Optimization of Best Responsive Explants for Disease Free Plantlet Development of Sugarcane Variety Coc 671

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\textbf{Abbreviations:}  
\%: Percentage, °C: Degree centigrade, 2,4-D: 2, 4 Dichlorophenoxy acetic acid, BAP: 6-Benzyl amino purine, CIM: Callus induction media, HCl: Hydrochloric acid, HgCl\textsubscript{2}: Mercaric chloride, hr : Hour, IBA: Indole-butyric acid, mg/l: Milligram per litre, ml : Mills litre, MS: Murashige and Skoog medium, NAA: α-Naphthalene acetic acid, NaOH: Sodium hydroxide, GA\textsubscript{3}: Gibberellic acid, pH: Negative logarithm of hydrogen ion concentration

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\textbf{Abstract}  
Sugarcane CoC 671 faced with poor stem propagation and poor tolerant to pest and diseases. This study was conducted to determine an efficient procedure for callus induction and regeneration from four different explants. Explants of 2-2.5 cm size were taken from direct leaf whorl, tissue culture leaf whorl, shoot tip and shoot base and cultured in different concentrations of 2,4-D for callus development. The callus obtained was further grown on MS + IBA (0.5 mg/l) + BAP (1.0 mg/l) and MS + NAA (0.5 mg/l) + BAP (1.0 mg/l) and resultant plantlets were multiplied in media MS + 1 mg/l BAP + 0.25 mg/l GA\textsubscript{3}. Optimal callus growth were obtained from direct leaf whorl explants in 3-4 mg concentration of 2,4-D that accumulated great amounts of biomass in 15 days under dark incubation. Callus proliferation occurred more in IBA + BAP than in NAA + BAP media. Multiplication of the plantlet was rapid in formulated media (MS + 1 mg/l BAP + 0.25 mg/l GA\textsubscript{3}) and good rooting of the shoot was achieved in 2 mg/l NAA. The study concluded that direct leaf whorl is a promising source of explant for sugarcane tissue culture using 3-4 mg/l of 2,4-D that accumulated great amounts of biomass in 15 days under dark incubation. Callus proliferation occurred more in IBA + BAP than in NAA + BAP media. Multiplication of the plantlet was rapid in formulated media (MS + 1 mg/l BAP + 0.25 mg/l GA\textsubscript{3}) and good rooting of the shoot was achieved in 2 mg/l NAA. The study concluded that direct leaf whorl is a promising source of explant for sugarcane tissue culture using 3-4 mg/l of 2,4-D in MS + IBA (0.5 mg/l) + BAP (1.0 mg/l) media.

\textbf{Citation:}  

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\textbf{1. Introduction}  

\textbf{1.1 World scenario}  
Sugarcane is main source of white crystal sugar and it contributes nearly 80% of the total sugar pool in the world. The leading sugarcane growing countries are Brazil, India, Cuba, USA, Philippines and China. In India sugarcane is cultivated over an area of 4.88 million ha, with an annual production of 342.4 million tonnes and productivity of 70.1 tonnes per hectare (Anonymous, 2012).

Although sugar is the most important product of sugarcane, various by-products utilization as industrial raw material for biofuel, citric acid production, paper pulp animal feeds among others has been reported (Wheals et al., 1999).

\textbf{1.2 Tissue Culture in Sugarcane.}  
Contributions of tissue culture technology to plant propagation are especially notable and today, this technology has emerged as a viable alternative to the conventional vegetative propagation with definite and indispensable advantages. The method of producing large numbers of identical clones by in-vitro culture is being routinely used for horticultural, ornamentals and woody species (Vasil and Vasil, 1980).
Common commercial sugarcane is a sexually sterile autopolyploid and it is propagated through stem cuttings consisting of 2-3 nodes, thus required a large number of stems for a plantation. Conventional propagation by stem is slow, non genetically uniform and produce crops that are less tolerance to biotic and abiotic stress with consequently reduced yield (Gill et al., 2004). Initial attempts to regenerate sugarcane through In vitro technique were made by Nickell (1964) and Heinz & Mee (1969). Micropropagation through tissue culture holds immense potential for mass multiplication and subsequent rejuvenation and quality production.

