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Effect of growth regulators concentrations on *in vitro* multiplication of three elite sugarcane (*Saccharum officinarum* L.) Genotypes using shoot tip culture

Gerema Amente^{1*}, Dr.Tileye Feyissa²

¹Adami Tulu Agricultural Research Center, Oromia Agricultural Research Institute, Batu, Oromia, Ethiopia

²Institute of Biotechnology, Addis Ababa University, Addis Ababa, Ethiopia

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***Corresponding Author:**
Gerema Amente,
E-mail: amente2015@gmail.com

ABSTRACT

Conventional vegetative propagation of sugarcane generally has low multiplication rate and allows distribution of diseases. Micropropagation is the only practical means of achieving rapid, large-scale production of disease-free quality planting material. Experiments on shoot tip culture initiation and shoot multiplication were laid out in completely randomized design with 2x3x3 and 4x5x3 factorial treatment arrangements respectively. Data was subjected to analysis of variance (ANOVA) and significant means were separated using Duncan's multiple range tests. With regard to shoot multiplication, genotype Q200 showed a maximum of 13.59 shoots per explant with 5.83cm shoot length on a medium fortified with 2 mg/l BAP alone, while genotype Q217 produced a maximum of 15.28 shoots per explant with 5.37cm mean shoot length on a medium supplied with 2.0 mg/l BAP and 0.25 mg/l kinetin. Likewise, Co-0238 produced maximum of 13.56 shoots per explant with mean shoot length 6.50 cm on medium fortified with 1.5 mg/l BAP + 0.5mg/l kinetin

KEYWORDS: Genotypes, 6-Benzylaminopurine, Kinetin, Invitro Micro propagation

INTRODUCTION

The sugar industry has great contributions to the socio-economy of the country, given its agricultural and industrial investments, foreign exchange earnings, its high employment, and its linkages with major suppliers, support industries and customers (Belay *et al.*, 2014). Hence, much research has been focused on sugarcane crop improvement through conventional breeding and quite recently, extensive attention has been paid to biotechnological approaches (Baksha *et al.*, 2003; Hoy *et al.*, 2003).

Conventional propagation of sugarcane in general suffered from low propagation rates, expensive labor, large land areas demand, time consuming and potential transmission of pathogens from the seed cane to the subsequent crop limits the efficiency of this method (Sandeep *et al.*, 2009). Much research has been focused on sugarcane crop improvement through conventional breeding and quite recently, extensive attention has been paid to biotechnological approaches (Baksha *et al.*, 2003; Hoy *et al.*, 2003). Therefore, efficient propagation system is required for mass multiplication of sugarcane in a short period of time.

Rapid clonal propagation of sugarcane planting materials depends on the genotype and the plant growth regulators combinations used and needs to develop plant growth regulators combinations for each genotype (Singh *et al.*, 2006, Melaku *et al.*, 2016). Plant growth regulators necessities for *in vitro* propagation responses differ from cultivar to cultivar in sugarcane (Pathak *et al.*, 2009). It is necessary that an efficient protocol is needed for every new variety or clone of sugarcane to get rapid shoot initiation and shoot multiplication. Thus, this research work was designed with the objective to study effect of growth regulators concentrations on *in vitro* multiplication of sugarcane genotypes (Q200, Q217 and Co-0238) using shoot tip explants.

MATERIALS AND METHODS

The study was conducted at Holeta National Agricultural Biotechnology Laboratory of the Ethiopian Institute of Agricultural Research. Three elite sugarcane genotypes (Q-200, Q-217 and Co-0238) were used for the study. They were obtained from the Ethiopian Sugar Corporation, Research and

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Training Division. The Sugar Corporation imported Q-200 and Q-217 varieties from Australia and Co-0238 variety from India. These genotypes are among the selected ones to be commercialized by the Ethiopian Sugar Corporation for their cane yield performance and sugar quality.

Culture Media Preparation and Sterilization

The basal medium employed for the culture is MS medium (Murashige & Skoog, 1962). The required amount of macronutrient, micronutrient, vitamins from their respective stock solution were dissolved in double distilled water along with 30 g/L sucrose (for initiation and multiplication).

