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Full Length Research Paper

Rapid micropropagation of three elite Sugarcane (*Saccharum officinarum* L.) varieties by shoot tip culture

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Studies were carried out for rapid micropropagation of three sugarcane varieties i.e., HSF-240, CP-77-400 and CPF-237. Shoot tip was used as explant source. Shoot initiation from explant of all three varieties was achieved at 1 mg/l Kin and 0.1 mg/l GA_3. For rapid multiplication the regenerated shoots were transferred on liquid Murashige and Skoog medium containing 2% sucrose, supplemented with BAP in combinations with GA_3. Optimum multiplication was observed at 1 mg/l BAP in combination with 0.1 mg/l GA_3 for variety HSF-240. Best response of multiplication for variety CP-77-400 was observed at 0.5 mg/l BAP with 0.1 mg/l GA_3. Variety CPF-237 was multiplied at 1.0 mg/l BAP with 0.5 mg/l GA_3. Rooting response was observed on half strength liquid MS medium with 6% sucrose containing different concentrations of IBA and NAA. The sugarcane plantlets were acclimatized in greenhouse.

Key words: Micropropagation, Sugarcane, Shoot tip, Hormone and Meristem culture.

INTRODUCTION

Plant tissue culture techniques have become a powerful tool for studying and solving basic and applied problems in plant biotechnology. During the last thirty years, micropropagation and other *in vitro* techniques have become more widely used in commercial horticulture and agriculture for the mass propagation of crop plants (George and Sherrington, 1984; Dodds, 1991; George, 1993; Das et al., 1996). Sugarcane (*Saccharum officinarum* L.) is an important industrial cash crop of Pakistan. It is an economically important, polysomatic, highly heterozygous, clonally propagated crop that accounts for more than 60% of the world s sugar production (Guimarces and Sobral, 1998). Lack of rapid multiplication has been a serious problem in Sugarcane breeding (Ali and Afghan, 2001). Time required and continuous contaminations by systemic diseases are the serious problems to multiply an elite genotype of sugarcane in the open field (Nand and Singh, 1994). Micropropagation is currently the only realistic means of achieving rapid, large-scale production of disease-free seed canes of newly developed varieties in order to speed up the breeding and commercialization process in Sugarcane (Feldmenn et al., 1994; Lal and Krishna, 1994; Lee, 1987; Lorenzo et al., 2001; Taylor and Dukie, 1993). Barba et al. (1978) reported that within 9 months callus culture of apical meristem produce planting material from a single spindle which was sufficient to plant a hectare of land. Sauvaire and Glozy, (1978) used auxiliary buds for micropropagation of Sugarcane. Lee, (1987) and Heinz et al. (1977) also reported shoot tip culture for mass propagation of Sugarcane. This study was carried out to develop protocol for multiplication of elite cultivars grown in Pakistan.

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Abbreviations: BAP, 6-Benzyl amino purine; GA_3, gibberellic acid; IBA, indole-3-butyric acid; Kin, Kinetin; MS, Murashige and Skoog; NAA, naphthalene acetic acid; P-value, probability value.

MATERIALS AND METHODS

Three sugarcane varieties HSF-240, CP-77-400 and CPF-237 were used in this study. The varieties were selected, based on their early maturity, high productivity and resistance to pests in different areas of Pakistan. The plant materials were provided by the Sugar crops programme at crop sciences Institute, National Agriculture Research Centre (NARC). All the experimental work was carried out at Agriculture Biotechnology Programme (ABP) at NARC, Islamabad during March to November 2005.

The explant materials were taken from six months old sugarcane plants. Size of the apical meristem taken was 4 - 6 mm. For surface sterilization of explants 50% clorox (commercial bleach containing 5.25% v/v sodium hypochlorite) for 30 minutes was used. After that the explants were washed with autoclaved distilled water three times each for 10 min. The explants were then cultured on MS (Murashige and Skoog`s medium (1962)) supplemented with various combinations of GA_3 and Kin for initiation of cultures, BAP and GA_3 in liquid medium for the multiplication of cultures and with various levels of NAA and IBA for the rooting of cultures. Data incase of shoot initiation were recorded 20 days after culturing, for shoot multiplication 30 and 20 days for rooting after culturing. Each individual experiment was carried out with three replications and MSTAT-C (1991) package was used for the statistical analysis of data.

