IN VITRO PROPAGATION PROTOCOL OF TWO ELITE SUGARCANE (*Saccharum officinarum* L.) GENOTYPES USING APICAL MERISTEM CULTURE

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We, the undersigned, member of the Board of Examiners of the final open defense by **Belete Getnet Tesfaye** have read and evaluated his/her thesis entitled **"*In Vitro* [illegible]:ation Protocol of two Elite Sugarcane (*Saccharum afficinarum* L.) Genotypes Using Apical Meristem Culture"** and examined the candidate. This is therefore to certify that the thesis has been accepted in partial fulfillment of the requirements for the degree Master of Science in **Plant Biotechnology.**

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DEDICATION

This thesis was dedicated for my mother Shibashwork Zegeye as she always eagers and prays to see the final fruitful of this work.

STATEMENT OF THE AUTHOR

First, I declare that this thesis is my original work and that all sources of materials used for this thesis is duly acknowledged. This thesis is submitted for partial fulfillment of the requirements for the award of the Degree of Master of Science in Plant Biotechnology at Jimma University and it can be deposited at the University Library to be made available to borrowers under rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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BIOGRAPHICAL SKETCH

Belete Getnet was born in Dec 1988 in Ebinat, South Gondar Zone of Amhara Regional State, Ethiopia. He attended his elementary school at Dega Melza Elementary School from 1998 to 2004. He pursued his secondary school education at Ebinat Secondary High School from 2005 to 2006, and at Addis Zemen Preparatory School from 2007 to 2008. He joined University of Gondar in 2009 and graduated with BSC. degree in Biotechnology in July 2011. After graduation, he was employed by Ethiopian Sugar Corporation, and he worked as assistant researcher at Omo Kuraz Sugar Project from May to September 2012. He joined the School of Graduate studies of Jimma University, College of Agriculture and Veterinary Medicine in September 2012 through the financial support of Ethiopian Sugar Corporation to pursue his MSc. study in Plant Biotechnology.

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LIST OF ABBREVIATIONS

ADS	Adenine hemisulfate
BAP	6- Benzyl Amino Purine
EDTA	Ethylene Di amine Tetra Acetic acid
ESC	Ethiopian Sugar Corporation
GA3	Gibberellic acid
HTM	High throughput multiplication
KIN	Kinetin
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
PGRs	Plant Growth Regulators

***In Vitro* Propagation Protocol of Two Elite Sugarcane (*Saccharum officinarum* L.) Genotypes Using Apical Meristem Culture**

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, the present study was initiated to optimize a protocol for rapid in vitro propagation of two sugarcane genotypes (B4906 and Pr1013) grown in Ethiopia. Multiplication of propagules were carried out in completely randomized design(CRD) with 2x5x5 and 2x6 factorial treatment arrangements of genotypes, BAP(0.5, 1.0, 1.5, 2.0, and 2.5 mg/l) and NAA(0, 0.2, 0.3 0.4, and 0.5 mg/l), and genotypes with sucrose(20, 30s, 30, 40, 50 and 60 g/l) in combination respectively. In vitro rooting was also carried out in completely randomized design (CRD) with 2x5 factorial treatment arrangements of genotypes and NAA(2.0, 3.0, 4.0, 5.0 and 6.0 mg/l) in combination. For shoot multiplication, the initiated shoots were cultured on a medium containing BAP in combination with NAA. The effect of table sugar concentration was also tested for multiplication. For rooting, separated shoots were cultured on 1/2 MS media supplemented with NAA. Number of shoots and leaves, shoot length, number and length of roots were recorded. The results showed that the interaction effects of genotypes and plant growth regulators significantly influenced in vitro sugarcane multiplication. The interaction of genotypes and table sugar concentration also significantly influenced in vitro sugarcane multiplication. The combination effects of NAA and genotypes significantly influenced in vitro rooting. On MS media with 1.5 mg/l BAP and 0.4 mg/l 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 2mg/l BAP and 0.5 mg/l 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. On MS medium with 50g/l table sugar, B4906 gave the highest (13.42±0.29) shoots with 4.09±0.08 cm shoot length and 8.92±0.14 leaves /shoot, whereas Pr1013 produced a maximum of 7.78 ± 0.19 shoots with 4.61±0.04 cm shoot length and 7.77±0.03 leaves /shoot at 60g/l table sugar. Half MS medium with 2mg/l NAA resulted in 91.67% rooted shoots with 12.58±0.23 roots and 2.54±0.04 cm root length in B4906 whereas 4mg/l resulted in 66.67% rooted shoots with 7.83±0.70 roots and 2.60±0.05 cm shoot length in Pr1013. Rooted plantlets acclimatized in greenhouse and 96.1% of plantlets survived successfully in 15days. It could be concluded that the optimized protocol is useful for rapid clonal multiplication of sugarcane planting materials. In vitro propagation through bioreactor using this optimized protocol could be recommended to increase multiplication rate and reduce agar cost. In addition, further studies will be required for protocol improvement using different hormone combinations with aim of increasing multiplication efficiency and cost reduction.

