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Effects of 6-Benzyl aminopurine and Kinetin on *In Vitro* Shoot Multiplication of Sugarcane (*Saccharum officinarum* L.) Varieties

Belay Tolera[1*], **Mulugeta Diro**[2] **and Derbew Belew**[3]

[1]*Ethiopian Sugar Corporation, Research and Training Division, Variety Development Directorate, Biotechnology Research Team, Wonji Research Center, Wonji, Ethiopia*

[2]*Capacity Building for Scaling up of Evidence-based Best Practices in Agricultural Production in Ethiopia (CASCAPE) Project, Addis Ababa, Ethiopia*

[3]*Jimma University, College of Agriculture and Veterinary Medicine, Jimma, Ethiopia*

***Corresponding author:** Dr. Belay T, Ethiopian Sugar Corporation, Research and Training Division, Ethiopia, Tel: +251-910181644; E-mail: belaytolera@yahoo.com

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Abstract

In vitro shoot multiplication of two commercial sugarcane varieties grown in Ethiopian sugar estates initiated using shoot tip explants was carried out with the objective to assess the multiplication responses of the sugarcane varieties (B41-227 and N14) under five levels of 6-benzylaminopurine (0, 0.5, 1, 1.5 and 2 mgL^{-1}) and kinetin (0, 0.1, 0.25, 0.5, and 1 mgL^{-1}) in a completely randomized design with 5x5x2 factorial treatment combinations arrangements. Analysis of variance (ANOVA) showed that the interaction effects of 6-benzylaminopurine, kinetin and the sugarcane genotypes on number of shoots per explant, average shoot length and number of leaves per shoot was very highly significant ($P < 0.0001$). Murashige and Skoog (MS) medium fortified with 1 mgL^{-1} 6-benzylaminopurine (BAP) and 0.5 mgL^{-1} kinetin for B41-227; and 2 mgL^{-1} 6-benzylaminopurine (BAP) without kinetin for N14 were found to give optimum shoot proliferation results. On these medium, B41-227 gave 34 ± 1.54 shoots per explant with 6.95 ± 0.01cm average shoot length and 12 ± 0.17 leaves per shoot while N14 produced 21 ± 0.58 shoots per explant with 5.63 ± 0.01 cm average shoot length and 5.4 ± 00 leaves per shoot after 30 days of culture transfer to multiplication medium. Thus, the optimized protocol is useful for rejuvenation, rapid *In vitro* propagation and production of large quantity quality sugarcane planting materials and hence help minimizes the major limitations hampering sugarcane production in the Ethiopian sugar Estates.

Keywords: *In vitro* Shoot multiplication; Sugarcane; 6-benzylamminopurine (BAP); kinetin.

Introduction

Sugarcane (*Saccharum officinarum* L.) is a monocotyledonous crop plant that belongs to the family of grasses, poaceae [1-4]. It is an octaploid crop with chromosome number of 2n = 80 [5-7]. It is a tall perennial tropical grass that tillers at the base, grows 3-4 meters tall and about 5 cm in diameter [8] with strong midrib, white and concave on the upper surface, convex and green below [9]. Sugarcane is one of the most efficient convertors of solar energy into sugar and other renewable forms of energy and hence produced primarily for its ability to store high concentrations of sucrose, or sugar, in the internodes of the stem [10]. Currently, the crop is cultivated in over 120 countries with estimated total annual global sugar production of 1.7 billion tonnes in the year 2011 [11] and the crop accounts for about 70% of the world's total sugar production [11-14] while the rest is produced from sugar beet. However, in Ethiopia, sugarcane is the sole base material for sugar production and thus the sugar industries in Ethiopia solely depend on the fate of this crop. The sugar industry in Ethiopia 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 [15,16].

However, the current sugar production in Ethiopia covers only 60% of the annual demand for domestic consumption while the deficit is imported from abroad. In spite of this fact, the country has huge production potentials and opportunities which include specifically identified irrigable suitable fertile areas of 1,390,000 ha [17], favorable weather conditions, cheap and productive labour force, high demand for sugar and other by-products and huge market outlets to the nearby countries [18]. To utilize these opportunities and reverse current situation: satisfy the local sugar demand and export the surplus; the Ethiopian Sugar Corporation is undertaking large scale expansion and new sugar development projects in different regions of the country. However, availability of adequate amount of quality and disease free planting materials within a short time is the major limiting factor to attain the intended plan using the conventional method of propagation. On the other hand, the yield of the existing few and old commercial cane varieties is declining and some productive sugarcane varieties were also obsolete due to lack of alternative technologies for disease cleansing and rejuvenation. Moreover, commercialization of improved introduced and adapted sugarcane varieties takes more than 10 years using the usual conventional route of propagation.