RESULTS

Shoot tips with apical meristem were found excellent starting material for the micropropagation of sugarcane. We also tested lateral buds and spindle leaves but unable to initiate shoots. Anita et al. (2000) also used shoot tip as explant source for mass micropropagation of sugarcane crop, which supported strongly our choice of explant selection. Rapid shoot growth occurred when shoot tips were used as explants for micropropagation inoculated on MS medium.

Interaction of shoot initiation with hormones Kin and GA_3

The different hormonal combinations attributed highly significant ($P < 0.01$) impact on growth percentages of shoot tips (Figure 1A-C). Growth percentages for different hormones ranged from 15 to 78.3%, where the control (TI_5) showed the lowest value 15% and TI_2 showed the highest value of 78% (Table 1). The interaction effect of different varieties and hormones combinations was also highly significantly ($P < 0.01$) different. The growth percentages for interaction between varieties and different hormones combinations ranged from 10 to 85%. The highest value (85%) was observed for HSF-240 with TI_2. Control (TI_5) showed significantly lower value (10%) as compared to all the treatments (Table 1).

The different hormones combinations showed highly significant ($P < 0.01$) impact on average shoot length of shoot tips. However the average shoot length ranged from 5.0 cm for TI_1 to 8.1 cm for TI_2 (Table 1). The interaction effect of different varieties and hormones combinations was non-significant ($P > 0.05$) on average shoot length. However the average shoot length for interaction between varieties and hormones combinations ranged from 2.5 to 9 cm. The highest value (9 cm) was observed for HSF-240 with TI_2. The control (TI_5) showed significantly lower value of 2.5 cm for all the three varieties (Table 1).

Similarly different hormones combinations showed highly significant ($P < 0.01$) impact on leaves number. The average leaves number ranged from 1.8 to 5.6 where the control (TI_5) showed the lowest value (1.8) and TI_2 showed the highest value of 5.6 (Table 1). Interaction effect of hormones and different varieties showed non-significant ($P > 0.05$) effect on leaves number. The leaves number for interaction between varieties and different hormones combinations ranged from 2 to 7. The highest value (7) was observed for HSF-240 with TI_2. Control showed significantly lower value (2) as compared to all the treatments (Table 1). A maximum of 9 cm shoot length and 7 leaves per plant were observed at 1 mg/l Kin and 0.1 mg/l GA_3 in 20 days.

Multiplication of varieties HSF-240, CP-77-400 and CPF-237

Multiplication of cultures, initiated on solid medium was carried out on liquid medium with four different combinations of BAP and GA_3 along with control for all the three varieties.

Effects of different concentrations and combinations of BAP and GA_3 on multiplication of sugarcane varieties

The different hormones combinations showed highly significant ($P < 0.01$) impact on average shoot length (Figure 1D-F). The shoot length ranged from 1.75 cm for control (TM_5) to 10.5 cm for TM_1 (Table 2). The interaction effect of varieties and hormones combinations was non-significant ($P > 0.05$) for shoot length averages. The shoot length for interaction between varieties and different hormones combinations ranged from 1.5 to 13 cm. The highest value (13 cm) was observed for variety HSF-240 on TM_1. The control showed significantly lower value (1.5 cm) as compared to all other treatments (Table 2).

The different hormones combinations also showed highly significant ($P < 0.01$) impact on number of tillers. The number of tillers ranged from 0 for control (TM_5) to 5.8 for TM_2 (Table-2). The interaction effect of varieties and different hormones combinations on average tillers number was highly significant ($P < 0.01$). The number of tillers ranged from 0 to 7.5. The highest value (7.5) was observed for variety HSF-240 on TM_2 while control (TM_5) showed no response (Table 2).

The different hormones combinations showed significant ($P < 0.05$) impact on average leaves number.