The final volume was made up to the required level with double distilled water and then divided into required volume of treatments, to which amount of PGRs from stock solution were added in combinations at different concentrations. The pH was adjusted in all cases to 5.8 by using 1N NaOH and/or 1N HCL before gelling with agar. Agar-Agar type I (4.5 g/l for culture initiation) and Phytigel (2.0 g/l for multiplication) were added to the nutrient and heated on magnetic hot plate till it was completely melted. Before autoclaving, the media was poured into washed and dried test tubes of 30 ml volume for initiation and 50 ml volume of culture jars for multiplication, then, capped and labeled properly. The dispensed medium was then autoclaved at 121° C for 15 minutes at 15-psi pressure.

Explant Collection and Sterilization

Actively growing shoot tips were taken from 4-months old greenhouse grown healthy mother plants. The intact leaves were removed, and the segments with leave sheath were taken to the laboratory for surface sterilization and explant preparation. Trimmed shoot tips were washed thoroughly under running tap water for 20 minutes, and resized to just about 10 cm length by cutting off at the two ends. Then the explants were further washed for 30 minutes with tap water containing a drop of liquid detergent (Largo liquid soap) solution plus two drops of tween-20 with continuous shaking and rinsed three times with double distilled water. Later, the explant was taken to a laminar air flow cabinet and immersed in 0.3% (w/v) Kocide fungicide solution, ascorbic acid (0.2% w/v) and citric acid (0.4% w/v) for 30 minutes followed by three times rinsing each for five minutes with autoclaved double distilled water.

The shoot tips were washed again with 70% ethanol for one minute and 30 seconds and rinsed with sterile double distilled water three times each for five minute to remove residual ethanol from the shoot tip surface. Finally, 2.5cm sized explants were surface sterilized with 50% (v/v) aqueous solution of Sodium hypochlorite bleach (5% v/v active chlorine) containing two drops of a wetting agent (tween-20) with gentle shaking for 25 minutes. After pouring out sodium hypochlorite solution, the explants were rinsed with sterile double distilled water three times for five minutes to remove all traces of the sterilant. All the steps of sterilization were carried out under aseptic condition

Shoot Initiation from Shoot Tip Explant

Shoot tip containing apical meristem of genotypes Q-200, Q-217 and Co-0238 were cultured on MS medium containing different concentrations and combinations of BAP (0.0, 1.5 and 2.0 mg/l) and IBA (0.0 and 0.5 mg/l), 30 g/l sucrose and 4.5g/l agar. The factorial treatment was 3x2x3 combinations arrangements. The experiment was carried out at growth room temperature of 25°C ± 2°C under 16 hours photoperiod with light intensity of 30 µmol m⁻²s⁻¹ provided by cool white fluorescent light and 70% to 80% relative humidity. Thirty test tubes with each having one shoot tip explant were cultured for each three genotypes (30 shoot tips per treatment). Sub culturing was done frequently (7 days). Data of shoot initiation were recorded three weeks after culture

In vitro Shoot Multiplication

Healthy micro-shoots having same size obtained from the subculture stage were used for shoot multiplication experiment after maintaining on plant growth regulator free semi-solid medium for two weeks. These micro-shoots were transferred to full MS semisolid media containing BAP (0, 0.5, 1.0, 1.5 & 2 mg/l) in combination with kinetin (0, 0.25, 0.5 & 1.0 mg/l) and supplemented with 30g/l sucrose. The experiment was laid out in CRD with three factor factorial combination of five levels of BAP (0, 0.5, 1.0, 1.5 & 2 mg/l); four levels of kinetin (0, 0.25, 0.5 & 1.0 mg/l) and three levels of sugarcane genotypes (Q200, Q217 and Co-0238) resulting in 3x5x4 factorial treatment combinations arrangement. Each treatment was replicated three times and four shoot bunches (12 shoot bunches per treatment) were cultured in each culture jar. Sub-culturing was carried out at 15 days interval to obtain shoot multiplication. Data on number of shoots per explants, length of shoots and number of leaves per shoot were carefully recorded during each sub culturing.

Data Analysis

The analysis of variance for different variables was performed by GenStat Version 15.1, (GenStat15.1 (64-bit Edition) 2012, VSN International Ltd.) and for significantly different treatments, mean separation was done with Duncan's multiple range tests at or below the probability level of 5%.

