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Plant Regeneration through Indirect Somatic Embryogenesis in Sugarcane and Evaluation of Regenerated Plantlets through ISSR Molecular Markers in Genotype CoM0265

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Abstract

Somatic embryogenesis and regeneration system has wide application in sugarcane crop improvement technology. In sugarcane tissue culture, response to the media component differs with varieties. CoM 0265 is popularly grown sugarcane variety in Maharashtra state, India. However, no protocol was published for the variety. Therefore, efficient and reproducible regeneration protocol was necessary for the selected sugarcane variety before application of biotechnological interventions. Leaf whorl disc from CoM 0265 shoot tops were used for the callus induction and further embryogenic callus induction. The callus was developed using 2 different concentrations of 2, 4-D (3 & 4 mg L^{-1}) and using different concentration of sucrose (20, 25 and 30 g L^{-1}). Embryogenic callus induction, shoot regeneration trough callus, direct shoot regeneration from leaf whorl disc and root induction was checked on different media. Plants were hardened in green house condition and genetic fidelity was checked using ISSR markers. Basal MS with 3 mg L^{-1} of 2, 4 D and 30% sucrose showed highest callus induction within 10 days. This media was used as callus maintenance medium (CM). The callus was then transferred to MS containing different casein highest embryogenic response on basal MS media containing 4 mg L^{-1} of 2, 4-D, 3% sucrose, 10% coconut water with other additives: Thiamine HCl-1 mg L^{-1}, Inositol-20 mg L^{-1}, Proline-500 mg L^{-1}, PVP-500 mg L^{-1}. The embryonic callus exposed to light gave maximum shooting on basal MS containing 1% casein, 30% sucrose. The roots were induced by using different concentration of MS (50%, 75% and 100%) and NAA (4 mg L^{-1} and 5 mg L^{-1}). Highest root induction was observed on 50% MS containing 5 mg L^{-1} NAA and 40 g L^{-1} sucrose. The rooted plantlets were hardened using sterilized soil mixture and maintained in greenhouse before transferring to the field. The plantlets were checked for genetic stability using ISSR molecular markers. The genetic similarity was observed to be 98% within regenerated population plantlets obtained through embryogenic callus.

***Key words*:** Sugarcane, Plant regeneration, Somatic embryogenesis, ISSR molecular markers, Genotype CoM0265

Sugarcane is one of the important cash crops in the world and India ranks second in the production of sugarcane. It is world's most productive crops, with biomass accumulation rates as high as 550 kg/ ha/ day. In addition to its use in the food industry as source of sucrose, sugarcane's significantly higher biomass generation capacity has made it the most promising energy crop due to sugarcane-based ethanol production when compared to sugar beet or sweet potato (https://sugarcane.icar.gov.in). Obtaining disease free and quality seed material is important for efficient crop management system which indirectly improves on agriculture yield by reducing losses. In this regard, tissue culture propagation systems have a wide range of applications for obtaining high-quality seed material in large quantities. These tissue culture plant regeneration systems are also essential in application of other biotechnological interventions such as transformation, gene editing and *In vitro* mutagenesis [1]. Where, an efficient and reproducible in vitro regeneration system is primary requirement for these applications. In sugarcane, different tissue culture techniques such as shoot tip culture [2], direct regeneration and regeneration through eye bud are used for the in vitro plant regeneration; regeneration through embryonic callus is preferred under several conditions such as cell suspension culture, transformation system [3-4]. *In vitro* mutagenesis and for producing artificial seeds which further can lead to automation [5-7]. The reason behind the preference in the embryonic callus regeneration system is that the somatic embryo-regenerated plantlets are considered to be single cell-originated [8]. Therefore, there are higher possibilities for the uniform expression of the embryo-acquired changes throughout the regenerated plantlets. In addition, the callus can be maintained on a long-term basis which reduces hurdles for obtaining required explant material for the purpose. However, it has been observed that the reactions of plants to

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tissue culture techniques may vary depending on the type of tissue and the variety used [9]. It is therefore necessary to optimize the protocol for the selective variety.

Somaclonal variation is commonly observed in somatic embryo regenerated plantlets [10] which may further lead to phenotypic variation due to mutations or epigenic changes. The genetic change can further lead to losses in yield and thus seriously limits the application of micro-propagation techniques in large scale production. Efficient and rapid identification of such genetic or cytological changes in the micro-propagated sugarcane population is a challenge as it may not be identifiable as phenotypic change at young plant age or at any growth stage but might affect yield characteristics in sugarcane. It is therefore desirable to test the genetic fidelity of micro-propagated plants, irrespective of the regeneration pathway of the derived plants, in order to optimize the conditions of cultivation for minimal variation with efficient production for large-scale applications [11]. Molecular markers are the most prominent technique for the analysis of the genetic uniformity of micro-propagated plantlets. PCR based molecular markers have several advantages over other methods and have been used for genotyping and detecting polymorphism or variability in plants. ISSR markers are routinely used to study polymorphism occurred within a simple sequence of repeat regions located abundantly within the genome. Higher sensitivity, reproducibility and ease of performance have made ISSR markers ideal for molecular analysis of polymorphism. In this manuscript, we have optimized organogenesis through somatic embryogenesis in the sugarcane variety CoM 0265 until acclimatization and also analyzed the genetic fidelity of regenerated plants using ISSR markers. A commercial sugarcane variety CoM 0265 was used as the experimental material. The variety has been developed from Co 87044GC and has been released in 2007. The variety is grown in fields of Vasantdada Sugar Institute, Manjari (Bk.) Pune. This is one of the prominent varieties commercially cultivated in Maharashtra state.

MATERIALS AND METHODS

Plant material

Sugarcane shoot apical meristem of CoM 0265 harvested from the fields maintained in Vasantdada sugar Institute, Manjari (Pune). The sugarcane top was removed manually from the base of lowermost leaf taking care not to break the culm disc of internode just below the apical meristem (Fig 1a).

Media preparation

Tissue culture grade chemical products as described in were purchased from Hi Media. The macronutrient and macronutrient stocks were prepared as per standardized laboratory protocols (Supplementary Table 1). All the prepared plant growth regulators: 2, 4-D, BAP, Kinetin and NAA were membrane sterilized and stored in sterile vials at -20°C till use. The coconut water was passed through filter paper to remove any particles mixed during breaking open the coconut and added into the media in a 10 % concentration of total volume prepared (100 ml L^{-1}) in callus regeneration and embryogenic callus media. Sucrose and plant hormones were added to the MS media and the pH was adjusted to 5.8 using 0.1 N HCl or NaOH. Agar agar 0.8% was added to the media as gelling agent. Media was heated to dissolve the agar and sterilized in an autoclave at 121°C for 20 minutes at 15 PSI. Antibiotic 'Cefotaxime' (100 mg L^{-1}) was added into the autoclaved media under aseptic conditions when the media cooled to around 40-50°C. Around 22-25 ml media were poured into the sterile glass Petri plates. Media plates were kept ready for at least 3 days before the experiment to check the contamination if any.

