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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

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Abstract

In vitro aseptic culture establishment of sugarcane varieties using shoot tip explants was carried out with the objective to evaluate the initiation response of sugarcane varieties B41-227 and N14 under four levels of Gibberellic acid (GA_3) (0.1, 0. 5, 1 and 1.5 mgL^{-1}) and kinetin (0.5, 1, 1.5, and 2 mgL^{-1}) in a completely randomized design with 4 * 4 * 2 factorial treatment combination arrangements. Data on percent shoot tip explant initiation, number of shoots per explant and average shoot length were collected after 30 days.

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Analysis of variance proved that the interaction effects of GA_3, kinetin and the sugarcane genotypes on percent initiation of shoot tip explants, number of shoots per explant and average shoot length was very highly significant ($P < 0.0001$). Murashige and Skoog (MS) medium containing 0.5 mgL^{-1} GA_3 and 1 mgL^{-1} kinetin for B41-227 and 1 mgL^{-1} GA_3 and 1.5 mgL^{-1} kinetin for N14 were found to be optimum. These media could produce 83.33% establishment of shoot tip cultures with 3.0 ± 00 shoots per explant and 4.8 ± 0.54 cm average shoot length in B41-227 and 70% shoot tip cultures establishment with 2.9 ± 0.20 shoots per explant and 5.45 ± 0.29 cm shoot length in N14. Thus, the optimized protocol can be used for rapid initiation of aseptic shoot tip cultures of the sugarcane varieties that can be propagated successfully in the subsequent stages and hence minimize the current challenges in shortage of adequate quantity quality sugarcane planting materials in the Ethiopian Sugar Estates.

Keywords: Conventional propagation; in vitro aseptic culture establishment**;** shoot tip explant; Sugarcane; GA_3 and Kinetin.

1. Introduction

Sugarcane (*Saccharum officinarum* L., Poaceae) is a monocotyledonous, tall, perennial, tropical and subtropical grass widely grown in a zone around the world within 30^0 of the equator [1]. It is an octaploid crop with 2n = 80 number of chromosomes [2] , tillers at the base and grows 3-4 meters tall and about 5 cm in diameter [3] . It is thought to be origin in the New Guinea region and distributed to the other regions and continents along the human migration routes [2] and today, cultivated in over 120 countries with estimated annual global sugar production of 1.74 billion tonnes in 2011 [4]. It is usually vegetatively propagated from axillary buds on stem (stock) cuttings. The first, "plant," crop is generally harvested from 12 to 24 months after planting; thereafter, "ratoon" crops may be harvested at shorter to equal time periods. It accounts for about 70% of the worlds' total sugar production [5] while the remaining is produced from sugar beet (*Beta vulgaris* L., Chenopodaceae). Sugarcane is one of the multipurpose commercial cash and industrial crops of Ethiopia. The sugar industry in Ethiopia has great contribution to the socio-economy of the country in many ways. The contributions are concerned with production and consumption of sugar, ethanol and biofertilizer production, income generation, employment creation, revenue contribution, electric power generation, skill and know-how development, capital formation, agriculture and other industries development, urbanization and market development benefit, provides access to health and clean water, education and road facilities. However, the current sugar production covers only 60% of the annual demand for domestic consumption while the deficient is imported from abroad. To reverse the current situation i.e. satisfy the local sugar market demand and export the surplus, the corporation is undertaking large scale expansion and new sugar development projects; nevertheless, availability of adequate quantity, quality and disease free planting materials of sugarcane within a short time period is the major limiting factor to attain the intended production plan using the conventional method of propagation. In

addition, the yield of the existing few and old commercial cane varieties is declining and some productive varieties were also obsolete due to lack of alternative technologies for disease cleansing and rejuvenation. Moreover, commercialization of improved introduced and adapted sugarcane varieties took several years using the conventional route of propagation.

