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In Vitro Plant Regeneration of Sugarcane (*Saccharum* spp.) Variety Inoculated Under Different Levels of Plant Growth Regulators

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Abstract

Sugarcane (*Saccharum* spp.) is an excellent candidate for mass propagation using tissue culture as seed multiplication using the conventional method is time consuming. The main objective of this study was to optimize an efficient and reliable protocol for direct propagation of sugarcane varieties under *in vitro* condition. Different levels of BAP along with kinetin were used for shoot proliferation. The proliferated shoots were subjected to MS medium supplemented with different levels of NAA. Results of the present study revealed both the BAP and Kinetin had a synergistic effect and none of them generated the maximum response while separately applied. The maximum number of shoots (10.3 ± 0.31), shoot length (3.77 ± 0.40) and number of leaves (3.87 ± 0.17) were recorded on the MS medium supplemented with 2 mg/l of BAP and 1.5 mg/L of Kinetin. In the rooting medium, highest rooting frequency (100%), root length (2.21 cm) and number of roots per shoots (20.13) were recorded on the half strength MS semi-solid media fortified with 5 mg/L NAA. The better rooted plantlets were transferred to green house for hardening under pot comprised of sand, soil and farm yard manure in 1:1:1 ratio and 100% of the well rooted plantlets survived under greenhouse conditions.

Keywords: Acclimatization; Fortified; Kinetin; Micro-propagation; Proliferation

Introduction

Sugarcane (*Saccharum officinarum* L.) is a tall perennial grass with 2n=80 chromosomes number and has distinctive feature of partitioning carbon into sucrose in the stem. Sugarcane has a sucrose content of 10-18% and a fiber content of 10-15% at harvest. It is an important industrial crop accounting for nearly 70% of sugar produced worldwide [1] and with an employment opportunity to over more than 100 million people across the world, both in rural and urban industrial sector. The significance of this crop has augmented in many folds in the globe because of ethanol, a biofuel which is derived from cane.

Sugarcane is conventionally propagated through stem cutting or setts containing two or three buds. This propagation method has a limitation in the availability of disease-free material for planting; slow rate of propagation (1:8) [2] and also requires large planting materials and area. Tissue culture technology is the best apt method for mass multiplication of vegetative propagated crops, including sugarcane. The technology offers best procedure to obtain quality seed material in shorter period of time [3,4] and can augment the propagation potential by 20-35 times [5,6]. Research on *in vitro* culture of sugarcane began in the 1960s and subsequently numerous protocols have been documented. Earlier, *in vitro* shoot tip culture for mass propagation of sugarcane was reported by several authors [7,8]. However, the nutritional and exogenous growth hormone requirements for *in vitro* culture vary according to genotype [9,10] as well as explants used.

In Ethiopia, the crop is commercially cultivated on about 93,000 ha at different agro-ecological conditions of the country. As part of the expanding Sugar Development in Ethiopia, the construction and installation of many Sugar Factories have been completed and the existing command area for sugarcane production is under increase accordingly. For this reason, many sugarcane varieties have recently been promoted for commercial purpose as alternative to the few dominant varieties under cultivation in Ethiopia. N52 is one of the recently released sugarcane varieties and fast seed multiplication system is required to commercialize it in short period of time. However, no protocol is developed for *in vitro* mass propagation of the variety as protocol for *in vitro* mass propagation in sugarcane is variety specific. Thus, optimization of *in vitro* propagation protocol for this variety is very important. Therefore, the present study was conducted to determine the appropriate level of growth regulators on *in vitro* mass propagation of N52 sugarcane variety.

Materials and Methods

The *in vitro* experiment work was undertaken at National Agricultural Biotechnology Laboratory (NABL), Holetta, Ethiopia, in 2015. Plant materials were provided by Sugar Corporation, Research and Development Center (SCRDC). Sugarcane elite variety N52 released by SCRDC, Ethiopia, was used as source of the explants. The two-budded young plant cane setts were treated with hot water at 50°C for 2 hours followed by immersing in fungicide (Bayleton® DF 50%) solution at rate of 1 g/l for 5 minutes. The treated setts were planted in plastic pots containing mixture of autoclaved forest soil, farmyard manure and river sand in the ratio of 1:1:1 and allowed to grow in a screen house of NABL.