Supplementary Table 1 Murashige and Skoog (1962): Media components and Stock preparations

Macronutrient (MS-1)		Quantity (mg/l)	10X (g/l)
Ammonium nitrate	NH_4NO_3	1650	16.5
Calcium chloride	$CaCl_2.2H_2O$	440	4.4
Magnesium sulphate	$MgSO_4.7\ H_2O$	370	3.7
Potassium phosphate	KH_2PO_4	170	1.7
Potassium nitrate	KNO_3	1900	1.9
Disodim EDTA	Na_2EDTA	37.2	3.72
Ferrous sulphate	$FeSO_4$	27.8	2.78
Micronutrients (MS-II)			1000X (mg/50ml)
Boric acid	H_3BO_3	6.2	310
Cobalt choride	$CoCl_2.6H_2O$	0.025	1.25
Cupric sulphate	$CuSO_4.5\ H_2O$	0.025	1.25
Magnetic sulphate	$MnSO_4.7H_2O$	22.3	1115
Potassium iodide	KI	0.83	41.5
Sodium molybdate	$Na_2MO_4.2H_2O$	0.25	12.5
Zink sulphate	$ZnSO_4.7H_2O$	8.6	430
Common organic addition			1000X (mg/50 ml)
Nicotinic acid		0.5	25
Pyridoxine HCl		0.5	25
Thiamine HCl		0.1	5
Glycine		2.0	100
Inositol		100	5000

10 ml each of 1000X stock mixed in distilled water and volume make up to 1 liter for MS-2 (10X) stock preparation

Explant collection and callus initiation

The tops of the 8-9 month old sugarcane were plucked manually in morning to avoid phenolic secretions. The leaves were cut and the old leaf cover was removed (Fig 1a). These sugarcane tops were hold under flowing tap water for the removal of superficial dust and then washed with detergent 'Teepol' followed by double washing with distilled water. About 1.5 cm thick cylindrical uppermost node with leaf whirls 12-14 cm in height was prepared (Fig 1b). Care was taken to keep the lower nodal disc intact. The cylinders were then placed in glass bottles containing 0.1% Bavistin, 0.4% Citric acid, and 0.2% Ascorbic acid in about 200 ml sterile distilled water and kept on

Gyro-rocker for 20 minutes. The process was repeated with 0.1% $HgCl_2$ for 20 minutes [12]. After incubation, under aseptic conditions the $HgCl_2$ was decanted and the cylindrical leaf whorl explants were transferred to bottles containing sterilized distilled water and stirred manually to wash $HgCl_2$. This distilled water rinsing step was repeated twice further and these cleaned cylindrical explants were transferred to another sterile empty glass bottles.

Supplementary Table 2 Percent callus induction from leaf whorl disc of sugarcane var. CoM 0265

Media code	Medium composition	Callus induction (%)
Control	MS + Sucrose (3.0 %) + 10 % CW	00.0 ± 0.0^{f}
MS 1	MS + 2,4-D (3.0 mg L^{-1}) + sucrose (2 0 %) + 10 % CW	75.0 ± 2.9^{d}
MS 2	MS + 2,4-D (4.0 mg L^{-1}) + sucrose (2 0 %) + 10 % CW	71.7 ± 1.7^{de}
MS 3	MS + 2,4-D (3.0 mg L^{-1}) + sucrose (2.5 %) + 10 % CW	80.0 ± 2.9^{c}
MS 4	MS + 2,4-D (4.0 mg L^{-1}) + sucrose (2.5 %) + 10 % CW	84.0 ± 1.0^{b}
MS 5	MS + 2,4-D (3.0 mg L^{-1}) + sucrose (3.0 %) + 10 % CW	91.7 ± 1.7^{a}
MS 6	MS + 2,4-D (4.0 mg L^{-1}) + sucrose (3.0 %) + 10 % CW	70.0 ± 2.9^{de}

Supplementary Table 3 Effect of different media compositions on embryogenic callus percent and Relative growth rate (RGR)

Media code	Medium composition	RGR	Embryogenic callus (%)
Control	MS + Sucrose (3.0 %)	0.50 ± 0.06^{e}	00.0 ± 0.00^{f}
MS-E1	MS + 2, 4-D (3.0 mg L^{-1}) + sucrose (2.0 %) + CH (1.0 g L^{-1})	0.97 ± 0.20^{cd}	00.0 ± 0.00^{f}
MS-E2	MS + 2, 4-D (3.0 mg L^{-1}) + sucrose (2.5 %) + CH (0.0 g L^{-1})	1.05 ± 0.22^{cd}	15.0 ± 2.24^{e}
MS-E3	MS + 2, 4-D (3.0 mg L^{-1}) + sucrose (2.5 %) + CH (0.5 g L^{-1})	1.38 ± 0.68^{c}	30.0 ± 3.16^{d}
MS-E4	MS + 2, 4-D (3.0 mg L^{-1}) + sucrose (2.5 %) + CH (1.0 g L^{-1})	1.85 ± 0.59^{bc}	44.0 ± 2.30^{dc}
MS-E5	MS + 2, 4-D (4.0 mg L^{-1}) + sucrose (2.5 %) + CH (1.0 g L^{-1})	2.45 ± 0.51^{a}	66.7 ± 2.40^{b}
MS-E6	MS + 2, 4-D (3.0 mg L^{-1}) + sucrose (3.0 %) + CH (1.0 g L^{-1})	1.80 ± 0.68^{bc}	70.0 ± 1.90^{b}
MS-E7	MS + 2, 4-D (4.0 mg L^{-1}) + sucrose (3.0 %) + CH (1.0 g L^{-1})	3.20 ± 1.14^{a}	81.0 ± 2.24^{a}

All the above media combinations contained 10 % coconut water, Thiamine HCl 1 mg L^{-1}, Inositol 20 mg L^{-1}, proline 500 mg L^{-1}, PVP-500 mg L^{-1}, and agar-agar 0.8 % in addition to the above-mentioned components. Values are represented in mean ± S.E., similar alphabets represent values are statistically non-significant, P<0.05

Callus induction and optimization for embryonic callus regeneration

After pre-treatment, one or two outer layers were removed again from the explant cylinders aseptically under laminar conditions. The disc segments of 1-2 mm thickness were prepared from the innermost compact tightly furled spindle leaves and the discs were kept on solidified media in an upright position. About 10-15 discs per plate were inoculated (Fig 1d) and the plates were placed under the dark conditions during incubation and temperature at 25 ± 2°C and relative humidity of 70-80% was maintained. Three such individual experiments were considered for the analysis. Six different combinations obtained by combination of two concentrations of 2, 4-D (2, 4 Dichlorophenoxyacetic acid), with three different sucrose concentrations (2%, 2.5% and 3%) (Table 1) in basal MS media were used for the callus induction. The observations for percent callus induction (% callus induction) were recorded after 30 days of incubation.

Embryogenic callus induction

The friable callus formed in three- four weeks of initial primary culture i.e. after around one month was sub-cultured onto various media combinations (Table 1) for optimizing embryogenic callus regeneration. The embryonic callus started to appear as compact off-white nodular structures among other different types of calluses. The percent of embryogenic callus was calculated as per Nikam *et al*. [13] using the below formula:

$$\text{Percent embryogenesis} = \frac{\text{No. of calli showing embryogenesis}}{\text{No. of calli initiated}} \times 100$$

Calculation of relative growth rate

The relative growth rate was calculated after 30 days of callus inoculation on embryogenic callus media by randomly selecting 20 plates and taking their average weights. The relative growth rate (RGR) calculations were done according to Santoso [14] using the formula given below:

$$\text{Relative growth rate (RGR)} = \frac{W_n - W_0}{W_0}$$

Where;

W_n is the weight of the callus at the end of the incubation period and W_0 is the weight of callus before experiment.