Keywords: apical meristem, BAP, multiplication, NAA, rooting, table sugar,

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 (Suprasanna *et al*., 2011). It is an octaploid crop with a chromosome number of 2n = 8x = 80 (Asano *et al*., 2004). Being highly cross-pollinated in nature, this crop requires specific, hot and humid climate for flowering (Gill *et al*., 2006). It has 4-6 m stem length and tillers at the base to produce unbranched stem. These solid unbranched stems clearly differentiate into joints; each comprises a node and internodes (Mengistu, 2013). It is one of the most efficient convertors of solar energy into sugar and other renewable forms of energy and hence produced primarily for its ability to store high concentrations of sucrose in the internodes of the stem.

The commercially cultivated crops of sugarcane have two geographic centers of origin via New Guinea and Northern India. Today, it is cultivated as a commercial crop in nearly 120 countries in the world (Belay *et al*., 2014a). Even though Brazil, India and China are the major growers of sugarcane accounting for more than 50% of world sugar production, the crop has been also commercially produced in many other countries, including Ethiopia. The total area under sugarcane cultivation in the world, Africa and Ethiopia is about 26.5 million ha, 1.58 million and 23000 ha with cane production of 1.9 billion, 97.17 million and 2.75 million tons, and also an average yield of 71.7, 56.81 and 126.9 tons/ha, respectively (FAOSTAT, 2014). Currently, Ethiopia produces 300, 000 tons sugar every year, which covers only 60% domestic consumption (ESC, 2014). The annual per capita sugar consumption in the world, Africa and Ethiopia was 24.3, 16.06 and 5.5 kg/year respectively (ISO, 2012).

It is a high valued cash crop and exclusive source of 75% world sugar production (Lakshmanan *et al*., 2006) whereas the remaining comes from sugar beet. It also provides many by-products for bio-factory to produce ethanol, butanol, acetic acid, plywood, industrial enzymes, animal feed, chipboard and paper production besides, sugar and energy (Garcia *et al*., 2007). Undoubtedly, ethanol is the key byproduct as oil prices are increasing and most of the developed countries are in favor of plant based ethanol production. Another important use of sugarcane is the production of press mud, which is used as a source of organic matter and

nutrients for crop production (Raja, 2006). In a typical sugar mill, 100 tons of sugarcane on an average produce 10 tons of sugar, 4 tons of molasses from which ethanol is produced, 3 tons of press mud which is converted into biofertilizer, 30 tons of bagasse used for cogeneration of power to yield 1,500 kW electricity and for manufacturing paper (Jalaja *et al*., 2008). Hence, improving sugarcane production can play a role in the economic prosperity of any country like Ethiopia.