RESULTS & DISCUSSION

Culture Initiation

The shoot tip explants on full MS media lacking of plant growth regulator (control) failed to grow. However, shoot initiation was observed in MS media supplemented with PGR. Healthy and vigorous shoots started appearing within 6-8 days in many of the culture tubes containing 2.0 mg/L BAP in combination with 0.5 mg/L IBA, in Q200 and Q217 varieties, while 8-10 days in Co-0238 variety on medium containing 1.5 mg/L BAP in combination with 0.5 mg/L IBA in the culture tubes. The percent establishment varied between different combinational

levels of BAP and IBA. Frequency of establishment was maximum for Q217 (86.6%) and Q200 (90%) genotypes at 2.0 mg/L of BAP and 0.5 mg/L of IBA hormone combination (Table.1, Figure 1(B) and Figure 1(A)), while it was maximum in case of Co-0238(93.3%) variety at 1.5 mg/L of BAP and 0.5 mg/L of IBA, on which shoots were active and healthy (Table.1 and Figure 1(C)). The study of Tilahun *et al.* (2014) indicated that normal shoot initiation and establishment was obtained in two cultivars Co449 and Co678 with 2.0 mg/L of BAP and 0.5mg/L of IBA, which was similar result with Q200 and Q217 variety. Godheja *et al.* (2014) reported that initiation of shoot tip explant of Co-86032 variety was maximum at 1.0 mg/L BAP. The study of Sawant and Meti (2016) showed that variety Co86032 shoot formation (96.67%) on MS medium supplemented with BAP (0.2 mg/L) and Kinetin (0.1 mg/L), on the other hand variety CoC671 showed maximum shoot formation (93.33%) on MS medium supplemented with only Kinetin(0.5mg/L). The study of Sandeep *et al.* (2008) also indicated shoot initiation and establishment was obtained in cultivars of CoC-671 at 2.0 mg/L of BAP. These observations showed that the concentration of BAP and IBA required for shoot initiation and establishment varies with genotypes. The amount of cytokinin applied depends on the genotype used and the micro propagation strategy employed

Shoot Multiplication

Analysis of variance revealed that the interaction effects of genotype, BAP and kinetin was very highly significant on the number of shoots per explant, average shoot length (cm) and number of leaves per shoot. The interaction of genotype, BAP and kinetin indicated that all the three factors are reliant on each other for *in vitro* shoot proliferation of sugarcane. Shoot multiplication was not observed within 4 weeks when shoot explants were cultured on MS medium without plant growth regulators in all the three genotypes. However, increasing the concentration of kinetin alone from 0.0mg/L to 0.25 mg/L resulted in 5.22, 6.51 and 5.39 shoots per explant for Q-200, Q-217 and Co-0238, respectively. In the same way, increasing BAP concentration alone from 0.0mg/L to 0.5 mg/L gave 5.50, 5.49 and 3.57 shoot per explant in genotype Q-200, Q-217 and Co-0238, respectively. This result indicates that the supplementation of exogenous plant growth regulators to MS medium is fundamental to develop multiple shoots. Certainly, cytokinins are capable of overcoming apical dominance and release lateral buds from dormancy thereby boost shoot multiplication (George *et al.*, 2008).

It was observed that the three genotypes responded differently to the same media for all parameters studied. A significantly highest average number of shoots per explant (13.59), with average shoot length 6.97cm and 5.83 number of leaves per shoot was observed in genotype Q200 on MS medium fortified with 2.0 mg/L BAP alone (Table 2 and Figure 2(A)), while Q217 and Co-0238 gave only 5.16 and 6.43 shoots per explant, respectively on the same medium composition. Among different combinations of BAP and Kin, genotype Q217 produced the highest number of shoots per explant (15.28) with mean shoot length of 6.30 cm and average number of leaves per shoot of 5.37 on MS medium fortified with 2.0 mg/L BAP and 0.25 mg/L Kinetin (Table 2 and Figure 2(B)) while the same medium composition resulted in only 5.47 and 6.76 shoots per explant in Q200 and Co-0238 genotypes respectively. Similarly, Co-0238 produced maximum of 13.56 shoots per explant with average shoot length of 6.67cm and mean number of leaves per shoot of 5.50 on MS medium fortified with 1.5 mg/L BAP + 0.5 mg/L kinetin (Table 2 and Figure 2(C)) while the same medium composition resulted in only 5.26 and 6.74 shoots per explant in Q217 and Q200, respectively. In three genotypes, maintaining Kin at 0.25 mg/L while increasing BAP levels from 0.5 to 2.0 mg/L, showed a marked increase in the number of shoots per explant from 5.50 to 13.59, 5.16 to 5.4 and 3.57 to 3.80, average shoot length from 2.93 cm to 5.80 cm, 2.87 cm to 4.13 cm, 2.30 cm to 3.43 cm and number of leaves per explant from 3.10 to 6.97, 3.63 to 4.87, 3.60 to 4.47 in Q200, Q217 and Co-0238 genotypes, respectively. Increase of kinetin from 0.25 to 1.0 mg/L without BAP showed a significant increase in the number of shoots per explant from 6.51 to 7.34 but, decrease the average shoot length from 4.37 to 3.60cm and number of leaves per shoot from 5.03 to 4.13 in Q217 genotypes. On the other hand, significant reduction of the same parameter with the above genotype was observed in Q200 and Co-0238 varieties.

