Advances in Life Science and Technology
ISSN 2224-7181 (Paper) ISSN 2225-062X (Online)
Vol.53, 2017

In Vitro Shoot Multiplication of Two Sugarcane (Saccharum officinarum L.) Genotypes using Shoot Apical Meristem

Belete Getnet*[1] Kassahun Bantte[2] Mulugeta Diro[3]
1.Ethiopian Sugar Corporation, Research and Training Division, Variety Development Directorate, Biotechnology Research Team, Wonji Research Center, Wonji, Ethiopia
2.Jimma University College of Agriculture and Veterinary Medicine, Jimma, Ethiopia
3.Capacity Building for Scaling up of Evidence - based Best Practices in Agricultural Production in Ethiopia (CASCAPE) Project, Addis Ababa, Ethiopia

Abstract
Conventional propagation of sugarcane (Saccharum officinarum L.) is limited due to low propagation rates, its time demand and potential transmission of pathogens through seed cane from generation to generation. In vitro propagation is the best alternative to overcome such limitations of conventional propagation. Hence, this study was initiated to optimize a protocol for rapid in vitro multiplication of two sugarcane genotypes (B4906 and Pr1013) grown in Ethiopia. This experiment was carried out in completely randomized design (CRD) with 2x5x5 factorial treatments arrangement of genotypes, BAP (0.5, 1.0, 1.5, 2.0, and 2.5 mgl^{-1}) and NAA (0, 0.2, 0.3 0.4, and 0.5 mgl^{-1}) in combination. Analysis of variance revealed that the interaction effects of genotypes, BAP and NAA were very highly significant ($p< 0.001$) for number of shoots/explant, shoot length and leaves/shoot. On MS media with 1.5mgl^{-1} BAP and 0.4mgl^{-1} NAA, B4906 gave the highest (16.88±0.5) numbers of shoots with 5.94±0.17 cm shoot length and 6.33±0.29 leaves/shoot. Whereas 2mgl^{-1} BAP and 0.5mgl^{-1} NAA resulted in a maximum of 11.70±0.28 shoots with 4.48±0.08 cm shoot length and 4.95±0.11 leaves/shoot for Pr1013. It could be concluded that the optimized protocol is useful for rapid clonal multiplication of sugarcane planting materials.
Keywords: apical meristem, BAP, multiplication, NAA

1. Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the most important perennial field crops widely cultivated in tropical and subtropical regions globally, which belongs to the Poaceae family [1]. It is an octaploid crop with a chromosome number of 2n = 8x = 80 [2]. It has 4-6 m stem length and tillers at the base to produce unbranched stem. It is one of the most efficient convertors of solar energy into sugar and hence produced primarily for its ability to store high concentrations of sucrose in the internodes of the stem. It is a high valued cash crop and exclusive source of 75% world sugar production [3] whereas the remaining comes from sugar beet. It also provides many by-products for biofactory to produce ethanol, butanol, acetic acid, plywood, industrial enzymes, animal feed, and chipboard and paper production besides, sugar and energy [4]. The production of press mud is also used as a source of organic matter and nutrients for crop production [5]. Hence, improving sugarcane production can play a role in the economic prosperity of any country like Ethiopia.

In commercial scale cultivation, stem cutting is the main propagation method. However, the amount of planting material provided by this method is limited as it has a 1:10 hectare ratio [6].It is very low and takes 10-12 months to use as a seed cane to get best sprouting [7, 8]. In addition, there is a high risk of disease transmission during seed cane preparation, distributions of seed cane within and among sugar estate were also there that leads to yield loss [9].The cost of laborer and planting materials transportation is also high [10]. In general, it requires large area, incurs high cost, consumes time and thus it is wasteful practical system.

Now, Ethiopian Sugar Corporation started to establish an advanced seed cane propagation system (plant tissue culture laboratories) to supplement the seed cane requirements of commercial propagation. Therefore, the use of micropropagation for sugarcane multiplication is a better alternative to the conventional methods, which also eradicates the risks of contamination by disease during seed production and ensures rapid multiplication [11]. Although *in vitro* propagation is very advantageous, genotype and type of explant source affecting genetic uniformity resulting in changed some yield components [12].

Apical meristem is the best choice for *in vitro* propagation as it produces genetically uniform plantlets that are identical to the mother plant and gives much more rapid multiplication rate [13]. It is suitable for production of seed cane as the canes derived from it, do not significantly differ for any measured yield trait from the source germplasm, and have 97% clonal fidelity [14]. Song *et al*. [15] obtained 1.77%, 1.56% and 0.31% variation incidence of plantlets generated from somatic embryogenesis, shoot tip and apical meristem respectively. Moreover, A new genotype that has to be propagated *in vitro*; its media protocol for regeneration is optimized first [16, 17]. *In vitro* multiplication and physiological response was dependent upon plant types of growth regulators, genotype and type of explants [18, 19].

So far, *in vitro* apical meristem culture offers an opportunity for genetically uniform *in vitro*

commercial propagation of sugarcane in some countries including India, United States, Brazil, Australia and Cuba [3]. However, there is no evidence on *in vitro* propagation using apical meristem, especially B4906 and Pr1013 genotypes in Ethiopia. Therefore, the present study was initiated with the objective to determine the optimum concentration of BAP and NAA combination for shoot multiplication of B4906 and Pr1013 genotypes.