Varieties of sugarcane are highly heterogeneous and generally multiplied vegetatively by stem cuttings with each cutting or sett having two or three buds and the rate of propagation is very slow, usually 1:10 in a year [19-22]. Lack of rapid multiplication procedures, time spent for multiplication and continuous contamination by systemic disease is the serious economic problem to multiply an elite genotype of sugarcane in the open field [23-26]. In addition, the conventional propagation method requires large quantity of seed and land demanding [27,28] and the cutting implements in seed cane preparation play a significant role in facilitating cross contamination during seedcane preparation [29]. Besides the costly transport of the

bulky cane cuttings, harbor many pests and diseases with accumulation of disease over vegetative cycles leading to further yield and quality decline over the years [20,23]. Attacks by pathogens cause 10 -77% yield loss in sugarcane [30-32].

Micropropagation Technology is a technique through which groups of genetically identical plants all derived from a selected individual multiplies vegetativelly and rapidly by aseptic culture of meristematic regions under controlled nutritional and environmental conditions in vitro. Nowadays, unlike the conventional propagation method, it is the only realistic means of achieving rapid and large scale production of disease free quality planting materials in sugarcane [33-35] and an alternative approach for fast multiplication of a variety in its original form [19,36]. It is very effective in entire disease cleansing, rejuvenation and subsequent mass propagation of well adapted and promising varieties facing gradual deterioration in yield, quality and vigor due to accumulation of pathogens during prolonged vegetative cultivation and hence sustains the productive potential of sugarcane crops for a longer period [37,38]. Furthermore, micropropagated sugarcane plants were reported to give superior in cane and sugar yield as compared to their donors under similar agronomic management systems [38-43].

Considering the diverse limitations of conventional method and potential of tissue culture techniques, researchers have developed protocols for sugarcane in vitro propagation using shoot tip explants [28,44-47]. Every new variety or clone needs an efficient protocol to get rapid in vitro propagation [44]. 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 [28]. The nutritional requirement for in vitro propagation protocol of sugarcane should be according to genotype and explant used [46]. Similarly, plant growth regulators requirements for in vitro propagation responses vary from cultivar to cultivar in sugarcane [47]. The nutritional requirement for every sugarcane variety is specific and exact [48]. In addition, report on tissue culture study of sugarcane varieties grown in Ethiopian Sugar Estates is scarce. Therefore, this study was carried out with the objective to optimize protocol for in vitro shoot multiplication of two commercial sugarcane (B4-227 and N14) varieties widely grown in the Ethiopian Sugar Estates to solve the current challenges of sugarcane production in the country.

Materials and Methods

The same batch of micro-shoots initiated from shoot tip explants having similar size were used for the experiment after maintaining the initiated cultures on plant growth regulator free medium to minimize the carry over effect of the initiation medium. Full strength [48], (MS) medium was used with different concentrations and combinations of BAP (0, 0.5, 1, 1.5 and 2 mgL^{-1}) and kinetin (0, 0.1, 0.25, 0.5, and 1 mgL^{-1}) in a factorial treatment combinations arrangements along with 30 g/l sucrose as a carbon source. The pH of the medium was adjusted to 5.8 before gelled with 8 g/l agar and autoclaved at 121°C and 15 psi for 20 minutes. The experiment was carried out at a temperature of 25 ± 2°C less than 16 hours light and eight hours dark photoperiod regimes maintained under fluorescent light having 2500-3000 lux light intensity with 75 to 80% relative humidity of the growth chamber. The experiment was laid out in completely randomized design with three factor factorial treatment combinations arrangements: sugarcane varieties (B41-227 and N14) and two Plant growth regulators (BAP and kinetin). Data on number of shoots per explant, average shoot length (cm) and number of leaves per shoot was collected after 30 days of culture [49].

The collected data were subjected to analysis of variance (ANOVA) using SAS statistical software (*version 9.2*) and treatments' means were separated using the procedure of REGWQ (Ryan-Einot-Gabriel-Welsch Multiple Range Test).