Regarding shoot length, Q200 produced the highest shoot length (5.83 cm) with the maximum number of leaves per shoot (6.97) on MS medium supplemented with 2.0 mg/L BAP alone, whereas only 4.13 cm and 3.43 cm shoot length with 4.87 and 4.47 leaves per shoot production in Q217 and Co-0238, respectively. This different shoot length growth response to the similar media composition might be as a result of difference in inherent endogenous growth hormone level among genotypes (George *et al.*, 2008).

It is evident from Table 2 that the use of 2.0 mg/L BAP alone produced only 5.16 shoots per explant, which was increased to

Table 1. Effect of BAP and IBA on establishment of shoots

PGR (mg/L)		Sugarcane Varieties					
		Q200		Q217		Co-0238	
IBA	BAP	shoots established	% of establishment	shoots established	% of establishment	shoots established	% of establishment
0	0	0	0%	0	0%	0	0%
0	1.5	12	40%	13	43.3%	14	46.6%
0	2	17	56.6%	15	50%	11	36.6%
0.5	0	3	10%	4	13.3%	2	6.6%
0.5	1.5	16	53.3%	18	60%	28	93.3%
0.5	2	26	86.6%	27	90%	17	56.6%

15.28 shoots by addition of 0.25 mg/L kinetin in genotype Q217. In similar way, increased shoot number per explant was observed

in genotype Co-0238 from 3.80 to 13.56 when 0.5 mg/L kinetin was added to MS medium containing 1.5 mg/L BAP. This positive effect implies the significance of adding the two growth regulators in combination rather than alone in shoot multiplication medium. Ali and Afghan (2001) reported that medium supplemented with BAP and Kinetin resulted in rapid multiplication of shoots.

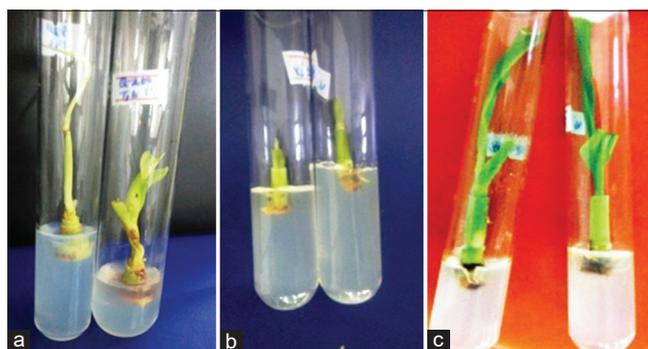


Figure 1: Shoot tip culture initiation and establishment of three genotypes A'-Q200 on MS medium containing 0.5 mg/L IBA +2.0 mg/LBAP; 'B'-Q217 on MS medium containing 0.5 mg/L IBA +2.0 mg/LBAP; 'C'-Co-0238 on MS medium containing 0.5 mg/l IBA+1.5 mg/l BAP

When the same concentration of Kinetin (0.5mg/L) and BAP (0.5mg/L) are used in combination for shoot multiplication, Q200 genotype produced more number of shoots (6.72) per explant than other genotypes. The same is also true when 1.0 mg/L Kinetin and 1.0 mg/L BAP were used in combination. This result also points out that different genotypes show different performance on the same hormone (cytokinins) concentration and combination.