2. Material and Methods

This experiment was carried out in the plant tissue culture laboratory, in Jimma University, College of Agriculture and Veterinary Medicine. Two genotypes (Pr1013 and B4906) were introduced in 2006 from India and Barbados, which are equatorial zones similar to Ethiopia. After evaluated in different agronomical practices in Wonji, Methara and Fincha`a Sugar Estates, B4906 genotype was released and gives 10.51 ton/ha/month cane yield and 14.59% sucrose recovery in light clay soil at Wonji Shoa Sugar Estate [20]. However, the evaluation of genotype Pr1013 is still ongoing but has better morphological and agronomical status under Fincha'a Sugar Estate agroclimatic condition. The stem cuttings of these genotypes were collected from Fincha`a Sugar Estate seed cane nurseries. Stems of these two genotypes were cut and prepared as a seed cane with two buds, and planted in greenhouse of College of Agriculture and Veterinary Medicine, Jimma University where the study were carried out. The setts were watered every three days and allowed to grow for three months.

Shoot tops containing apical meristem were collected from actively growing shoots of 2-3 months old. The apical portions were cut from stock plants close to the first node; the mature leaves were removed after bringing into the laboratory. The apical stem portions were initially washed thoroughly in running tap water and removed outer leaf sheath. After cut off the shoots into 5 cm length by locating the bud somewhere on the shoot, they were washed three times each for 10 min in sterile /distilled water with liquid soap solution and three drops of Tween-20. The explants were then taken into sterilized laminar airflow cabinet, and then rinsed using 0.3% (w/v) mancozuim (fungicide) solution for 30 min using gently shaking to assure proper submerging and rinsed in sterile distilled water three times each for 5min. They were also sterilized by 70% ethanol for 10 min, and washed using sterile distilled water three times each for 5 min. Subsequently, they were rinsed into 25% (v/v) commercial bleach (Berekina) solution for 20 min followed by washing using sterile distilled water 3 times each for 5min with gentle shacking to remove the chemical residue. Then after, the remaining whorls of leaves were removed from apical stem portions until the apical meristem with two to three primordial leaves were left.

The apical meristems (4-6 mm) were excised and isolated using sterile blade and forceps then cultured one explant/jar on jelled MS basal medium supplemented with BAP+KIN+NAA, 0.5mgl^{-1} each [21], 1mg/l methylene blue, 0.08 mg/l Adenine hemisulfate,0.3% PVP and 0.5 mg/l each of antibiotic (kanamycin and streptomycin) for shoot establishment. To minimize the exposure contamination, all steps were done aseptically in the laminar airflow cabinet.

Effects of BAP and NAA on shoot multiplication

After 30 days of culture, the same batch of shoots having above 3cm height, were taken and excised aseptically, then cultured on hormone free MS basal medium for two weeks to avoid carryover effects for the next circumstances. In this experiment, full strength MS [22] media were used supplemented with various concentrations and combinations of BAP (0.5, 1.0, 1.5, 2.0, and 2.5 mgl^{-1}) and NAA (0, 0.2, 0.3 0.4, and 0.5 mgl^{-1}) in a factorial combinations arrangement along with 30gl^{-1} sucrose as a carbon source. Methylene blue and adenine hemisulfate were added as the same rate of initiation medium for shoot growth and multiplication enhancement respectively. The PH of the medium was adjusted 5.8 before jelled with 8g/l agar and autoclaved at 121˚C and 15 psi for 20 minutes. The experiment was laid out in completely randomized design (CRD) factorial arrangement (2x5x5- two genotypes and two PGRs) with nine regenerated shoots per treatment.

The cultures were placed in white florescent light room having 25 µmolm^{-2}s^{-1} light intensity and adjusted at 16/8 hrs light/dark photoperiod regimes at 25±2˚C room temperature.

Data on number of shoots, shoot length and numbers of leaves were recorded 30 days after culturing. SAS software (SAS, 2008 version 9.2) was used for the analysis of variance of collected data and treatments` means were separated using the procedure of DMRT (Duncan multiple range test) at 5 % probability.

3. Results and Discussion

Shoot Initiation and Establishment of Explants

B4906 gave 84% shoots establishment while Pr1013 produced only 56% regenerated shoots. This regenerated percentage difference may becomes from genotypic difference, and from the effect of phenolics oxidation that is highly serious in Pr1013 clones. Jahangir *et al*. [23] reported that hormonal supplementation was not the only factor for regeneration but potential of a specific variety is equally affecting. For shoot establishment, the antibiotics are important to prevent bacterial contamination if the mother or source plant has systemic disease. Besides genotypes, genetically depended phenolics oxidation determined *in vitro* survivality and regeneration of explants at the stage of micropropagation shoot establishment. This means that the cultures free from phenolic oxidation had better regeneration and changed to green within a week but that have not free became dry and die.

