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Effect of NAA on Ex vitro Rooting of In vitro Derived Microshoots of Elite Sugarcane (Saccharum officinarum L.) Genotypes

Melaku Tesfa[1*] Belayneh Admassu[2] Kassahun Bantte[3]
1.Ethiopian Sugar Corporation Research and Training Division, Biotechnology Research Team, Wonji Research Center, P.O Box 15, Wonji, Ethiopia
2.National Agricultural Biotechnology Research Program Holetta Agricultural Research Center
3.Jimma University College of Agriculture and Veterinary Medicine, Jimma, Ethiopia

Abstract
Root formation of rootless *in vitro* microshoots can be induced *ex vitro* with auxin during *ex vitro* acclimatization period to shorten the procedure and to reduce plantlet production cost of micropropagation. Hence, this study was carried out using a completely randomized design to determine the effect of different concentrations of auxin (0, 10, 20, 30, 40 mg/l) on *ex vitro* root development of sugarcane microshoots. The basal end of the shoots was dipped in auxin solution overnight before the shoots were transferred into a plastic tray containing a mixed growing medium in green house. The results showed that for genotype N52, best root formation was found on the shoots treated with 20 mg/l NAA by which rooting frequency was 76% 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 frequency was 70% with 5.42±0.11cm 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*, Auxin, *in vitro,* Acclimatization

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 (FAOSTAT, 2013). 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 (ESC, 2014).

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 (Jalaja *et al*., 2008). 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 (Lorenzo *et al*., 2001). 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 (ESC, 2014). To do so, *in vitro* propagation protocols have been developed using apical meristem and callus explant for several sugarcane cultivars of Ethiopian Sugar Estates (Tilahun *et al*., 2013; Belay *et al*., 2014; Dereje *et al*., 2014; Gemechu *et al*., 2014).

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 in *in vitro* propagation (Leva, 2011). In addition, roots of plantlets raised *in vitro* are generally very fragile and not have root hairs (Hazarika 2006). 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 (Feyissa *et al*., 2007; Shekafandeh 2007). Pandey *et al*. (2011) revealed that *ex vitro* rooting reduced more than 50% cost of sugarcane plantlet raised by conventional micropropagation. Besides, the plantlets produced after *ex vitro* rooting have better developed root system than the ones produced after *in vitro* rooting (Bozena 2001; Yan *et al*., 2010). 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 (Martin 2003a; Feyissa *et al*., 2007, Shekafandeh 2007), 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) (Martin 2003a; Shekafandeh 2007; Pandey *et al*., 2011). 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 which is located at 28 km west of Addis Ababa, Oromia region.

Plant Materials

Two elite sugarcane genotypes were used from Ethiopian Sugar Corporation, Research and Training Division, Wonji. The genotypes were N-52 and N-53. These genotypes were selected based on their higher yield performance and sugar quality. Genotype N52 gives 206.3 ton cane/ha with 15.36% sucrose content while N53 produces 166.9 ton cane /ha with 13.36% sucrose content.

Exvitro rooting

For present investigation, healthy micro-shoots having 3–4 cm in height, obtained from 4 weeks old multiplication cultures were used. 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 micro shoots. 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 25–30ºC 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 x 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.

Statistical Analysis

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).

Table 1: ANOVA summary for effect of NAA on *ex vitro* rooting

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 $P\leq0.0001$, DF = Degree of freedom, NAA = α-naphthalene acetic acid, MS = mean square CV= Coefficient of variation

In the control treatment reduced rooting frequency of 36% and 28% were obtained in genotypes N52 and N53, respectively. However, in NAA treated microshoots than 50% of the shoot developed roots regardless of the NAA concentration (Table 2). Shekafandeh (2007) also 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 Riyadh (2011) in oil palm (*Elaeis 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) average root length and 8.06 average number of roots per shoot on microshoots dipped in 20 mg/l concentration of NAA (Table 2 & Fig.3a). At the same concentration of NAA, N53 had only 60% rooting frequency with 4.34 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 and 4.52 numbers of roots per shoot on microshoots treated with 30 mg/l concentration of NAA (Table 2 and Fig.3b). At this concentration of NAA, genotype N52 gave almost equal rooting frequency (70%) with comparable root length (5.04 cm) and higher (6.36) root number per shoot than N53.The result of this experiment revealed that genotype N52 was more responsive than N53 for different NAA concentrations.