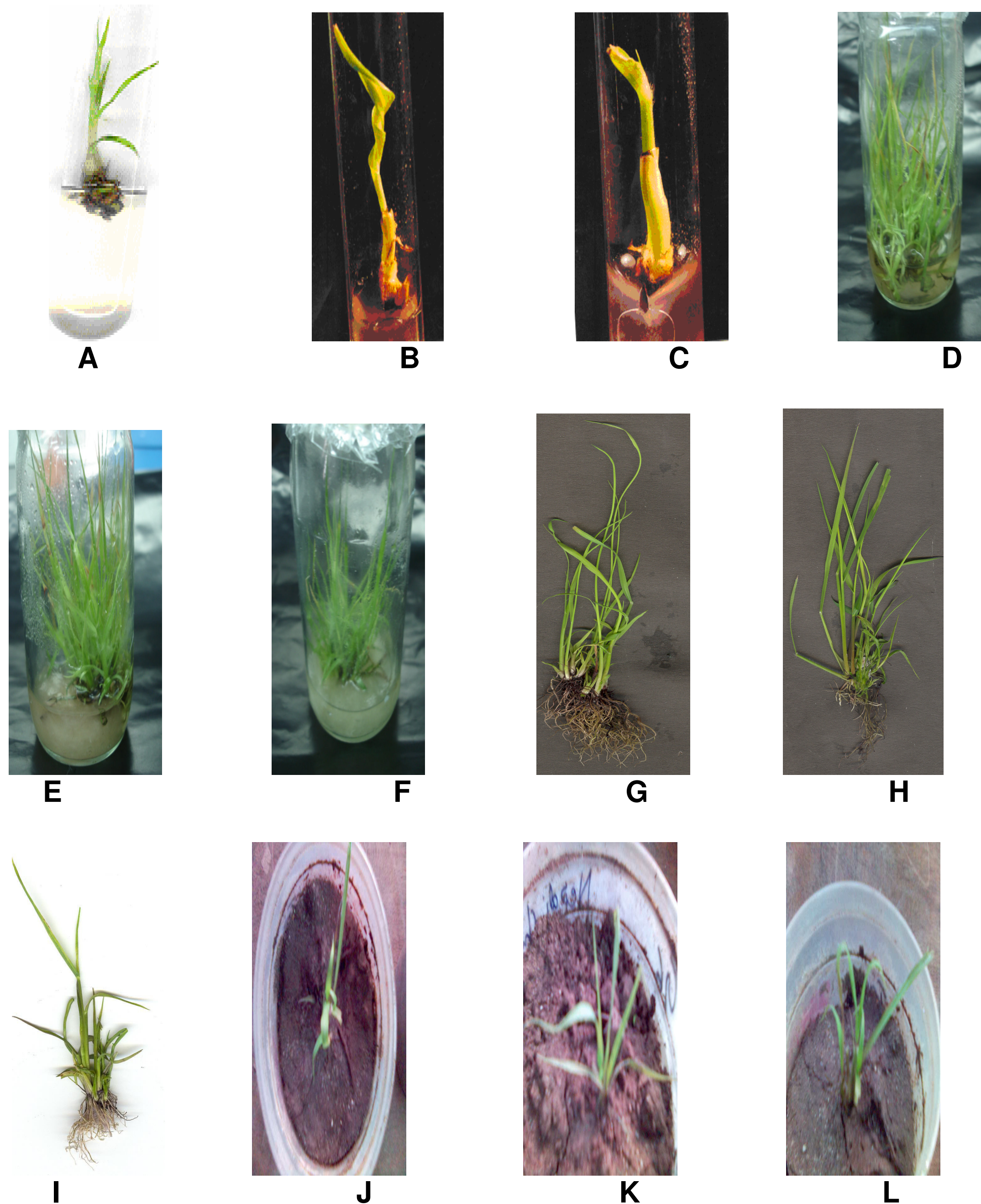

Figure 1. A. *In vitro* shoot tip growth of var. HSF-240 at 1.0 mg/l Kin + 0.1 mg/l GA_3 after 20 days. **B.** *In vitro* shoot tip growth of var. CP-77-400 at 1.0 mg/l Kin + 0.1 mg/lGA_3 after 20 days. **C.** *In vitro* shoot tip growth of var. CPF-237 at 1.0 mg/l Kin + 0.1 mg/l GA_3 after 20 days. **D.** In *vitro* shoot multiplication of var. HSF-240 at 1.0 mg/l BAP + 0.1 mg/l GA_3 after 30 days. **E.** In *vitro* shoot multiplication of var. CP-77-400 at 0.5 mg/l BAP + 0.1 mg/l GA_3 after 30 days. **F.** *In vitro* shoot multiplication of var. CPF-237 at 1.0 mg/l BAP + 0.5 mg/l GA_3 after 30 days. **G.** *In vitro* rooting of var. HSF-240 at 0.5 mg/l IBA after 20 days. **H.** In *vitro* rooting of var. CP-77-400 at 1.0 mg/l IBA after 20 days. **I.** *In vitro* rooting of var. CPF-237 at 1.5 mg/l IBA after 20 days. **J, K and L.** Acclimatization of sugarcane varieties. CPF-237, CP-77-400 and CPF-237, respectively.

Average leaves number for different hormones combinations ranged from 2.5 for control (TM_5) to 16.17 for TM_2 (Table 2). The interaction effect of different varieties and hormones combinations was highly significantly ($P < 0.01$) different. The leaves number for different varieties and hormones combinations ranged from 2 to 26. The highest value (26) was observed for HSF-240 with TM_2 while the lowest value (2) was recorded at control (TM_5) for both CP-77-400 and CPF-237 (Table-2). The leaves were dark green in color initially but changed to light

Table 1. Effects of hormonal combinations/concentrations (Kin + GA_3) on initiation of sugarcane varieties (HSF-240, CP-77-400, CPF-237).

Hormones combinations	Mean growth percentage	Mean shoot length (cm)	Mean no. of leaves