Because the compound makes the media dried and blocks the osmosis system used for nutrient uptakes of the explants from the media. However, it could be controlled by using polyvinyl pyrolidone (PVP) and activated charcoal. Shimelis *et al*. [24] reported the optimization of PVP and activated charcoal for two genotypes. The authors obtained 2 gl^{-1} and 3 gl^{-1} PVP has gave 100% and 80% survived explants of C86-56 and C86-12 genotypes respectively while 0.4 and 0.3 gl^{-1} of activated charcoal resulted in 46 % and 40% survived explants of C86-56 and C86-12 respectively. For this work, 0.3% PVP used in Pr1013 culture was reduced phenolic oxidation or browning effect and increased the explants survivality to get enough shoot establishments.

Table 1. Percentage of shoot initiated explants

Genotype	No. of explants cultured	No. of explants regenerated	%age of regenerated shoot
B4906	50	42	84
Pr1013	50	28	56

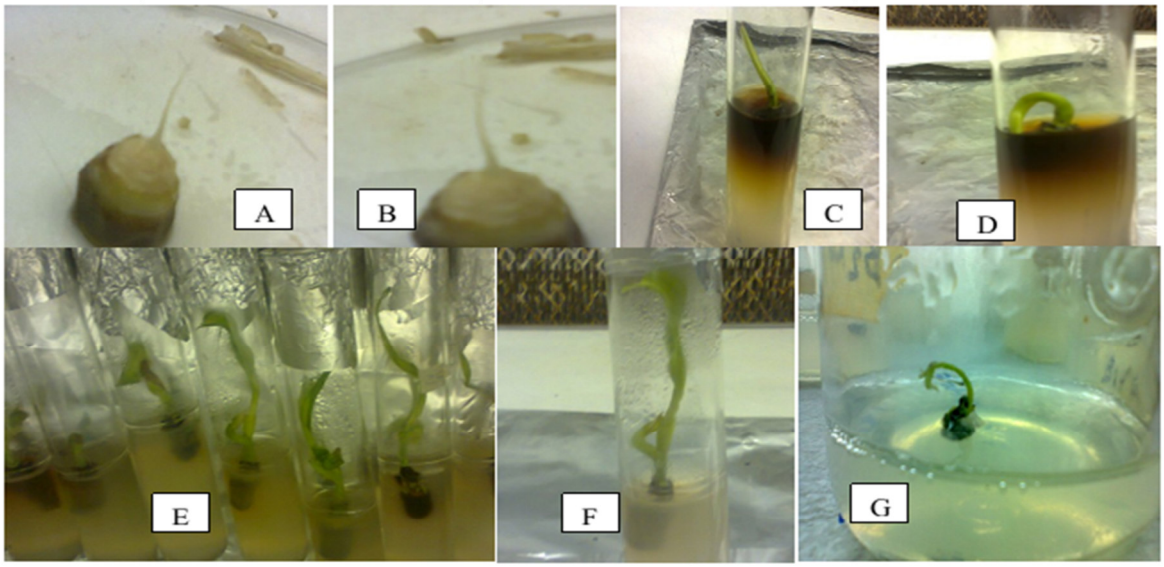


Figure 1: A&B). Size of explant preparation, C&D). Shoot initiation and establishment of Pr1013 using 3% PVP to reduce browning affect after two weeks, E-G). Shoot initiation and establishment of B4906 after two weeks of culture.

Effects of BAP and NAA on Shoot Multiplication of Two Genotypes

Analysis of variance revealed that the interaction effects of genotypes, BAP and NAA were very highly significant ($p< 0.001$) for number of shoots/explant, shoot length and leaves/shoot. On MS media devoid of BAP and NAA, young shoots were developed from the primary shoot and showed shoot elongation in both genotypes after being cultured for a month (control, data was not taken). This might be due to the presence of methylene blue, which stimulates shoot growth and increasing survival [25, 26], and adenine hemisulfate enhanced shoot multiplication [27]. B4906 gave the highest (16.88 ±0.54) shoots/explant with 5.94±0.17 cm average shoot length and 6.33±0.29 leaves/shoot on MS media with 1.5 mgl^{-1} BAP and 0.4 mgl^{-1} NAA (Table 1; Fig. 2). Whereas, Pr1013 produced maximum of 11.70 ±0.28 shoots/explant and 4.48±0.08 cm shoot length with 4.95±0.11 leaves/shoot on MS media fortified by 2 mgl^{-1} BAP and 0.5 mgl^{-1} NAA (Table1; Fig.3). This multiplication rate difference might be due to genotypic difference, which affects the frequency of shoot organogenesis [28], and also endogenous cytokinin and auxin concentration differences [29, 30]. The performance of each cultivar is expected to be different in *in vitro* culture as a field response regarding shoot number and shoot length as described by [31]. This requires that novel or modified *in vitro* regeneration procedures must be developed for each genotype because of the significant variations in response to hormone combinations.