Although, Taylor et al. (1992) observed four types of callus tissues in sugarcane micropropagation, only embryogenic and organogenic calluses were capable of producing plants and suggested that for establishment and maintenance of embryo culture, lower levels (0.5-1.5 mg/l) 2,4-D are essential for long term cultures. Tarique et al., (2010) determined the optimal concentrations of BAP in combination with NAA or IBA for shoot initiation and multiplication of the sugarcane varieties Isd-16, Isd- 36 and Isd-37. The morphogenic capacity of the tissues after few months in cultures declines rapidly. The loss of morphogenic potential has been restricted with effective utilization of tissue culture techniques for genetic improvement. Nonetheless, plants can still be obtained from callus cultures maintained for as long as two years (Dobariya, 1994).

Induction of callus and regeneration of plants using sugar-cane varieties from India were reported (Islam et al., 1996; Reetu et al., 2012). However, reports are scanty on shoot tip and leaf whorl culture in sugarcane varieties cultivated in Gujarat; an area with highest sugarcane plantations and sugar factories in India. The present study seek to regeneration and develop plantlets from sugarcane variety CoC 671; a high yielding variety but with poor conventional vegetative propagation and poor resistance to biotic and abiotic stress. In addition, the study includes optimization of 2, 4-D concentration and different combinations of IBA, BAP and NAA for callus induction and subsequent plantlet establishment for variety CoC 671.

1.3 Objectives of Research
To identify best responsive explants for callus culture in variety CoC 671.
Optimization of 2, 4-D concentration for callus induction in variety CoC 671.
Optimization of different combination of IBA, BAP and NAA for callus regeneration.
Establishment of sugarcane callus culture for further shoots and roots development.

2. Materials and Methods
Sugarcane CoC 671 faced with poor stem propagation and poor tolerant to pest and diseases. This variety contain higher amount of sugar comparing to other sugarcane variety. Therefore this study was conducted to determine an efficient procedure for callus induction and regeneration from four different explants.

The present study on “Regeneration and plantlet development from somatic tissue in sugarcane (S. officinarum) variety CoC 671 was carried at the Sugarcane Tissue Culture Laboratory, Main Sugarcane Research Station, Navsari Agricultural University (NAU), Navsari, Gujarat, India. All tissue culture work was performed under aseptic condition and standard practice. The cultures were maintained at a constant temperature 23±1 °C and exposed to a 12 hours photoperiod regime of 88 µ mol m² light intensity.

2.1 Plant Material

Figure 1: Mother plant part used as explants
Plate 1: Different explants sources of Sugarcane variety CoC 671 used for the study

- Shoot base
- Direct leaf (D.L.) whorls
- Shoot tips
- Tissue culture (T.C.) leaf whorls
The sugarcane cultivar CoC 671 grown in Gujarat was used for this study. The explants were obtained from Main Sugarcane Research Station, Navsari Agricultural University, Navsari. Healthy and disease free direct leaf whorl, shoot tip and shoot base of sugarcane were used as explants (Fig 1 and Plate 1).

2.2 Laboratory Ware
All glass and plastic wares were of high quality from Genaxy, Tarsons Ltd, Axygen and Eppendorf (USA). Glasswares were first soaked in chromic acid solution, cleansed with Labolene, rinsed with distilled water and oven dried for overnight at 120°C before used. While plasticwares (non sterile) were thoroughly cleansed with Labolene, rinsed with distilled water and autoclaved before used.

2.3 Chemicals
Chemicals used were of analytical/molecular grade and were obtained from Sisco Research Lab. (SRL), India; Sigma-Aldrich, Germany; Hi-Media, Mumbai, India; E-Merck, Germany; Qualigens, Bangalore, India and QIAGEN, Netherland.