Explants Preparation and Sterilization

Stem segments with 10 cm size, together with meristem tip were excised from healthy five months old sugarcane plants were used as explant. The spindle segments were thoroughly washed under running tap water for 30 minutes. Subsequently, the explants were washed

gently with 2% detergent solution with two drops of tween 20 for 30 minutes and then rinsed with sterile DDH_2O (double distilled water) three times. The explant is then transferred to laminar air flow cabinet. The washed explants were treated with 70% alcohol for 30 second to one minute, followed by another treatment in 2% (w/v) sodium hypochlorite (NaOCl) for another 20 minutes. Finally, rinsed thoroughly 3 to 5 times with sterile distilled water and then 1.5 cm sized shoot tips were aseptically excised before the inoculation into sterilized nutrient agar media pre-packed in culture jars.

Culture Media Preparation Under Aseptic Conditions

Throughout the experiment, the MS medium [11] was supplemented with 3% w/v (for shoot regeneration and multiplication) and 5% w/v (for rooting) sucrose (table sugar); and 0.45% agar (w/v) (agar type-II) was used as culture medium throughout the experiment. Different plant growth regulators, singly and in combinations were added to the medium as specified below. pH was adjusted to 5.8 using 1N NaOH or 1N HCl prior to autoclaving at 120°C for 15 min. All the cultures were maintained at 25 ± 2°C under 16/8 h light/dark regime, a photo flux density of 30 μ mol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes and at 55-60% relative humidity in culture room.

Treatment Combinations and Culture Mediums

The experiment was laid out in completely randomized design (CRD) and replicated five times. For shoot induction, shoot tip explants with 1.5 cm size were placed on MS medium supplemented with BA, Kin and NAA at 0.5 mg/l, each. For shoot multiplication, healthy micro-shoots having the same size obtained from the initiation stage were cultured on the media supplemented with different concentrations and combinations of BAP (0, 0.5, 1.0, 1.5 and 2 mg/l) and kinetin (0, 0.5, 1.0 and 1.5 mg/l). The experiment was laid out in completely randomized design (CRD) and each treatment was replicated four times. Elongated healthy shoots (3-4 cm) were excised and cultured on root induction media comprising half-strength MS medium augmented with NAA at different concentrations (3, 5 and 7 mg/l).

Acclimatization of Well Rooted *In Vitro* Regenerated Plantlets

Well-developed plantlets were removed from rooting medium and washed thoroughly in running tap water and then transferred to plastic tray containing sterile sand, soil and farm yard manure (in 1:1:1 ratio) to acclimatize under *in vivo* condition. Moreover, the plantlets were covered with transparent plastic sheet and red cheese cloth ensures high humidity and watered every day. After 2 weeks, the plastic was removed, and the plastic trays were left open. After four weeks, acclimatized plants were transferred to the pots containing normal forest soil, farm yard manure and river sand in 1:1:1 ratio and maintained in greenhouse under normal day length conditions.

Data Collection and Analysis

At the end of shoot regeneration, data for shoot induction and multiplication were recorded four weeks after inoculation. Four weeks after shoot culture, data for percent of explants (shoots) regenerated roots, number of roots and average root length per explant were collected. The data were subjected to analysis of variance (ANOVA) using SAS program Version 9.2 (SAS software package, 2009). Treatments' means were separated ($P \leq 0.05$) using the procedure of REGWQ (Ryan-Einot-Gabriel-Welsch).

Results and Discussion

Effect of 6-benzylaminopurine (BAP) and kinetin (Kn) on shoot regeneration

In the analysis of variance (Table 1), results show the BAP, Kn and the interaction effects were all highly significant ($P<0.01$) for number of shoots per explant, average shoot length and number of leaves. The difference among the levels of BAP and Kn effects for number of shoots per explant demonstrated the increasing or decreasing levels of BAP and Kn brought a significant change on the performance of shoot regenerants. Moreover, the significance of the interaction between both effects suggested that the performance of the shoot regenerants were inconsistent across levels of BAP and Kn in the MS medium.

Sources of variance	Number of Shoots/ explant	Shoot length (cm)	No. of leaves/ shoot
Rep	ns	ns	ns
BAP	24.82**	1.19**	0.13**
Kinetin	6.48**	0.82**	0.18**
BAP*Kinetin	3.89**	0.84**	0.13**
Error	0.40	0.32	0.21
CV (%)	5.89	10.56	5.79
Means	6.74	3.08	3.70
**=highly significant at 1% CV=Coefficient of Variation ns=non-significant			

Table 1: Mean squares for Number of Shoots/explants, shoot length (cm) and No. of leaves/shoot evaluated under MS medium supplemented with different levels of BAP and Kinetin.