Increasing NAA from 0.3 to 0.4mgl^{-1} at 1.5mgl^{-1} BAP showed a significant increase from 13.42±0.38 to 16.88±0.54 shoots/explant, from 2.08±0.25 to 5.94±0.17 cm shoot length and from 4.75±0.45 to 6.33±0.29 leaves/shoot in B4906. However, further increase of NAA to 0.5 mgl^{-1}, significantly reduced number of shoots/explant, shoot length and number of leaves/shoot from 16.88±0.54 to 11.00±0.50, 5.94±0.17 to 2.06±0.07 and 6.33±0.29 to 5.17±0.29 respectively (Picture 1). In addition, proliferation and shoot length increased (from 4.83±0.38 to 8.17±0.44 and 1.83±0.16 to 4.63±0.34 respectively) with increasing of BAP from 0.5 to 1.5 mgl^{-1} at 0.0 mgl^{-1} NAA, but further increase of BAP to 2 mgl^{-1} led to decrease of shoot number and length, and aggregation of shoots in B4906 (Table 1, Picture 2). The shoots became inseparable and stunted, which are generally unusable due to high dosage of hormone that disorders the metabolism of the shoot. Hence, this shows

that higher concentration of cytokinin inhibits cell division and hence multiplication, whereas low concentration of cytokinin promotes shoot multiplication and elongation in sugarcane as reported previously [32-34].

In addition, Pr1013 showed continuously increased proliferation from 2.42±0.09 to 8.53±0.36 when BAP was increased from 0.5 mgl^{-1} to 2.5 mgl^{-1} at 0.0 mgl^{-1} NAA (Picture 2). This also indicates that it needs further increase of BAP to get the optimum proliferation. Jalaja *et al*. [35] obtained maximum shoot multiplication on MS medium with high levels of BAP (6mgl^{-1}) and 0.5mgl^{-1} NAA for several genotypes. Khan *et al*. [36] obtained a higher shoot multiplication on a medium containing high concentration of BAP (4.5mgl^{-1}) for clone NIA-98. However, addition of exogenous BAP +NAA resulted in increased rate of propagules multiplication than using BAP alone. This suggests possible synergistic effect of these hormones on adventitious shoots. Thus, the ratio of cytokinin and auxin balance is proved to be more important with respect to morphogenesis in sugarcane. Although cytokinins are known in stimulating cell division, they do not induce DNA synthesis. Nevertheless, addition of auxin at low concentration is very important to promote cell division and elongation, and has an ability to induce DNA synthesis [32]. Hence, the presence of auxin with cytokinin stimulates cell division and control morphogenesis thereby influences adventitious shoot production.

The result from B4906 are different from the reports of [37, 38] who obtained 15.5-17.2 shoots/explant on MS supplemented with 1.5mgl^{-1} BAP and 0.5mgl^{-1} NAA, in which only 11.00±0.50 shoots/explant were obtained in the current study. The difference could be due to differences in genotypes and type of explant used. Besides, the addition of adenine hemisulfate might have contributed for the difference. The result from Pr1013 is in line with [39] who obtained the highest (8.2) shoots/explant on MS with 2mgl^{-1} BAP and 0.5 mgl^{-1} NAA, where 11.70±0.28 shoots/explant were obtained in the current study.

Table 1. Mean Values of Shoot Number, Shoot Length and Leaf Number of B4906 And Pr1013 Genotypes Under Influence of 6-BAP And α-NAA Combination.