2.4 Sterilization of Glasswares
Sterilization of glasswares was carried out as described by Patel, 2007 and Sathyanarayana and Varghese (2007) with modifications. Cleaned glasswares were washed in Chromic acid, rinsed in running tap water and detergent (Labonin a product of Qualigen Fine Chemicals Limited). The glasswares were finally washed with double glass distilled water and oven dried at 150°C for 30 min.

2.5 Preparation of Media
The culture medium used in this study was MS medium (Murashige and Skoog, 1962). Composition and preparation of the MS media and other stock solutions for the study were as described by Patel, 2007. The basal media was prepared form the stock solution and the pH of the media was adjusted to 5.7 ± 0.1 using 0.1 N NaOH or 0.1 N HCl.

2.6 Growth regulators
Benzy1 Amino Purine (BAP) 20 mg of BAP powder was dissolved in 2-3 ml of 1N NaOH and was made up to 100 ml with distilled water. Naphthalene Acetic Acid (NAA) and Gibberellic Acid (GA3) were prepared by dissolving 20 mg of each in 2-3 ml of 1N NaOH and made up to 100 ml with distilled water.

2.7 Explant Sterilization
The explants were sterilized as described by Verma et al., 2011 and Oyebanji et al, 2009. Explants were sterilized in laminar airflow chamber with 80% ethanol for 45 sec and in 0.1% mercuric chloride for 30 sec and finally washed 3 times with double distilled water.

2.8 Callus induction and shoot establishment
The sterilized explants were inoculated in sterile glass bottle in MS medium supplemented with different concentration of 2,4-D (1 mg to 5 mg). The cultures were incubated in dark for callus induction at 23±1°C temperature for 15 days. Two successive sub-cultures were performed at interval of 10 days. During every sub-culturing, developed portion of the callus was transferred to fresh media. In 10-12 days the resultant callus were transferred into proliferation media for shoot initiation. The proliferated shoots were multiplied in multiplication media and the shoots obtained grown on rooting medium. The plantlets were allowed to grow in bottles to a height of about 8-10 cm and shoots with 3-4 roots were prepared for hardening.

3. Results
The contamination and survival percentage of explants from different meristem cells are shown in Table 1. High level of contamination was observed in shoot base and shoot tip cultures with 36 and 23% contamination respectively. Moderate contamination of 18% was obtained in tissue culture leaf and low contamination from direct leaf whorl cultures (8%) which also had highest callus survival of 70%. Tissue cultured leaf and shoot tip recorded low survival rate of 30% each while 50% callus from shoot base survived (Table 1).

Brown coloration was observed in the medium at the base of the explants after 12-14 days. Callus initiation started within 21 days in dark condition and 30 days in light condition.

![Table 1: Survival of different explants callus culture in variety CoC 671](image-url)
The per cent establishment varied with different concentrations of 2,4-D as shown in Table 2. The explants turned pale in colour with 2-4 mg/l concentration of 2,4-D (Plate-2b-d).

Callus initiation started from the cut edges or injured sites and gradually occurs completely over the explants. A non-compact, mucilaginous whitish callus was first to develop. Subsequently a compact creamish white, morphogenic callus developed over the entire explants within 30-35 days (Plate-3a-e).

**Plate 2:** Callus initiation on different concentrations of 2,4-D media using different explant sources from sugarcane CoC 671

![Callus initiation on different concentrations of 2,4-D media using different explant sources from sugarcane CoC 671](image-url)
Plate 3: Callus development of sugarcane CoC 671 explants on different concentrations of 2,4-D media. 3 and 4 mg/l of 2,4-D showed 70-75% and 75% callus initiation response respectively, whereas 2 mg/l recorded 50-55%. Other callus initiation characteristics are summarized in Table-2.

