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IN VITRO PLANT REGENERATION OF SUGARCANE (*Saccharum officinarum* L.); THE INFLUENCE OF VARIETY, EXPLANT, EXPLANT POSITION AND GROWTH REGULATORS

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ABSTRACT

Different sugarcane varieties (CP 77/400, CP 85/1491 and Punjabi) were checked for its callus induction and regeneration capabilities using different growth regulators and explants (eye bud, upper portion of inner leaf whorls and lower portion of inner leaf whorls). Seven callus induction media i.e. CIM-1 to CIM-6 and control media, five shoot induction media i.e. SIM-1 to SIM-4 and control media were used. All the varieties showed best results in terms of callus induction on CIM-6 (5 mg L^{-1},4.D + 10% Coconut water) with all the selected explants but the most promising results were observed using lower portion of inner leaf whorls. However CP 77/400 resulted in maximum callus induction (77.403%) on CIM-6 using lower portion of inner leaf whorls. Calli with different morphological characteristics were observed. Whitish/compact calli was observed using eye bud and upper portion of inner leaf whorls as explant while lower portion of inner leaf whorls gave yellowish/friable calli. The calli induced from different explant were subjected to regeneration media. Amongst these calli, the calli induced from lower portion of inner leaf whorls responded efficiently to regeneration in all the varieties. However the best result was observed in CP 77/400 i.e. (46.665%). The regenerated shoots were subjected to root induction media (½ MS media + 3.5 mg L^{-1}NAA). All the varieties showed efficient root induction however maximum root induction was observed in variety CP 77/400.

Keywords: sugarcane, callus induction, regeneration, root, shoot.

1. INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) a vital member of the family Poacae is reputed worldwide for its high sugar producing capacities. About 65% of the world's sugar is contributed by sugarcane (Alam*et al.,* 1995.). It is amongst the leading cash crop which contributes a considerable share to the country's GDP. It is cultivated on 20 million hectares in more than 90 countries of the world (Naz, 2003). Guris another popular product of sugarcane which is used sweetener used in our daily life. In addition to sugar, other industries such as biofuel are also dependent on sugarcane crop. According to (Menéndez *et al*., 1994) certain pharmaceutical products are being isolated from sugarcane. Sugarcane is an efficient and cheaper biomass for lactic acid production (Rashid and Altaf, 2008). Sugarcane in symbiotic association with G.diazotrophicus fixes atmospheric nitrogen hence increasing soil fertility. Sugarcane crop is under severe threat by various biotic and abiotic factors which reduces its production considerably. Its lengthy juvenile period, heterozygosity and improper flowering conditions make varietal improvement very difficult by conventional breeding (Khan *et al*., 2008).Therefore alternative methodologies should be practiced to develop competent sugarcane varieties having strong agronomic characteristics.

Tissue culture has been practiced over the past few decades for crop improvement which enables the scientists to develop new varieties in a short time span Heinz and Mee (1969) and Nickell (1964). Diseases free sugarcane plants and its mass propagation using shoot tip culture and leaf culture offers the maximum availability of germplasm for the farmers throughout the year (Roy and Kabir, 2007). Eye spot disease resistant sugarcane somoclonal variants has been reported by (Leal *et al*., 1996). Genetic transformation is also dependent on the development of efficient tissue culture protocols. The present research is aimed to established effective tissue culture protocols for sugarcane in order to meet the increasing demand of disease free sugarcane germaplasm and to suggest tissue culture responsive variety for further transgenic programs.

2. MATERIAL AND METHODS

The research was performed at Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture Peshawar, Pakistan in 2014.

2.1 Plants collection

Selected Sugarcane varieties i.e. Punjabi, CP 85/1491 and CP 77/400 were collected from Sugar Crop Research Institute (SCRI) Mardan and sown in Green house. After sprouting the plants were transferred to fields for further growth.