PGRs		B4906			Pr1013		
BAP	NAA	No. of Shoots /explant (mean±SD)	Shoot length /shoot (mean±SD)	No. of leaves /shoot (mean±SD)	No. of Shoots /explant (mean±SD)	Ava. of Shoot length (mean±SD)	No. of leaves /shoot (mean±SD)
0.5	0	4.83^{lmn}±0.38	1.83^{qrs}±0.16	4.67^{p-s}±0.38	2.42^{p}±0.09	1.84^{qrs}±0.08	3.73^{u-x}±0.21
0.5	0.2	7.33^{hi}±0.29	2.41^{no}±0.10	5.58^{h-k}±0.29	2.36^{p}±0.14	2.05^{pq}±0.06	5.75^{g-j}±0.33
0.5	0.3	5.33^{lm} ±0.17	3.45^{f}±0.49	4.92^{op}±0.38	2.27^{p}±0.23	1.36^{t}±0.08	5.86^{ghi}±0.25
0.5	0.4	8.67^{g}±0.29	2.53^{mn}±0.20	7.58^{b}±0.38	2.77^{p}±0.31	1.20^{t}±0.08	4.73^{pqr}±0.32
0.5	0.5	7.17^{hi}±0.58	3.96^{d}±0.37	5.33^{k-n}±0.29	2.34^{p}±1.85	1.85^{qrs}±0.05	4.45^{qrs}±0.28
1	0	7.44^{h}±0.10	3.17^{g}±0.02	3.67^{u-x}±0.76	3.53^{o}±0.28	1.84^{qrs}±0.08	6.25^{ef}±0.21
1	0.2	9.83^{ef}±0.29	3.83^{de}±0.50	6.00^{efg}±0.43	2.71^{p}±0.45	2.19^{op}±0.05	7.07^{dc}±0.26
1	0.3	12.00^{c}±0.50	3.54^{f}±0.57	5.42^{jkl}±0.52	5.13^{lm}±0.19	2.12^{p}±0.05	4.94^{nop}±0.14
1	0.4	13.50^{b}±0.5	2.98^{g-j}±0.17	7.17^{dc}±0.38	4.74^{mn}±0.51	2.24^{op}±0.08	3.76^{uvw}±0.21
1	0.5	10.33^{e}±0.17	3.62^{ef}±0.55	5.92^{fgh}±0.14	6.28^{k}±0.14	2.83^{hij}±0.05	3.46^{vwx}±0.21
1.5	0	8.17^{g}±0.44	4.63^{b}±0.34	4.83^{opq}±0.38	4.34^{n}±0.28	2.13^{p}±0.12	3.36^{wx}±0.11
1.5	0.2	11.42^{c}±0.38	2.79^{jkl}±0.21	5.33^{k-n}±0.38	5.26^{lm}±0.09	3.12^{gh}±0.14	4.63^{p-s}±0.12
1.5	0.3	13.42^{b}±0.38	2.08^{pq}±0.25	4.75^{pq}±0.43	5.22^{lm}±0.08	3.07^{ghi}±0.12	5.35^{klm}±0.25
1.5	0.4	16.88^{a}±0.54	5.94^{a}±0.17	6.33^{e}±0.29	5.20^{lm}±0.08	2.99^{g-j}±0.09	3.54^{u-x}±0.33
1.5	0.5	11.00^{d}±0.50	2.06^{pq}±0.07	5.17^{l-o}±0.29	6.69^{ijk}±0.28	2.53^{mn}±0.05	3.35^{x}±0.11
2	0	6.39^{jk}±0.35	2.55^{lmn}±0.39	5.00^{m-p}±0.43	5.51^{l}±0.28	2.08^{pq}±0.08	3.94^{tu}±0.13
2	0.2	9.67^{f}±0.58	4.70^{b}±0.65	3.75^{u-x}±0.25	7.28^{hi}±0.22	2.91^{h-k}±0.12	4.85^{opq}±0.17
2	0.3	9.50^{f}±0.50	2.91^{h-k}±0.54	3.83^{uv}±0.52	7.32^{hi}±0.29	3.60^{f}±0.08	4.30^{st}±0.32
2	0.4	11.44^{c}±0.99	3.15^{gh}±0.59	6.83^{d}±0.14	6.78^{h-k}±0.16	2.67^{klm}±0.08	4.62^{p-s}±0.10
2	0.5	10.05^{ef}±0.64	3.90^{d}±0.53	5.50^{i-l}±0.25	11.70^{c}±0.28	4.48^{bc}±0.08	4.95^{nop}±0.11
2.5	0	4.92^{lmn}±0.14	2.20^{op}±0.28	5.67^{g-k}±0.63	8.53^{g}±0.36	2.00^{pqr}±0.08	4.30^{st}±0.32
2.5	0.2	5.06^{lm}±0.59	2.67^{klm}±0.19	4.75^{pq}±0.43	6.76^{h-k}±0.18	4.37^{c}±0.12	7.25^{bc}±0.42
2.5	0.3	5.17^{lm}±0.29	1.98^{pqr}±0.03	4.33^{rs}±0.14	7.25^{hi}±0.28	2.00^{pqr}±0.08	3.37^{wx}±0.10
2.5	0.4	7.00^{hij}±0.50	2.00^{pqr}±0.33	8.25^{a}±1.32	2.75^{p}±0.34	1.97^{pqr}±0.05	2.90^{y}±0.24
2.5	0.5	7.33^{hi}±0.58	1.69^{s}±0.28	5.83^{ghi}±0.63	2.51^{p}±0.17	1.76^{rs}±0.08	4.30^{st}±0.32
CV		5.31	6.38	4.05	5.31	6.38	4.05

Note: PGRs=Plant growth regulators. *Values for number of shoots/explant, shoot length and number of leaves/shoot are given as mean ± SD. *Values in the same column with different letter (s) are significantly different from each other at $p \leq 0.05$.

