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Optimization of an efficient system for the *In Vitro* Organogenesis of Sugarcane (*Saccharum Officinarum* L.) from apical shoot explant using different plant growth regulators

Naveed Ahmad[1,2], Muhammad Ishaq[3], Ajmal Mandozai[4], Asif Ali Khan[5], Noor al amin[4], Muhammad Inam[6], Raza Ullah[4], Hina Shaukat[2], Muhammad Abrar[7], Ismail Khan[2], Raham Sher Khan[2*]

[1]*College of Life Sciences, Jilin Agricultural University, Changchun 130118, China*

[2]*Department of Biotechnology, Faculty of Chemical and life sciences, Abdul Wali Khan University Mardan (AWKUM), Mardan, 23390, Pakistan*

[3]*College of Food Science and Engineering, Jilin Agricultural University, Changchun 130118, PR China*

[4]*College of Agronomy, Jilin Agricultural University, Changchun 130118, China*

[5]*Engineering Research Centre of Chinese Ministry of Education for Edible Mushroom and Medicinal Fungi, Jilin Agricultural University, Changchun 130118, China*

[6]*Department of Animal Sciences, Shaheed Benazir Bhutto University Sheringal Dir Upper, Pakistan*

[7]*National Centre for Bioinformatics, Quaid-i- Azam University, Islamabad, Pakistan*

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Abstract

An efficient *in vitro* system provides considerable opportunities to regenerable target tissues in plants. The large scale multiplication of newly released sugarcane varieties still remains inexplicit. Here, we investigate the response of three different sugarcane varieties (CP 77400, CP2086, and M 93) towards the onset of callus induction and adventitious organogenesis. Young leaves of apical shoot were subjected to Murashige and Skoog (MS) medium with various combination and concentrations of growth regulators for callus induction, adventitious shoot morphogenesis and rooting. Our results indicated that highest percent callus induction was observed in variety CP77400 which was 80% when explants were cultured on MS medium supplemented with 3.5 mg/l auxin after 14 days of incubation. The maximum adventitious shoot induction was observed in CP 77400 variety incubated with MS medium supplemented with 2.5 mg/l auxin and 1 mg/l cytokinin. Morover, sugarcane variety CP 77400 showed highest rate of *in vitro* root induction when incubated with half MS basal medium supplemented 3 mg/l auxin alone. Regarding the mean number of roots per shoots and the average length of the roots, the variety CP77400 showed the highest number of roots per shoots (8.4) and the highest root length (6.5 cm) on half-strength MS medium supplemented with auxin (3 mg/L). Thus, these findings can be used to promote mass production of different varieties of sugarcane which may overcome the present trend of demand future.

* **Corresponding Author:** Raham Sher Khan ✉ rahamsher@awkum.edu.pk

Introduction

Sugarcane (*Saccharum officinarum* L.) is an important agro-industrial crop which serves as a primary source of sugar across the world (Raza *et al.,* 2010). It is a C4 plant belongs to genus *Saccharum*, which represent not only the major source of world's sugar but possess potential to be a key crop in bio-factory evolution as it generates high yield of important products e.g. paper, biofibers, acetic acid, industrial enzymes, animal feed, renewable source of energy, biofuel and bioplastic (Nonato *et al.,* 2001). Every year product losses in sugarcane production account for up to 70%, due to many biotic and abiotic stresses as bacteria, fungi, viruses (Parmessur *et al.*, 2002) and drought and salinity stresses. Approximately 100 types of diseases caused by different types of pests and insects concerned with sugarcane have been reported worldwide in different countries, but most predominant diseases of sugarcane are fungal pathogen which leads to the appearance of spots on the leaves of sugarcane. However, various other physiological factors which comprise major constrains to the production of sugarcane, including high aneuploidy, low fertility, large genome size and complex environmental interactions. Consequently, lack of suitable propagation system is considered serious threats in sugarcane cultivation procedures (Tiwari *et al.,* 2010). In order to resolve the problems related to the climate changes and the need of a novel varieties, the techniques of plant tissue culture are followed nowadays (Yadav *et al.,* 2012). For many other species, sugarcane may benefit of unconventional breeding mediated by *in vitro* techniques to speed up the genetic improvement (Rugini *et al.*, 2016). The micropropagation and establishment of aseptic tissue culture have gained enormous position throughout last thirty years. Genetic engineering can significantly integrate transgenic traits or somaclonal variation for the introduction of interesting traits as salt and drought tolerance, pest and disease resistance (Ahmed *et al.*, 2019; Rugini *et al.*, 2016; Silvestri *et al.*, 2016). Furthermore, it has been observed that plant tissue culture has overcome many problems related with traditional breeding systems by confirming disease free propagation of sugarcane and reduced the time frame which is a pre requisite for plant proliferation (Khan *et al.*, 2006). The aim of current study was to develop an efficient and reliable protocol for the *in vitro* callus initiation and regeneration of sugarcane (*Saccharum officinarum* L.) using apical shoots as explants.