As the interaction was significant, multiple mean comparisons were conducted to separate the interaction means for numbers of shoots, shoot length and number of leaves/shoot (Table 2). Results from the mean separation revealed that the MS medium supplemented with 2 mg/lt BAP and 1.5 mg/lt kinetin produced significantly highest number of shoots per explant (10.73 ± 0.35) while the lowest shoot number per explant (3.60 ± 0.20) produced on MS medium fortified with a combination of BAP and kinetin at 1 mg/l and 1.5 mg/l, respectively. From these combinations, we can conclude that highest numbers of shoots can be regenerated when the explants were inoculated in to the MS medium with 2 mg/lt BAP and 1.5 mg/lt kinetin. On the contrary, the shoot length measured under the MS medium supplemented with 2 mg/lt BAP and 1.5 mg/lt kinetin was not significantly different from the shoot length measured in MS medium supplemented with 0 mg/lt BAP and 0.5 mg/lt Kn. Similarly, the numbers of leaves/shoot (3.87 ± 0.17) recorded at MS medium with 2 mg/lt BAP and 1.5 mg/lt was similar (means are statistically the same) to the numbers of leaves (3.69 ± 0.38) at recorded at MS medium supplemented with no BAP and Kn (0 mg/lt BAP and 0 mg/lt Kn). Generally, only the numbers of explants were more affected by the increasing levels of BAP and Kn in the MS medium. This is the most important trait that determines the numbers of explants for root

regeneration. Results of the present investigations were consistent with reports of many workers [12,13] who observed rapid shoot multiplication when the levels of BAP and Kin in the MS medium was increased.

BAP (mg/l)	Kn (mg/l)	No. of shoots /explant	Shoot Length (cm)	No. of leaves /shoot
0	0	$4.33^{ij} \pm 0.42$	$3.04^{b-e} \pm 0.29$	$3.69^{ab} \pm 0.38$
0	0.5	$5.80^{fgh} \pm 0.26$	$3.67^{ab} \pm 0.30$	$3.69^{ab} \pm 0.32$
0	1	$6.27^{efg} \pm 0.06$	$3.80^{ab} \pm 0.20$	$3.62^{ab} \pm 0.41$
0	1.5	$5.00^{hi} \pm 0.36$	$4.00^{a} \pm 0.20$	$3.55^{ab} \pm 0.04$
0.5	0	$5.20^{ghi} \pm 0.26$	$2.55^{de} \pm 0.05$	$3.84^{a} \pm 0.17$
0.5	0.5	$6.50^{ef} \pm 0.26$	$3.37^{a-d} \pm 0.17$	$3.52^{ab} \pm 0.34$
0.5	1	$8.13^{cbd} \pm 0.23$	$3.56^{abc} \pm 0.34$	$3.85^{a} \pm 0.17$
0.5	1.5	$7.13^{de} \pm 0.31$	$2.69^{cde} \pm 0.23$	$3.42^{ab} \pm 0.04$
1	0	$6.60^{ef} \pm 0.20$	$2.25^{e} \pm 0.50$	$3.87^{a} \pm 0.14$
1	0.5	$7.10^{de} \pm 0.36$	$2.39^{e} \pm 0.53$	$3.53^{ab} \pm 0.06$
1	1	$6.27^{efg} \pm 0.76$	$2.66^{cde} \pm 0.37$	$3.41^{ab} \pm 0.08$
1	1.5	$3.60^{j} \pm 0.20$	$2.70^{cde} \pm 0.57$	$3.78^{a} \pm 0.03$
1.5	0	$7.93^{cbd} \pm 0.50$	$3.02^{b-e} \pm 0.27$	$3.93^{a} \pm 0.24$
1.5	0.5	$8.53^{b} \pm 0.31$	$2.55^{de} \pm 0.16$	$3.80^{a} \pm 0.00$
1.5	1	$8.67^{b} \pm 0.70$	$2.27^{e} \pm 0.26$	$3.75^{ab} \pm 0.39$
1.5	1.5	$7.40^{cde} \pm 0.87$	$3.70^{ab} \pm 0.39$	$3.14^{b} \pm 0.11$
2	0	$5.45^{fgh} \pm 0.12$	$3.16^{a-e} \pm 0.17$	$3.78^{a} \pm 0.04$
2	0.5	$6.47^{efg} \pm 0.42$	$3.17^{a-e} \pm 0.28$	$3.99^{a} \pm 0.01$
2	1	$8.50^{bc} \pm 0.10$	$3.19^{a-e} \pm 0.20$	$3.89^{a} \pm 0.14$
2	1. 5	$10.73^{a} \pm 0.35$	$3.77^{ab} \pm 0.40$	$3.87^{a} \pm 0.17$

*Means with different letters in each column represent significant difference based on Tukey's multiple comparison tests at α=0.05

