

Ex Vitro Rooting of Sugarcane (*Saccharum officinarum* L.) Plantlets Derived from Tissue Culture

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Abstract

A study was conducted at Holetta National Agricultural Biotechnology Laboratory with the objective to determine the effect of different concentrations of NAA on *ex vitro* root development of sugarcane microshoots. Five levels of NAA (0, 10, 20, 30 and 40 mg/l) and two levels of genotypes were combined in factorial arrangement. The basal end of the shoots was dipped in NAA solution overnight before the shoots were transferred into a plastic tray containing a mixed growing medium in green house. The results showed that interaction effect of genotype and NAA was highly significant ($p < 0.0001$) on rooting percentage, number of roots per shoot, root length. In genotype N52, best root formation was found on the shoots treated with 20 mg/l NAA by which rooting percentage was 76 ± 5.48 with 5.88 ± 0.04 cm root length and 8.06 ± 0.13 number of roots per plantlets. While in genotype N53 maximum, root formation was recorded on the shoots dipped in 30 mg/l NAA by which rooting percentage was 70 ± 7.07 with 5.42 ± 0.11 cm root length and 4.52 ± 0.19 number of roots per plantlets. Shoots rooted through this method exhibited 100 % survival in both genotypes.

Keywords: Microshoot; *Ex vitro*; NAA; *In vitro*; Medium

Introduction

Sugarcane (*Saccharum officinarum* L.) is a monocotyledonous crop that is grown in the tropical and subtropical regions of the world, and almost cultivated on over 23.8 million ha for its sucrose rich stalk [1]. In Ethiopia, it is cultivated commercially on around 96,000 ha with annual sugar production of 370,000 ton and is a solely raw material for sugar production in the country. Recently, in Ethiopia, sugarcane production has gained attention, allied with its important potential for an environment-friendly bio-fuel (ethanol) production, in creating job opportunity to the nation and generating huge electric power [2].

Sugarcane, since commercially propagated vegetatively by stem cutting, has a low seed multiplication rate (1:10) which resulted in slow seed production of newly released improved varieties. Furthermore, the seed builds up diseases and pests during several cycles of field production, which leads to further yield and quality declines over years [3]. Thus, unavailability of disease-free, true to type planting material is a major limitation in improving sugarcane productivity.

Recently, tissue culture technology plays a leading role in rapid multiplication of disease-free and quality planting material of sugarcane [4]. Accordingly, Ethiopian Sugar Corporation has established its own tissue culture laboratory at Metahara, Kuraz, Tendaho and Fincha sugar factories/projects to produce about 55 million disease free plantlets per year [2]. To do so, *in vitro* propagation protocols have been developed using shoot tip and callus explant for several sugarcane cultivars of Ethiopian Sugar Estates [5-8].

In vitro propagation involves four crucial steps namely, initiation, multiplication and rooting of microshoots and acclimatization of plantlets. However, *in vitro* rooting process is an expensive, labour consuming process and can even double the final price of micropropagated plants. Earlier report shown that *in vitro* rooting may account for 40% total cost of the intensive manipulation needed during *in vitro* propagation [9]. In addition, roots of plantlets raised *in vitro* are generally very fragile and not have root hairs [10]. Therefore, during early acclimatization period, the roots do not function properly

to support the plantlets to absorb water and nutrients from the potting medium. *Ex vitro* rooting is more advantageous than *in vitro* rooting in reducing cost of labour, chemicals and equipments, and the time of establishment from laboratory to soil revealed that *ex vitro* rooting reduced more than 50% cost of sugarcane plantlet raised by conventional micropropagation [11-13]. Besides, the plantlets produced after *ex vitro* rooting have better developed root system than the ones produced after *in vitro* rooting [14,15]. Furthermore, rooting and acclimatization phase can be carried out simultaneously; hence, it is more time efficient.

Ex vitro rooting has been applied in micropropagation of various plants species [11,12,16], but there are very limited reports yet on *ex vitro* rooting of sugarcane plantlets. Most reports of *ex vitro* rooting of plant species have involved treatment with exogenous auxin such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 1-naphthalene-acetic acid (NAA) [12,13,16]. Auxin is applied singly or in a combination at different concentrations to improve rooting frequency of plantlets in the acclimatization period. Therefore, this study was aimed to determine the effect of different concentrations of auxin, NAA on *ex vitro* rooting of two elite sugarcane genotypes.