Material and methods

Plant material

Three varieties (CP77400, CP2086 and M-93) were kindly provided by Sugar Crop Research Institute (SCRI) Mardan and the research was carried out at Institute of Biotechnology and Genetic Engineering (IBGE), the University of Agriculture, Khyber Pakhtunkhwa, Peshawar. Apical regions of sugarcane (*S. officinarum* L.) were used as explants which were 2 to 3 months old (Fig. 1).

Surface sterilization of explants

Approximately 8 cm long roll of apical meristems of sugarcane were thoroughly soaked in 70% (v/v) ethanol for 10 minutes followed by three washing with distilled water. Explants were then treated in mercuric chloride(0.2%) for 5 minutes and washed with double distilled water in order to remove the remaining mercuric chloride. leaf was cut into small sections of 5 mm slices and transferred to callus induction media for callus initiation.

Culture medium and conditions

The sterilized explants were cultured on modified Murashige and Skoog (MS) medium containing 30 g/L sucrose, 0.5 g/L casein hydrolysate and solidified with 8 g/L agar (Technical Agar). Different plant growth regulators (PGRs) were added to callus induction medium (CIM), shoot induction medium (SIM) and root induction medium (RIM) as described in Table1; pH of the media has been adjusted to 5.8 with NaOH 1M before autoclaving at 121 °C for 20 min at1 atm pressure.

Callus induction

Six types of culturing media (CIM1-CIM6) with various concentrations of auxins and cytokinins were

examined for initiation of callus formation in the present study in which CIM1 was kept as negative control (Table 1), and placed in a growth chamber at 27 ± 1°C with a 16-h photoperiod of 40 µmol m^{-2} s^{-1} provided by fluorescent lamps for 3 weeks. At the end of the culture period, the percent of callus formation and the morphology of neo-formed calli have been investigated.

Shoot induction

After 3 weeks of culture in callus induction media, different types of callus with different morphology were obtained and transferred to four different shoot induction media (SIM1-SIM4) consisting in Murashige and Skoog (MS) medium supplemented with different concentration of auxin and cytokinin (Table 1) in order to get *in vitro* adventitious shoot organogenesis. SIM1 has been used as control. The callus culture have been kept in a growth chamber at 27 ± 1°C with a 16-h photoperiod. Regeneration rate, duration of shoot induction and length of adventitious shoot formation have been investigated.

Rooting and acclimatization

Shoots succeeded as 4-5 cm in length were transferred to rooting media. For these purposes rooting media consisting of half-strength MS has been supplemented with four different concentrations of NAA (RIM1-RIM4), where RIM1 was kept as control (Table 1). The callus culture has been kept in a growth chamber at 27 ± 1°C with a 16-h photoperiod. Rooting rate, mean number of roots per explants and root length has been collected and analyzed. Plantlets which were successfully differentiated into shoots and roots were removed from the root induction medium and washing twice in running tap water and then transferred to plastic bags which contain soil. Plastic bags were kept in greenhouse for further growth and hardening. After 20 days transfer of soil the regenerated plantlets were ready to transfer them into field.

Statistical analysis

Experiments were conducted in Randomized Block Design (RBD) and all experiments had 3 replicates and were repeated two times. 10 to 15 explants were evaluated per treatment in each culture.

The data were subjected to the analysis of variance (ANOVA). The means were separated according to Duncan's test (P B 0.05), using R software package (http:// cran.rpoject.org).

Results

Callus induction

Any explants employed leads to the callus formation. However, there are several other factors affecting the formation of callus induction.

Table 1. Different hormonal supplementation on onset of callus induction, shoot induction and root induction on the inner leaf whorls explants of sugarcane varieties including (CP 77400), CP 2086 and M 93).