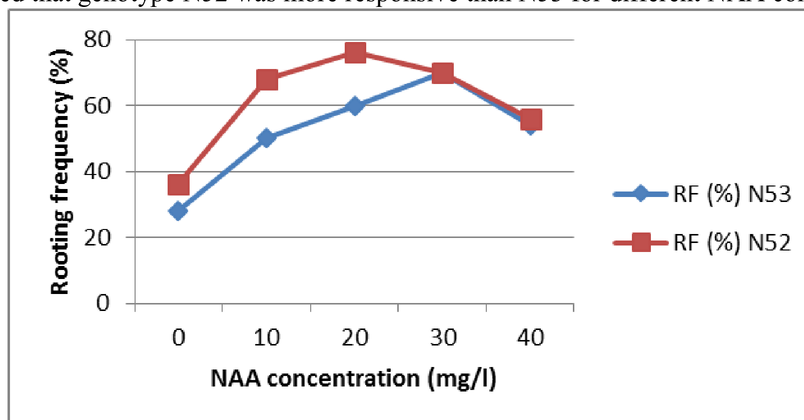

Figure.1 Effect of NAA on rooting frequency of both genotypes

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(Fig.1). Similarly, average root length and average number of roots increased from 4.44 cm and 2.14 to 5.88 cm and 8.06, 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 cm and 5.68, 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 and 1.74 to 5.42 cm and 4.52 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 and 3.88, respectively (Fig.1-3). This reduction in rooting response could be due to the fact that higher concentrations of NAA promote the biosynthesis and build up of ethylene at the basal end of the shoot, which have inhibitory effect on the overall rooting response of sugarcane microshoots (Biradar, 2009).

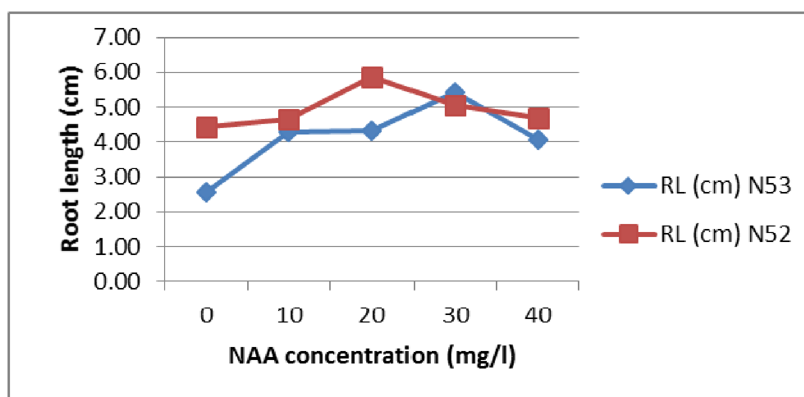

Figure 2. Effect of NAA concentration on root length of both genotypes

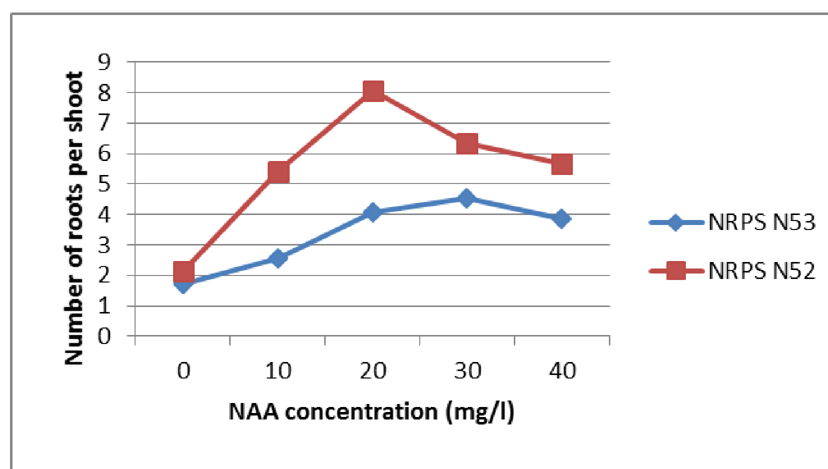

Figure 3. Effect of NAA on number of root per shoot in both genotypes

The result of the present study on genotype N52 were in agreement with earlier results by Pandey *et al.* (2011), who obtained the highest 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 (2003a) 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. Sumaryono and Riyadh (2011) found best root formation on shoot treated with 2 mM NAA with 80% rooting frequency in oil palm. However, other authors (Martin, 2003b; Chinnu *et al.*, 2012) 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: l: 1) exhibited 100 % survival in both genotypes.