In conventional propagation method where stem cuttings with two to three nodes used as a planting material have various limitations. A bud produces 4 to 5 shoots [6] and the rate of propagation is 1:10 in a year [7-8]. In contrast, if estimated conservatively, micropropagation can produce 10,000 identical plants from a single bud in about 3 to 4 months [9] and the rate of propagation is 1:22 to 1:25 in 8 to 10 months [10]. Propagation from stem cuttings facilitates spread of pathogens with accumulation of disease over vegetative cycles leading to reduction in yield and quality [7-8]. Unlike the conventional propagation method, micropropagation using shoot tip or apical meristem culture has been widely used to produce virus-free plants [9-12] with rapid multiplication of new variety [13-15] and for rejuvenation and mass production of true to type and uniform planting materials from old diseased sugarcane plants. Moreover, tissue culture raised sugarcane plants were reported to give superior cane and sugar yield as compared to their donors from conventional seed source under similar climatic conditions and agronomic management practices [16-22]. Thus, it is crucial to optimize *in vitro* aseptic culture establishment protocol for successful subsequent *in vitro* propagation to minimize the challenges of conventional propagation method and utilize the merits of micropropagation technology. In addition, information on tissue culture study of sugarcane varieties grown in Ethiopian Sugar estates is scarce. Therefore, this study was carried out with the objective to evaluate the response of the two sugarcane varieties to different levels GA_3 and kinetin on *in vitro* aseptic culture establishment using shoot tip explants.

2. Materials and Methods

Hot water treated setts of the two sugarcane varieties (N14 and B41-227) were obtained from Metahara and Wonji sugar estate seedcane nurseries to use as source of explant for the study. These varieties were selected as they are well adapted and have higher cane and sugar yield and are among a few very productive ones. N14 gives 176.44 t/ha cane yield with 12.11% sucrose content while B41-227 produces 165.35 t/ha cane yield with 11.15% sucrose content. In addition, N14 is characterized by thick green colored cane with broad dark-green leaves, spreading type of growth while B41-227 has thin yellowish-green cane with light-green leaves, erect type of growth. The stem cuttings of these two sugarcane varieties with two node cuttings or setts were planted in the greenhouse of Jimma University College of Agriculture and Veterinary Medicine (JUCAVM) where the study was carried out. The setts were watered every three days and allowed to grow for two to three months after which actively growing shoot tops with apical meristem collected and used as source of shoot tip explants. [23] MS medium, in full strength was used with different concentrations and combinations of GA_3 and kinetin. The MS medium contained 30 g/l sucrose as a carbon source and the pH of the medium was adjusted to 5.8 using 1N NaOH and / or 1N HCl before gelled with 8 g/l agar and autoclaved at 120^o C and 15 psi for 20 minutes and molten medium of 40 ml was dispensed per culture jar. The method of explant preparation and surface sterilization was adopted from [24] and [8] with some modifications. Shoot tops were cut from stock plants at the base with some nodes and the leaves were trimmed, taken to the laboratory for surface sterilization

and explant preparation. Trimmed shoot tops were washed thoroughly under running tap water, outer leaf sheath removed and cut in to about 10 cm length.

Thereafter, the shoot tips were further washed three times each for 15 minutes with tap water containing a drop of liquid soap solution (Top) and three drops of Tween-20. This step was done twice. Then, explants taken to laminar airflow chamber, immersed in 0.3% (w/v) Kocide solution for 30 minutes followed by three times washing each for five minutes with sterile distilled water. The shoot tips were again, rinsed in 70% ethanol for one minute and washed with sterile distilled water three times each for five minutes. Finally, the explants treated with 10% (v/v) sodium hypochlorite solution (4% w/v active chlorine) for 20 minutes. After discarding the sodium hypochlorite solution, the explants were washed with sterile distilled water three times each for five minutes. The surface sterilized explants were excised and sized to 1 cm long and 0.5 cm diameter cultured on initiation medium. The experiment was carried out at a temperature of 25 ± 2 ^{o}C under 16-hours light and eight hours dark photoperiod regimes maintained under fluorescent light having 2500-lux light intensity with 75 - 80% relative humidity of the incubation chamber. The experiment was laid out in a factorial treatment combination in a completely randomized design. The experiment was a three-factor factorial treatment combinations arrangement; sugarcane varieties (B41-227 and N14) and two Plant growth regulators (GA_3 and kinetin). Data were subjected to analysis of variance (ANOVA) using SAS statistical software *(version 9.2)*. Treatments' means were separated using the procedure of REGWQ (Ryan-Einot-Gabriel-Welsch Multiple Range Test).