TI_1	48.333 c	5.000 b	4.333 b
TI_2	78.333 a	8.167 a	5.667 a
TI_3	46.667 c	5.833 b	4.667 ab
TI_4	60.000 b	5.833 b	5.333 ab
TI_5	15.000 d	2.500 c	1.833 c
Interaction of varieties and hormones combinations			
HSF-240 × TI_1	45.000 ef	6.000 cdef	5.000 abcd
HSF-240 × TI_2	85.000 a	9.000 a	7.000 a
HSF-240 × TI_3	45.000 ef	5.000 ef	5.000 abcd
HSF-240 × TI_4	65.000 cd	6.500 bcde	6.500 a
HSF-240 × TI_5	20.000 g	2.5 g	2.0 ef
CP-77-400 × TI_1	45.000 ef	4.5 f	3.000 def
CP-77-400 × TI_2	70.000 bc	7.5 abc	4.000 bcde
CP-77-400 × TI_3	55.000 de	5.5 def	3.500 cdef
CP-77-400 × TI_4	60.000 cd	5.5 def	4.000 bcde
CP-77-400 × TI_5	15.000 g	2.5 g	1.500 f
CPF-237 × TI_1	55.000 de	4.5 f	5.000 abcd
CPF-237 × TI_2	80.000 ab	8.00 ab	6.000 ab
CPF-237 × TI_3	40.000 f	7.000 bcd	5.500 abc
CPF-237 × TI_4	55.000 de	5.5 def	5.500 abc
CPF-237 × TI_5	10.000 g	2.5 g	2.000 ef

Legends: TI_1 = 1.0 mg/l Kin + 0.0 mg/l GA_3; TI_2 = 1.0 mg/l Kin + 0.1 mg/l GA_3; TI_3 = 2.0 mg/l Kin + 0.0 mg/l GA_3; TI_4 = 2.0 mg/l Kin + 0.1 mg/l GA_3; TI_5 = 0.0 mg/l Kin + 0.0 mg/l GA_3.

green and in some cases yellowish after some time.

Rooting of sugarcane plantlets

The shoots of size 8 to 10 cm with multiple leaves were transferred to rooting medium. Two growth hormones i.e., NAA and IBA with six different concentrations were used (Figure 1G-I). We found that root initiation started in all the three varieties of sugarcane after 6 to 10 days.

Rooting response of the three varieties of sugarcane were observed on half strength liquid Murashige and Skoog medium supplemented with various concentrations of NAA and IBA with 60 g/l sucrose. There were differences in the response of different varieties towards different concentrations however NAA 0.5 - 3.0 mg/l and IBA 0.5 - 2.0 mg/l responded well.