Result and Discussion

Analysis of variance (ANOVA) revealed that the interaction effects of genotype, BAP and kinetin was very highly significant (Genotype *BAP* kinetin = $p<0.0001$) on the number of shoots per explant, average shoot length (cm) and number of leaves per shoot. Formation of multiple shoots did not occur within 30 days when explants were cultured on MS medium without plant growth regulators (Table 1). Increasing the concentration of kinetin alone from 0 to 0.1 mgL^{-1}, resulted in 3.5 ± 0.12 and 4.23 ± 0.12 shoots per explant for B41-227 and N14, respectively, while increasing levels of BAP alone from 0 to 0.5 mgL^{-1} gave 12.11 ± 0.22 and 4.53 ± 0.00 shoot per explant, respectively. Among the different concentration and combinations of BAP and kinetin used, B41-227 gave the highest average number of 34 ± 1.54 shoots with 6.95 ± 0.01 cm average shoot length and 12 ± 00 leaves per shoot on MS medium fortified with 1 mgL^{-1} BAP and 0.5 mgL^{-1} kinetin (Table 1 and Figure 1). However, N14 gave maximum of 21 ± 0.58 shoots per explant with 5.63 ± 0.01 cm average shoot length and 5.4 ± 00 leaves per shoot on MS medium supplemented with 2 mgL^{-1} BAP (Table 1 and Figure 2). Increase of kinetin from 0.25 to 0.5 mgL^{-1} at 1 mgL^{-1} BAP showed a significant increase in the number of shoots per explant (from 21.5 ± 0.27 to 34 ± 1.54), average shoot length (from 4.5 ± 0.00 to 6.95 ± 0.01 cm) and number of leaves per shoot (from 8.3 ± 0.00 to 12 ± 0.17) in B41-227. However, further increase in kinetin to 1 mgL^{-1}, significantly reduced the number of shoots per explant, average shoot length and number of leaves per shoot to 27.21 ± 0.29, 3.24 ± 0.00 cm and 8.3 ± 0.01, respectively. In the same way, increase in kinetin from 0 to 0.1 mgL^{-1} at 2 mgL^{-1} BAP, showed a marked reduction in number of shoots per explant (from 21 ± 0.58 to 16.5 ± 0.25), average shoot length (from 5.63 ± 0.01 to 2.51 ± 0.01cm) and number of leaves per shoot (5.4 ± 0.00 to 3.0 ± 0.15) in N14. Similarly, increase in levels of BAP from 1.5 to 2 mgL^{-1} at 0 mgL^{-1} kinetin showed a significant reduction in number of shoots per explant (from 17 ± 0.58 to 14.07 ± 0.25), average shoot length (from 2.7 ± 0.01 to 1.44 ± 0.28 cm) and number of leaves per shoot (from 7.23 ± 0.11 to 1.73 ± 0.11) in B41-227. This indicates that higher concentrations of cytokinins inhibit cell division and hence multiplication while low concentrations are suitable for cell division and elongation in sugarcane.

However, maintaining the concentration of kinetin at 0 mgL^{-1} and increase in BAP from 1.5 to 2 mgL^{-1} significantly increased the number of shoots per explant (from 12 ± 0.17 to 21 ± 0.58), average shoot length (from 5.46 ± 0.00 to 5.63 ± 0.01 cm) and number of leaves per shoot (from 3.57 ± 0.00 to 5.4 ± 0.00) in N14. This indicates the need to investigate the shoot multiplication responses in N14 at higher (more than 2 mgL^{-1}) levels of BAP. The current result in B41-227 is in contrast with the findings of [22] and [50]. On MS medium fortified with 1 mgL^{-1} BAP and 0.5 mgL^{-1} kinetin, sugarcane varieties CP-77-400 and BL-4 gave 24 ± 1.2490 and 19.6 ± 1.1833 shoots per explant [22]. Similarly, it was reported that 7.0 shoot per explant with 8.5 cm average shoot length and 24 leaves per shoot on the same treatment combination (i.e. MS +1 mgL^{-1} BAP +0.5 mgL^{-1} kinetin) for

sugarcane variety CP-77-400 after 30 days [50]. The result obtained in N14 is in line with the findings of [51].