The best result achieved in genotype Co-0238 is consistent with the result obtained by Khan *et al.* (2009). They reported variety



Figure 2: *In vitro* shoot multiplication of three sugarcane genotypes 'A'-Q200 on 2.0 mg/l BAP; 'B'-Q217 on 2.0 mg/l BAP and 0.25 mg/l kinetin; 'C'-Co-0238 on 1.5 mg/l BAP and 0.5 mg/l kinetin

Table 2: The effect of BAP and Kinetin on number of shoots per explant, shoot length and number of leaves per shoot

PGRs (mg/L)		Sugarcane Varieties								
Kin	BAP	Q200			Q217			Co-0238		
		#of shoots per explant	#of leaves per shoot	Shoot length (cm)	#of shoots per explant	#of leaves per shoot	Shoot length (cm)	#of shoots per explant	#of leaves per shoot	Shoot length (cm)
0	0	0.00 ^A	0.00 ^r	0.00 ^s	0.00 ^A	0.00 ^r	0.00 ^s	0.00 ^A	0.00 ^r	0.00 ^s
0	0.5	5.50 ^{h-o}	3.10 ^q	2.93 ^{k-r}	5.49 ^{j-o}	3.63 ^{pq}	2.87 ^{l-r}	3.567 ^{xy}	3.60 ^{pq}	2.30 ^r
0	1	7.05 ^f	4.10 ^{l-q}	3.90 ^{d-m}	5.35 ^{l-q}	3.57 ^q	3.27 ^{f-r}	5.470 ^{lp}	4.00 ^{lq}	2.93 ^{k-r}
0	1.5	9.15 ^c	4.80 ^{d-p}	4.33 ^{b-g}	4.87 ^{rs}	4.17 ^{j-q}	3.83 ^{d-n}	5.647 ^{l-l}	4.13 ^{j-q}	3.73 ^{d-n}
0	2	13.59 ^b	6.97 ^a	5.83 ^a	5.16 ^{pq}	4.87 ^{d-p}	4.13 ^{c-j}	3.80 ^x	4.47 ^{f-q}	3.43 ^{f-r}
0.25	0	5.22 ^{o-q}	5.03 ^{c-o}	4.37 ^{b-q}	6.51 ^{g-i}	6.00 ^{a-e}	3.97 ^{d-m}	5.39 ^{l-q}	4.83 ^{d-p}	3.63 ^{f-o}
0.25	0.5	6.36 ⁱ	4.07 ^{k-q}	3.17 ^{g-r}	5.63 ^{j-l}	4.87 ^{d-p}	3.50 ^{f-q}	4.657 st	5.67 ^{b-i}	3.77 ^{d-n}
0.25	1	8.44 ^d	4.37 ^{l-q}	5.50 ^a	6.46 ^{g-i}	4.43 ^{i-q}	4.20 ^{c-h}	5.767 ^{jk}	5.10 ^{c-o}	3.30 ^{f-r}
0.25	1.5	9.27 ^c	4.37 ^{l-q}	4.80 ^{a-e}	7.30 ^e	5.83 ^{a-g}	4.83 ^{a-d}	5.873 ^j	5.30 ^{c-l}	2.53 ^{o-r}
0.25	2	5.47 ^{i-p}	5.43 ^{b-k}	5.13 ^{a-c}	15.28 ^a	6.30 ^{a-c}	5.37 ^{ab}	6.763 ^g	4.13 ^{j-q}	3.63 ^{f-o}
0.5	0	4.60 ^{s-u}	4.40 ^{l-q}	3.80 ^{d-n}	8.45 ^d	5.47 ^{b-j}	3.40 ^{f-r}	5.600 ^{l-m}	3.80 ^{n-q}	2.30 ^r
0.5	0.5	6.72 ^{gh}	5.27 ^{c-l}	3.33 ^{f-r}	4.27 ^{vw}	4.50 ^{f-r}	3.30 ^{f-r}	6.43 ^{hi}	5.37 ^{b-l}	3.50 ^{f-q}
0.5	1	6.65 ^{h-i}	4.10 ^{l-q}	2.97 ^{j-r}	5.44 ^p	5.17 ^{c-n}	3.67 ^{e-o}	6.553 ^{g-i}	5.83 ^{a-f}	3.50 ^{f-q}
0.5	1.5	5.26 ^{n-q}	5.03 ^{c-o}	3.57 ^{f-p}	6.74 ^g	5.20 ^{c-m}	3.67 ^{e-o}	13.56 ^b	6.67 ^{ab}	6.50 ^a
0.5	2	4.327 ^{u-w}	3.73 ^{a-q}	4.17 ^{c-i}	7.13 ^{ef}	3.60 ^{pq}	2.67 ^{n-r}	8.623 ^d	6.27 ^{a-c}	2.80 ^{m-r}
1	0	4.28 ^w	4.13 ^{l-q}	3.60 ^{f-o}	7.34 ^e	5.80 ^{a-h}	3.33 ^{f-r}	4.270 ^w	5.47 ^{b-j}	3.00 ^{j-r}
1	0.5	6.35 ⁱ	6.13 ^{a-d}	4.40 ^{b-f}	5.30 ^{m-q}	5.03 ^{c-o}	4.10 ^{c-k}	5.557 ^{l-n}	5.27 ^{c-l}	4.00 ^{d-h}
1	1	5.08 ^{qr}	5.47 ^{b-j}	3.53 ^{f-q}	4.41 ^{lv}	4.30 ^q	3.77 ^{d-n}	4.76 ^s	5.13 ^{c-n}	3.50 ^{f-q}
1	1.5	4.39 ^{u-v}	4.73 ^{a-p}	3.83 ^{d-n}	3.63 ^{xy}	4.00 ^{l-q}	2.97 ^{j-r}	3.480 ^y	4.30 ^{j-q}	3.03 ^{h-r}
1	2	4.06 ^w	3.87 ^{m-q}	3.70 ^{d-o}	3.18 ^z	4.00 ^{l-q}	2.47 ^{p-r}	3.593 ^{xy}	3.83 ^{m-q}	2.40 ^{qr}
%Cv		8.2	14.8	16.5	8.2	14.8	16.5	8.2	14.8	16.5
SE		0.274	0.389	0.332	0.274	0.389	0.332	0.274	0.389	0.332