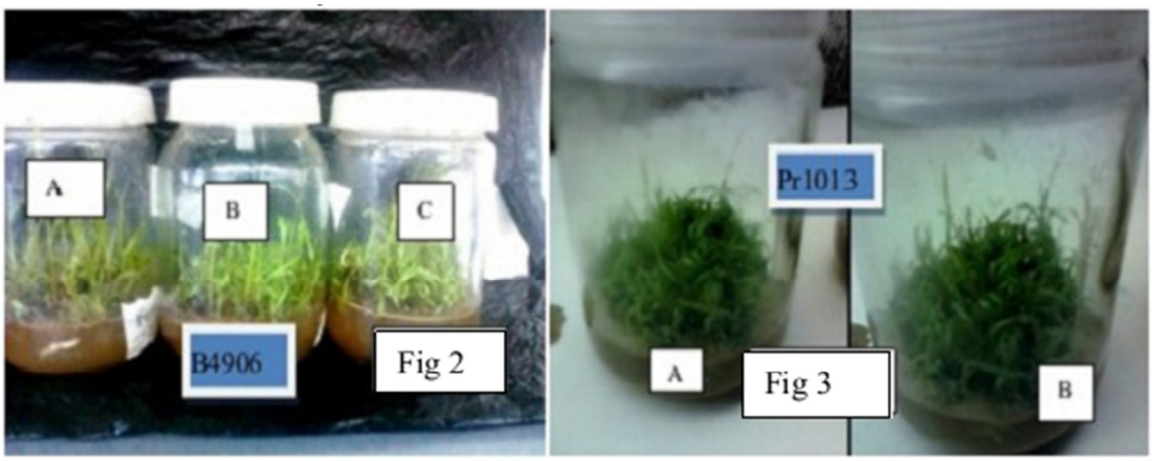
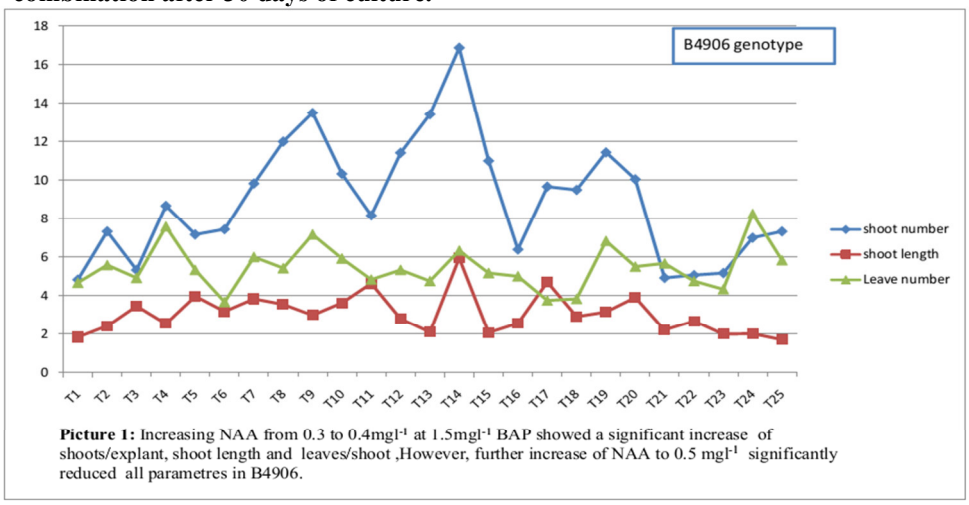
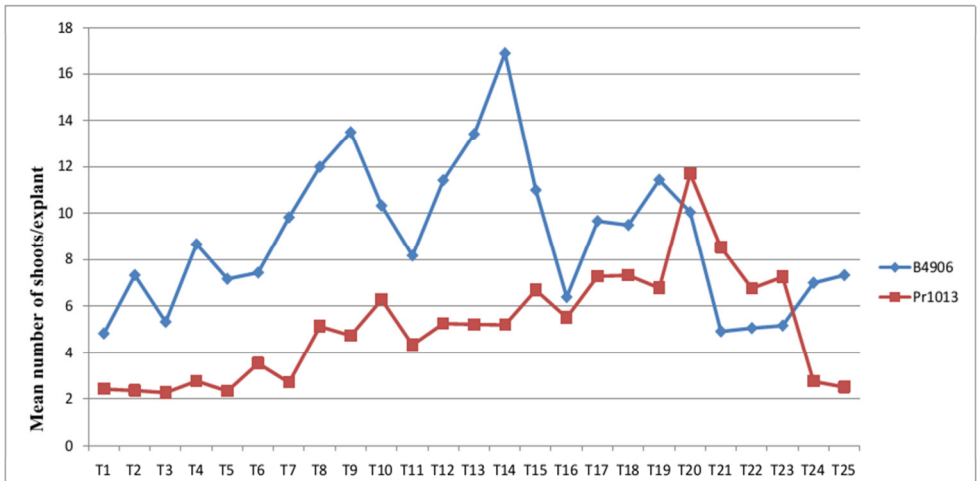


Figure 2. ***In vitro*** **shoot multiplication of B4906 on MS medium containing 1.5mg/l BAP and 0.4mg/l NAA combination after 30 days of culture.**

Figure 3. ***In vitro*** **shoot multiplication of Pr1013 on MS medium containing 2mg/L BAP and 0.5mg/L NAA combination after 30 days of culture.**



Picture 1: Increasing NAA from 0.3 to 0.4mgl^{-1} at 1.5mgl^{-1} BAP showed a significant increase of shoots/explant, shoot length and leaves/shoot ,However, further increase of NAA to 0.5 mgl^{-1} significantly reduced all parametres in B4906.



Picture 2: Proliferation increased with increasing of BAP from 0.5 to 1.5 mgl^{-1} at 0.0 mgl^{-1} NAA, but further increase of BAP to 2 mgl^{-1} led to decrease of shoot number and length in B4906. whereas Pr1013 showed continuously increased proliferation when BAP from 0.5 mgl^{-1} to 2.5 mgl^{-1} at 0.0 mgl^{-1} NAA.