After 4 weeks, it was observed that initial mass of cells appears to increase with each sub culturing of 2-4 times same media composition. Callus that was cut into smaller units regenerated faster than whole callus and optimal multiplication was observed in MS + IBA (0.5 mg/l) + BAP (1.0 mg/l). It was observed that media combination MS + BAP(1.0 mg/l) + IBA(0.5 mg/l) showed better proliferation rate than MS + BAP (1.0 mg/l) + NAA (0.5 mg/l).
Table 2: Callus induction in different 2, 4-D concentration

<table>
<thead>
<tr>
<th>Media</th>
<th>Callus Details</th>
<th>Callus Initiation</th>
<th>Callus size</th>
<th>Callus colour</th>
<th>Callus type</th>
<th>Callus initiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>Conc (mg/l)</td>
<td>Dark (Days)</td>
<td>Light (Days)</td>
<td>S</td>
<td>M</td>
<td>L</td>
</tr>
<tr>
<td>1</td>
<td>25 30</td>
<td>M 30</td>
<td>20 40 60 50</td>
<td>60 70 50</td>
<td>50 60 50</td>
<td>50 60 50</td>
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<tr>
<td>1</td>
<td>25 30</td>
<td>M 30</td>
<td>20 40 60 50</td>
<td>60 70 50</td>
<td>50 60 50</td>
<td>50 60 50</td>
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<tr>
<td>2</td>
<td>21 30</td>
<td>M 70</td>
<td>30 70 30 75</td>
<td>70 30 75</td>
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<td>3</td>
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<tr>
<td>5</td>
<td>25 30</td>
<td>M 10</td>
<td>30 60 10 70</td>
<td>30 70 50</td>
<td>50 30 70</td>
<td>50 30 70</td>
</tr>
</tbody>
</table>

Key: S = Small callus = 2-4 cm, M = Medium callus = 4-5 cm, L = Large callus = above 5 cm.

Figure 2: Effect of cut on callus regeneration response in media

![Percentage callus growth](image)

Media combinations

composed media after 14 days of culture period (Fig 2).

In both the cases of media combinations, the shoots formed showed moderate growth in height but numbers of shoots per bottle were higher in MS + IBA (0.5 mg/l) + BAP (1.0 mg/l) comparing MS + NAA (0.5 mg/l) + BAP (1.0 mg/l) as shown in Plate 5 & 4 respectively.

The response of callus grown on 3 mg/l 2,4-D and regenerated in shoot initiation media containing 1 mg/l BAP + 0.5 mg/l NAA and 1 mg/l BAP + 0.5 mg/l IBA is presented in Table 4.

Proliferation and multiplication response was successful for all the explants (Plates 6-9). Adequate rooting was achieved with media combinations of half MS + 2 mg/l NAA + 40 gm/L sugar and 8 gm/L agar maintained at optimum conditions of pH of 5.7 and 23°C ± 2°C for Direct Leaf Whorl culture (Plate 10)

Discussion

The present study attempt regeneration and develop of plantlets from different meristem parts of CoC
Plate 4a: Response of callus (grown on 3 mg/l 2, 4-D) regeneration in shoot initiation media containing 1 mg/l BAP + 0.5 mg/l NAA

Plate 4b: Response of callus (grown on 3 mg/l 2,4-D) regeneration in shoot initiation media containing 1 mg/l BAP + 0.5 mg/l IBA

Plate 5a: Response of callus (grown on 4 mg/l 2,4-D) regeneration in shoot initiation media containing 1 mg/l BAP and 0.5 mg/l NAA
Plate 5b: Response of callus (grown on 4 mg/l 2,4-D) regeneration in shoot initiation media containing 1 mg/l BAP and 0.5 mg/l IBA

Plate 6: Shoot regeneration and multiplication in 1 mg/l BAP and 0.25 mg/l GA₃.

Plate 7: Shoot regeneration and multiplication in 1 mg/l BAP and 0.25 mg/l GA₃.

Plate 8: Shoot regeneration and multiplication in 1 mg/l BAP and 0.25 mg/l GA₃.

Plate 9: Shoot regeneration and multiplication in 1 mg/l BAP and 0.25 mg/l GA₃.
Plate 10: Root initiation in sugarcane variety CoC 671 developed from Direct Leaf Whorl (D.L.W)

671 sugarcane variety. High incidence of contamination was observed in some cultures which were probably due to contaminants from explants that were not removed by surface sterilization, contaminants introduced by handling process or contaminants introduced to media during preparation. A similar opinion was expressed by Arafa et al. (1999) who opined that surface sterilization method and chemicals determines the degree of contamination incidence in tissue culture works. In corroboration of this, Hussein (2002) reported that surface sterilization with HgCl2 followed by Chlorex resulted in highest decontamination and survival percentage of Aglaonema spp.