2.2 Callus Induction Media (CIM) preparation

MS basal salt media (Murashige and skoog, 1962) was prepared which was augmented with different combination and concentrations of growth regulators and was solidified with 6-8% agar. pH of the media was kept at 5.84. Media after being autoclave was poured in petri plates.

2.3 Shoot Induction Media (SIM) preparation

MS basal salt media (Murashige and skoog, 1962) having different combination and concentration of growth regulators was prepared and poured in jars after autoclave. 30% D-Sorbitol was also added to SIM along with 30% sucrose. For SIM 6% agar was used for solidification. pH of the media was kept at 5.84. The autoclaved media was poured in jars.

2.4 Root Induction Media preparation

For root induction ½ strength MS media (Murashige and skoog, 1962) was supplemented with 3.5 mg L^{-1}NAA.

2.5 Explant preparation

Explants i.e. Eye Buds, inner immature leaf whorls were collected from 7-9 months old plant and were sterilized. In case of eye buds sterilization was performed by soaking them in absolute ethanol for about 30-50 sec followed by two rinses with sterilized water. Then the eye buds were rinsed thoroughly with 15% bleach for about 20 minutes followed by three rinses with sterilized water. In case of immature leaf whorls two portions were selected as explant. One portion i.e. upper portion of inner leaf whorls were collected from the region near the upper most portion of the fully mature expanded leafs while the second portion i.e. lower portion of inner leaf whorls were collected from the region about 12 -16 inches below the upper expanded leafs. The leaf whorls were chopped into 2 inches pieces and were sterilized with absolute ethanol for 30 minutes. The upper whorls were removed inside the laminar flow hood and the inner immature 3-4 leafs were sliced into 3-4 mm slices. The sterilized explants i.e. eye buds, upper portion of inner leaf whorls and lower portion of inner leaf whorls were inoculated on callus induction media (CIM) plates having different combination and concentration of growth regulators (Table-1). The plates after inoculation were kept in a complete dark for 7 days and later after callus initiation were exposed to 12 hours photo period.

The induced calli from all the three explants after three subcultures were inoculated separately on the shoot induction media (SIM) contained in jars, supplemented with different combination and concentration of growth regulators (Table-2). The regenerated shoots were allowed to mature enough and then were transferred to root induction medium for root initiation.

Table-1. Shows callus induction media (CIM) with different combination and concentrations of growth regulators.

Callus Induction Media	**Media composition**	**Concentration** (mgL^{-1})
Control	MS+0.0	0.0
CIM-1	MS + 2,4-D+ BAP	2.0 + 2.0
CIM-2	MS + 2,4-D+ BAP	2.0 + 0.5
CIM-3	MS + 2,4-D+ BAP	3.0 + 0.25
CIM-4	MS + 2,4-D	3.0 + 0.0
CIM-5	MS + 2,4-D+ BAP	3.0 + 0.75
CIM-6	MS + 2,4-D + CW	5.00 + 10%

Table-2. Shows shoot induction media (SIM) with different combination and concentrations of growth regulators.

Shoot Induction Media (SIM)	**Media compositions**	**Concentration** (mgL^{-1})
Control	MS	0.0 + 0.0
SIM-1	MS + NAA + BAP	2.0 + 1.0
SIM-2	MS + NAA + BAP	1.0 + 1.0
SIM-3	MS + NAA + BAP	3.0 + 2.0
SIM-4	MS + 2,4-D + BAP	1.0 + 1.0

2.6 Statistical analysis of data

Each of the experiment had three replicates and the data was analysed using Statistical package Statistix 8.1. LSD test was performed in case of significant differences ($P \leq 0.05$) were observed.

