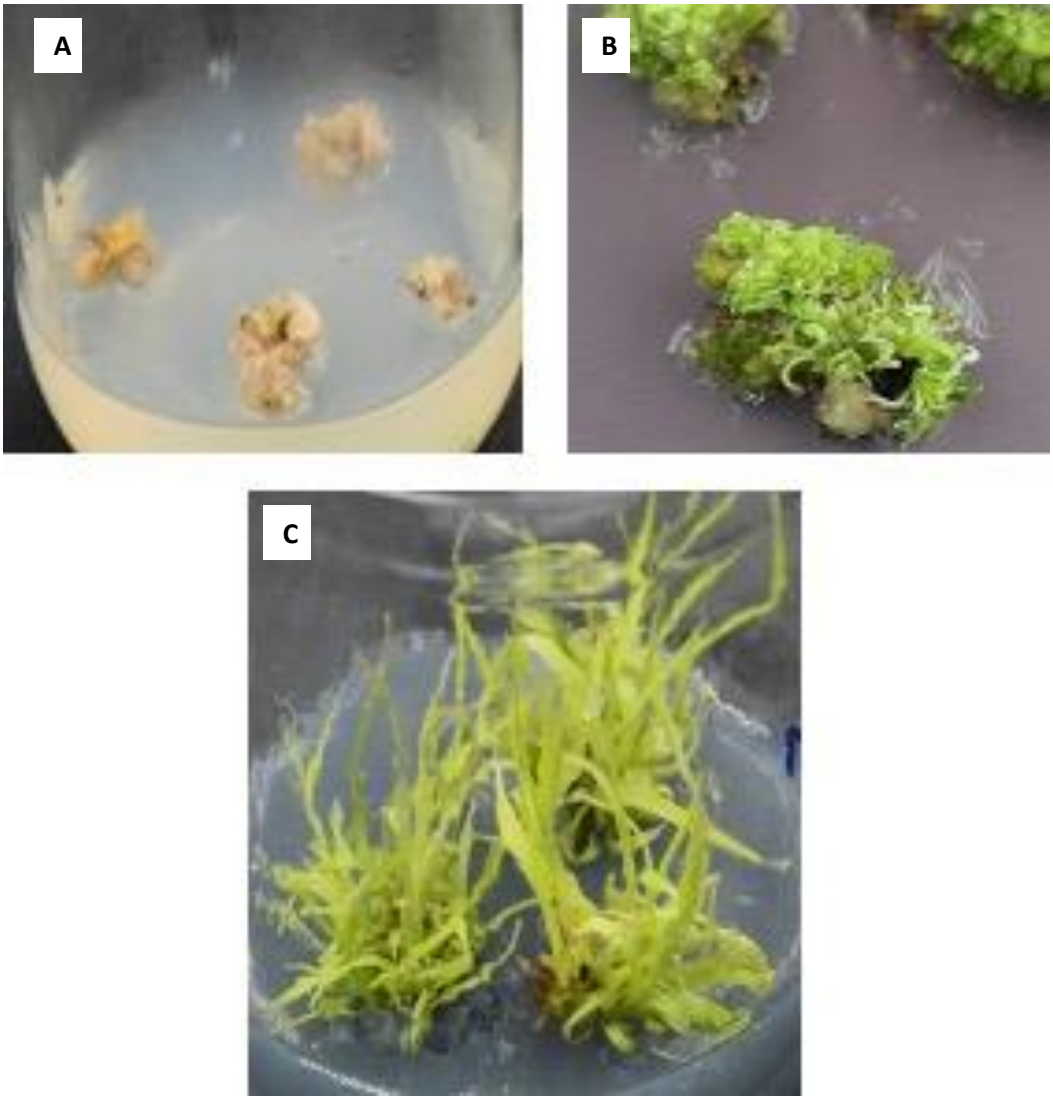


Fig.4. (A), (B), (C) Shoot initiation and regeneration

Table 3. Effect of different concentrations of BAP or Kinetin on shoot regeneration