Table 1 Media Composition used for callus induction and embryogenic callus formation

	Media composition used for callus induction						
	Control	MS-1	MS-2	MS-3	MS-4	MS-5	MS-6
2.4-D (mg/ L)	0	3	4	3	4	3	4
Sucrose (g/L)	20	20	20	25	25	30	30
Coconut water (%)	10	10	10	10	10	10	10
Casein hydrolysate	0	0	0	0	0	0	0

	Media compositions used for embryogenic callus induction							
	Control	MS-E1	MS-E2	MS-E3	MS-E4	MS-E5	MS-E6	MS-E7
2.4.-D (mg/ L)	0	3	3	3	3	4	3	4
Sucrose (g/L)	20	20	25	25	25	25	30	30
Coconut water (%)	10	10	10	10	10	10	10	10
Casein hydrolysate	1	1	0	0.5	1	1	1	1

Initiation for plant regeneration from embryogenic callus

The embryonic callus was placed on different combinations of shoot induction media. The different compositions of media mentioned earlier in literature [15-18] were used for deciding growth hormone concentrations for shoot regeneration from the callus. Approximately 1 cm^2 bunches were put on callus induction media and the plates were incubated under 2400-3000 lux cool white fluorescent light in 16h/ 8h light and dark photoperiod. The temperature of the culture room was maintained at 26 ± 1°C. The shoot primordial started to appear in the first week of transfer onto the shooting media. Percent shoot formation and the number of plantlets formed from the callus on different media was recorded after 15 days and 30 days of inoculation into the media. The different compositions used for the shoot regeneration is mentioned below in (Table 2).

Table 2 Media composition for shoot initiation from embryogenic callus

	CH	Proline	2,4-D	BAP	Kinetin
	g L^{-1}	mg L^{-1}	g L^{-1}	mg L^{-1}	mg L^{-1}
SM-1	0.5	-	-	-	-
SM-2	0.5	-	0.6	-	-
SM-3	1.0	0.5	-	-	-
SM-4	0.5	-	-	0.20	-
SM-5	0.5	-	-	0.25	-
SM-6	0.5	-	-	0.50	-
SM-7	0.5	-	-	0.25	0.25
SM-8	0.5	-	-	0.50	0.25

Media optimization for rooting

Well-grown shoots were transferred to different combinations of root induction media. Only plants with similar height were chosen for the experiment to minimize the error Three different strengths of the MS media (50%, 75% and 100%) with two different concentrations of α-naphthalenic acetic acid (4 mg L^{-1} and 5 mg L^{-1}) in media containing 4% sucrose were used for observing root induction as mentioned in (Table 3) [19]. The cultures were incubated under the same condition as for shoot induction. The root length, number of shoots showing root formation (% root generation), and the number of roots per shoot were recorded and number of shoots with longer length per tube were recorded.

Table 3 Media compositions used for optimization of rooting

	Control	RM-1	RM-2	RM-3	RM-4	RM-5	RM-6
NAA (mg/l)	0	4	4	4	5	5	5
MS salts (%)	100	100	75	50	100	75	50
Sucrose (g/L)	40	40	40	40	40	40	40

Hardening and maintaining the plantlets into greenhouse

For hardening red soil, black soil, vermicomposting and coco peat were mixed in ratio (1:1:1:1) dried and put in small plastic bags of 10 cm height. The transplantation was done for the well rooted plantlets to the plastic bags containing soil mixture, watered and incubated under normal lab condition for a week with and then transferred to the green house for around 45 days. After preliminary hardening in the greenhouse, the plantlets were kept in the open nursery under natural sun light with daily irrigation for another 45 days.

DNA extraction from plantlets

DNA extraction was done as per described by Aljanabi *et al*. [20] with minor modifications. 100 mg leaf tissue from five different randomly selected plants were pulled together and was crushed to powder using liquid nitrogen using clean sterile mortar and pestle. The powdered tissue was put into pre-warmed 1 ml buffer containing 4.6% CTAB, 45 mM EDTA, 2.4 M NaCl, 1.8% PVP, 0.2 M Tris HCl and 1.3% SDS and kept at 65 ºC for 1 hour with intermittent inverting. The solution was cooled to room temperature (26 ± 3 ºC) and treated with 800 µl of (25:24:1) phenol, chloroform, Isoamyl alcohol mixture with invert mixing for 10 minutes followed by centrifugation at 12000 rpm/10 min. The upper aqueous layer was collected into 1.5 ml Eppendroff tube. About 10 µl of RNase (10 mg/ml) was added to the aqueous layer; mixed and the tubes were incubated at 37 ºC for 1 hour. The DNA was precipitated by addition of 800 µl of chilled Isopropanol into the aqueous layer and the tubes were incubated at -20 °C for 1 hour. The precipitated DNA was collected at bottom of Eppendroff tubes by centrifugation at 14,500 rpm at 4°C for 15 minutes. DNA was washed with 80% ethanol twice, dried at 37 °C and mixed with Tris EDTA buffer (10 mM Tris HCl containing 1mM EDTA.Na_2, pH-8.0). It was stored at -20 °C until further analysis.

ISSR PCR analysis

A set of 10 UBC series ISSR markers of were synthesized from Sigma®. About 90 ng of DNA was used for the analysis. 0.5 mM primer, 2.5 mM dNTPs, 1X buffer containing 1.5 mM KCL and 2.5 mM $MgCl_2$ (Sigma) and 1 U Taq polymerase (Sigma Aldrich, USA) were used in total 25 µl reaction mixture. PCR was performed using Himedia Prima Duo™ PCR. The initial denaturation was done at 95 ºC for 5 min followed by 30 cycles of denaturation at 95 ºC for 1 minute, annealing at 48-52 ºC (depending upon primer selected) for 1 minute and extension at 72 ºC for 2 minutes. This was then followed by final extension at 72 ºC for 15 minutes. The PCR products were stored at 4 ºC till loading. For analysis, the PCR product was loaded along with loading dye on 2 % agarose gel in 1 X TAE containing 1 mg/ ml EtBr. The electrophoresis as performed at 90 V for 2 hours was applied. The gel images were captured using UVi Tech® UV trans-illuminator and gel documentation system. The amplified profiles for all the primers were compared with each other and bands of DNA fragments were scored manually as '1' for absence and '0' for presence, generating '0' and '1' matrix. Molecular weights of the bands were estimated by using 1 kb DNA ladder as standards. All amplification was repeated at least twice and only reproducible bands were considered for analysis. Very light bands difficult for the analysis were not considered for fidelity analysis. However light amplification of the sharp mobility

band was considered and scored as '0' in preparation of matrix. Percent polymorphism was calculated by using the below formula:

$$\text{Percent polymorphism} = \frac{\text{No. of polymorphic bands}}{\text{Total No. of bands}} \times 100$$

Statistical analysis

For the statistical analysis of the responses of 20 individual plates showing best responses were used. Treatment means and standard deviation of the observed values were calculated using MS excel 2010. SPSS 10.0 software for windows (version 16) was used for the one-way analysis of variance and Duncan's new multiple range test was used to establish significance of the treatments at $p<0.05$. The results were expressed as mean ± S.E. In molecular marker analysis the amplified bands of particular base pair location were designated as '1' for the presence and '0' for the absence.

RESULTS AND DISCUSSION

Callus induction and embryogenic callus formation

The callus initiation was carried out on MS-1 to MS-6 having two different 2, 4-D concentrations (3 mg L^{-1} and 4 mg L^{-1}) in combination with three different sucrose concentrations (2.0%, 2.5%, and 3.0%) respectively. Leaf whorl disc of sugarcane was used as an explant. The callus started to appear on the lower edges of the leaf whorl disc after 12-15 days. After subculture of the leaf whorl discs on fresh media plates after 20 days (Fig 1e); the callus also appeared on the upper edges and the whole leaf-whorl disc explant turned to the callus in 40-45 days (Fig. 1f). The results obtained for percent callus induction on different media compositions are represented below in (Fig 2). MS-5 media containing 3.0 mg L^{-1} of 2, 4-D and 3% sucrose gave maximum callus induction followed by MS-4 containing 4 mg L^{-1} of 2, 4 -D and 2.5% sucrose. MS-1 and MS-2 containing lower sucrose (2%) showed lower callus induction. On control medium without hormone, there was no callus induction (Fig 2a).