4. Conclusion

In Ethiopia, *in vitro* mass propagation of sugarcane is used to supplement conventional propagation by producing disease free and the required amount of quality planting material for newly established sugar factory projects. Hence, new and old high yield varieties protocol optimization is developed to make the technology

successful. In the present study, it was observed that the genotypes responded differently and needed different concentration in terms of shoot number, shoot length and leaf number. In this result, MS medium containing 1.5mgl^{-1} BAP and 0.4mgl^{-1} NAA produced the highest shoot numbers and shoot length in B4906 while MS medium supplemented with 2mgl^{-1} BAP and 0.5mgl^{-1} resulted in maximum shoot number and shoot length in Pr1013. However, the maximum number of leaves per shoot recorded at different concentrations means 2.5mgl^{-1} BAP and 0.4mgl^{-1} NAA in B4906 while 2.5mgl^{-1} BAP and 0.2mgl^{-1} NAA in Pr1013. Hence, the optimized protocol could be used for mass *in vitro* proliferation of these genotypes to supplement the conventional propagation.

Acknowledgements
The authors would like to thanks Ethiopian Sugar Corporation funding the research and College of Agriculture and Veterinary Medicine, Jimma University giving an access of plant tissue culture laboratory

Reference

[1]. Suprasanna, P., Patade, V., Desai, N., Devarumath, R. and Kawar, P., 2011. Biotechnological developments in sugarcane improvement an overview. *Sugar Technology*.***13*** *(4):322-335*.
[2]. Sreenivasan T, Ahloowalia B., Heinz D., 1987. Cytogenetics. In: Heinz DJ (ed.) Sugarcane Improvement through Breeding. Amsterdam: Elsevier.
[3]. Lakshmanan, P., Geijskes, R., Wang, L. and Smith, G., 2006. Developmental and hormonal regulation of direct shoot organogenesis and somatic embryogenesis in sugarcane (*Saccharum spp. interspecific hybrids*) leaf culture. *Plant cell reports.* ***25****(10):1007-1015*.
[4]. Garcia, R., Cidade, D. and Castellar, A., 2007. *In vitro* morphogenesis patterns from shoot apices of sugar cane are determined by light and type of growth regulator. *Plant Cell Tissue and Organ Culture.* ***90****(2):181–190*.
[5]. Raja, N. and Abbas, H., 2006. Sugarcane Overview: htp://www.pakistan/com/English/allabout/
[6]. Sood, N., Gupta, P., Srivastava, R. and Gosal, S., 2006. Comparative studies on field performance of micropropagated and conventionally propagated sugarcane plants. *Plant Tissue Culture and Biotechnology.* ***16****(1): 25-29*.
[7]. Feyissa, T., Tariku, G., Yohannes, B. and Girama, W., 2010. Performance of Ethiopian sugar estates. *Second Biennual Conference, ESDA*, p. 1-3, Adama, Ethiopia. 22-23 Dec 2010.
[8]. Sime M., 2013. The effect of different cane portions on sprouting, growth and yield of sugarcane (*Saccharum spp* L.). *International Journal of Science Research.Publication.****3*** *(1):338-341*.
[9]. Yohannes, Z., Leul, M. and Teklu, B., 2010. Yield loss assessment due to ratoon stunting disease of sugarcane in the Ethiopian sugar estates. *ESDA,* Research Directorate, Wonji, Ethiopia, P.2-3.
[10]. Ayele N., Getaneh A., Negi T. and Mequanent Y., 2014. Effect number of buds per sett and sett spacing on yield of sugarcane at Metahara Sugar Estate. *Journal of Agriculture and Natural Resources Science.* ***1****(4): 238-244*.
[11]. Khan, I., Khatri, A., Raza, S., Shah, G. and Naqvi, M., 2004. Effects of Different phytohormones on Sugarcane (*Saccharum Spp*.) Regeneration. *Pakistan Journal of Biotechnology*. ***1****(2):17-22*
[12]. Hoy, J., Bischoff, K., Milligan, S. and Gravois, K., 2003. Effect of tissue culture explant source on sugarcane yield components. *Euphytica*. ***129****(2):237-240*.
[13]. Ali, A., Naz, S., Siddiqui, F. and Iqbal, J., 2008. An efficient protocol for large scale production of sugarcane through micropropagation. *Pakistan Journal of Botany. 40(1):139-149.*
[14]. Devarumath, R., Doule, R., Kawar, P., Naikebawane, S. and Nerkar, Y., 2007. Field performance and RAPD analysis to evaluate genetic fidelity of tissue culture raised plantsvis-ã -vis conventional setts derived plants of sugarcane. *Sugar Technology.* ***9****(1), 17-22*.
[15]. Song, L., Kun-Xing, Y., LI-min, L., Ming, D., Hong-jian, L. and Liu, Y., 2010. Sugarcane micropropagation through shoot tip embryogenesis. *Sugar Crops of China*. ***4*** *(2)*.
[16]. Singh, N., Kumar, A. and Garg, G., 2006. Genotype dependent influence of phytohormones combination and subculturing on micropropagation of sugarcane varieties. *Indian Journal of Biotechnology*. ***5****(1): 99-106.*
[17]. Cheema, L. and Hussain, M., 2004. Micropropagation of sugarcane through apical bud and axillary bud. *International Journal of Agriculture and Biology*. **6**(2): 257-259.
[18]. Uzma, M., Khan A., Muhammad, I., Hussain, S., Shah, T., Kumar, S. and Inam, M., 2012. Rapid *in vitro* multiplication of sugarcane elite genotypes and detection of sugarcane mosaic virus through two steps RT-PCR. *International Journal of Agriculture and Biology.****14*** *(6):870–878.*
[19]. Malik, S., Chaudhury, R. and Kalia, R., 2005. Rapid in vitro multiplication and conservation of Garcinia indica: a tropical medicinal tree species. Science and Horticulture. 106(4), 539-553.
[20] Getaneh, A., Taddesse, F., and Ayele, N., 2013. Agronomic performance evaluation of ten sugarcane

varieties under wonji-shoa agro-climatic conditions. *Annual research conference, ESDA,* Pp 1-7, 22-23 Nov 2013, Sugar corporation Research and training center, wonji shoa, Ethiopia.