Code	Medium+ Plant growth regulators (mg/L)	Days to shoot initiation	Number of shoots per calli	Shoot length (cm)
M0	Control	22.00±1.00^{e}	6.33±0.33^{f}	5.30±0.17^{d}
M1	MS +BAP(1.0)	9.67±0.88^{a}	17.33±0.66^{b}	8.20±0.23^{b}
M2	MS+ BAP (2.0)	11.67±0.67^{ab}	13.33±0.33^{d}	7.33±0.88^{c}
M3	MS +BAP (3.0)	13.00±1.00^{bc}	10.33±0.33^{e}	5.57±0.14^{d}
M4	MS +BAP (4.0)	15.00±0.57^{cd}	5.66±0.33^{f}	3.27±0.07^{f}
M5	MS +Kin (1.0)	9.67±0.33^{a}	20.66±0.33^{a}	8.69±0.10^{a}
M6	MS+ Kin (2.0)	12.67±0.67^{bc}	18.33±0.81^{b}	4.00±0.05^{e}
M7	MS+ Kin(3.0)	14.67±0.67^{cd}	15.66±0.33^{c}	3.67±0.03^{e}
M8	MS+ Kin (4.0)	16.00±0.58^{d}	10.66±0.33^{e}	2.67±0.09^{g}
	Mean	13.81	13.14	5.44
	CD	1.99	1.450	0.32
	SEm(+)	0.67	0.48	0.10
	SED	0.94	0.68	0.15
	C.V.%	9.12	6.01	3.00

Each value represents the mean ± SE of three replicates. 10 calli /replication
Different letters in the same column indicated the significant differences at p ≤ 0.05.(Duncan's multiple range test

The number of shoots regenerated per callus in two different regeneration media with different concentrations of BAP and KIN was significantly different (p<0.05) and furnished in (**Fig. 4 C and Table 3**). The mean number of shoots regenerated per callus ranged from 6-21. A maximum number of 21 shoots per callus got regenerated on MS medium fortified with 1 mg/l Kin which was recorded as the single best treatment. Next to the best combination, MS medium supplemented with BAP (1.0 mg/l or Kin (2.0 mg/l) was observed to produce 17-18 shoots/callus. The lowest number of shoots, 6 per callus was documented on MS medium supplemented with 4 mg/l BAP.

There was a reduction in the number of shoots (from 17 to 6) as the BAP concentration was increased from 1 to 4 mg/l. A similar trend was observed when the Kinetin concentration increased from 1 to 4 mg/l with a significant reduction in no. of shoots per calli from 21 to 11. The reduction due to higher concentrations of cytokinins (4 mg/l) was 50 per cent in the case of KIN and 75 per cent in the case of BAP. MS medium supplemented with 3 mg/l BAP and 4 mg/l Kin were non– significant with a mean value of 10 and 11 shoots per callus, respectively.

The shoot length recorded for different treatments was significantly different (p<0.05) and furnished in **Table 3**. The shoots from MS medium fortified with 1 mg/l KIN recorded a maximum shoot length of 8.69 cm, followed by (MS + 1mg/l BAP) with 8.20 cm and (MS+ 2 mg/l BAP) with 7.63 cm. As the concentration of cytokinins was increased, in both the cases, there was a reduction in shoot length and the reduction was higher to the tune of 75 per cent when compared to the lowest concentration. Very short shoots with a shoot length of 2.67 cm were observed in MS medium fortified with 4 mg/l KIN.

In the regeneration experiments, the best combination for shoot regeneration was MS supplemented with KIN (1.0 mg/l) which could initiate 21 shoots in 10 days with an optimum shoot length of 8.69 cm. Though MS with BAP (1.0 mg/l) also initiated shoots in the shortest duration of 10 days (which was on par with the previous treatment), the shoots regenerated were only 17.33 with a shoot length of 8.20 cm. Although, MS medium with BAP (2.0 mg/l) produced the lengthier shoots but the number of shoots induced was only 13/calli in 13 days. However, in sugarcane variety Isd-31, Karim *et al*. (2002) reported that the MS medium supplemented with 1 mg/l BAP and 0.5 mg/l IBA performed best for shoot formation. Furthermore, Khan *et al*. (2009) reported 11 shoots with 16.5 cm shoot length for variety HSF- 240 cultured on MS medium fortified with 1.5 mg/L BAP, 0.5 mg/L KIN. Tarique *et al*. (2010) determined the optimal concentrations of BAP with NAA or IBA for shoot initiation and multiplication of the sugarcane varieties Isd-16, Isd- 36 and Isd-37, and found that 1.0 mg/l BAP + 0.5 mg/l NAA showed the best result for induction and multiplication of shoots.