The Federal Government of Ethiopia has planned to establish sugarcane plantation on 538,343 ha area in all six-sugar estates within five years (2011/12 to 2015/16), which needs about 646,011.6 to 807,514.5 tons of planting material (Belay, 2012). However, only 37,000 hectares has been accomplished until now; one of the major reasons is shortage of planting material, which needs more rapid multiplication methods to produce sufficient and quality planting material supplementing conventional propagation. This is because sugarcane requires large quantities of seed cane (1.5-2 t/ha) with 3-bud setts planting under subtropical conditions. The total seed canes required for 538,343ha plantation covers 21.53% of the cane produced for sugar production, which means that approximately 0.07 million tons sugar would be buried in the soil annually to accomplish this plan.

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 (Sood *et al*., 2006).It is very low and takes 10-12 months to use as a seed cane to get best sprouting (Feyissa *et al*., 2010; Mengistu, 2013). Hence, once a desired clone is identified, it usually takes 6-7 years to produce sufficient seed material (Yadav *et al*., 2012). Because of this limited availability of seed cane of a newly released variety at the time of its release, reaching to the desired area for commercial cultivation takes long time. 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 (Yohannes *et al*.,2010).The cost of laborer and planting materials transportation is also high (Abiy *et al*., 2014). 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) in two sugar estates, namely, Metehara and Omo Kuraz to supplement the seed cane requirements of commercial propagation. Therefore, the

use of *in vitro* technology 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 (Khan *et al*., 2004). Micropropagation is the first major and widely accepted practical application to *in vitro* techniques of plant propagation in the plant tissue culture (Lyam *et al*., 2012). This technique is used as a tool for rapid clonal propagation and production of cost effective planting materials, and also reducing the time taken by half through producing large amount of plantlets within a short period of time (Khan *et al*., 2004).

Although *in vitro* propagation is very advantageous, genetic variation has been observed in different plant species, including sugarcane (Taylor *et al*., 1995; Zucchi *et al*., 2002).This type of instability often results by genetic instability that leads to somaclonal variations. In the case of sugarcane, shoot tip, direct regeneration and callus culturing induces genetic variability (Burner and Grisham, 1995; Dash *et al*., 2011). Hoy *et al*. (2003) also showed genotype and type of explant source affecting genetic uniformity resulting from tissue culture that changed some yield components. Further, its viability for multiplication also reduced after second subculture (Saini *et al*., 2004; Sahoo *et al*., 2009). However, genetic and phenotypic instability among *in vitro* raised plants of a single donor clone is not required and is undesirable when the purpose is only commercial propagation of plants like sugarcane (Ali *et al*., 2012).

Hence, micropropagation through apical meristem is the best choice for propagation as it produces genetically uniform plantlets that are identical to the mother plant and gives much more rapid multiplication rate (Ali *et al*., 2008). 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 (Devarumath *et al*., 2007). Song *et al*. (2010) obtained 1.77%, 1.56% and 0.31% variation incidence of plantlets generated from somatic embryogenesis, shoot tip and apical meristem respectively. In general, plantlets derived from *in vitro* apical meristem culture are considered to be more genetically and phenotypically uniform. Therefore, considering the limitations of shoot tip and callus culture techniques, researchers have developed protocols for sugarcane *in vitro* propagation using apical meristem explants (Cheema and Hussian, 2004; Singh *et al*., 2006;Uzma *et al*.,2012). Singh *et al*. (2006) and Cheema and Hussian (2004) showed that a new genotype has to be

propagated *in vitro*; its media protocol for regeneration is optimized first. Uzma *et al*. (2012) reported that *in vitro* multiplication was dependent upon plant growth regulators, genotype and type of explants. Malik *et al*. (2005) reported different genotypes had different physiological requirement of plant growth regulators for *in vitro* shoot regeneration and thus different types of plant growth regulator has been given different physiological response. Moreover, the elevated concentration of PGRs leads to metabolic inhibition of the shoot and hence decreases multiplication, whereas low concentration promotes shoot multiplication and elongation in sugarcane (Gopitha *et al*., 2010; Abdu *et al*., 2012; Belay *et al*., 2014b).