PGRs (mg/l)		B41-227			N14		
BAP	**Kin**	**Number of shoots per explant**	**Shoot length (cm)**	**Number of leaves per shoot**	**Number of shoots per explant**	**Shoot length (cm)**	**Number of leaves per shoot**
0	0	$0.00^{t} \pm 0.00$	$0.00^{z} \pm 0.00$	$0.00^{v} \pm 0.00$	$0.00^{t} \pm 0.00$	$0.00^{z} \pm 0.00$	$0.00^{v} \pm 0.00$
	0.1	$3.50^{qr} \pm 0.12$	$1.62^{y} \pm 0.02$	$2.51^{r} \pm 0.00$	$4.23^{pq} \pm 0.12$	$2.50^{t} \pm 0.00$	$4.22^{gh} \pm 0.00$
	0.25	$5.82^{no} \pm 0.06$	$1.75^{x} \pm 0.01$	$3.37^{lmn} \pm 0.54$	$4.40^{pq} \pm 0.00$	$1.63^{y} \pm 0.01$	$4.40^{fg} \pm 0.12$
	0.5	$5.00^{op} \pm 0.20$	$2.33^{u} \pm 0.01$	$3.00^{op} \pm 0.17$	$5.31^{op} \pm 0.8$	$1.51^{xz} \pm 0.00$	$3.50^{klm} \pm 0.00$
	1	$1.80^{s} \pm 0.06$	$5.21^{e} \pm 0.00$	$2.62^{qrs} \pm 0.00$	$4.20^{pq} \pm 0.17$	$1.05^{yz} \pm 0.03$	$3.20^{hi} \pm 0.16$
0.5	0	$12.11^{jk} \pm 0.21$	$4.80^{g} \pm 0.00$	$5.12^{e} \pm 0.14$	$4.53^{pq} \pm 0.00$	$2.30^{u} \pm 0.80$	$4.53^{f} \pm 0.00$
	0.1	$13.40^{i} \pm 0.06$	$4.63^{h} \pm 0.05$	$4.50^{f} \pm 0.00$	$5.20^{op} \pm 0.12$	$2.73^{pq} \pm 0.00$	$5.11^{e} \pm 1.57$
	0.25	$14.20^{hi} \pm 0.29$	$5.42^{d} \pm 0.17$	$4.09^{hi} \pm 0.58$	$9.20^{lm} \pm 0.00$	$2.53^{st} \pm 0.01$	$3.52^{klm} \pm 0.00$
	0.5	$9.20^{lm} \pm 0.06$	$5.04^{f} \pm 0.38$	$3.84^{ij} \pm 0.17$	$8.51^{m} \pm 0.00$	$2.80^{p} \pm 0.05$	$2.72^{qr} \pm 0.00$
	1	$8.53^{m} \pm 0.00$	$2.17^{v} \pm 0.00$	$3.70^{jk} \pm 0.00$	$6.00^{no} \pm 0.11$	$2.68^{qr} \pm 0.02$	$2.50^{r} \pm 0.57$
1	0	$13.50^{i} \pm 0.12$	$1.88^{w} \pm 0.01$	$5.00^{e} \pm 0.00$	$11.25^{k} \pm 0.00$	$3.54^{m} \pm 0.0i$	$2.42^{s} \pm 0.00$
	0.1	$19.41^{e} \pm 0.00$	$4.31^{j} \pm 0.00$	$5.15^{c} \pm 0.01$	$9.42^{lm} \pm 0.00$	$3.90^{k} \pm 0.01$	$3.33^{lmn} \pm 0.00$
	0.25	$21.50^{d} \pm 0.27$	$4.50^{i} \pm 0.00$	$8.30^{b} \pm 0.00$	$9.37^{m} \pm 0.11$	$3.76^{kl} \pm 0.00$	$3.84^{ij} \pm 0.58$
	0.5	$34.00^{a} \pm 1.54$	$6.95^{a} \pm 0.01$	$12.00^{a} \pm 0.17$	$9.33^{lm} \pm 0.16$	$3.73^{l} \pm 1.13$	$2.51^{r} \pm 0.00$
	1	$27.21^{b} \pm 0.29$	$3.24^{no} \pm 0.00$	$8.30^{b} \pm 0.01$	$4.50^{pq} \pm 0.00$	$2.61^{rs} \pm 0.00$	$2.40^{s} \pm 0.00$
1.5	0	$17.00^{fg} \pm 0.58$	$2.70^{qr} \pm 0.01$	$7.23^{c} \pm 0.11$	$12.00^{k} \pm 0.17$	$3.16^{o} \pm 0.00$	$2.82^{pq} \pm 0.58$
	0.1	$24.17^{c} \pm 0.11$	$2.50^{t} \pm 0.00$	$3.11^{no} \pm 0.00$	$13.05^{ij} \pm 1.64$	$3.64^{l} \pm 1.80$	$3.54^{klm} \pm 0.02$
	0.25	$19.23^{e} \pm 0.23$	$4.52^{i} \pm 0.17$	$4.20^{gh} \pm 0.17$	$13.13^{ij} \pm 0.09$	$4.50^{i} \pm 1.43$	$3.00^{op} \pm 0.00$
	0.5	$13.33^{i} \pm 0.17$	$6.23^{b} \pm 0.00$	$2.82^{pq} \pm 0.03$	$12.18^{jk} \pm 0.3$	$3.33^{n} \pm 0.00$	$3.30^{mn} \pm 0.00$
	1	$12.00^{k} \pm 0.17$	$5.51^{d} \pm 0.12$	$3.22^{no} \pm 0.17$	$12.13^{jk} \pm 1.4$	$5.46^{d} \pm 0.00$	$3.57^{kl} \pm 0.00$
2	0	$14.07^{hi} \pm 0.25$	$1.44^{xz} \pm 0.28$	$1.73^{u} \pm 0.11$	$21.00^{d} \pm 0.58$	$5.63^{c} \pm 0.01$	$5.40^{d} \pm 0.00$
	0.1	$3.53^{qr} \pm 0.06$	$1.15^{yz} \pm 0.58$	$1.50^{u} \pm 0.11$	$16.50^{g} \pm 0.25$	$2.51^{t} \pm 0.01$	$3.00^{op} \pm 0.15$
	0.25	$3.50^{qr} \pm 1.27$	$1.80^{wx} \pm 0.00$	$2.44^{s} \pm 0.28$	$13.00^{ij} \pm 0.28$	$2.50^{t} \pm 0.00$	$2.43^{s} \pm 0.00$
	0.5	$1.80^{m} \pm 0.21$	$1.59^{xz} \pm 0.00$	$3.00^{op} \pm 0.17$	$5.28^{op} \pm 0.17$	$2.50^{t} \pm 0.05$	$2.13^{t} \pm 0.28$
	1	$1.82^{m} \pm 0.21$	$1.15^{yz} \pm 0.00$	$1.51^{u} \pm 0.00$	$4.50^{pq} \pm 0.00$	$2.19^{v} \pm 0.24$	$3.00^{op} \pm 0.47$
CV (%)		4.65	3.62	5.34	4.65	3.62	5.34