On transfer of the callus cultures on different media (MS E1-E7) with different combinations of auxin (2, 4-D), sucrose, and casein hydrolysate, the embryogenic callus regeneration was observed after two weeks on transfer to the media. Mixed type of callus (Fig 3) containing i) Separable (friable) callus where fresh callus was observed more of off white translucent type with texture which can be easily separated, ii) Chalky white colored dry degenerative type callus which produced root-like growths which later could not grow (Fig 3b), iii) Watery type of callus which had spongy type appearance, iv) Smooth, sticky type of callus, which turned brown even after sub-culturing onto fresh media (Fig 3c) and v) Embryogenic callus containing an opaque compact structure of embryos (Fig 3a) were observed on different combinations. However, the prominence of every type of callus was observed to vary with the change in media composition. Higher RGR of the callus was observed on MS-E7 and MS-E5 (3.20 ± 1.14 and 2.45 ± 0.51 respectively) (Fig 2c). On MS-E7 containing 4 mg L^{-1} of 2, 4-D, 3% sucrose, 10% coconut water and 1 g L^{-1} casein hydrolysate, a maximum of 81% embryogenic callus induction observed (Fig 2b).

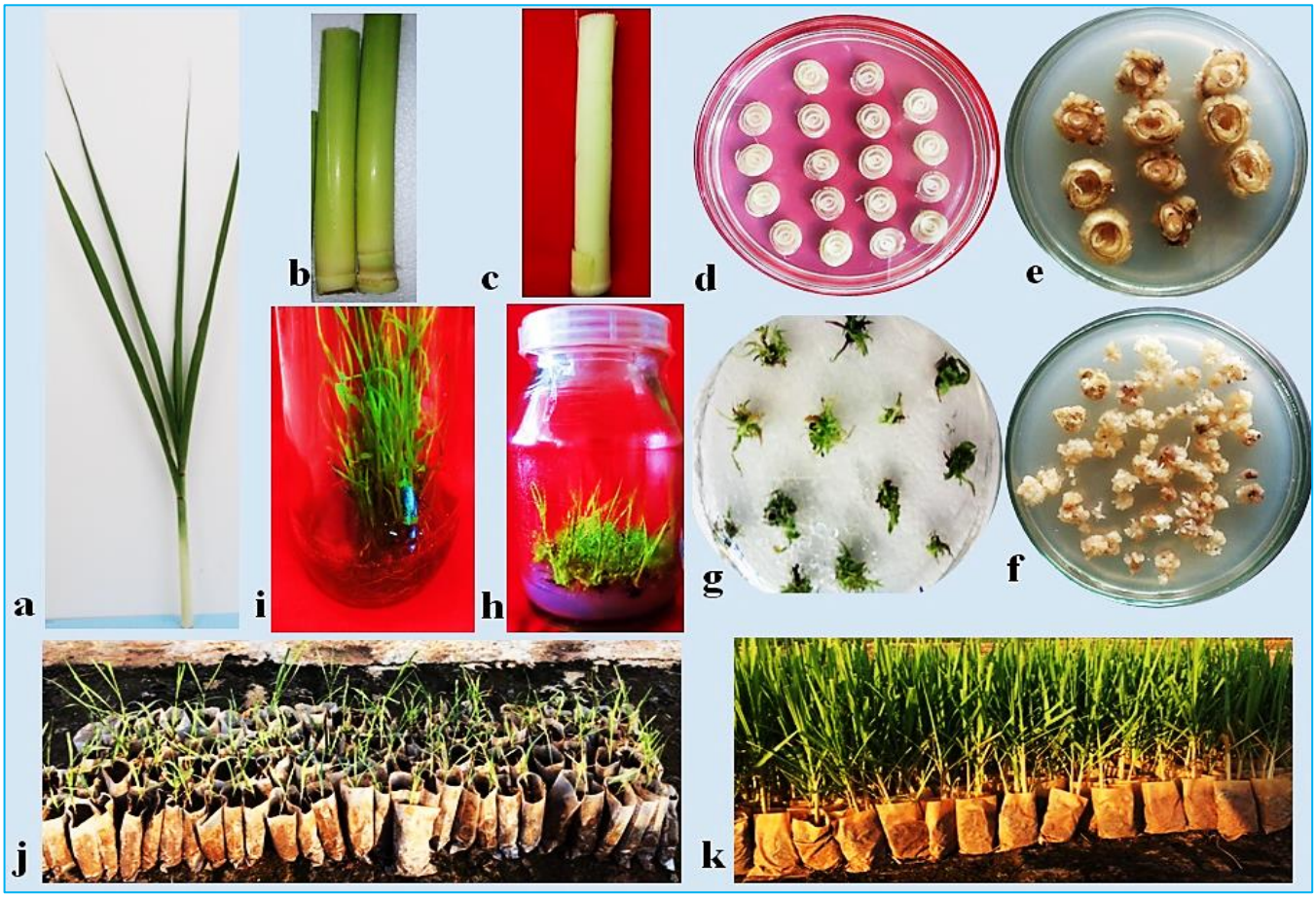

Fig 1 a-Sugarcane top removed from the field, b-removal of upper leaves and shoots to form cylindrical explant, c- Explant used for primary culture by further removal of outer leaf layers after surface sterilization. d- Leaf whorl disc inoculated on callus induction media made by horizontal cuts in cylindrical explant of sugarcane top, e-formation of the callus on leaf whorl discs after 10-15 days of incubation under dark. f-formation of embryogenic callus, g-shoot initiation from embryogenic callus, h-transfer of shoots in bottles for growth, i-Transfer of shoots for rooting, j-transfer of the rooted plantlets to the plastic bags containing sterile soil mixture, k-hardening and growth of the raised plantlets

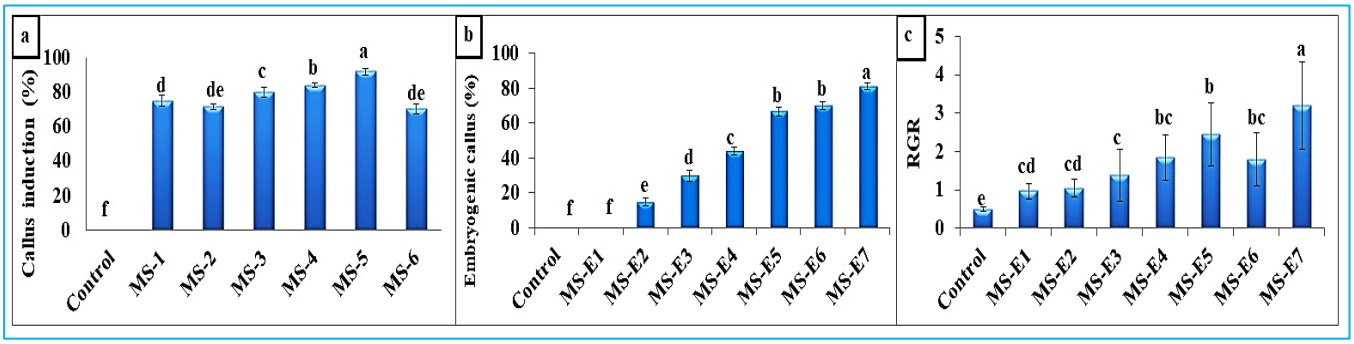

Fig 2 Response of sugarcane variety CoM 0265 to different media combinations for a) callus induction and (b, c) Embryogenic callus induction. Values are represented in mean ± S. E, similar alphabets represent values in rows are statistically non-significant at P ≤ 0.05 in Duncan's multiple replication test