Media code	Auxins	Cytokinins
	(2,4-D)	(BAP)
CIM1 (CONTROL)	MS + 0 mg/l	0
CIM2	MS + 2.5 mg/l	MS + 1.5 mg/l
CIM3	MS + 3 mg/l	MS + 1 mg/l
CIM4	MS +3.5 mg/l	0
CIM5	MS +4 mg/l	MS + 2.5 mg/l
CIM6	MS +4.5 mg/l	MS + 3 mg/l
	(NAA)	(BAP)
SIM1 (CONTROL)	MS + 0 mg/l	0
SIM2	MS +2 mg/l	MS + 1.5 mg/l
SIM3	MS + 2.5 mg/l	MS +1 mg/l
SIM4	MS + 3 mg/l	MS + 0.5 mg/l
	(NAA)	(BAP)
RIM1 (CONTROL)	½ MS + 0 mg/l	0
RIM2	½ MS + 2 mg/l	0
RIM3	½ MS + 3 mg/l	0
RIM4	½ MS + 3.5 mg/l	0

Table 2. Response of three sugarcane varieties towards each callus induction media.

Sl. No.	Media	Sugarcane varieties			Morphology
		Percentage of callus formation			
		CP 77400	M 93	CP 2086	
1	CIM1	0	0	0	---
2	CIM2	36.18±0.74^{c}	19.7±0.44^{g}	28.09±0.74^{f}	Green/Friable
3	CIM3	56.75±0.40^{ab}	30.50±0.54^{d}	43.76±0.35^{b}	Yellowish/Compact
4	CIM4	80.01±0.55^{a}	42.62±0.33^{b}	61.84±0.30^{a}	Yellowish/Friable
5	CIM5	34.6±0.54^{c}	19.02±0.17^{g}	27.73±0.31^{f}	Light Green/Friable
6	CIM6	11.6±0.14^{i}	9.86±0.31^{i}	10.07±0.50^{i}	Whitish/Compact

During this study the callus formation was induced with all concentrations of 2,4-D, however, the response of CP77400 resulted higher than the others experiments when the explants were inoculated in CIM6 medium using inner leaf whorls as explants. Prominent and yellowish/friable callus was recorded with 2,4-D at 3.5 mg/L among all three varieties of sugarcane supplemented with CIM6 medium as shown in Fig. 2. But, the percentage of callus formation of CP 77400 on CIM6 medium reached the 80% 14 days after the start of the experiment (Table 2, Fig.3).

Table 3. Length of *in vitro* shoots (cm) observed in three varieties of sugarcane when induced to different concentration of growth hormones in MS basal Medium.

Sl. No.	Media	Sugarcane varieties		
		Length of shootlets (cm)		
		CP 77400	M 93	CP 2086
1	SIM1	0	0	0
2	SIM2	4.1±0.14^{cd}	1.3±0.44^{f}	1±0.17^{f}
3	SIM3	7.4±0.51^{a}	2.6±0.37^{ef}	1.4±0.14^{f}
4	SIM4	6.2±0.32^{b}	3.8±0.49^{d}	2.1±0.44^{ef}

Table 4. Time period of shoot formation in different sugarcane varieties when induced to different concentrations of growth hormones + MS basal medium.

Sl. No.	Media	Sugarcane varieties		
		Days taken to shoot sprouting		
		CP 77400	M 93	CP 2086
1	SIM1	0	0	0
2	SIM2	17±1.5^{ef}	20±2.0^{h}	19±1.5^{g}
3	SIM3	11±1.0 ^{a}	16±0.5^{c}	18±1.7^{e}
4	SIM4	15±0.5^{c}	17±1.5^{cd}	18±2.0^{e}

In vitro shoot induction

After 10-15 days of incubation, adventitious shoots appeared inside the callus which was further elongated to normal leaves and stem with repeated subculture. The Calli produced from CP 77400 variety of sugarcane showed best response to the adventitious shoot organogenesis in medium SIM3, consisting of NAA at 2.5 mg/L NAA and BAP 1mg/L (Table 3 and 4, Fig. 4).

Table 5. Number of roots obtained per shoot when subjected to various concentrations of growth hormones.