Table 1: Effect of 6-Benzylaminopurine (BAP) and Kinetin on in vitro shoot multiplication of B41-227 and N14 after 30 days

PGRs= Plant growth regulators. *Values for number of shoots per explant, average shoot length and number of leaves per shoot given as mean ± SD. *Numbers with in the same column with different letter(s) are significantly different from each other at $p \leq 0.05$ according to REGWQ Multiple Range Test.

Figure 1: *In vitro* shoot multiplication of B41-227 at 1 mgL^{-1} BAP and 0.5 mgL^{-1} Kin after 30 days

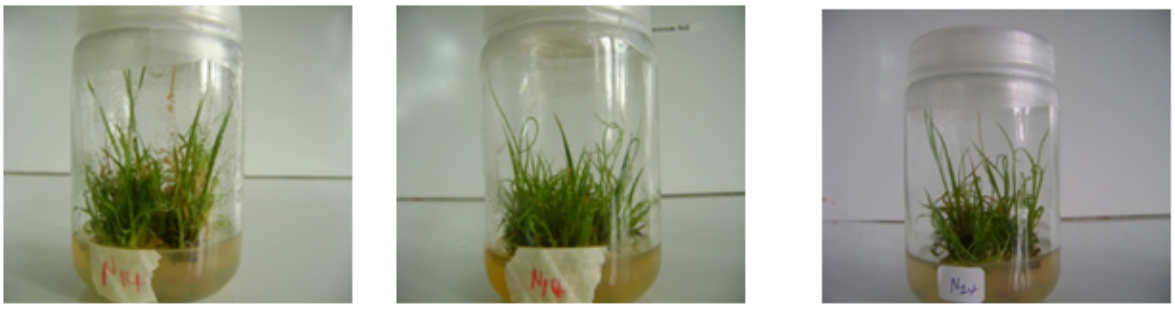

Figure 2: In vitro shoot multiplication of N14 at 2 mgL^{-1} BAP after 30 days

Conclusion

In vitro shoot multiplication of two commercial sugarcane varieties 'B41-227' and 'N14' has been developed. The result indicated that in vitro shoot multiplication of sugarcane is highly dependent on the interaction effects of BAP, kinetin and genotype. Murashige and Skoog (MS) medium fortified with 1 mgL^{-1} BAP and 0.5 mgL^{-1} kinetin for B41-227 and 2 mgL^{-1} BAP without kinetin for N14 were found to be optimum. On these medium, B41-227 gave 34 ± 1.54 shoots per explant with 6.95 ± 0.01 cm average shoot length and 12 ± 0.17 leaves per shoot while N14 produced 21 ± 0.58 shoots per explant with 5.63 ± 0.01 cm average shoot length and 5.4 ± 00 leaves per shoot after 30 days of first culture transfer to multiplication medium. Thus, the developed protocol will help minimize the current challenges of sugarcane production by rejuvenating and availing adequate amount of quality disease free planting material of the sugarcane varieties within a short time.

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