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

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

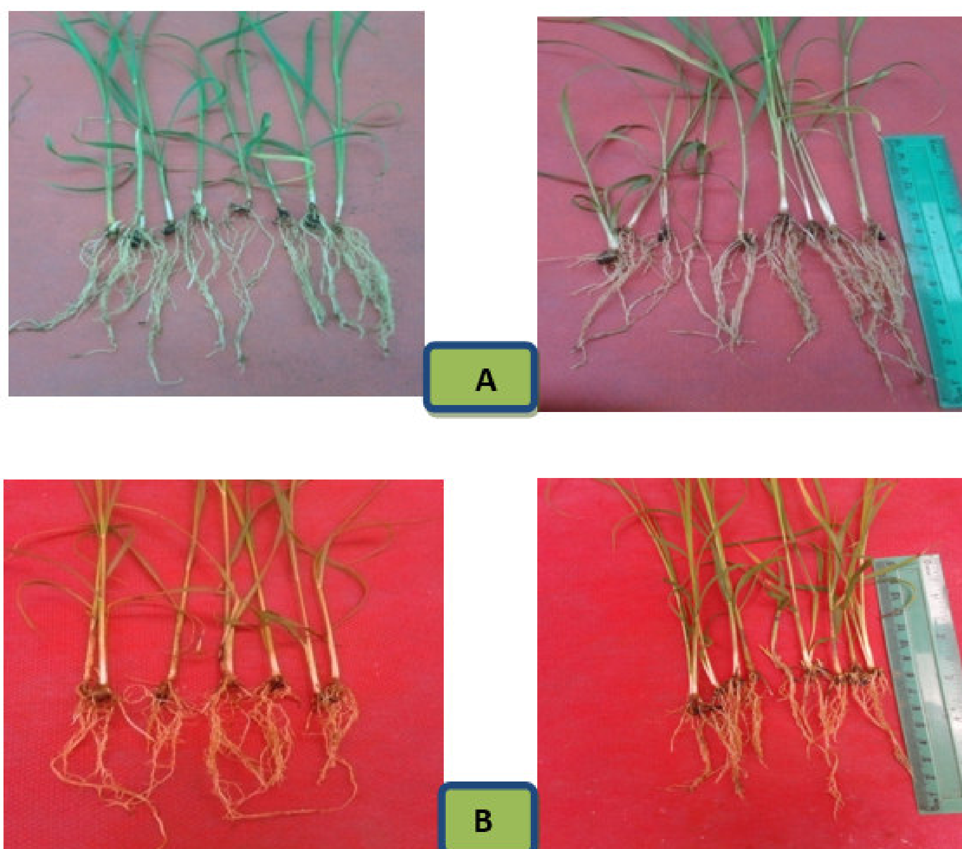

Figure 4. *Ex vitro* rooting of sugarcane micro-shoots. A). genotype N52 at 20 mg/l NAA.
B). genotype N53 at 30 mg/l NAA

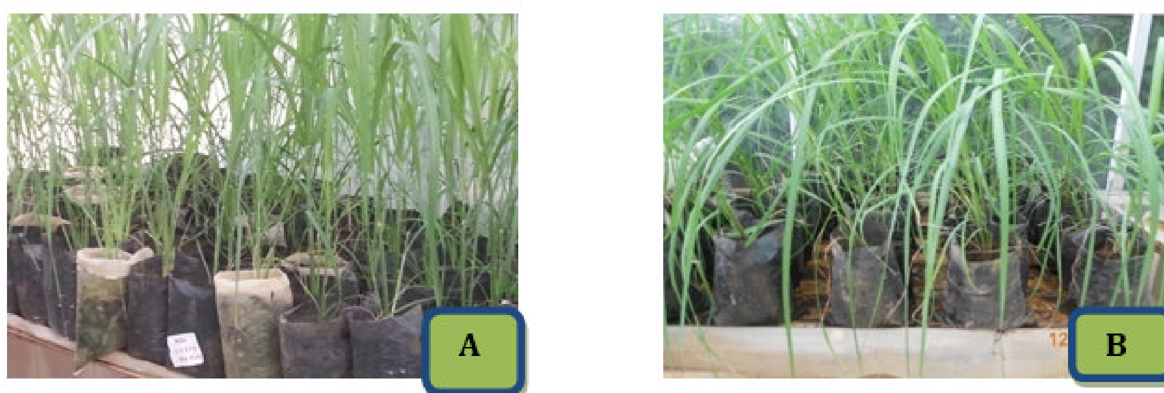

Figure 5. Acclimatized plantlets. A) Genotype N52 B) Genotype N53

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.

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