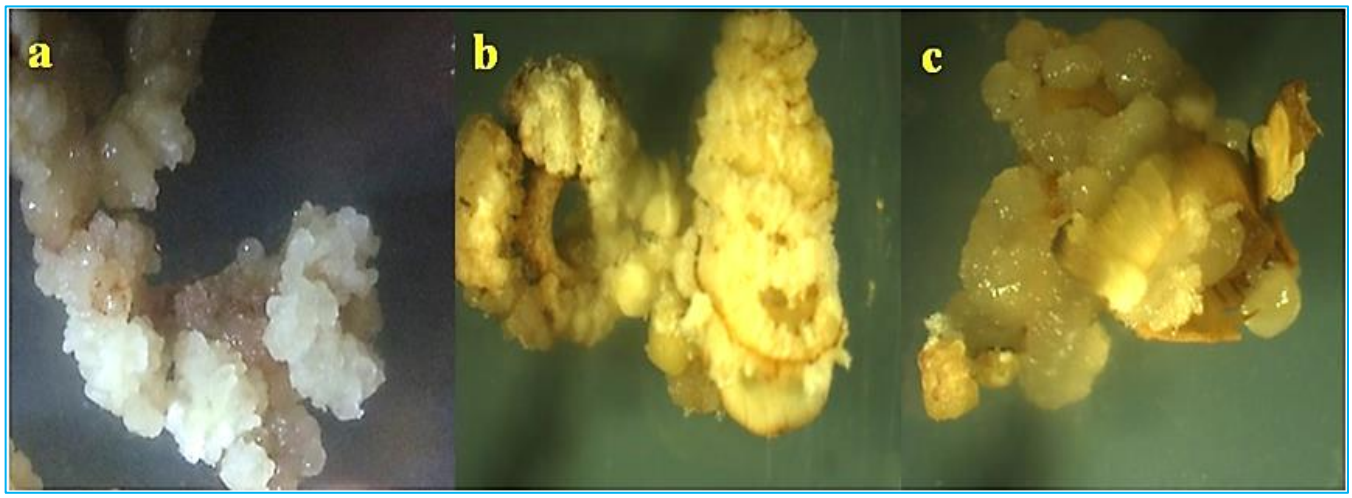

Fig 3 Types of callus: a-friable callus with compact embryonic callus, b-chalky white degenerative callus, c-loose sticky watery callus

Table 4 Analysis of genetic fidelity in sugarcane plant samples derived through embryogenesis by ISSR markers

S. No.	Primer code	Sequence (5′→3')	Temp (°C)	Range	NSb	NMb	NPb
1	UBC 807	$(AG)_8T$	49	250-1500	12	11	1
2	UBC 810	$(GA)_8T$	49	375-1200	7	7	0
3	UBC 811	$(GA)_8C$	49	250-1500	12	12	0
4	UBC 818	$(CA)_8G$	49	250-1500	11	11	0
5	UBC 825	$(AC)_8T$	49	500-2000	10	10	0
6	UBC 828	$(TG)_8A$	52.3	250-1500	12	12	0
7	UBC 836	$(AG)_8CA$	53.5	100-1000	7	7	0
8	UBC 842	$(GA)_8TG$	49	250-1500	11	11	0
9	UBC 857	$(AC)_8CG$	53.5	250-1000	5	5	0
10	UBC 859	$(TG)_8TC$	52.3	750-2000	7	6	1
		Total			94	92	2
		Percentage (%)				97.87	2.13

Sb-Number of scorable bands, Pb. -Number of polymorphic bands, Mb- Number of monomorphic bands. A-Adenine, C-Cytosine, G-Guanine, T-Thiamine

Supplementary Table 4 Effect of different combinations of growth regulators on shoot initiation and shoot multiplication of sugarcane cv. CoM 0265

Media code	% Shoot initiation		Shoot length 30 days (cm)	Number of shoots / plate at 30 days
	15 days	30 days		
SM-1	40.00 ± 0.00 ^{ab}	67.14 ± 8.92 ^{a}	3.287 ± 0.18 ^{b}	30.75 ± 1.29 ^{ab}
SM-2	30.00 ± 5.77 ^{b}	36.67 ± 8.82 ^{c}	1.807 ± 0.27 ^{e}	19.00 ± 1.97 ^{d}
SM-3	53.33 ± 3.33 ^{a}	72.67 ± 3.33 ^{a}	5.176 ± 0.28 ^{a}	40.00 ± 3.41 ^{a}
SM-4	23.33 ± 6.67 ^{b}	40.00 ± 6.67 ^{bc}	3.075 ± 0.25 ^{b}	25.25 ± 3.99 ^{bcd}
SM-5	23.33 ± 8.82 ^{b}	66.67 ± 8.82 ^{a}	3.363 ± 0.21 ^{b}	26.75 ± 1.85 ^{bcd}
SM-6	53.33 ± 8.82 ^{a}	63.33 ± 8.82 ^{ab}	2.903 ± 0.20 ^{bc}	32.25 ± 3.56 ^{ab}
SM-7	23.33 ± 8.82 ^{b}	70.00 ± 8.82 ^{a}	2.495 ± 0.27 ^{cd}	21.25 ± 3.58 ^{cd}
SM-8	23.33 ± 3.33 ^{b}	63.33 ± 3.33 ^{ab}	2.217 ± 0.10 ^{de}	33.75 ± 3.52 ^{ab}

Values are represented in mean ± standard error (S.E.)
Similar alphabets represent values in columns are statistically non-significant at (P<0.05)

Shoot formation

The embryogenic callus was inoculated on different media compositions. The different parameters observed are graphically represented in (Fig 4) and numerical values of the results are mentioned in (Supplementary Table 4). On media SM-3 (containing full strength MS, 3% sucrose, 1 g L^{-1} of casein hydrolysate, and 0.5 g L^{-1} proline) and SM-6 (containing full strength MS components, 3% sucrose, 0.5 g L^{-1} casein hydrolysate, 0.25 mg L^{-1} BAP and 0.25 mg L^{-1} kinetin) shoot initiation took place early resulting into highest shoot initiation in 15 days. However, at 30 days after subculture, nearly equivalent percent of shoot initiation was observed on media SM-1, SM-3, SM-5 and SM-7 (Fig 4c). The media SM-2 containing 0.6 mg L^{-1} 2, 4-D showed the lowest percent shoot initiation and also the lowest average shoot length and a number of shoots. SM-3 produced a significantly higher number of shoots (40 numbers/ plate) (Fig 4b) and the shoots produced on SM-3 also showed a higher average length (5.176 cm) (Fig 4a). Considering the results obtained for early shoot regeneration and higher number of shoots, media SM-3 was used further in the experiment for shoot regeneration from callus.