Sl. No.	Media	Sugarcane varieties		
		Number of roots/plantlet		
		CP 77400	M 93	CP 2086
1	RIM1	0	0	0
2	RIM2	4 ± 2.0^{d}	2 ± 1.53^{e}	1.6 ± 1.3^{g}
3	RIM3	8.4 ± 1.75^{a}	5.2 ± 1.8^{c}	2.3 ± 0.75^{e}
4	RIM4	3.9 ± 0.57^{cd}	2.2 ± 1.59^{e}	1.4 ± 0.55^{g}

Table 6. Various lengths of roots conceded by sugarcane varieties when induced on different concentrations of growth hormones in MS basal Medium.

Sl. No.	Media	Sugarcane varieties		
		Length of roots (cm)		
		CP 77400	M 93	CP 2086
1	RIM1	0	0	0
2	RIM2	2.2 ± 0.35^{e}	1.5 ± 0.17^{fg}	0.9 ± 0.35^{i}
3	RIM3	6.5 ± 0.18^{ab}	3.4 ± 0.72^{d}	2 ± 0.75^{ef}
4	RIM4	3.4 ± 0.75^{d}	1.8 ± 0.15^{f}	1.6 ± 0.35^{fg}

Rooting and acclimatization

Surprisingly it has been observed that there are no specific growth hormones required for *in vitro* root of sugarcane, since the regenerated shoots started to root on the hormone free half-strength MS medium. However, in this study the best rooting ability of the varieties CP77400, CP2086 and M93) showed different root traits depending on the root induction medium (RIM) used. CP7740 showed highest root number (8.4) and highest root length (6.5 cm) on half-strength MS medium supplemented with NAA 3 mg/L. Sugarcane variety CP77400 showed highest rate of *in vitro* rooting, in contrast to CP2086 and M93. Plantlets have been transferred to the acclimatization phase and successfully transplanted to soil with an average of acclimatization up to 85% (Table 5 and 6, Fig. 5).

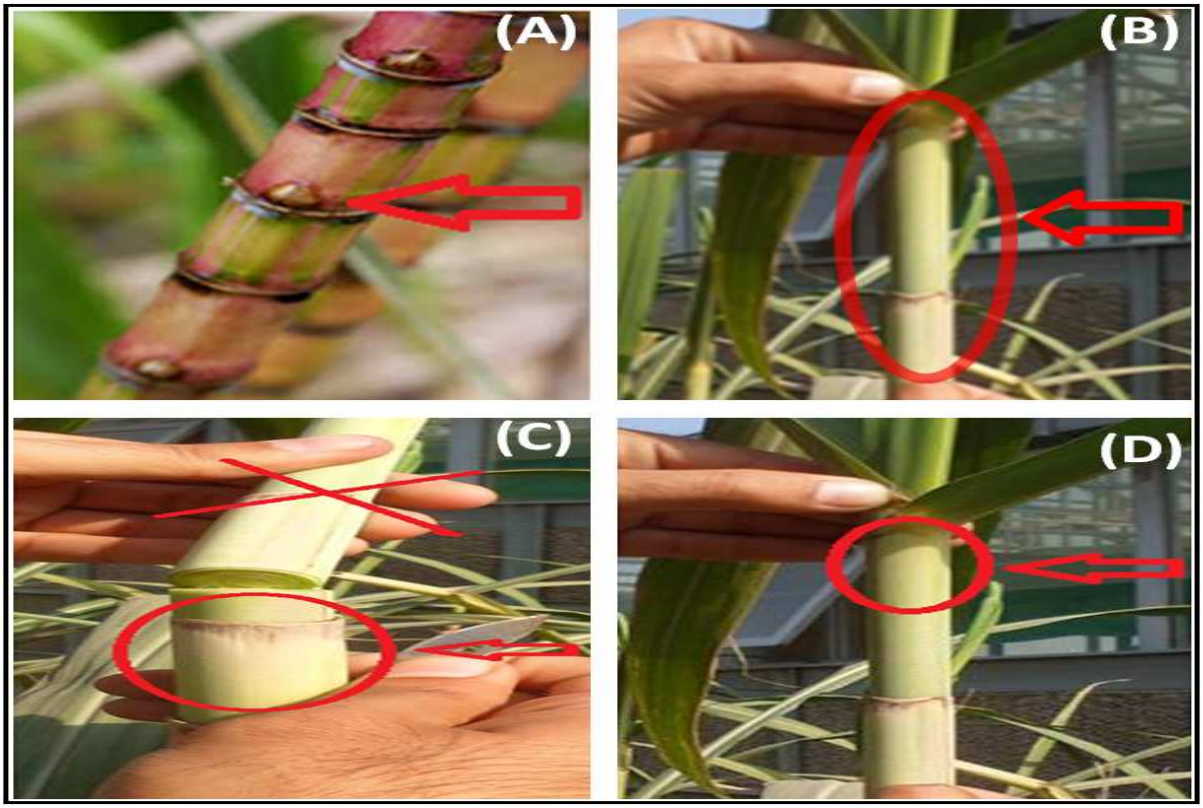

Fig. 1. The selection of sugarcane explants. (A) Eye bud (B) Inner leaf whorls (C) Inner leaf whorls (D) Upper leaf whorls.