3. RESULTS

3.1 Callus induction

Differential responses of callus induction were observed amongst the used explants. In case of eye buds callus initiation was observed after about 20 days which was too slow as compared to upper portion of inner leaf whorls and lower portion of inner leaf whorls where callus initiation was observed after 8-10days. Also the amount of calli produced was different. Calli with different

morphological characteristics were observed with different explants (Table-3). Eye bud and upper portion of inner leaf whorls resulted in whitish compact calli (Table-3A and B and Figure-1) whereas yellowish friable calli were observed using lower portion of inner leaf whorls as an explant (Table-3C and Figure-1). For all the three varieties lower portion of inner leaf whorls was noticed to be the best explant which resulted in maximum callus induction for all the three varieties (Table 3C). Analysis of variance (($P \leq 0.05$) revealed significant differences amongst the varieties and CIM used (Table-3).CIM-6 showed maximum callus induction in all the varieties with all the explant used (Table-3).But maximum callus induction (77.403%) was observed when lower portion of inner leaf whorls was used as explant (Table-3C), while eye bud resulted in minimum callus induction (31.477%) (Table-3A). Control media showed zero response to callus induction for all the three varieties using different explants. Amongst the varieties, the CP 77/400 resulted in maximum and Punjabi resulted in minimum callus induction with all the three explant used (Table 1). However, maximum callus induction was observed in CP 77/400 (47.108%) when lower portion of inner leaf whorls was used as an explant (Table-3C) while minimum callusing (19.664%) was achieved when eye bud was used as explant (Table-3A).

Table-3. Callus induction in sugarcane varieties on different CIMs using eye bud as explant (**A**), Callus induction in sugarcane varieties on different CIMS using upper portion of inner leaf whorls as explant (**B**), Callus induction in sugarcane varieties on different CIMS using lower portion of inner leaf whorls as explant(**C**).

A

Callus Induction Medium	Varieties			Means	Morphology
	Punjabi	CP 77/400	CP 85/1491		
Control	0.0000 k	0.0000 k	0.0000 k	**0 f**	-
CIM-1	11.107 i	15.553 gh	14.440 h	**13.700d**	Whitish/compact
CIM-2	11.110 i	12.107 i	12.220 i	**11.812e**	Whitish/compact
CIM-3	14.440 h	21.110 e	16.663 fg	**17.404c**	Whitish/compact
CIM-4	17.773 f	36.663 a	33.330 b	**29.256b**	Whitish/compact
CIM-5	8.8867 j	15.550 gh	12.220 i	**12.219e**	Whitish/compact
CIM-6	26.660 d	36.663 a	31.107 c	**31.477a**	Whitish/compact
Means	**12.854 c**	**19.664 a**	**17.140 b**		

B

Callus Induction Medium	Varieties			Means	Morphology
	Punjabi	CP 77/400	CP 85/1491		
Control	0.0000 k	0.0000 k	0.0000 k	**0.0000 f**	-
CIM-1	28.887 j	42.220 e	37.773 fghi	**36.293de**	Whitish/compact
CIM-2	30.107 j	38.883 fg	36.663 hi	**35.218 e**	Whitish/compact
CIM-3	36.773 ghi	46.683 bc	42.883 de	**42.107 c**	Whitish/compact
CIM-4	38.440 fgh	46.663 bc	45.107 cd	**43.403 b**	Whitish/compact
CIM-5	35.550 i	39.550 f	37.330 fghi	**37.477 d**	Whitish/compact
CIM-6	45.550 bc	49.553 a	47.777 ab	**47.627 a**	Whitish/compact
Means	**30.758 c**	**37.648 a**	**35.362 b**		

C

Callus Induction Medium	Varieties			Means	Morphology
	Punjabi	CP 77/400	CP 85/1491		
Control	0.0000 k	0.0000 k	0.0000 k	**0.0000 f**	-
CIM-1	28.887 j	42.220 e	37.773 fghi	**36.293de**	Whitish/compact
CIM-2	30.107 j	38.883 fg	36.663 hi	**35.218 e**	Whitish/compact
CIM-3	36.773 ghi	46.683 bc	42.883 de	**42.107 c**	Whitish/compact
CIM-4	38.440 fgh	46.663 bc	45.107 cd	**43.403 b**	Whitish/compact
CIM-5	35.550 i	39.550 f	37.330 fghi	**37.477 d**	Whitish/compact
CIM-6	45.550 bc	49.553 a	47.777 ab	**47.627 a**	Whitish/compact
Means	**30.758 c**	**37.648 a**	**35.362 b**		