Root induction was noticed when plant growth regulators *viz.,* IBA and NAA were added to MS medium. The effect of different concentrations of IBA and NAA on the number of days to root initiation, the number of roots and root length was significantly different at p<0.05 (**Fig. 5A, 5B and Table 4**). The number of days taken for root induction ranged from 6 to 16. A minimum number of days (6) to root induction was observed on MS medium

with IBA 3 mg/l while it took twice the number of days (12) on MS medium with NAA, 0.5mg/l and IBA 2 and 3 mg/l). Sughra *et al*. (2014) reported that root induction on 9 days, 10 days and 11.50 days in BL-4, Thatta-10 and Larkana-2001, respectively with NAA (3 mg/l) and also recorded an average number of 7 and 5 roots per shoot in BL-4, Thatta-10 and Larkana-2001 respectively with 3 mg/l NAA. The highest number of roots (9) was initiated on MS medium supplemented with 3 mg/l NAA, while the least number of roots (2) were seen with MS basal medium. The highest root length (3.63 cm) was observed on the MS medium fortified with 3 mg/l NAA, while the least (1.46 cm) was seen with the MS basal medium. The rest of the combinations recorded a root length of 1.00-2.08 cm. Tolera (2016) obtained an average root length of 2.92 ± 0.18 cm and 2.58 ± 0.00 cm in B41-227 and N14, respectively. Behera and Sahoo (2009) obtained an average root length of 4.0 ± 0.94 cm for the variety Nayana on MS media supplemented with 2.5 mg/l of NAA. In the rooting experiments, the performance of NAA at 3.0 mg/l was better compared to other combinations inducing 9 roots in 9 days with an average root length of 3.63 cm. MS medium with NAA combinations was superior with respect to the number of roots and root length when compared to IBA combinations. Trippathy *et al.* (2020) also noticed satisfactory rooting

Table 4. Effect of different concentrations of NAA and IBA on *in vitro* root induction

Code	Medium with plant growth regulators (mg/L)	Days to root initiation	Number of roots per shoot	Root length (cm)
R0	Control	24.33±0.33^{e}	2.00±0.00^{c}	1.46±0.33^{e}
R1	MS +NAA (1.0)	12.33±0.33^{d}	4.00±0.33^{c}	2.63±0.09^{c}
R2	MS +NAA(2.0)	9.67±0.88^{bc}	6.00±0.58^{b}	2.90±0.058^{b}
R3	MS +NAA (3.0)	**8.67±0.33^{b}**	**9.00±0.33^{a}**	**3.63±0.88^{a}**
R4	MS +NAA (4.0)	10.33±0.33^{c}	7.00±0.33^{b}	2.50±0.14^{c}
R5	MS+ IBA (1.0)	9.67±0.33^{bc}	3.00±0.67^{c}	1.53±1.00^{e}
R6	MS +IBA(2.0)	12.33±0.33^{d}	4.00±0.33^{c}	1.77±0.14^{d}
R7	MS +IBA (3.0)	6.33±0.33^{a}	7.00±0.67^{b}	2.90±0.06^{b}
R8	MS +IBA (4.0)	15.67±0.33^{de}	5.33±0.33^{b}	1.66±0.03^{ed}
	Mean	10.25	7.00	2.33
	CD	1.32	1.41	1.09
	SEm(+)	0.44	0.47	0.36
	SED	0.62	0.66	0.516
	C.V.%	7.45	11.52	35.30

Each value represents the mean ± SE of three replicates. 10 shoots/replication.
Different letters in the same column indicated the significant differences at p ≤ 0.05.(Duncan's multiple range test)