Materials and methods

The study was undertaken at the National Agricultural Biotechnology Laboratory of the Ethiopian Institute of Agricultural Research, in Holetta. The study was undertaken with two elite sugarcane genotypes

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Received February 04, 2016; Accepted March 17, 2016; Published March 23, 2016

Citation: Tesfa M, Admassu B, Bantte K (2016) *Ex Vitro* Rooting of Sugarcane (*Saccharum officinarum* L.) Plantlets Derived from Tissue Culture. Adv Crop Sci Tech 4: 215. doi:10.4172/2329-8863.1000215

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viz. N-52 and N-53 obtained from Ethiopian Sugar Corporation, Research and Training Division. The genotypes were selected based on their higher yield performance and sugar quality. In order to carry out explant sterilization and preparation, actively growing shoot tops were excised from 5-months-old screen house grown healthy mother plants of genotype N52 and N53. The trimmed shoot tops (segment) were washed carefully under running tap water for 30 minutes, and reduced to 10 cm length by cutting off at the two ends. Then washed thoroughly for 30 minutes with tap water containing a drop of liquid detergent solution and two drops of tween-20 and rinsed three times with double distilled water. Subsequently, the explant was taken to a laminar air flow cabinet and immersed in 0.1% (w/v) Bavistin® DF 50 % (Carbendizem) 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 sterile double distilled water. The shoot tips were washed again with 70% ethanol for one minute and rinsed with sterile double distilled water three times each for five minute to remove residual ethanol from the shoot tip surface. Finally, surface sterilized with 50 % (v/v) aqueous solution of Sodium hypochlorite (5.25% w/v active chlorine) containing a few drops of tween-20 for 25 minutes. After pouring out sodium hypochlorite solution, the explants were rinsed with sterile double distilled water three times each for five minutes to remove all the trace of the sterilant.

Culture were initiated on [17] medium fortified with BAP, Kinetin and NAA (0.5 mg/l each) [18] and 2% sucrose (w/v) and solidified using agar (agar agar, type I) (0.45 %; w/v) for induction of shoots. The pH of the medium was adjusted to 5.8 followed by autoclaving at 121°C at 105 Kpa pressure for 20 minutes. After 30 days of inoculation the regenerated shoots were transferred to multiplication medium supplemented with 2 mg/l BAP + 0.5 mg/l Kinetin (N52) and 1.5 mg/l BAP and 0.5 mg/l Kinetin (N53) [19]. After 30 days of incubation, shoots were maintained on plant growth regulators (PGRs) free MS medium with 2 g/l activated charcoal for two weeks before transferring rooting stage in order to avoid the carry over effect of hormones from the multiplication media on *ex vitro* rooting of the plantlets.

After 45 days, healthy micro-shoots having 4 cm heights were employed for *ex vitro* rooting study. The clumps of *in vitro* shoots were separated to obtain single micro shoot. The basal portion of these rootless microshoots was dipped in distilled aqueous solution containing auxin, NAA at different concentrations i.e. 0, 10, 20, 30, & 40 mg/l overnight to induce rooting under *ex vitro* condition. The experiment was arranged in completely randomized design (CRD) with five replications and each treatment had 50 microshoots. After treated with auxins, the shoots were transferred to polystyrene trays containing autoclaved mixture of river sand and forest soil in 2:1 ratio. Subsequently, maintained in greenhouse, which uses Fan-Pad evaporative cooling system providing 25–30°C temperature. During experimenting, high humidity level (80 %–85 %) was maintained by covering the tray with moisten polyethylene sheet and red shade cloth and then sprinkled with water three times a day as necessary and sprayed with quarter strength MS basal medium at weekly interval.

After 4 weeks, the plantlets were carefully removed from the soil mix and data on number of rooted shoots, total number of primary roots and root length were recorded. All microshoots that remain green were considered living and used in calculating rooting percentage. Successfully rooted plantlets were subsequently transferred in medium polyethene bags (15 cm × 20 cm) containing mixture of sand, farm yard manure and soil in 1:1:1 ratio for further hardening and data on survival rate of the plantlets was recorded 4 weeks after transplanting. The collected data were subjected to analysis of variance (F test) using SAS program (Version 9.2). The differences among treatment means were determined by REGQ multiple range test at P<0.05.