Supplementary Table 5 Effect of differential MS strength with NAA on rooting of sugarcane cv. CoM 0265

Media Code	% Root formation	Number of roots/Shoot	Root length (cm)	Number of longer shoots (> 5 cm)
Control (100% MS + 0.0 mg L^{-1} NAA)	11 ± 0.20 ^{b}	0.02 ± 0.00^{e}	0.070 ± 0.00 ^{c}	0.0 ± 0.00 ^{e}
RM-1 (50% MS + 4.0 mg L^{-1} NAA)	85 ± 5.00 ^{a}	3.20 ± 0.58^{b}	0.115 ± 0.023^{b}	6.6 ± 0.33 ^{ab}
RM-2 (75% MS + 4.0 mg L^{-1} NAA)	90 ± 6.12 ^{a}	4.00 ± 0.29^{ab}	0.285 ± 0.034^{a}	5.0 ± 0.49 ^{cd}
RM-3 (100% MS + 4.0 mg L^{-1} NAA)	95 ± 15.0 ^{a}	3.38 ± 0.31^{b}	0.255 ± 0.024^{a}	5.6 ± 0.40 ^{bc}
RM-4 (50% MS + 5.0 mg L^{-1} NAA)	95 ± 15.8 ^{a}	5.15 ± 0.47^{a}	0.283 ± 0.028^{a}	7.6 ± 0.56 ^{a}
RM-5 (75% MS + 5.0 mg L^{-1} NAA)	100 ± 0.00 ^{a}	4.00 ± 0.33^{ab}	0.140 ± 0.015^{b}	6.6 ± 0.54 ^{ab}
RM-6 (100% MS + 5.0 mg L^{-1} NAA)	75 ± 5.00 ^{a}	2.60 ± 0.69^{c}	0.098 ± 0.017^{b}	3.6 ± 0.46 ^{d}

Values are represented in mean ± standard error (S.E.)
Similar alphabets represent values in columns are statistically non-significant at (P<0.05)

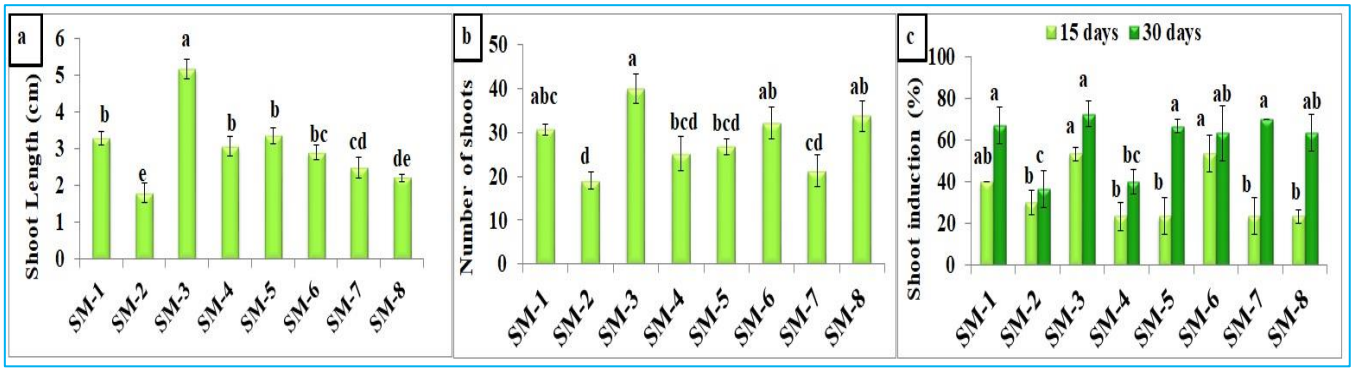

Fig 4 Responses of embryogenic callus of CoM0265 to different shooting media composition in terms of a) Shoot length, b) Number of shoots, and c) Percent shoot induction. Values are represented in mean ± standard error (S.E.); similar alphabets represent values in columns are statistically not significant at (P<0.05)

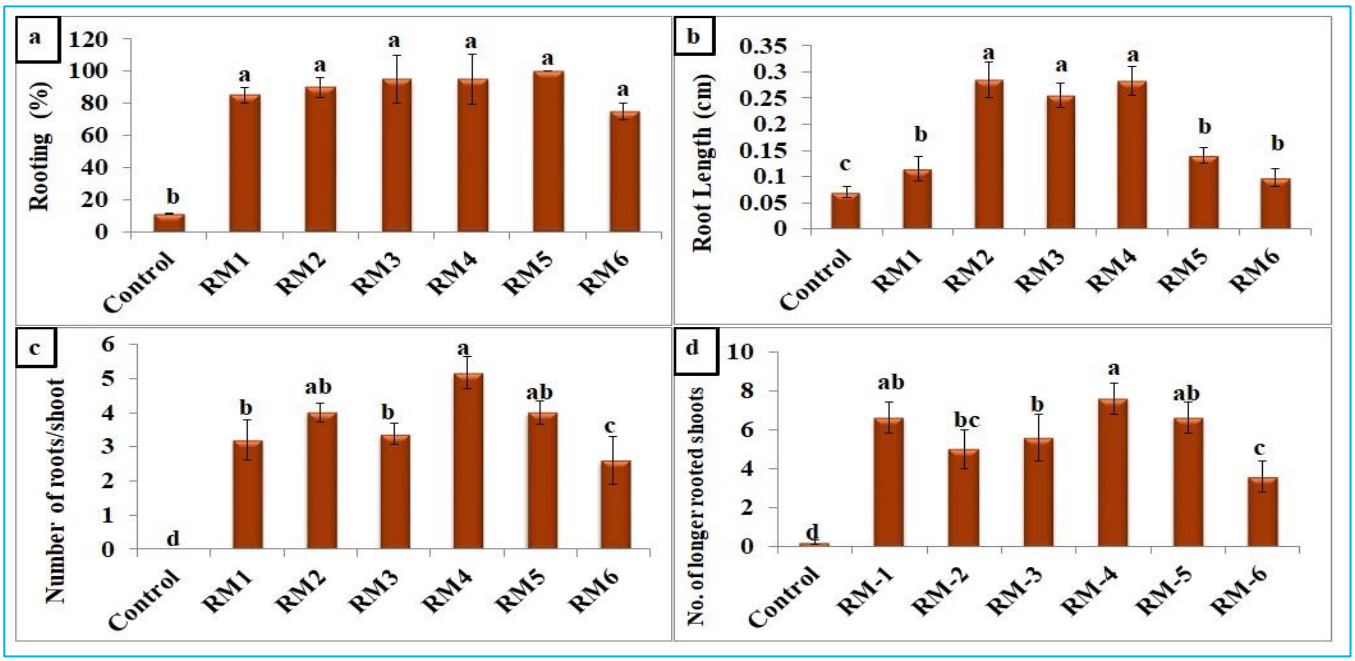

Fig 5 Responses of sugarcane *in vitro* shoots to different media combinations for root induction in terms of a) percent rooting, b) Root length, c) Number of roots per shoot and d) Number of longer shoots. Values are represented in mean ± standard error (S.E.); similar alphabets represent values in columns are statistically non-significant at (P<0.05)

Responses of the shoots to different MS strengths with NAA for rooting

All shoots of nearly similar height (5 cm) regenerated from the shoot optimization experiment were aseptically transferred on rooting media differing in MS strength and NAA concentration (Table 3). Roots started to appear from the base of the shoots at around 10 days. The difference in percent root initiation was not significantly different in the different media combinations except for control which showed significantly lower root initiations (Fig 5). However, the number of roots, length of the roots, and the number of longer shoots varied with a change in media composition (Fig 5a). The highest ratio of the number of roots to the number of shoots was observed in RM-4 media containing 50% MS components, 4% sucrose, and 5 mg L^{-1} NAA. RM-4 also showed the highest length of the plantlets.