Effects of different concentrations of NAA and IBA on rooting of variety HSF-240

The average roots number for variety HSF-240 were highly significantly ($P < 0.01$) different. The average roots number ranged from 11 to 35. The highest value (35) was observed at 0.5 mg/l IBA while the lowest value (11) was for that of 1.5 mg/l NAA (Table 3). The control for both hormones (NAA and IBA) failed to produce any root in HSF-240. Root length for variety HSF-240 was highly significant ($P < 0.01$)) for different treatments. The root length means ranged from 1.2 cm at 0.5 mg/l NAA and 1.5 mg/l IBA to 3.05 cm at 0.5 mg/l IBA and 2.95 cm at 2.0 mg/l NAA. The means for 0.5 and 1.5 mg/l NAA were non-significant. Similarly 1.0 mg/l NAA, 3.0 mg/l NAA and 3.0 mg/l IBA were also non-significant. The impact of 1.5, 2.0 and 3.0 mg/l IBA was non-significantly (Table 3) different. No roots were observed for medium without any hormone.

Effects of different concentrations of NAA and IBA on rooting of variety CP-77-400

The roots number means for variety CP-77-400 were highly significantly ($P < 0.01$) different. The roots number averages ranged from 9.0 as the lowest value at 3.0 mg/l IBA to 41 as the highest value at 1.0 mg/l IBA (Table 3). The root length means for variety CP-77-400 were highly significantly ($P < 0.01$) different. The root length ranged from 0.75 to 2.15 cm. The highest value (2.15 cm) was observed for 2.5 mg/l NAA while the lowest value (0.75 cm) was recorded for 3.0 mg/l IBA (Table 3). The medium without any hormone failed to give any rooting response.

Table 2. Effects of hormonal combinations/concentrations (BAP + GA_3) on multiplication of sugarcane varieties (HSF-240, CP-77-400, CPF-237).

Hormones combinations	Mean shoot length (cm)	Mean no. of tillers	Mean no. of leaves
TM_1	10.50 a	5.167 ab	15.83 a
TM_2	9.583 ab	5.833 a	16.17 a
TM_3	8.667 ab	5.333 ab	14.33 a
TM_4	7.200 b	3.500 b	10.50 ab
TM_5	1.750 c	0.0000 c	2.500 b
Interaction of varieties and hormones combinations			
HSF-240 × TM_1	13.00 a	6.000 ab	18.50 ab
HSF-240 × TM_2	13.25 a	7.500 a	26.00 a
HSF-240 × TM_3	10.50 abc	5.000 ab	16.50 abc
HSF-240 × TM_4	9.100 abcd	3.500 b	13.00 abcd
HSF-240 × TM_5	2.500 ef	0.0000 c	3.500 cd
CP-77-400 × TM_1	11.50 ab	5.500 ab	17.50 abc
CP-77-400 × TM_2	8.500 bcd	5.000 ab	12.50 abcd
CP-77-400 × TM_3	6.500 cde	4.500 ab	14.00 abcd
CP-77-400 × TM_4	5.000 def	3.500 b	10.50 bcd
CP-77-400 × TM_5	1.250 f	0.0000 c	2.000 d
CPF-237 × TM_1	7.000 cd	4.000 b	11.50 bcd
CPF-237 × TM_2	7.000 cd	5.000 ab	10.00 bcd
CPF-237 × TM_3	9.000 abcd	6.500 ab	12.50 abcd
CPF-237 × TM_4	7.500 bcd	3.500 b	8.000 bcd
CPF-237 × TM_5	1.500 f	0.0000 c	2.000 d

Legends:
TM_1 = 0.5 mg/l BAP + 0.1 mg/l GA_3, TM_2 = 1.0 mg/l BAP + 0.1 mg/l GA_3
TM_3 = 1.0 mg/l BAP + 0.5 mg/l GA_3, TM_4 = 1.5 mg/l BAP + 0.5 mg/l GA_3
TM_5 = 0.0 mg/l BAP + 0.0 mg/l GA_3

Table 3. Effects of different concentrations of hormones (NAA, IBA) on roots induction in sugarcane varieties HSF-240, CP-77-400 and CPF-237.