Results and Discussion

Statistical analysis of variance showed that the main effect of genotype and NAA and the interaction effect of genotype and NAA highly significant (p<0.0001) on rooting percentage, number of roots per shoot, root length of the two sugarcane genotype (Table 1). The present result also showed that rooting was induced *ex vitro* over the entire range of NAA concentration tested including the control shoots in both sugarcane genotypes (Table 2).

In the control treatment reduced rooting frequency of 36% and 28% were obtained in genotypes N52 and N53, respectively (Figure 1A and Figure 1B) However, in NAA treated microshoots than 50% of the shoot developed roots regardless of the NAA concentration (Table 2). Shekafandeh [12] observed increased rooting frequency and number of roots from zero percent in untreated shoots to 91.7% and 3.3 roots per shoot, respectively, when the basal end of the shoots were dipped in a solution of 1.5 mg/l IAA and 0.3 mg/l IBA for 24 h before culturing in soil mixture in Myrtle (*Myrtus communis* L.) plant. Similar results were also reported by Sumaryono and Riyadi [20] in oil palm (*Elaeis*

Source of variation	DF	Rooting Percentage	Root length (cm)	Number of roots per shoot
		MS	MS	MS
Genotype	1	968***	7.76***	59.19***
NAA	4	2307***	4.73***	25.56***
Genotype*NAA	4	163***	2.08***	4.46***
CV %		10.38	5.60	2.72

*** = Very highly significant at P≤0.0001; DF = Degree of freedom; NAA = α-naphthalene acetic acid; MS = Mean square; CV= Coefficient of variation

Table 1: Effect of different concentration of NAA on *ex vitro* rooting.

Treatment	Genotypes							
	NAA mg/l	N52			N53			
		Rooting Percentage	Root length (cm)	Number of root per shoot	Rooting Percentage	Root length (cm)	Number of root per shoot	per shoot
0	36 ^d ± 5.48	4.44 ^{cd} ± 0.30	2.14 ^h ± 0.13	28 ^d ± 0.47	2.58 ^f ± 0.54	1.74 ⁱ ± 0.05		
10	68 ^{ab} ± 4.47	4.64 ^{cd} ± 0.29	5.42 ^d ± 0.08	50 ^c ± 7.07	4.32 ^{ed} ± 0.24	2.56 ^g ± 0.13		
20	76 ^a ± 5.48	5.88 ^a ± 0.04	8.06 ^a ± 0.13	60 ^{bc} ± 7.07	4.34 ^{ed} ± 0.21	4.08 ^f ± 0.08		
30	70 ^{ab} ± 7.07	5.04 ^{bc} ± 0.05	6.36 ^b ± 0.11	70 ^{ab} ± 7.07	5.42 ^b ± 0.11	4.52 ^e ± 0.19		
40	56 ^c ± 5.48	4.68 ^{cd} ± 0.24	5.68 ^c ± 0.13	54 ^c ± 5.48	4.08 ^e ± 0.19	3.88 ^f ± 0.13		
CV%	10.38	5.60	2.72	10.38	5.60	2.72		

*NAA =α-naphthalene acetic acid. Values in the same column and variables with different letters are significantly different from each other according to REGWQ at P<0.05.

Table 2: The effect of NAA on rooting percentage, root length and number of roots per shoot.

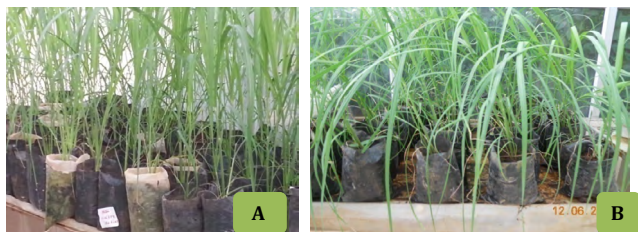


Figure 1: Acclimatized plantlets. (A) Genotype N52 (B) Genotype N53.