Discussion

During our investigations, the response of callus formation of CP77400 was induced at a higher level than the other sugarcane varieties using inner leaf whorls as explants. Prominent and yellowish/friable callus was recorded with 2,4-D at 3.5 mg/L among all three varieties of sugarcane supplemented with CIM6 medium as shown in Fig. 2. But, the percentage of callus formation of CP 77400 on CIM6 medium reached to 80% in 14 days after the start of the experiment (Table 2, Fig.3).

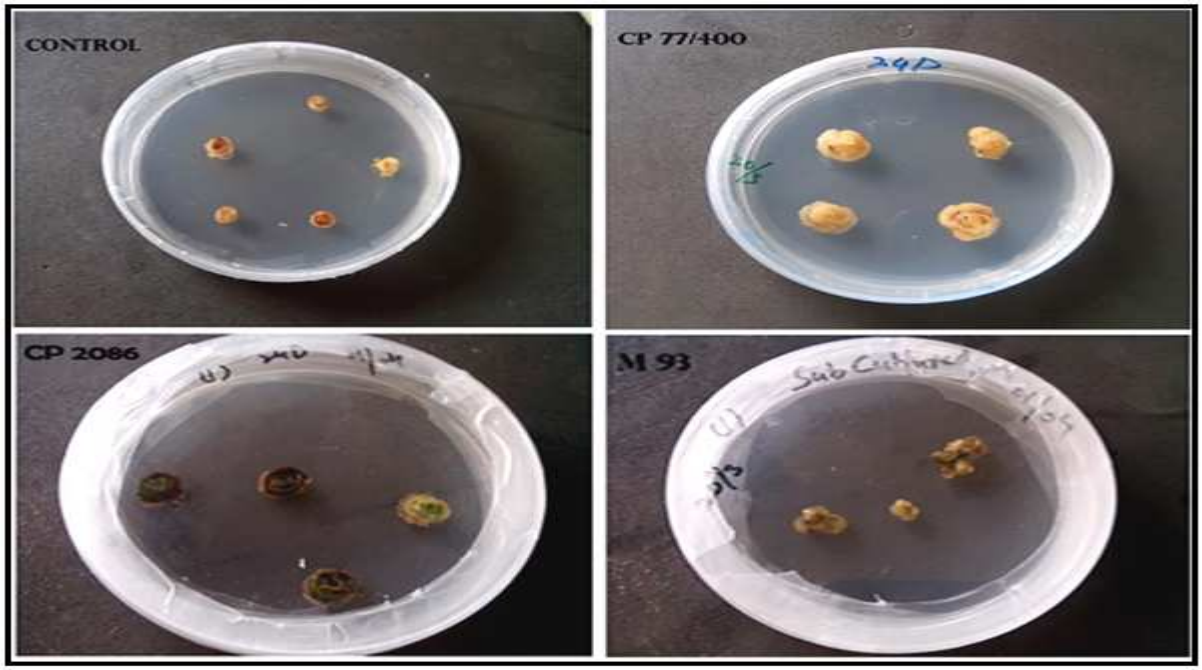

Fig. 2. (a) Response of all three varieties on negative control (media without hormones) media. (b) Prominent callus induction of CP 77400 on optimized media with 3.5mg/l 2, 4-D. (c) CP 2086 response on selected media. (d) M 93 response on selected media.