In addition, the combination of BAP (0.2-6 mg/l) or BA(0-3mg/l) and NAA (0.1- 1 mg/l) were reported better for shoot multiplication and controlling morphogenesis influencing factor (Bhor and Mungse, 2005; Roy and Kabir, 2007; Behera and Sahoo *et al*., 2009; Song *et al*., 2010; Abdu *et al*., 2012). Different concentrations of table sugar were also tested as best alternative of grade sucrose to minimize the cost of media besides its morphogenesis and growth importance (Khan *et al*.,2006; Demo *et al*.,2008; Swamy *et al*.,2010; Buah *et al*., 2011). For rooting, NAA (0.5- 7 mg/l) alone were reported best for vigorous rooting (Biradar *et al*., 2009; Behera and Sahoo, 2009; Sahoo *et al*., 2009; Gopitha *et al*., 2010; Yadav and Ahmad, 2013; Dereje *et al*., 2014). 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 (Lakshmanan *et al*., 2006). 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 following objectives.

General objective

To develop a suitable protocol for *in vitro* mass propagation of two elite sugarcane clones using apical meristem culture

Specific objectives

- To determine the optimum concentration of BAP and NAA combination for shoot multiplication.
- To determine optimum concentration of table sucrose for shoot multiplication.
- To determine the optimum concentration of NAA for rooting.

2. LITERATURE REVIEW

2.1. Sugarcane Propagation

Sugarcane plants exhibit both sexual and asexual modes of reproduction. It reproduces asexually by three or two buds stem cutting called setts, and *in vitro* propagation by taking parts of it such as shoot tip, apical meristem, axillary shoot and bud. It also reproduces sexually via flower (fuzz), which is used for breeding purposes. Currently in Ethiopia, the breeding program involves only the import of fuzz and selection of exotic lines. However, most of the time the flower of sugarcane is not viable, and the reason behind this might be highly variable. This variation source is not required to maintain continuity of the variety to be stable for commercial propagation.

2.1.1. Conventional propagation

Sugarcane is propagated commercially by vegetative method, which involves the planting of the stem cuttings of premature cane about 8 to 12 months old grown with special care are recommended for seed cane (Mengistu, 2013). The seed cane that is used as planting material may be either whole stalks or stalks` cut up in shorter segments called setts (Garside and Braunack,2001). The growth of sugarcane has different phases: emergency, tillering, stalk growth, and maturation. The germination is a critical event in the plant life to assure a good harvest. It is initially dependent on the set nutrients and water till developing its own root system for three weeks under proper conditions, though, the initial growth of sugarcane is influenced by several internal and exogenous factors such as set age, cultivar, setts nutrients, temperature, soil aeration; setts position on the stalk and humidity (Mengistu, 2013).

A cane sett is the main conventionally propagation system for sugarcane growing countries in the world. In some instances, the buds scooped out of the cane using a bud-chipping machine or knives are used for raising the seed nursery (Jalaja *et al*., 2008). These seed canes involve a three-tier system after treating with hot water or aerated steam therapy to kill pathogens and pests harboring the seed pieces and treating the setts with a fungicide. The treated setts planted in seed nurseries are used to raise primary seed, the primary seed is used as planting material to grow a foundation seed nursery and the foundation seed in turn is used to raise the

certified seed nurseries. In addition, higher seed rate of 75,000 two-bud setts per hectare is needed for raising breeder's seed to compensate for germination loss due to heat therapy (Jalaja *et al*., 2008).

In addition, one shoot bud produces four to five shoots in a year (Khan *et al*., 2009) and one hectare of seed cane is only sufficient to plant 10 ha commercial fields (1:10) in 7- 10 months (Biradar *et al*., 2009). Thus, The newly released varieties take 6-10 years to produce enough quantity of breeder seed material for the required vast area (Sengar *et al*., 2009; Sughra *et al*.,2014),while its seed multiplication rate ranging from 1:7 to 1:10 (Sood *et al*., 2006; Dash *et al*., 2011). However, the seed accumulates diseases and pests during several cycles of field production. In general, non-availability of quality and true to type planting material of newly released varieties is a major constraint in their quick adoption for commercial use, and improving sugarcane productivity. Further, traditional method of cultivation using three-budded set requires large quantity of seed, which is costly, time consuming and land demanding (Singh *et al*., 2006).