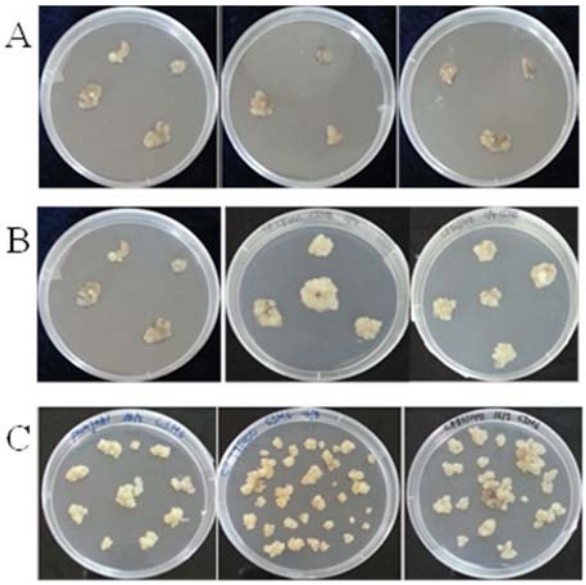

Figure-1. Callus induction of different sugarcane varieties on CIM-6 using eye bud (A), upper portion of inner leaf whorls (B) Lower portion of inner leaf whorls (C) explants.

3.2 Regeneration

The calli induced from each of the explant was inoculated on regeneration media separately to check the tendency of the induced calli of different explant to regeneration. The calli responded differently to regeneration. Maximum regeneration was observed in lower portion of inner leaf whorls calli for all the three varieties (Table-4C). Anova suggested significant difference ($P \leq 0.05$) among the varieties and SIM used. Amongst the variously used media SIM-1 showed maximum regeneration for all the three varieties using the calli induced from all the three explant (Table-4 and Figure-2). However maximum regeneration was observed when lower portion of inner leaf whorls calli of all the varieties were subjected to SIM-1 (74.071%) (Table-4C) while eye bud calli resulted in minimum shoot induction for all the varieties (20.366%) (Table-4A). Control media resulted in zero shoot induction. CP 77/400 showed efficient shoot induction amongst all the three varieties using the induced calli from all the three explants, but the maximum shoot induction in CP 77/400 (46.665%)was observed when lower portion of inner leaf whorls calli was subjected to SIM (Table-4C) while minimum shoot induction (15.551%) was observed in eye bud calli (Table-4A).

Table-4. Shoot induction in sugarcane varieties by subjecting calli induced from eye bud explant to different SIMs (A), shoot induction in sugarcane varieties by subjecting calli induced from upper portion of inner leaf whorls explant to different SIMs (B), shoot induction in sugarcane varieties by subjecting calli induced from lower portion of inner leaf whorls explant to different SIMs (C).

A

Shoot Induction Medium	Varieties			Means
	Punjab	CP 77/400	CP 85/1491	
Control	0.0000 f	0.0000 f	0.0000 f	**0.0000 e**
SIM-1	11.107 d	27.773 a	22.217 b	**20.366 a**
SIM-2	11.107 d	22.217 b	16.660 c	**16.662 b**
SIM-3	11.107 d	16.660 c	16.660 c	**14.809 c**
SIM-4	5.5533 e	11.107 d	11.107 d	**9.2556 d**
Means	**7.775 c**	**15.551 a**	**13.329 b**	