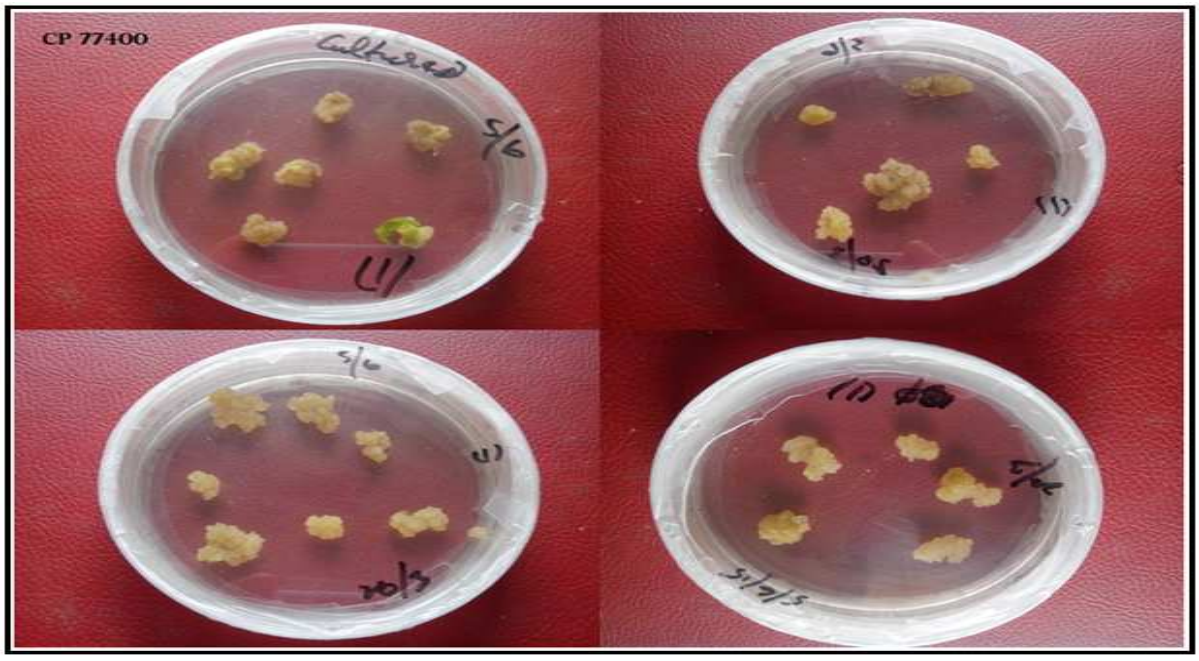

Fig. 3. The formation of yellow friable callus of CP 77400 sugarcane variety on Callus induction medium (CIM4) medium supplemented with MS +3.5 mg/l auxin (2,4-D).

Sugarcane varieties are asexually propagated by stem cutting. Serious threats are the lake of appropriate multiplication procedures as sugarcane is characterized by high heterozigosity (Nand and Singh, 1994). Explants from the inner young leaves of sugarcane were found good for induction of callus through tissue culture (Khatri *et al.*, 2002). Our results also coincides with the findings of (Brisible *et al.*, 1994) who proved that 2, 4-D is a pre-requisite in sugarcane for callus induction and even embryogenesis.