Therefore, development of tissue culture technology for rapid multiplication of disease-free planting material has been an important step towards sufficient, true to type and quality seed production in sugarcane. Australia, India and the Philippines in the Asia-Pacific region have already applied this technology for commercial seed production and the benefits have become evident through rapid multiplication and distribution of elite varieties and increased sugarcane production (Jalaja *et al*., 2008). The experiences of these countries would be of considerable benefit to all those who are in the process of adopting micropropagation for their seed production programs.

2.1.2. *In vitro* propagation

Conventional propagation of sugarcane suffered from low propagation rates, expensive labour, time consuming and potential transmission of pathogens through seed cane from generation to generation, which limits the efficiency of this method (Lakshmanan *et al*., 2006). This long time taken of propagation causes a major bottleneck in commercial propagation and breeding programmers (Siddiqui *et al*., 1994). Thus, the growing demand of newly released varieties could not be fulfilled by only conventional propagation methods.

Therefore, application of plant tissue culture techniques provides an alternative method for multiplication and improvement of sugarcane (Sengar *et al*., 2011). Plant tissue culture offers the best methodology through micropropagation of sugarcane for quality and phytosanitary planting material at a faster rate in a shorter period. Tissue culture can increase the propagation potential by 20-35 times (Snyman *et al*., 2006). About 18, 520 plants, produced from a single shoot through micropropagation, were required as compared to 8.8 tons of cane seed in conventional methods for planting in one hectare. Thus, multiplication ratio was 100-150 times using tissue culture plants as compared to 11-12 using conventional cane setts, leading to drastic reduction in seed cane requirement (Sandhu *et al*., 2009). Kuar and Sandhu (2014) showed the shoot multiplication rates were ranged from 4 to 25 fold in CoPb 91 and CoJ 83 cultivars, respectively and the complete plantlets were produced in 157 days with 97 percent survival rate. The fidelity of this protocol for agri-business industry was tested by producing approximately 0.1 million saleable HTM sugarcane plantlets in a small-scale (150 m^2) tissue culture unit.

In addition, plants can be disease indexed and healthy material multiplied in half time compared to the conventional route (Snyman *et al*., 2007). Hence, methods of more efficient regeneration protocol for propagation have been developed through micropropagation (Jalaja *et al*., 2008; Sahoo *et al*., 2009). Micropropagation is currently the only realistic means of achieving rapid, large-scale production of disease-free seed canes of newly developed varieties in order to speed up the breeding and commercialization process in sugarcane. It is an important tool for the production of thousands of genetically uniform and safe plantlets, and its usefulness in germplasm storage (Khan *et al*., 2009). Khan *et al*. *(*2006) stated that *in vitro* propagation produce millions of plantlets from single shoot tip within a short period in contrast to conventional method where one bud produces, 4-5 shoots. Lee (1987) and Lal *et al*. (1996) produced around 10,000 identical plantlets in about 3-4 months and 75600 shoots from a single shoot apex explant in a period of about 5.5 months.

Hendre *et al*. (1983) and Biradar *et al*. (2009) estimated that it is possible to produce some 260,000 shoots in four months and $2x10^8$ plantlets in a year over a 4-5 weeks micropropagation cycles from single shoot tips of sugarcane respectively. Moreover, the micropropagated plants (CV. Co 83) grown in the field had up to 44.96% more canes/plots and up to 22.9% greater cane yield/plot than plants conventionally propagated from three-

budded setts (Gosal *et al*., 1998). Benisheikh *et al*. (2012) also stated that Gross yield obtained from *in vitro* multiplication derived plantlets becomes over than their source plants.