B

Shoot Induction Medium	Varieties			Means
	Punjab	CP 77/400	CP 85/1491	
Control	0.0000 g	0.0000 g	0.0000 g	**0.0000 e**
SIM-1	22.217 c	38.887 a	27.773 b	**29.626 a**
SIM-2	11.107 e	27.773 b	16.660 d	**18.513 b**
SIM-3	11.107 e	22.217 c	16.660 d	**16.661 c**
SIM-4	5.5533 f	16.660 d	11.107 e	**11.107 d**
Means	**9.997 c**	**21.107 a**	**14.440 b**	

C

Shoot Induction Medium	Varieties			Means
	Punjab	CP 77/400	CP 85/1491	
Control	0.0000 m	0.0000 m	0.0000 m	**0.0000 e**
SIM-1	66.663 d	79.997 a	75.553 b	**74.071 a**
SIM-2	48.887 g	68.887 c	55.550 e	**57.774 b**
SIM-3	24.443 k	51.110 f	35.550 h	**37.034 c**
SIM-4	13.3301	33.330 i	28.883 j	**25.181 d**
Means	**30.665 c**	**46.665 a**	**39.107 b**	

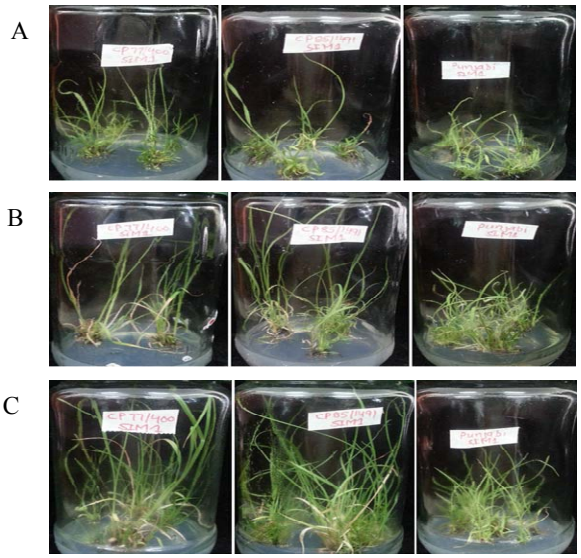

Figure-2. Shoot induction in different sugarcane varieties on SIM-1 using eye bud calli (A), upper portion of inner leaf whorls (B) and lower portion of inner leaf whorls (C) explants.

3.3 Root induction

After the regenerated shoots became mature enough, they were transferred to root induction media (RIM). ½ MS Media + 3.5mg L^{-1} was proven to be the efficient root induction media for all the three sugarcane varieties however differential responses was observable among the three varieties. Root induction was noticed after 10 days of plantlets transfer to the root induction media. CP 77/400 showed maximum number of roots which were longer in length as compared to the other two sugarcane varieties (Figure-3).

Figure-3. Root induction in different sugarcane varieties on ½ strength MS media supplemented with 3.5 mg L^{-1}NAA.