Treatment	HSF-240		CP-77-400		CPF-237	
	Means for roots number	Means for roots length (cm)	Means for roots number	Means for roots length (cm)	Means for roots number	Means for roots length (cm)
0.5 mg/l NAA	14.50 fg	1.250 e	9.500 gh	1.050 e	10.50 g	1.050 g
1.0 mg/l NAA	16.50 ef	1.550 d	12.00 fg	1.200 d	15.50 f	1.450 c
1.5 mg/l NAA	11.00 g	1.250 e	15.50 e	1.000 e	15.50 f	1.250 e
2.0 mg/l NAA	29.50 b	2.950 a	19.50 d	1.550 b	30.00 b	1.410 cd
2.5 mg/l NAA	19.00 de	2.150 b	24.50 c	2.150 a	26.00 c	1.250
3.0 mg/l NAA	15.00 f	1.400 de	37.00 b	1.500 b	20.50 d	0.8000 h
0.5 mg/l IBA	35.00 a	3.050 a	20.50 d	2.050 a	10.50 g	1.300 de
1.0 mg/l IBA	23.00 c	1.750 c	41.00 a	1.550 b	15.50 f	1.200 ef
1.5 mg/l IBA	21.00 cd	2.050 b	14.50 ef	1.250 cd	34.50a	1.800 a
2.0 mg/l IBA	17.00 ef	2.050 b	9.500 gh	1.050 e	30.50 b	1.650
2.5 mg/l IBA	15.50 ef	2.000 b	9.500 gh	1.350 c	15.50 f	1.100 fg
3.0 mg/l IBA	16.00 ef	1.400 de	9.000 h	0.7500 f	17.50 e	1.050 g

Effects of different concentrations of NAA and IBA on rooting of variety CPF-237

Roots number averages for variety CPF-237 were highly significant ($P < 0.01$) at different treatments of two hormones. However the roots number means ranged from 10.5 to 34.5. The highest value (34.5) was observed for 1.5 mg/l IBA. The lowest value (10.5) was for 0.5 mg/l NAA and 0.5 mg/l IBA, while the effects of 1.0 , 1.5 mg/l NAA, 1.0 and 2.5 mg/l IBA were non-significantly different (Table 3). The root length for variety CPF-237 was highly significantly ($P < 0.01$) different. The root length means ranged from 1.05 to 1.8 cm. The highest value (1.8 cm) was observed for 1.5 mg/l IBA, while the lowest value (1.05 cm) was recorded for two concentrations i.e., 0.5 mg/l NAA and 3.0 mg/l IBA (Table 3).

The total number of roots produced per plant ranged from 9.0 to 41. A maximum of 41 roots were recorded for var. CP-77-400 at 1.0 mg/l IBA, minimum roots number of 9 was observed again for CP-77-400 at 3.0 mg/l IBA. The root length for different concentrations of IBA and NAA ranged from 0.75 cm for var. CP-77-400 at 3.0 mg/l IBA to 3.0 cm for var. HSF-240 at 0.5 mg/l IBA. No rooting response was observed for medium without hormones.

Acclimatization

The rooted plantlets were transferred in flasks containing plain water and covered with polythene bags for high humidity for 2 - 3 days. After that these were transferred to soil in growth room for few days and then were transferred to green house to assess their potential for further hardening. The acclimatization potential was 70 - 80%.

DISCUSSION

In this article, we reported an easy and efficient protocol for sugarcane mass micropropagation which can be easily repeated for the improvement of sugarcane crop. Gosal et al. (1998) established shoot cultures from spindle explants on MS medium instead of shoot tips, supplemented with IAA (0.5 mg/l), BAP (0.5 mg/l) and Kin (0.5 mg/l), these results are different from those of our results as we found shoot tip better than spindle leaves, but our results are in line with those of Anita et al. (2000), because they also reported shoot tip as explant source for *in vitro* mass micropropagation of sugarcane crop. The reason might be that shoot tip is much safer and fast growing portion of the plant.