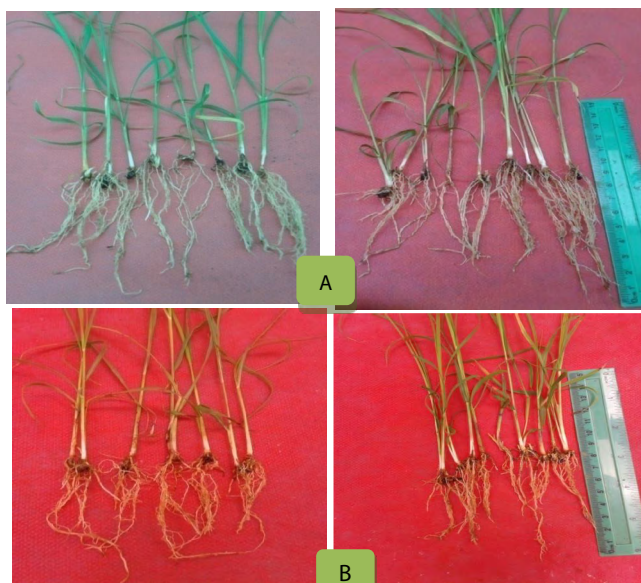


Figure 2: *Ex vitro* rooting of sugarcane micro-shoots. (A) Genotype N52 at 20 mg/l NAA (B) Genotype N53 at 30 mg/l NAA

guineensis Jacq.). These results indicated the significance of treating of microshoots with plant growth regulators during *ex vitro* rooting before culturing in soil medium.

There was a significant response variation in rooting between the two genotypes. Genotype N52 had the highest (76 %) rooting frequency with a maximum (5.88 cm ± 0.04 cm) average root length and 8.06 ± 0.13 average number of roots per shoot on microshoots dipped in 20 mg/l concentration of NAA (Table 2 and Figure 2A). At the same concentration of NAA, N53 had only 60 % rooting frequency with 4.34 cm ± 0.21 cm average root length and 4.08 ± 0.08 average roots number per shoot. On the other hand, genotype N53 showed a maximum rooting frequency (70 %) with 5.42 cm ± 0.08 cm and 4.52 ± 0.19 numbers of roots per shoot on microshoots treated with 30 mg/l concentration of NAA (Table 2 and Figure 2B). At this concentration of NAA, genotype N52 gave almost equal rooting frequency (70 %) with comparable root length (5.04 cm ± 0.05cm) and higher (6.36 ± 0.11) root number per shoot than N53. The result of this experiment revealed that genotype N52 was more responsive than N53 for different NAA concentrations.

The rate of rooting frequency increased from 36 % in control shoots to 76 % when the basal ends of shoots were dipped in a solution of 20 mg/l NAA overnight (Figure 3). Similarly, average root length and average number of roots increased from 4.44 cm ± 0.30cm and 2.14 ± 0.13 to 5.88 ± 0.04cm and 8.06 ± 0.13, respectively, in genotype N52.

However, when NAA concentration was further elevated to higher concentration (40 mg/l), rooting frequency, average root length and average number of roots per shoot, reduced significantly to 56 %, 4.68 ± 0.24 cm and 5.68 ± 0.13, respectively. The same trend was observed in genotype N53, in that rooting frequency increased from 28% in control shoots to 70 % in treated shoots with a solution of NAA at 30 mg/l. The average root length and average roots number also increased from 2.58 cm ± 0.54 cm and 1.74 to 5.42 cm ± 0.08cm and 4.52 ± 0.19 respectively, as the concentration of NAA increased from 0.0 mg/l to 30 mg/l but as the concentration of NAA was increased to 40 mg/l the rooting frequency, root length and root number reduced markedly to 54 %, 4.08 cm ± 0.19 cm and 3.88 ± 0.13 respectively (Figures 3-5). This reduction in rooting response could be due to the fact that higher concentrations of NAA promote the biosynthesis and buildup of ethylene at the basal end of the shoot, which have inhibitory effect on the overall rooting response of sugarcane microshoots [21].

The result of the present study on genotype N52 were in agreement with earlier results by Pandey et al. [13], who obtained the highest

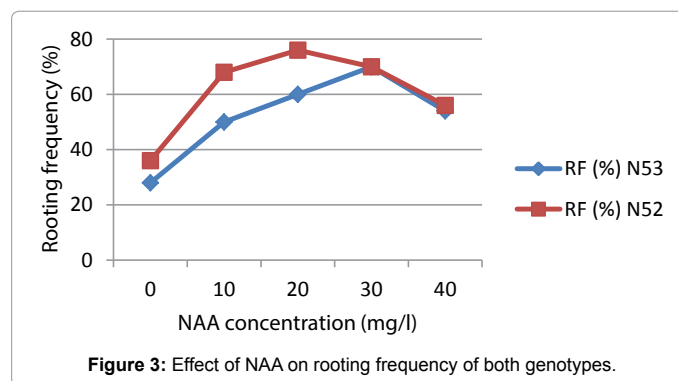


Figure 3: Effect of NAA on rooting frequency of both genotypes.