2.1.2.1. Apical/Axillary meristem culture

Plant tissue cultures are initiated from tiny pieces, called explant, taken from any part of a plant. Practically all parts of a plant have been used successfully as sources of explant. Plant segments used in tissue culture as explant are shoot tip, apical meristem, axillary bud, root tip, leaf, flower, ovule, cotyledon and hypocotyls. In sugarcane, shoot tip from 1-2 cm (Benisheikh *et al*., 2012; Belay *et al*., 2014a; Dereje *et al*., 2014; Tilahun *et al*.,2014), apical meristem from 1-6mm (Ali *et al*., 2007& 2008; Khan *et al*., 2008, Abbas *et al*.,2013), leaf roll disk (Khan *et al*., 2009 Ali *et al*., 2012; Pandey *et al*., 2012 and Nawaz *et al*., 2013). These explants form direct and indirect organs, embryos, though, shoot tips and meristems give successful results for direct shoot regeneration (Yildiz, 2012). Apical meristem is a small group of cells that develops to shoot, and communicates signals to the rest of the plant (Medford, 1992). Apical Meristem tips are perhaps the most popular source of explant to tissue cultures (Adilakshmi *et al*., 2014; Sughra *et al*., 2014; Jahangir *et al*., 2014). Because the apical meristem is the origin of the shoot, it has four functions; initiating new organs and tissues, communicating signals to the rest of the plant, and maintaining itself as a formative region (Viet, 2009).

It is the most distal to outer portion of the shoot and comprises two groups of cells: the initial/source cells and the cells that are progenitors for tissues and lateral organs or it is a region just proximal to the meristem where lateral organ primordia are formed. Moreover, *in vitro* propagation through apical and axillary meristem shoot is the most common technique in India and Australia for commercial mass production (Jalaja *et al*., 2008). This is due to the cells of apical and axillary meristems that are uniformly diploid and least susceptible to genotype changes, ensures genetic stability of the clones (Kuar, 2014). Eight sugarcane clones plantlet derived using meristem culture method were phenotypically uniform and 4mm size of meristem was the most suitable for establishment of culture while meristems were treated with a solution of ascorbic acid (100 mg/l) + citric acid (150 mg/l) for 10-15 minutes, phenolics could be controlled (Karim *et al*., 2002).

2.1.2.2. Size of explants

The size of explants were determined by the purpose of the experiment and the efficiency of regenerating multiple shoots. Virus free *in vitro* derived plantlets were successfully regenerated from 0.07 to 2 mm meristem size (Parmessur *et al*., 2002; Tiwari *et al*., 2008; Jahangir *et al*., 2014). On otherwise 0.03 to 2cm size of apical meristem and shoot tip (Ali *et al*., 2008 & 2009; Sahoo *et al*., 2009;Tiwari *et al*., 2011; Abbas *et al*., 2013; Belay *et al*., 2014a) for genetically uniform and potential *in vitro* propagation across the world. However, the culture of small meristems exhibits lower rates of survival and regeneration during shoot initiation than larger blocks of meristem. This happens probably because meristems larger sizes provide more amounts of readily available nutrients that require for initiation of shoot primordia than those of smaller sizes (Tiwari *et al*., 2011). They obtained higher at 4 mm size (60%) than in smaller ones at size of 2 and 3 mm (40%) during initiation.

Ali *et al*. (2009) also obtained high regeneration of apical meristems at 4 mm (100%) for two genotypes. Ali *et al*. (2008) obtained 100% survival with 90% regeneration potential at 3 mm size within 12 days, and the time for shoot formation was increased by decreasing the size of the meristem. However, when the explants became extremely large, contaminations are highly serious (Tiwari *et al*., 2011). In contrary, successful results from highly small explants are reported in previous works. Parmessur *et al*. (2002) reported Larger meristems (>1 mm) are likely to be dying, whereas smaller ones (< 0·3 mm) are unlikely to develop into plantlets, and the success resides in the ability to isolate the meristematic with one or two leaf primordia. Jahangir *et al*. (2014) reported successfully regenerates of apical meristem at 0.07mm length size for disease free and rapid mass production of sugarcane cultivars. Generally, the efficient genetically stable *in vitro* propagation to produce potential multiple shoots, 4-6mm size shoot apical meristem was the best in sugar cane (Abbas *et al*., 2013).