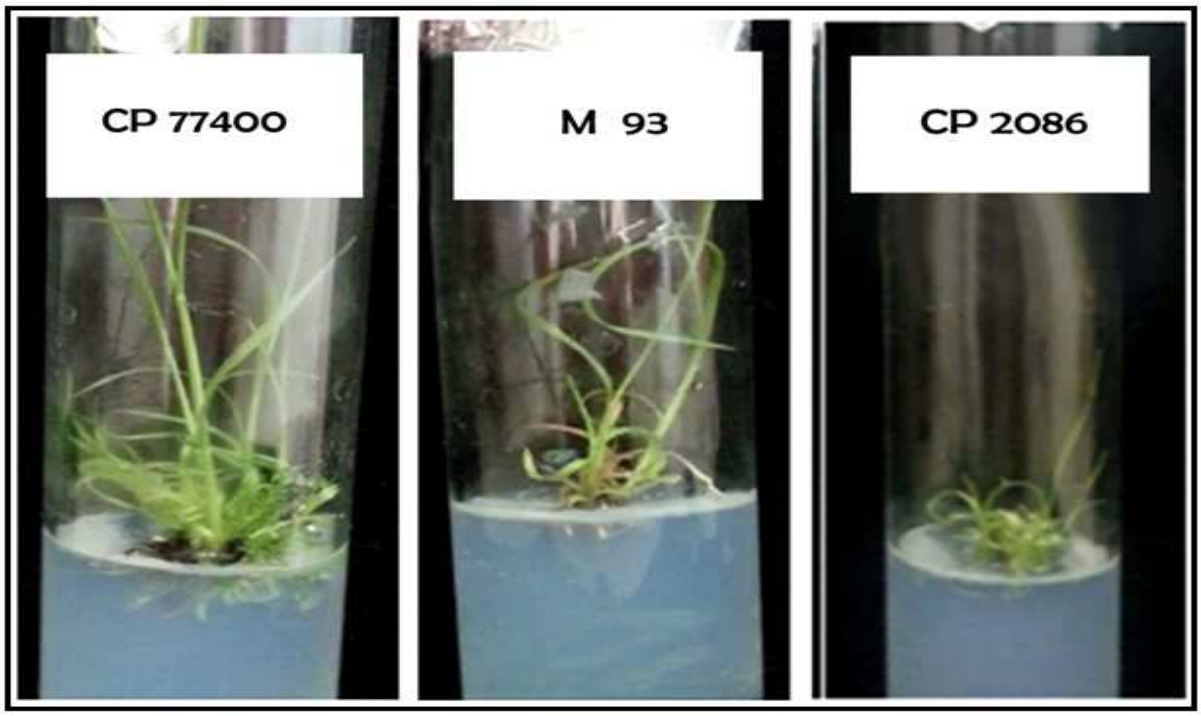

Fig. 4. Response of shoot induction in all three varieties of sugarcane on Shoot induction medium 3 (SIM3) (A) Prominent shoot induction of CP 77400 on SIM3 medium supplemented with 2.5 mg/l NAA and 1 mg/l BAP. (B) Shoot induction of CP 2086 on SIM3 medium supplemented with 2.5 mg/l NAA and 1 mg/l BAP (C) M 93 response to shoot induction on SIM3 medium supplemented with 2.5 mg/l NAA and 1 mg/l BAP.

In addition, a variety of sugarcane named as Nagabari has been reported for highest callus production while using 3 mg/l concentration of 2, 4-D with the MS basal media and did not show any result at all in 0.5-1.0 mg/l (Begum *et al.*, 1995). Similarly, using 0.5-5.0 mg/l of 2,4 –D resulted in various types of callus formation with MS basal media (Islam *et al.*, 1982).

In case of adventitious shoots regeneration inside the callus biomass which was further grown into normal leaves and stem with repeated subculture. We confirmed that the Calli formed by sugarcane variety CP 77400 showed efficient adventitious shoot organogenesis when incubated on shoot induction medium 3 consisting of NAA at 2.5 mg/L NAA and BAP 1mg/L (Table 3 and 4, Fig. 4). These results confirm previous findings of shoot proliferation in sugarcane varieties (CP 77400, CPF-237 and HSF-240) which was highly influenced by the effect of cytokinins, it was observed that highest number of shooting were examined at 1.5 mg/l BAP, 0.5 mg/l Kin (Khan *et al.*, 2009). Cytokinins influenced the development of SAM (shoot apical meristem) which is regulated by the gene expression. SAM inititiation is controlled by homeobox gene family (kn1). These are only expressed in the presence of cytokinin thus they help in the maintenance and development of SAM (Kerstetter, 1997). Geetha and Padmanadhan, (2001) suggested that different concentration of auxins used in the culture media for callogenesis showed variable regeneration capacity and proliferation. Tarique *et al.*, (2010) conducted their work on the effect of BAP

(cytokinin) with NAA or IBA (auxins) and observed that 1 mg/l (BAP) plus 0.5 mg/l (NAA) gave maximum percentage for initiation and proliferation of shootlet among varieties Isd-16, Isd- 36 and Isd-37. In this study the best rooting ability was found in CP7740 which showed highest root number (8.4) and highest root length (6.5 cm) on half-strength MS medium supplemented with NAA 3 mg/L in contrast to CP2086 and M93 varieties (Table 5 and 6, Fig. 5). These results also agree with the work of (Gosal *et al.*, 1998) they used 70 g/l sucrose and 5 mg/l (NAA) with liquid MS basal medium for generation of in vitro shoot which showed multiple roots of various lengths. Poor quality and less tendency of the in vitro root formation was found when ½MS medium was used with 1 - 0.5 mg/l IBA in combination with 0.5 - 2.0 mg/l BAP (Baksha *et al.*, 2002).