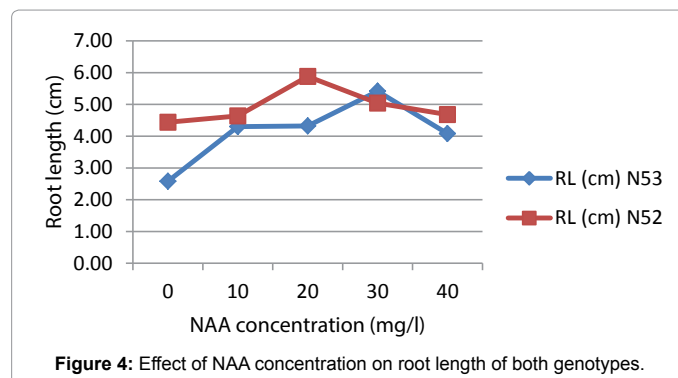


Figure 4: Effect of NAA concentration on root length of both genotypes.

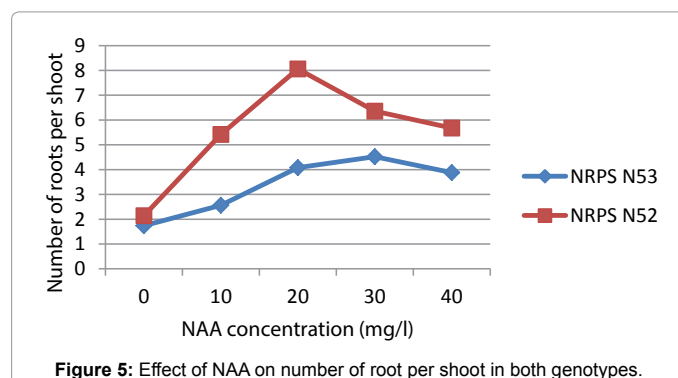


Figure 5: Effect of NAA on number of root per shoot in both genotypes.

rooting frequency, root length and number of roots per shoot from shoots treated in 20 mg/l of NAA concentration in sugarcane genotype CoS96268. Similarly, Martin [16] obtained an average of 5.6 roots per shoot after the microshoots of *Rotula aquatica* Lour were dipped in 0.5 mg/l NAA for 25 days. Biradar et al. [20] found best root formation on shoot treated with 2 mM NAA with 80% rooting frequency in oil palm. However, other authors Martin [22] and Chinnu [23] obtained best result of *ex vitro* rooting by using IBA. In present study, shoots rooted through this method transplanted to small pots containing soil, sand and farmyard manure (1: 1: 1) exhibited 100 % survival in both genotypes.

Conclusion

Generally, it appears that rooting of tissue culture-derived rootless sugarcane plantlets can be induced during *ex vitro* acclimatization by dipping in the auxin (NAA) solution overnight. From the five concentrations of NAA tested for *ex vitro* rooting, 20 mg/l NAA was found to be the optimal concentrations for *ex vitro* rooting of genotype N52. It produced the highest rooting frequency (76 %) with an average of 8.06 roots per shoot while in genotype N53, 30 mg/l NAA gave a maximum of 70 % rooting frequency with 4.52 average root numbers per shoot. *Ex vitro* rooting reduces the time of acclimatization and labor cost.

Acknowledgements

We would like to express our thanks to Ethiopian Sugar Corporation (ESC) for financing the study and Holetta National Agricultural Biotechnology Laboratory (HNABL) for provision of Tissue Culture Laboratory with all required consumable chemicals and facilities.

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Citation: Tesfa M, Admassu B, Bantte K (2016) *Ex Vitro* Rooting of Sugarcane (*Saccharum officinarum* L.) Plantlets Derived from Tissue Culture. Adv Crop Sci Tech 4: 215. doi:10.4172/2329-8863.1000215

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