HSF-240 produced 11 shoots per explant; 16.5 cm mean shoot length and 32 leaves per shoot on MS medium fortified with 1.5 mg/L BAP+ 0.5 mg/l kinetin. Dereje *et al.* (2014) reported maximum multiplication from C86-12 genotype with 33.8 ± 0.837 shoots per explant, 8.4 ± 0.008 cm mean shoot length and 13.04 ± 0.089 leaves per shoot on MS medium fortified with 1.5 mg/L BAP+ 0.5 mg/l kinetin. The present result in Q217 is also in agreement with the results reported by Tilahun *et al.* (2014) who obtained best result from Co-449 genotype on MS medium fortified with 2.0 mg/L BAP + 0.25 mg/L Kinetin with 7.87 ± 1.06 number of shoots, 6.33 ± 0.21 cm shoot length and 5.44 ± 0.19 leaves per shoot. The result in Q200 is similar with the report of Belay (2014) who found optimum result from N14 genotype on MS Medium fortified with 2.0 mg/L BAP without kinetin with 21 ± 0.58 numbers of shoots, 5.63 ± 0.01 cm shoot length and 5.4 ± 00 leaves per shoot.

In the three cases, the observed difference in number of shoots per explant, number of leaves and shoot length could be due to genotypic difference. Similar results were also reported by Sandeep *et al.* (2008) who observed an average of 19.91 shots on MS medium fortified with 2.0 mg/L BAP alone. However, there are also reports (Yadav *et al.*, 2012; Sawant & Meti, 2016) that indicated higher multiplication rate of sugarcane varieties at lower concentration of BAP and kinetin than obtained in this study. Result of the present study indicated that 1.5 mg/L BAP + 0.50 mg/l kinetin was the optimum and best hormone concentration and combination for maximum shoot multiplication of sugar cane genotype Co-0238. The present result showed that 2.0 mg/L BAP + 0.25 mg/L kinetin, and 2.0 mg/L BAP alone was found to be the best for sugarcane genotype Q217 and Q200, respectively. Comparison of the three genotypes showed that Q217 was a better responsive than Q200 and Co-0238 for in vitro multiplication in full MS medium.

CONCLUSIONS

From the result obtained in the study, it is concluded that the developed protocol is helpful for rapid in vitro propagation of the sugarcane planting materials and hence enhance the availability of healthy and true to type planting materials in Ethiopian sugarcane plantations

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interests to declare.

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