4. DISCUSSIONS

Establishment of efficient callus induction and regeneration protocols prior to transformation is an essential step. Callus induction is highly dependent on the variety of the sugarcane selected. Different sugarcane variety responds differently to callus induction. The present research confirms the variable response of varieties to callus induction which shows similarities with the result achieved by Ali *et al*. (2008a). Amongst the analyzed varieties CP 77/400 was found to the most responsive variety to callus induction as compared to CP 85/1491 and Punjabi. Other studies carried on callusing by Nawaz *et al*. (2013) and Gandonou *et al*. (2005) in sugarcane shows similarity with results achieved in the present research. In addition to different genetic background the effect of other factors such as proper combination and concentration of growth hormones and explant, explant position used was also observed on callus induction. The present results on differential response of sugarcane to callus induction to different combination and concentrations of growth regulators confirm the findings of Khattak *et al*. (2014). CIM-6 (5mg L^{-1} 2.4, D+10% Coconut water) resulted in maximum callus induction for all the verities using all the explant. Maximum callus was obtained by Ali *et al*. (2008a) when the media was supplemented with 3 mg L^{-1} 2.4, D alone. The present study suggest an enormous increase in callus when an increased concentration of 2.4, D (5 mg L^{-1}) in combination with coconut water was used. These findings are parallel to the results reported by Karim *et al*. (2002) however they used 3 mgL^{-1} 2.4,D which show differences with the concentration used in the present studies(5 mgL^{-1} 2.4,D). Type of explant also affected the rate of callusing in the present study. Amongst the three explants used, lower portion of inner leaf whorls proved to be the best explant for callus induction. These results are similar to the results achieved by Ali *et al*. (2012) and Bisht *et al*, (2011) however nothing have been mentioned about the position of the selected leaf whorls. While the present studies suggest remarkable increase in callus when the lower young, soft and fleshy portion of inner immature leaf whorls i.e. lower portion of inner leaf whorls was used for callus induction. These finding are parallel to the one reported by Mehdi *et al*. (2011) suggesting explant age as a vital factor affecting callus induction. Differential response of different explant was observed by Khadiga *et al*. (2015) to callus induction. According to the studies of (Wang and Juang, 1971) eye bud is an efficient explant for callus induction. The result reported by Mehdi *et al*. (2011) also illustrates the differential effects of explants on callus induction as an important factor affecting the callus induction capabilities. Different explant resulted in calli with different morphological characteristics. The findings of Khadiga *et al*. (2015) support our results which also suggest different morphological calli induced from different explant (cotyledon segments and roots). However the study of Ali and Iqbal (2012) contradicts our finding suggesting the morphology of the induced callias media and other physical factors dependent. In addition to media and other physical factors, type of explant is also an unavoidable factor which should be considered while studying the morphology of induced calli. The present study suggests that the amount of callusing can be increased when other factors such as choice of explant,

media composition in addition to highly responsive are also taken under consideration.

The induced calli from different explant was subjected separately to regeneration media. Media and varietal effect on the regeneration capabilities was observed. CP 77/400 showed maximum regeneration capacity. The results of Nawaz *et al*. (2013) also confirmed the differential response of varieties in terms of regeneration. The outcomes achieved by Ali *et al*. (2008b) also support our results. SIM-6 (2mg L^{-1}NAA + 1mg L^{-1} BAP) is observed to be an optimum growth regulator concentration for regeneration. Our findings show similarities with results achieved by Behera and Saho (2009) however they observed maximum regeneration with and increased value of BAP (2 mgL^{-1}) instead of NAA (0.5 mgL^{-1}). Maximum regeneration was achieved on IBA (0.5 mgL^{-1}) and NAA (1 mgL^{-1}) supplemented media Karim *et al*. (2002). The calli induced from lower portion of inner leaf whorls exhibit high level of regeneration capacities. Kumar and nandi (2014) observed the capacity of induced calli from intermodal region to regeneration which shows similarities with our result which suggests differential response of different morphological calli induced from different explant to regeneration. However Yadav and Ahmad (2013) suggest regeneration capacity fully dependent on the type of growth regulators and their combination used in the regeneration media. The present research confirms the effect of morphology on the regeneration capacities of the induced calli.

Best root induction was observed on ½ strength MS media supplemented with 3.5 mgL^{-1}NAA.The results of Yadav and Ahmad (2013)are parallel to or results however they used 3 mgL^{-1} NAA suggesting that auxins concentration can affect the root induction efficiency. Ali *et al*. (2008b) suggests that for promising root induction the use of auxins and cytokinin in combination is vital.

CONCLUSIONS

Conclusively, in addition to genetic background of sugarcane, an appropriate concentration and combination of growth regulators is vital for callus induction and regeneration in sugarcane. Also the selection of best explant and explant position is necessary for efficient callus induction. Callus with different morphological characteristics may influence the ability of regeneration.

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