3. Result and Discussion

Analysis of variance (ANOVA) revealed that the interaction effect of genotypes, GA_3 and kinetin was very highly significant (Genotype * GA_3 * kinetin = $p < 0.0001$) on percent shoot tip cultures initiation, number of shoots per explant and average shoot length of the sugarcane varieties tested. The two sugarcane varieties also showed statistically significant variation in all the responses tested: percent shoot tip explants initiated, number of shoots per explant and average shoot length. There was no culture initiation when shoot tip explants were cultured on MS medium lacking the plant growth regulators GA_3 and kinetin within 30 days, resulting in 100% death or did not show sign of growth. This result might be related to the fact that addition of GA_3 with cytokinin caused high frequency bud break and shoot multiplication in apical shoot buds and nodal explants [25-26]. Gibberellic acid is involved in a wide range of developmental responses. These include promotion of elongation in stems and grass leaves, due in part to activation of intercalary meristems. Even if the early response in gibberellic acid signal transduction is unknown, the later steps involve selective gene transcription and de novo protein synthesis [27] and hence morphogenesis while cytokinins activate RNA synthesis, stimulate protein synthesis and the activation of some enzymes [28] leading to morhogenic responses.

Higher levels or supra-optimal concentrations of both Gibberellic acid and cytokinin - kinetin hampers cell division and hence deleterious to cells. Among the different concentrations and combinations of GA_3 and kinetin tested, sugarcane vareity B41-227 gave the highest (83.33%) shoot initiation response on MS medium supplemented with 0.5 mgL^{-1} GA_3 and 1 mgL^{-1} kinetin (Fig.1 & 4a.) while only 63.33% of N14 shoot tip explants were initiated on this treatment combination. N14 gave the highest (70%) initiation of shoot tip

explants on MS medium supplemented with 1 mgL^{-1} GA_3 and 1.5 mgL^{-1} kinetin (Fig.1 and 4b) while B41-227 gave only 43.33% shoot tip explants initiation on this medium composition. B41-227 also gave the largest shoot multiplication (3 ± 0.00 shoots per plant) result with 4.8 ± 0.02 cm average shoot length when MS medium was supplemented with 1 mgL^{-1} kinetin and 0.5 mgL^{-1} GA_3 (Fig. 2, 3 and 4a). N14 produced relatively larger number of shoots (2.9 ± 0.20 shoot per explant) and maximum shoot length (5.45 ± 0.19 cm) when MS medium was supplemented with 1 mgL^{-1} GA_3 and 1.5 mgL^{-1} kinetin (Fig. 2, 3 and 4b). In B41-227, increasing the concentration of kinetin from 0.5 to 1 mgL^{-1} at GA_3 0.5 mgL^{-1} showed a significant increase in the percent of initiated shoots (from 63.33% to 83.33%).

Similarly, increase in the concentration of kinetin from 0.5 to 1 mgL^{-1} at 0.5 mgL^{-1} GA_3 significantly increased the number of shoots per explant (from 1.10 ± 0.40 to 3.00±0.00) and average shoot length (from 1.0 ± 0.87 to 4.80 ± 0.54 cm). However, further increase in kinetin concentration levels to 1.5 mgL^{-1} significantly reduced the percent initiated shoots, number of shoots per explant and average shoot length to70%, 1.33 ± 0.27, and 2.9 ±0.01 cm, respectively. The results of both sugarcane varieties (B41-227 & N14) were in consistent with the findings of [29-30] who found 70-85% shoot tip cultures initiation for three varieties of sugarcane on MS medium containing 1 mgL^{-1} kinetin + 0.1 mgL^{-1} GA_3 after 20 days of culture. Similarly, [6] also reported 78.33% shoot tip culture initiation on MS medium supplemented with 1 mgL^{-1} kinetin and 0.1 mgL^{-1} GA_3. In the present study, use of activated charcoal to reduce phenolic oxidation along with the two plant growth regulators; GA3 and kinetin showed 100% root induction in the shoot tip explants of sugarcane vareity N14 but not in sugarcane vareity B41-227. In addition, the rate of contamination is higher in sugarcane variety N14 and this might be due to the high sugar content and its long years stay under commercial propagation.