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

Effect of Naphthalene Acetic Acid on *In Vitro* Rooting of Micro-Shoots

Like in the shoot regeneration, the effect of NAA was strong and high significant ($P<0.01$) on days for root formation, rooting frequency, number of roots per shoot and root length (Table not presented). Table 3 presents means of plantlets evaluated under MS medium supplemented with different levels of NAA were separated for root emergence, rooting percentage, number of roots per shoot and root length. Earlier root formation was observed on half strength MS medium fortified with 5 mg/lt of NAA (7 days) followed by 9 days of root formation at 7 mg/l NAA. Among the given concentrations, percent of shoot explants evaluated in MS medium with less than 3 mg/l NAA was lowest (87.5%) followed by 7 mg/l (97.5%) while all of the shoots (100%) inoculated in MS medium supplemented with 5 mg/l of NAA were successfully rooted. Generally, results of the mean separation showed smallest numbers of days to emergence (earlier root formation, highest percent of explants regenerated roots, highest numbers of roots/shoot and longest root length in MS medium fortified with 5 mg/L of NAA.

NAA level	Days for root Emergence	Rooting Percentage	No of roots per shoot	Root length
3 mg/L	10^{C}	87.5^{b}	13.30^{b}	1.77^{b}
5 mg/L	7^{a}	100^{a}	20.13^{a}	2.21^{a}
7 mg/L	9^{b}	97.5^{a}	12.68^{b}	1.38^{c}
LSD	0.93	8.06	1.91	0.43
CV	5.44	4.3	6.29	12.29

Table 3: Effect of Naphthalene acetic acid (NAA) on days for root emergence, rooting percentage, number of roots per shoot and root

length. *Means with different letters in each column represent significant difference based on LSD test at α=0.05.

This could be because of the auxins stimulate plant cell to produce ethylene and this resulted in retardation of root elongation [15]. The increase or decrease in concentration of this hormones led to variability of root induction percentage. Results of the present study are consistent with the results of previous studies [16-18].

Acclimatization of *in vitro* regenerated plantlets

Regenerants identified as well rooted under *in vitro* condition (Figures 1a-1c) survived well at greenhouse (Figure 1d); an indication of the validity of our *in vitro* experiment. After four weeks of acclimatization in green house, plantlets were transferred to the plastic pots containing normal forest soil, farm yard manure and river sand in 1:1:1, ratio, maintained in greenhouse under normal day length conditions and the hardened plantlets were exhibit 100% survival (Figure 1e).

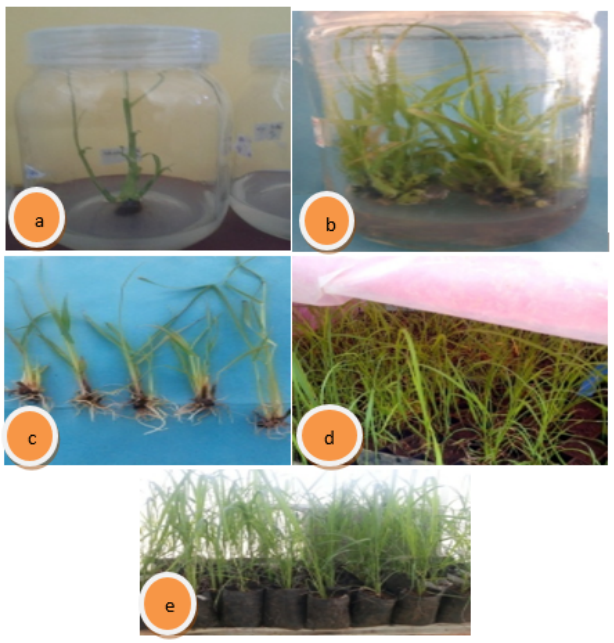

Figure 1: *In vitro* propagation of sugarcane: (a) Shoots regenerated from shoot tip on MS medium supplemented with BAP, Kn and NAA 0.5 mg/l each (b) Multiple shoot formed on MS medium fortified with 1 mg/l BAP and 1.5 mg/l Kn after first subculture (c) *In vitro* rooted shoots on MS supplied with 5 mg/l NAA (d) well rooted plantlets under greenhouse acclimatization (e) Tissue culture raised plants in pots 3 months after acclimatization.

Conclusion

Results of the present study indicated the protocol for mass propagation of sugarcane variety under investigation (N52) was successfully developed. 2 mg/l BAP and 1.5 mg/l Kinetin growth regulators was the best media combination for shoot regeneration and multiplication. On the other hand, half strength MS semi-solid medium fortified with 5 mg/l NAA+50 g/l sucrose was the combination for root regeneration. Therefore, micro-propagations aimed at increasing the seed rate of this sugarcane variety should use the above media combinations. As the variety was responsive to the different levels of the growth hormones, future *in vitro* experiments should be conducted to see the response of this variety to different types of growth hormones.

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