[21]. Pathak, S., Lal, M., Tiwari, k. and Sharma, L., 2009. Effect of growth regulators on *in vitro* multiplication and rooting of shoot cultures in sugar cane. *Sugar Technology*. ***11****(1):7–9*.

[22]. Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiology of Plant*. ***15****(3):473–497*.

[23] Jahangir, G., Nasir, I., and Iqbal, M., (2014). Disease free and rapid mass production of sugarcane cultivars. *Advance Life Science. **1**(3): 171-180*.

[24] Shimelis, D., Bantte, K., and Feyissa, T.,(2015). Effects of polyvinyl pyrolidone and activated charcoal to control effect of phenolic oxidation on in vitro culture establishment stage of micropropagation of sugarcane (Saccharum officinarum L.). Advances in Crop Science and Technology. Vol, **3**(4).

[25]. Lage, S. and Esquibel, A., 1997. Growth stimulation produced by methylene blue treatment in sweet potato. *Plant Cell Tissue and Organ Culture*. ***48****(2):77-81*.

[26]. Ramgareeb, S., Snyman, S. and Rutherford, R., 2010. Elimination of virus and rapid propagation of disease-free sugarcane (*Saccharum spp.* cultivar NCo376) using apical meristem culture. *Plant Cell Tissue Organ Culture. **100**(2):175-181*.

[27]. Visessuwan, R., Chiemsombat, P. and Naritoom, K., 1999. Role of growth regulators in meristem culture and production of virus-free sugarcane germplasm. *Sugar Technology*. ***1****(3): 82-88*.

[28]. Jahangir, G., Nasir, I., and Iqbal, M., (2014). Disease free and rapid mass production of sugarcane cultivars. *Advance Life Science. **1**(3): 171-180*.

[29]. Viet, B., 2009. Hormone mediated regulation of the shoot apical meristem. *Plant molecular and Biology. **69**(4):397-408*.

[30]. George, E., Hall, M. and De Klerk, G., 2008. Plant tissue culture procedure-background. In Plant propagation by tissue culture. *Springer*, 3rd edition, pp 175-205.

[31]. Ogero, O., Mburugu, G., Mwangi, M., Ngugi, M. and Ombori, O., 2012. Low cost tissue culture technology in the regeneration of sweet potato *(Ipomoea batatas* L). *Research Journal of Biology. **2**(2):51-58*.

[32]. Gopitha, K., Bhavani, L. and Senthilmanickam, J., 2010. Effect of the different auxins and cytokinins in callus induction, shoot, root regeneration in sugarcane. *International Journal of Pharmacology and Biotechnology Science. **1**(3):1-7*.

[33]. Abdu, S., Yahaya, M. and Shehu, U., 2012. *In vitro* regeneration of commercial sugarcane (*Saccharum spp*.) Cultivars in Nigeria. *Journal of Life Science*. ***6****(7): 721-725*.

[34]. Tolera B., Diro M. and Belew D., 2014. Effects of gibberellic acid and kinetin on *in vitro* aseptic shoot tip culture establishment of sugarcane (*Saccharum officinarum* L.) Varieties grown in Ethiopian sugar estates. *International Journal Science: Basic and Application Research.16 (1), 496-504*.

[35]. Jalaja, N., Neelamathi, D. and Sreenivasan, T., 2008. Micropropagation for quality seed production in sugarcane in Asia and the Pacific. *FAO of the UN*, sugarcane publication. *pp13-60.*

[36]. Khan, I., Dahot, U., Yasmin, S., Khatri, A., Seema, N. and Naqvi, M., 2006. Effect of sucrose and growth regulators on the micropropagation of sugarcane clones. *Pakistan Journal of Botany*. ***38****(4): 961.*

[37]. Roy, P. and Kabir, M., 2007. *In vitro* mass propagation of sugarcane (*Saccharum officinarum* L.) var. Isd 32 through shoots tips and folded leaves culture. *Biotechnology. **6**(4):588-592*.

[38]. Ali, S., Khan, M. and Iqbal, J., 2012. *In vitro* direct plant regeneration from cultured young leaf segments of sugarcane (*Saccharum officinarum* L.). *Journal of Animal and Plant Science. **22**(4), 1107-1112*.

[39]. Behera, K. and Sahoo, S., 2009. Rapid *in vitro* micropropagation of sugarcane (*Saccharum officinarum* L.) cv-Nayana through callus culture. *Nature and Science*. ***7****(4): 1-10.*