Fig.5. (A) Poor rooting (B) Well rooted plants

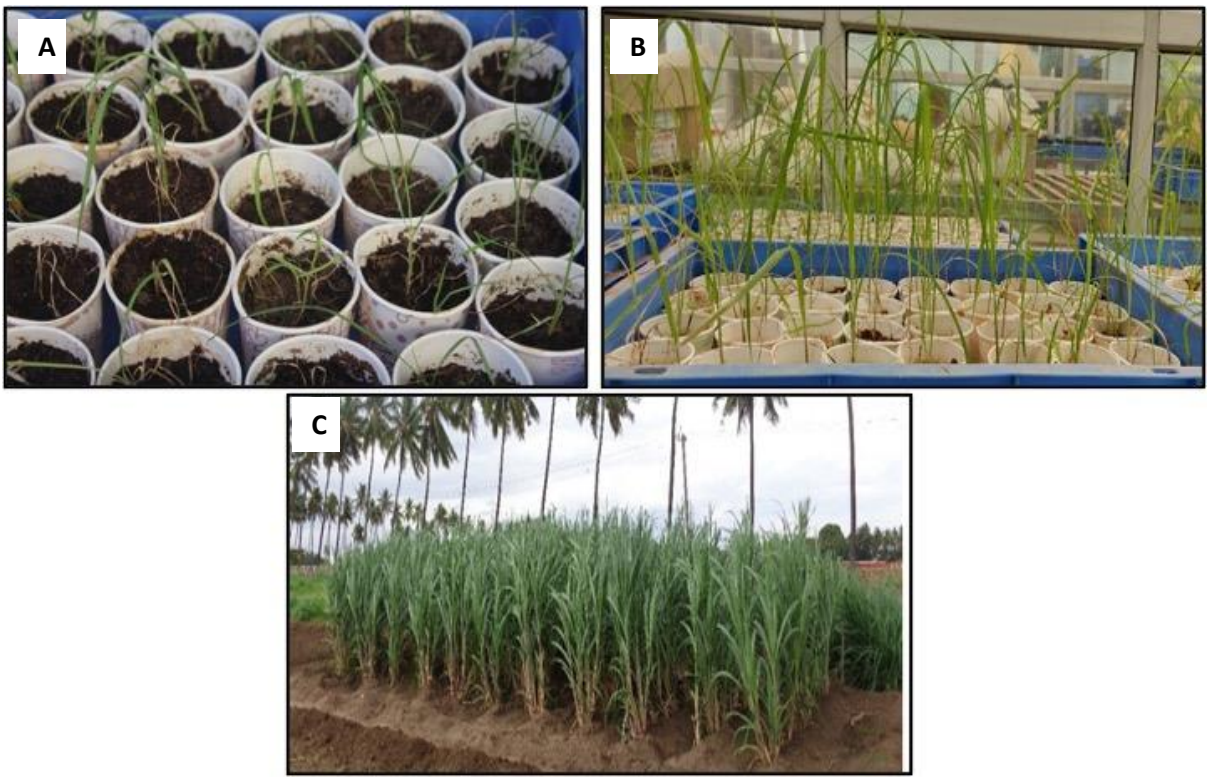


Fig.6. (A) Primary hardening (B) Secondary hardening (C) Field establishment

on MS medium supplemented with 3.0mg/L of NAA within 14 days. A different concentration of auxins (NAA and IBA) and their role in root induction from the micro shoots was observed. These results clearly witnessed that auxin hormones have a decisive role in rooted shoots of sugarcane callus. It gives maximum results when fortified at 3 mg/L NAA. Well rooted plants were taken out for hardening, (**Fig. 6 A, B & C**) weaned off from agar after proper washing and transferred to paper cups initially maintained in the culture room and later transferred to the green house for secondary hardening for 30 days. Later, the successful establishment was done in the field.

Gopitha *et al*. (2010) studied the response of different growth regulators on callus induction, regeneration and rooting in CoC 671 and noticed the best callus induction on MS medium supplemented with 3.0mg/L, 2,4-D with 10 per cent coconut milk (CM). Best regeneration of shoot was achieved when they were cultured on MS medium supplemented with BAP 1.0 mg/l and IBA 0.5 mg/l. Among the different media tested with 3 mg/l NAA and 5% sucrose supplemented media proved the best production of roots. Compared to previous reports, the current results were economical in terms of time and cost dues to its early response to callus induction and optimal regeneration either from MS medium fortified with BAP or KIN alone.

Our findings revealed that genotype and culture media affect callus induction capability, embryogenic callus response and plantlet regeneration, shoot elongation, and root induction in sugarcane. In the whole experiment, the best medium for callus induction was MS medium supplemented with 2,4-D (2.0 mg/l) with a callus induction frequency of 100 per cent in 8 days with a maximum callus fresh weight of 13.08 g/10 calli. The embryogenic calli of both the compact and friable nature could well be regenerated in MS medium supplemented with KIN (1.0 mg/L) with 21 shoots in 9 days with a shoot length of 8.6 cm. The well grown shoots were rooted in MS medium supported with NAA at 3.0 mg/l with 9 roots in 9 days which had an average root length of 3.63 cm. The study of callus mediated regeneration has given an advantage over conventional techniques for developing somaclonal variants, *in vitro* mutants, genetic transformation studies and germplasm conservation of elite sugarcane varieties. The present optimized protocol for callus induction and whole plant regeneration would be used for the development of red rot resistant somaclones in sugarcane *cv*. CoC 671.

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