The brown coloration observed at the base of explants in the media was due to the release of phenolics in the medium. This could slightly hinder the callus or shoot growth, therefore sub-culturing was imperative to reduce the adverse effect of browning of tissues and the release of pigments in the medium.

Table 3: Response of callus (grown on 3 mg/l 2,4-D) and regenerated on two different shoot initiation media.

<table>
<thead>
<tr>
<th>Set number</th>
<th>Shoots regeneration</th>
<th>Shoot initiation (Day)</th>
<th>% Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regenerated</td>
<td>Non-regenerated</td>
<td>Dark</td>
</tr>
<tr>
<td>Callus grown on NAA (0.5 mg/l) + BAP (1.0 mg/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Callus grown on IBA (0.5 mg/l) + BAP (1.0 mg/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>2</td>
<td>8</td>
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<tr>
<td>3</td>
<td>10</td>
<td>5</td>
<td>8</td>
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</table>

Table 4: Response of callus (grown on 4 mg/l 2, 4-D) and regenerated on two different shoot initiation media.

<table>
<thead>
<tr>
<th>Set number</th>
<th>Shoots regeneration</th>
<th>Shoot initiation (Day)</th>
<th>% Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regenerated</td>
<td>Non-regenerated</td>
<td>Dark</td>
</tr>
<tr>
<td>Callus grown on NAA (0.5 mg/l) + BAP (1.0 mg/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Callus grown on IBA (0.5 mg/l) + BAP (1.0 mg/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>3</td>
<td>7</td>
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<tr>
<td>3</td>
<td>10</td>
<td>5</td>
<td>7</td>
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</table>

Dark condition promotes callus initiation since callus growth was observed within 21 days in dark condition and 30 days in light condition. Ho and Vasil (1983), also reported that morphogenic callus was obtained from leaf explants incubated in the dark. The result showed that direct leaf whorl culture was the most regenerative among the explants sources studied. Chen et al. (1988) had earlier reported callus growth in leaf explant from the innermost whorl(s) cultured on MS media and supplemented with 0.5-1.5 mg/l 2, 4-D at 27° C in dark.

The callus subcultures on MS supplemented with BAP (1.0 mg/l) and IBA (0.5 mg/l) supported rapid callus growth than MS+ BAP (1.0 mg/l) + NAA (0.5 mg/l). This showed that composition of MS+ BAP (1.0 mg/l) + NAA (0.5 mg/l) favoured callus proliferations. A similar result was obtained by Ali et al. (2008). Callus that was sub-cultured in whole
without cuts grew much slowly compared to callus that were cut into smaller piece. The present finding concurred with the report of Siddiqui (1993) who had shown that size of the meristem shoot and callus plays a role in proliferation. Optimal shoot initiation and regeneration was achieved with the combinations of NAA (0.5 mg/l) + BAP (1.0 mg/l) compared with IBA (0.5 mg/l) + BAP (1.0 mg/l) and effective multiplication was achieved with MS basal 1 mg/l BAP + 0.25 mg/l GA\textsubscript{3} + 20 g/l sucrose + 7 g/l agar which is in agreement with Nadgauda (2002) who reported high concentrations (5.0 mg/l) of NAA or combination of NAA + IBA for optimum shoot formation in sugarcane. The use of kinetin combined with IBA for shoot formation in sugarcane has been reported (Geeta and Padmanabhan 2001; Dhillon, 2002)

Furthermore, the best cultured of shoot initiation was obtained from direct leaf whorl explant as substantiated by Chen et al, (1988), that the combinations of full strength media, supplemented with 0.5 mg/l of NAA and 0.5 mg/l of IBA promotes rooting. Although Lal and Singh (1999) reported that 1.0 mg/l produced best response in sugarcane, in contrast, low concentration of auxin has been reported as the best for rooting in sugarcane (Cooke, 2002).