Razi-ud-Din et al. (2004) reported maximum shoot growth of 83.3% with average shoot length of 3.7 cm when using MS medium supplemented with 5.0 mg/l BAP and 1.0 mg/l GA_3, while we reported here maximum growth of 85% for variety HSF-240 at 1.0 mg/l kinetin and 0.1 mg/l GA_3 without BAP, also we used very low concentration of GA_3. Khan and Rashid (2003) also reported shoot tip initiation at the same combination/ concentrations of above two growth hormones (Kin, GA_3) with 5 cm shoot length per plant. Saini et al. (2004) used 1.0 mg/l GA_3 and 1.0 mg/l Kin for the organogenesis of sugarcane on MS medium. These results are very much in line with those of our's regarding Kin concentration but they used a bit higher concentration of GA_3. Patel et al. (2001) also recorded highest shoot growth on 1.5 mg/l Kin with maximum length of main shoot and number of leaves.

Hendre et al. (1983) obtained shoots of sugarcane cultivars on different BAP and Kinetin concentrations, while Chattha et al. (2001) and Jadhav et al. (2001) reported that different genotypes give shoots on different media. Wongkaew and Fletcher (2004) used MS medium containing 0.5 mg/l NAA, 0.5 mg/l BAP and 15% coconut water for the growth initiation of meristem tips. These results are not in line with our results, while Khan and Rashid (2003) reported shoot tip initiation on Kin and GA_3 with 5 leaves per plant which is very much similar to our results. Baksha et al. (2002) recorded maximum of 4.5 ± 0.01 shoot length using 2.0 mg/l BAP and 0.5 mg/l IBA in MS medium, while Cheema and Hussain (2004) observed maximum of 7 cm shoot length at 0.4 mg/l Kin and 0.4 mg/l BAP. This strongly supports the use of cytokinin for multiple shoot formation but we recorded low level of cytokinin compared to them. Cheema and Hussain (2004) observed 29 shoots per plant at 0.4 mg/l BAP in combination with 0.4 mg/l Kin. We reported here the combinations of Gibberellic acid and cytokinins for sugarcane micropropagation, which is previously reported by only few authors and also not reported for varieties used in this research.

Baksha et al. (2002) used 5.0 mg/l NAA for best response of rooting in half strength MS medium. This suggests use of high concentration of NAA for rooting purpose while we observed 1.0 mg/l IBA as the best root initiation growth hormone with highest number of 41 roots per plant. Baksha et al. (2002) also got rooting response at 0.1 - 0.5 mg/l IBA along with 0.5 - 2.0 mg/l BAP but these were of poor quality. These findings also agree well with the previous findings of Nadar and Heinz (1977). Alam et al. (2003) reported best rooting response at 2.5 mg/l IBA with 16 number of roots/explant having 1.1 cm root length. Mamun et al. (2004) obtained best results of rooting on MS medium supplemented with auxins (NAA + IBA) 0.5 mg/l for each one. We also found that NAA 3.0 mg/l was best for optimum root growth for variety CP-77-400 but at the same concentration of NAA the other two varieties produced only 15 - 20 roots per plant. The other two varieties rooted well at IBA 0.5 - 1.5 with maximum 35 root per plant for variety HSF-240 and 34 for variety CPF-237. Similarly, Sing et al. (2001) observed best rooting response (75%) when using MS medium with 60 g/l sucrose and NAA, while Kale et al. (2004) observed best rooting at MS medium with 2 mg/l IAA.

Alam et al. (2003) found better rooting response at 2.5

mg/l IBA and 2.5 mg/l NAA. He observed maximum of 25 roots and 2.0 cm root length per plant. It proved the role of auxins in rooting, but he reported high concentration of IBA as compared to us, this may be due to different varietal response. Gosal et al. (1998) obtained rooting on liquid MS medium containing NAA (5 mg/l) and 70 g/l sucrose. Ali and Afghan (2001) observed only 6 - 7 roots after 3 weeks on MS medium containing 2 mg/l IBA and 6% sucrose while we reported 41 roots per plant which are much higher number as compared to their results. Here we suggested low level of IBA for rooting.

Micropropagation of sugarcane from shoot tip may become the successful method to cope with the present day demand. It will be an easy way for obtaining intensive number of plants in limited time under controlled conditions. Through the use of tissue culture technique it may be easy to obtain disease free plants. The protocol used in the present study can be used for rapid multiplication of sugarcane.

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