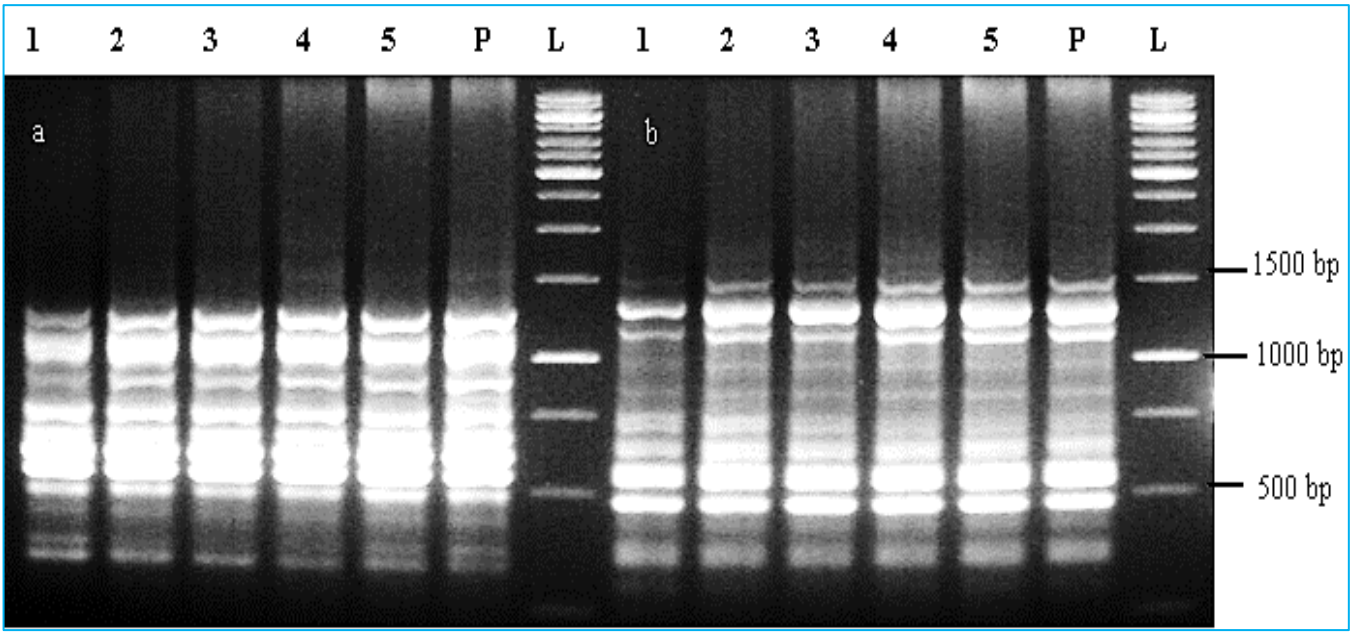

Fig 6 Agarose gel profile of ISSR marker analysis in sugarcane var. CoM0265 plants obtained through somatic embryogenesis using a) UBC-807, b) Agarose gel profile using UBC 811, P- DNA of field grown parent sample, L-ladder

Molecular marker analysis

ISSR molecular markers were used to check genetic uniformity or variation. Ten molecular markers which produced clear distinct profile were used to check uniformity in banding pattern. The primers used for the molecular analysis showed amplification in the range of 100-2500 base pairs. A total of 564 amplicons were amplified in the analysis. ISSR primer UBC-807, UBC-811 and UBC-812 has produced maximum number 12 bands each in the analysis followed by UBC-818 and UBC-842 which has produced 11 bands each. However polymorphic bands were observed only in UBC-807 and UBC-859 analysis.

Optimizing regeneration protocol through the selected explant is a primary and essential objective as the success of the experimentation procedures in biotechnological interventions are largely dependent on it. The regeneration through the embryogenic callus has been advocated to aid into the mutagenesis as the chance of additional variation through in vitro regeneration on combining with cellular level selections followed by formation of plantlets help obtaining true mutant population with improved desired characteristics (Suprasanna et al. 2023). The primary objective of this study was to develop a practically applicable efficient callus induction and regeneration protocol for sugarcane CoM 0265 variety.

Callus induction and embryogenic callus formation

The sugarcane top containing apical meristematic region and leaf whorls surrounding it from 8-10 months old sugarcane plant was used for the formation of the callus. The plant age at this stage was reported to have high regeneration ability and has been used for commercial production using the apical meristematic region as well as for the formation of callus for various transformation experiments [21-22] . The commercial sugarcane varieties have 12-14 months of maturity period and 150-180 days (5^{th}-8^{th} months) considered as the grand growth phase. At this phase, the sugarcane tip portion is still under division and new internodes can be formed implying a high rate of multiplication in this portion. Fitch and Moore [23] demonstrated the tissues in sugarcane tip comprising the apical meristematic region, newly formed leaves around the meristem and pith show superior callus formation and have been widely used as explant in several studies for the formation of callus [24-27]. Wounding or mechanical damage is a common stimulus for the formation of callus in plants. The discs of leaf whorls are cut from sugarcane tops and were placed in different concentrations of auxin: 2, 4 di-chlorophenoxyacetic acid (2, 4-D) containing media. Callus formation in plants is found to be governed by complex regulatory mechanisms. In terminally differentiated plant cells the progression of the mitotic cycle is suppressed for the callus formation aiming to regain the competence for cell proliferation under callus formation [28]. Generally intermediate to high auxin-to-cytokine ratio is required for callus formation. The response to the auxin concentration often varies with the experimental variety and the type of the explant [29]. For the sugarcane callus induction, 3-4 mg L^{-1} of 2, 4 D was reported previously [30-33]. Thus, these two concentrations along with different sugar concentrations were used to analyze the amount of callus formation. The callus initiated on average after two weeks of incubation at the lower cut surface of the leaf whorl disc which is in contact with the media. The whorls started to get separated with callus formation as callus generation progressed. The percentage of callus formation ranged from 70-91% concerning different media combinations. Maximum callus induction (91.7 ± 1.7%) was observed on media MS-5 containing 3 mg L^{-1} of 2, 4-D, 10% coconut water and 3% sucrose whereas lowest callus induction was observed on media MS-2 containing 4 mg L^{-1} of 2, 4-D,

with 10% coconut water and 2% sucrose (70 ± 2.9 %). Jamil *et al*. [34] observed 2 mg L^{-1} of 2, 4-D with 30 g L^{-1} sucrose optimum for sugarcane callus induction.

Somatic embryo development through callus is considered indirect embryogenesis and the development of an embryo occurs from induced-embryonic determined cells (IEDCs) [35]. The callus cells undergo reprograming of metabolic, physiological and transcriptional pathways and developed into pro-embryonic cell mass followed by somatic embryos [36]. In the experiment, MS-7 containing 4 mg L^{-1} 2, 4-D, 10% coconut water, 30 g L^{-1} sucrose, and 1 g L^{-1} casein hydrolysate along with other additives such as thymine hydrochloride (1 mg L^{-1}), proline (500 mg L^{-1}) and PVP (500 mg L^{-1}) was observed to be efficient in generating embryogenic callus from CoM 0265 variety. Use of coconut water has previously been reported to be beneficial for embryo formation in sugarcane [37-38] and it was observed to produce similar effect as auxin [39]. Casein hydrolysate being a rich source of various amino acids and also minerals such as calcium and phosphate has also reported improvement in embryogenic callus induction on addition into the media [40-42]. Proline has long been reported as osmoprotectant. Polyvinyl pyrrolidone (PVP) is non-toxic and helps in controlling phenolic secretions by plant-wounded surfaces. It was observed that in absence of PVP, the media turned brown in 2-3 days, if continued to be grown on the same media stopped growing or turned into a degenerative type. The inositol in phosphorylated form has been observed to play role in calcium channeling and has also been observed in somatic embryos of plants [43] whereas, Thymine serves as a co-factor for various enzymes involved in decarboxylation reactions and also observed to be important for somatic embryo formation from callus [44]. These additives were also observed to help in the formation of embryogenic cell suspension of sugarcane callus in Co 86032 and CoC 671 varieties [45].