Higher concentrations of auxin may inhibit root elongation as a result of possible deposit of ethylene since auxin would stimulate cells to produce ethylene Yi et al. (2004). Therefore presence of synthetic auxin hormone in high concentration in the media would enhance ethylene formation which would retard root development. The present study achieved good rooting response in media combinations of half MS + 2.0 mg/l NAA + 40 gm/L sugar and 8 gm/L and also reported the combined use of BAP and GA\textsubscript{3} for shoots elongation in a very short time.

**Conclusion**

Though there are several problems associated with sugarcane cultivation in India, identifying suitable variety in different agro geographical region is a panacea to sugarcane cultivation. In this study, CoC 671 variety with potential for high cane and sugar yield as well as early maturation was regenerated by culturing of explants from different meristem of the plants to determine which meristem region optimize plant regeneration that could be utilized to make available disease free planting materials with improved agronomic characters for the ultimate benefit of the farmers.

The study concluded that regeneration of sugarcane is not only feasible but could be employed as tool for micropropagation of the crop as a viable alternative to conventional use of several stem in plantation. The direct leaf whorl explant could be used in mass regeneration of sugarcane CoC 671 variety by tissue culture.

Growth regulators used in tissue culture of sugarcane variety CoC 671 for induction of callus, somehow genetic mutation in callus.

Browning of media in cultured bottle was also observed. Due to this, a chance of contamination in cultured bottle was increased.

No such recommendation was applied to avoid genetic mutation caused due to plant growth regulators. Because rate of genetic mutation was not so high or it is feasible.

Contamination due to media browning was avoided by keeping all the cultured bottles in dark condition. The size of explants was also reduced to diminish chances of contamination.

All the laboratory work and research design were performed by Naik, K. H. and Patel, V. S. Guideline for the research work were provided by Patel, D. U. and Mehta, R. D. Guideline for paper formatting and compiling was provided by Animasaun, D. A. We express our sincere thanks to Agriculture University of Navsari, Sugarcane Department, Navsari, Gujarat, India, for providing me tissue culture laboratory, and Dr. S. C. Mali. for providing me help, constant attention and useful suggestions. My deepest thank to Dr. Diwakar Singh (Associate professor (Biochemistry), Department of Plant Molecular Biology and Biotechnology, Navsari Agricultural University) for manuscript preparation.

**Research Highlights**

Combined use of BAP and GA\textsubscript{3} is the first report from this study for successful shoot elongation in short time.

Optimization of best responsive explants was main objective of this study.

Initiation and proliferation of callus from different explants.
Then further growth development of best responsive explant on two different growth media (IBA + BAP and NAA + BAP).

Developed plantlets were then transferred to rooting media.

Direct leaf whorl is concluded as best responsive explant for tissue culture of sugarcane CoC 671.

**Recommendation**

In this present study, attempts were made to work on tissue culture protocol for sugarcane, which is an important crop for Indian farmers. Though there are several problems associated with sugarcane in relation with cultivation, identification of suitable variety in different agro geographical regions, sale of sugarcane to the mills, minimum support price for the farmers, and a declining trend of sugarcane cultivation in India, we hope that with a renewed approach in combination of better package of services, better planting material and combination of Plant Biotechnological approaches for whole plant regeneration will certainly bring a change in the whole scenario. It is believed that whole plant regeneration of sugarcane through tissue culture approach will definitely result in availability of disease free vigorous planting material dissemination of newer variety with improved agronomic characters for the ultimate benefit of the farmers.

Future work possibility includes First, Testing of genetic diversity of plantlets which were derived from different callus of same explant through RAPD technique. After the findings of RAPD, result can be concluding as if presence or absence of genetic diversity. As all the callus were derived through direct organogenesis, chances of genetic diversity is very low. Second, if there is no diversity and plantlets are uniform then it can be taken under field trial for growth yield study and to test susceptibility against various diseases and resistance next to plant pathogens.

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