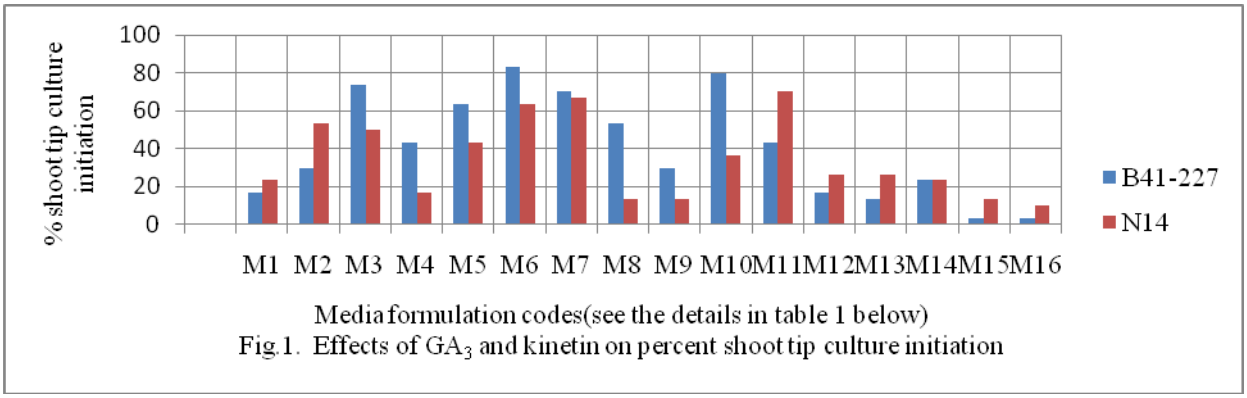

Fig.1. Effects of GA_3 and kinetin on percent shoot tip culture initiation

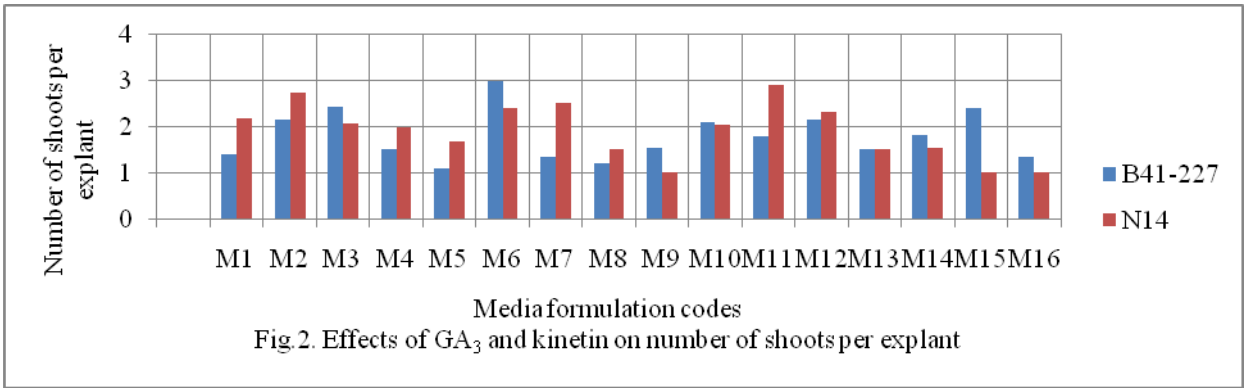

Fig.2. Effects of GA_3 and kinetin on number of shoots per explant

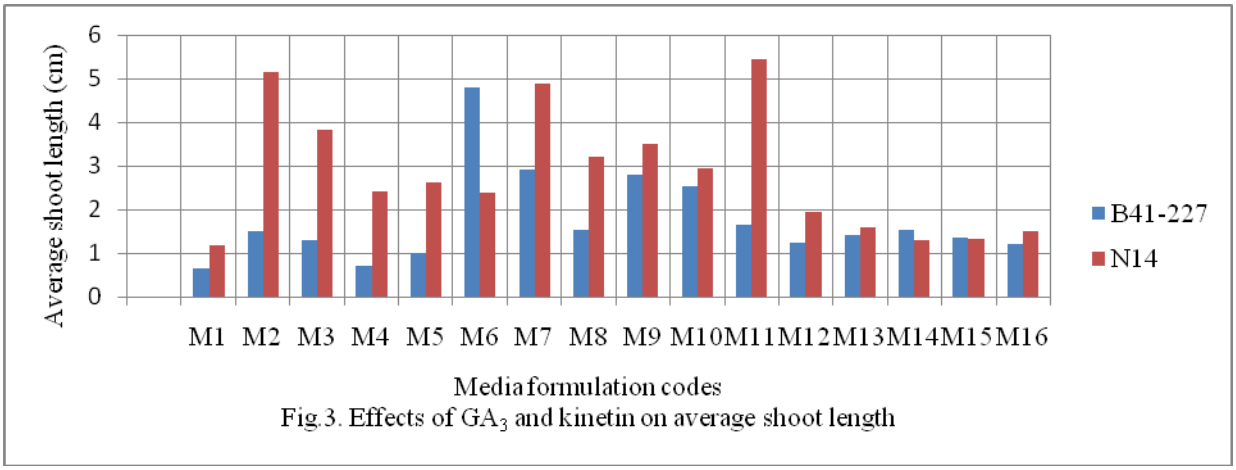

Fig.3. Effects of GA_3 and kinetin on average shoot length

Table 1. Description of Media formulation codes

PGRs (mg/l)	Details of Media formulation codes															
	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16
GA_3	0.1	0.1	0.1	0.1	0.5	0.5	0.5	0.5	1	1	1	1	1.5	1.5	1.5	1.5
kinetin	0.5	1	1.5	2	0.5	1	1.5	2	0.5	1	1.5	2	0.5	1	1.5	2

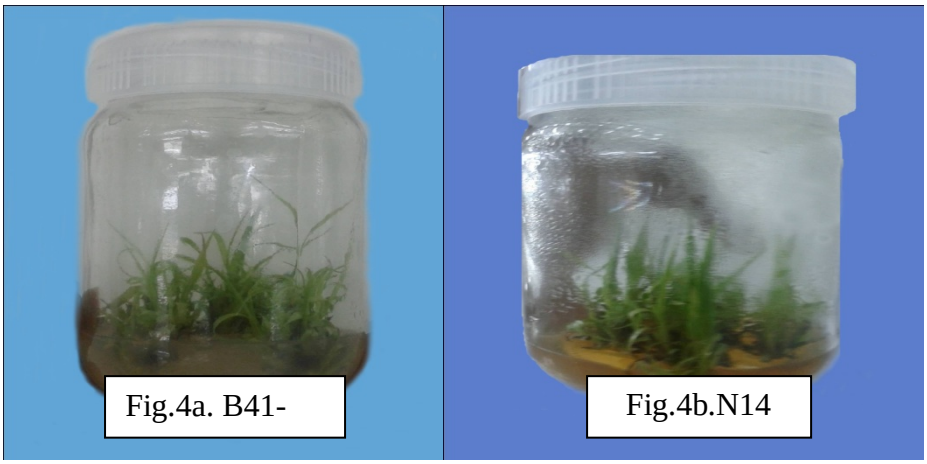

Fig.4. Effects of GA_3 and kinetin on *in vitro* shoot tip culture establishment of B41-227 and N14.

4. Conclusion

From the present result, it is possible to conclude that, MS medium supplemented with 0.5 mgL^{-1} GA_3 and 1 mgL^{-1} kinetin for B41-227 and 1 mgL^{-1} GA_3 and 1.5 mgL^{-1} kinetin for N14 were found to give optimum results for aseptic *in vitro* shoot tip culture establishment of the sugarcane varieties tested. These media could establish 83.33% shoot tip cultures with 3.0 ± 00 shoots per explant and 4.8 ± 0.54 cm shoot length in B41-227 and could establish 70% shoot tip explants with 2.9 ± 0.20 shoots per explant and 5.45 ± 0.29 cm shoot length in N14 after 30 days of culture on initiation medium. Thus, the optimized media composition can be used for rapid

establishment of aseptic shoot tip explants *in vitro* and hence used to avail adequate quantity and quality starter cultures for subsequent *in vitro* propagation of sugarcane planting materials in the Ethiopian Sugar Estates.

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