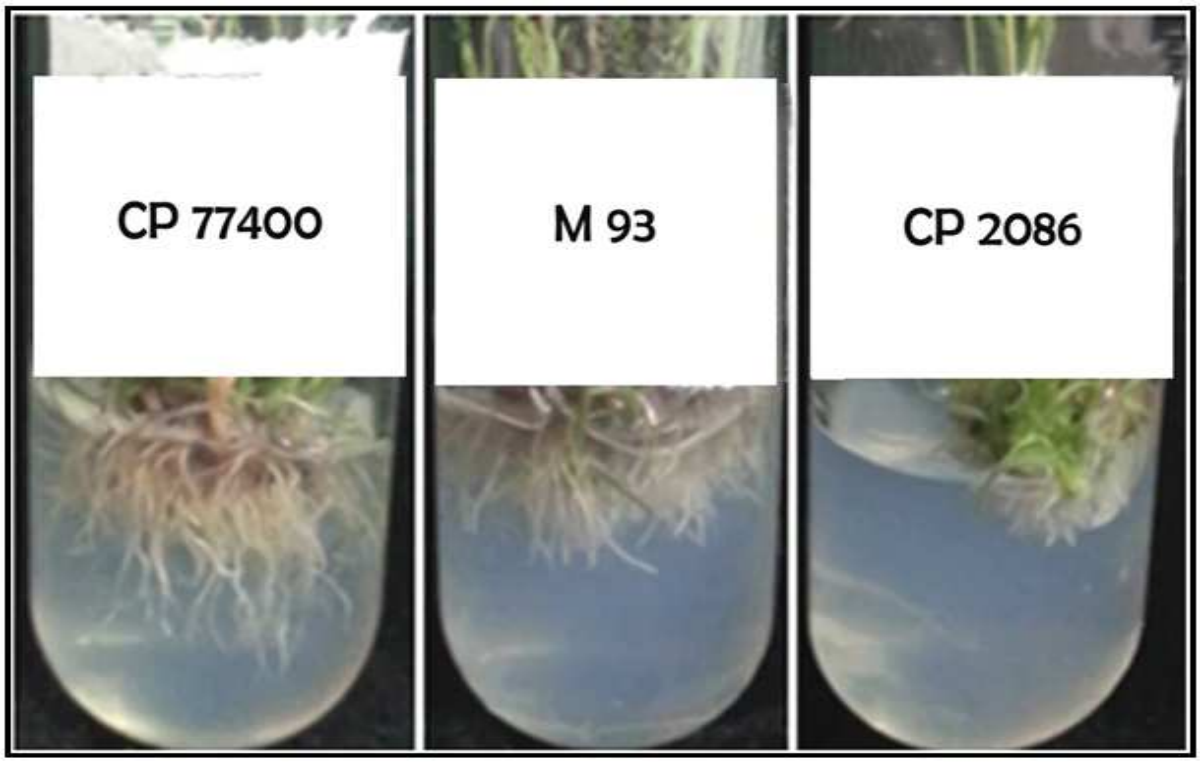

Fig. 5. Response of root induction in all three varieties of sugarcane on root induction medium 3 (RIM3) (A) Prominent root induction of CP 77400 on RIM3 medium supplemented with ½ MS + 3 mg/l (B) Root induction of CP 2086 on RIM3 medium supplemented with ½ MS + 3 mg/l (C) M 93 response to root induction on RIM3 medium supplemented with ½ MS + 3 mg/l.

It was investigated that response of root formation is directly influenced by increasing or decreasing the concentration of Naphthalene Acetic Acid (NAA) the rate of root initiation and number of roots per plantlet was decreased with minimizing (NAA) concentration (Ali *et al.*, 2008). Root induction was found on ½ strength MS media in combination with 6% sucrose supplemented with various concentrations of NAA and IBA (Khan *et al.*, 2008). Lal and Sing, (1994) observed adventitious roots formation in the regenerated plantlets by inducing them into the ½MS medium with or without NAA.

Conclusion

During the present work our results suggested that CP 77400 variety of sugarcane respond successfully to callus induction and regeneration media compared to other varieties (CP 2086 and M 93).

The success rate of CP 77400 towards callus induction media, shoot induction media and root induction was observed highest among all varieties. Our findings also demonstrated that different varieties of sugarcane posses different callus initiation and regeneration potential. Explants and their position can also affect the rate of callus induction and regeneration properties. It was observed that deviation from optimum concentrations of growth hormones minimize callus formation and regeneration capabilities of sugarcane.

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