Shoot formation

Three aspects of shoot regeneration were studied to optimize the media for shoot regeneration from embryogenic callus viz: percent regeneration, number of shoots, and average shoot length (Fig 4). Percent regeneration ranged from 36 to 72% on 30th day. 15 days after transfer of the callus to the medium and exposure to light, the highest regeneration was observed on media SM-3 having an absence of growth hormones along with 1 g L^{-1} casein hydrolysate and 500 mg L^{-1} proline and SM-6 containing 0.5 mg L^{-1} BAP. Whereas, on the 30th day, no significant differences were observed in percent shoot regeneration within different media compositions. However, SM-3 was selected since it showed the highest shoot length on the 30th day and the highest number of plantlets.

The addition of proline in media has also been found to increase shoot regeneration from rice callus [46-47]. Proline is a known biotic component known for imparting stress tolerance against various biotic and abiotic stresses and improving vigor [48]. Proline provides buffering effect in the medium [49] and was also reported to aid in somatic embryogenesis [50-51]. In this experiment, the combined effect of increasing proline and casein hydrolysate was observed to improve shooting emergence from the callus in media SM-3 as compared to the control. Small secondary shoots also started to appear from the callus near the bases of primary shoots after an average period of 10-15 days. Although more than 80% shoot regeneration from callus has been reported in sugarcane [52]; the CoM 0265 embryogenic callus showed comparatively lower shoot regeneration this might be due to a variety of specific responses in sugarcane [53-54].

SM-3 also produced the highest number of plantlets from the callus. It was observed that the use of kinetin along with BAP increased the number of shoots emerging from the callus as like in media SM-6 and SM-7 and also the length of shoots to some extent. However, we observed the length remained static if the same media was continued for the growth; rather shifting these regenerated shoots to SM-3 helped in improving length. BAP is commonly used synthetic cytokinin and 0.25-2.0 mg L^{-1} has been used for the shoot regeneration from sugarcane callus [55-56]. We found plain MS with 1 g L^{-1} casein hydrolysate, 500 mg L^{-1} proline and 3 % sucrose gave optimum shoot regeneration. Our reports are per previous reports of [57] who observed best shoot regeneration from cv. Co 740 and Co 86032 callus on media containing no phytohormone and Gandonou *et al*. [58] also reported efficient shoot regeneration from embryogenic callus of different sugarcane varieties on hormone-free media containing casein hydrolysate. We also observed a low number of albino shoots in SM-3.

Rooting is an important step and determines the success of in vitro propagation method. All the media combinations used for rooting were found to be equivalent in terms of percent root inductions (Fig 5a) as nearly all the inoculated shoots showed the formation of the roots 15 days after inoculation. Naphthalene acetic acid is auxin and involved in various plant biochemical mechanisms such as cell division and formation of adventitious roots. Although some researchers have reported root initiation in sugarcane without the addition of auxin in media [59-60]; these were reported for commercial propagation of sugarcane by meristem cultures where cytokinin (BAP) were added during regeneration and multiplications; sudden absence of cytokinin in media, therefore, might have promoted root formation [61]. Whereas, in our experiment, shoot cultures were grown on a hormone-free medium and negligible root formation was observed within 30 days. Rather, exposure to NAA-containing media showed initiation of rooting from the base of the shoots from nearly all the shoots within 15 days of culturing. The strength of the MS medium was also observed to affect the rooting. RM-4 containing 5 mg L^{-1} of NAA in 50% MS media with 4% sucrose produced optimum root induction. NAA was only auxin chosen for the optimum root induction. Nadar and Heinz [62] had reported it to be preferred auxin for root induction and little higher (4-5%) sucrose than that required for shoot initiation media (2-3%) is required for root initiation. 4% sucrose was reported to be optimum for root initiation for micro propagated plantlets in various sugarcane varieties [63]. Although RM-2 and RM-3 were also found equivalently effective in root length (Fig 5b), RM-4 showed a higher number of longer shoots/tubes (Fig. 5c). The effect of different NAA concentrations on shoot length has also been previously reported by Jamil *et al*. [34] who reported 0.5 mg L^{-1} NAA optimum for root induction and shoot elongation sugarcane CPF-246, CPF-247, CPF-248, and CPF-249 varieties. Our observations are as per a previous report from Tiwari *et al*. [64] who reported 100% rooting on shoots in media containing 5 mg L^{-1} NAA, ½ strength MS and 5% sucrose within 15 days. It was also observed that the rate of survival of the plantlets grown in RM-5 was highest when plants are transferred to the sterile soil: coco-pit: vermin-compost mixture in acclimatization in lab-controlled conditions for a week. The healthy rooting helps the plantlets for surviving and to overcome stress implied due to change in conditions. Implying the RM-5 had proven good rooting medium for the sugarcane variety CoM 0265. When the healthy plantlets were transferred to the plastic bags size 10 cm containing soil mixture for another 45 days under greenhouse conditions and then to the open conditions the plants were survived well in the conditions.

Thus, these optimized media can be used successfully for further in experiment involving shoot regeneration from callus of CoM 0265.

The regenerated plantlets did not show any recognizable variation in morphology except some albino shoots in minor quantity appeared during first week of shoot initiation and did not turned green even after long period of incubations. Further, to ascertain genetic fidelity of the regenerated plantlets; ISSR analysis was performed for the tissue culture plantlets by comparing the pattern of amplification to that with DNA of field plant of the same variety at Vasantdada Sugar Institute obtained by traditional method of sugarcane eye bud sowing. ISSR marker analysis showed high similarity with respect parent and among rest population regenerated through somatic embryogenesis (Table 4). However, tissue culture procedures involving callogenesis are prone for inducing somaclonal variations. Some changes which might not be recognized at phenotypic level but might affect overall growth and vigor of the plant. Assessment of genetic uniformity is required to prevent or reduce the chances of such variation in regenerated population as these may aid in losing the varietal characteristics in the population. This technique of molecular analysis is been applied in studies conducted to screen somaclonal variations produced in tissue cultured plants such as in *Aloe barbandesis* [65], *Chlorophytum borivilianum* [66], *Dendrocalamus strictus* [67]. Although our results are in accordance with earlier publication stating presence of polymorphism in callus culture derived plants, it was observed that percent similarity was higher [12].

CONCLUSION

Media optimization is basic still most important aspect for callus induced regeneration since this regeneration route has most application in modern biotechnological techniques. Based on above observations; somatic embryogenesis was efficiently regenerated for the sugarcane variety CoM 0265. High number of plantlets could be regenerated efficiently from these somatic embryos which consequently successfully hardened in the greenhouse. ISSR markers were observed to help in identifying genetic variation wherein higher similarity was observed among the plants indicating this protocol can be used successfully for the organogenesis of variety CoM 0265 through embryogenic callus. To the best of our knowledge this is first paper which has described complete organogenesis till hardening and further evaluation of regenerated plantlets in terms of molecular markers for the sugarcane var. CoM 0265. The optimized protocol has also been utilized for plantlets generation through embryogenic callus exposed to gamma radiation followed by *In Vitro* NaCl salt selection confirming application in biotechnological interventions.

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