2.1.2.3. Adventitious shoot proliferation

The multiplication of shoots are a crucial stage in the propagation of any species for commercial exploitation and the most rapid rates are required. The most common additives to standard media are cytokinins usually as BAP, BA and Kinetin with combination of low amount auxin like NAA, IBA and IAA. Typically, the same medium and environmental conditions are used for both shoot initiation and multiplication (Singh, 2003). The rate of

shoot multiplication mainly depends on a number of factors. These are type and combination of plant growth regulators, explants type, culture medium composition, and genotype. Jagadeesh *et al*. (2011) showed that high ratio of cytokinin and auxin was essential and better for production of adventitious shoots rather than cytokinin alone in sugarcane. The authors found the highest multiple shoot on MS medium with 3 mg/l BAP + 2 mg/l IAA + 2 mg/l Kin.

Bhor and Mungse (2005) obtained the maximum number of shoots (9.8 in Co-86032 and 8.1 in CoM-88121) on MS medium+1.0mg/I BAP+0.5mg/l NAA. Koy and Kabir (2007) obtained the maximum of 17.2 shoots and 7.2 shoot length on MS +1.5mg/l BA with 0.5mg/l NAA in Isd32 genotype. Abdu *et al*. (2012) reported the highest number, length and vigor of shoots in all the genotypes on MS media containing 1.0 and 1.5 mg/L BA with 0.2 mg/L NAA. Gopitha *et al*. (2010) also achieved best regeneration of shoots on MS medium fortified with BAP 1.0 mg/L and IBA 0.5 mg/L. Molina *et al*. (2005) obtained 24 shoots per bud for cv. Mex 68-P23 in four weeks and 29 shoots for cv. MY 55-14 in six weeks on 2 mg/l Kin with 1mg/l NAA. Mamun *et al*. (2004) obtained best shoots for Isd-28 and Isd-29 on MS medium fortified with 1.5 mg/l BA and 0.5 mg/l NAA. Wongkaew & Fletcher (2004) obtained best multiplication on 0.5 mg/l BAP with 0.5 mg/l NAA and 15% CW. In addition, Yadav *et al*. (2012) reported best response of multiplication on MS medium with BAP, Kin and NAA (0.5mg/l each). Sahoo *et al*. (2009) obtained multiple shoots from meristems on MS medium with 1.0 mg/L BA, 0.5 mg/L Kin and 0.25 mg/L NAA.

Furthermore, there are also many reports in cytokinin combinations. Khan *et al*. (2009) reported the optimum multiplication for var. HSF-240, CP-77-400 and CPF-237 at 1.5 mg/l BAP with 0.5 mg/l Kin, 1.0 mg/l BAP with 0.5 mg/l Kin, and 1.0 mg/l BAP with 0.1 mg/l Kin. Abbas *et al*. (2013) reported the optimum multiplication for HSF-240, CP-77-400 SPF-213, HSF-242 and CP-43-33 genotypes on MS with 1.5 mg/l BAP and 0.5 mg/l Kin, 0.5 mg/l BAP and 1.0 mg/l Kin, 1.5 mg/l BAP and 0.1 mg/l Kin, 1.5 mg/l BAP and 0.1 mg/l Kin, and 1.0 mg/l BAP and 0.1 mg/l Kin respectively. Ali *et al*. (2008) obtained maximum shoot multiplication in CP 77400 and BL-4, found 29 shoots on MS medium with 1.0 mg/l BAP, and 0.25 mg/l BAP and Kin respectively.

2.1.2.4. Rooting

The success of *in vitro* propagation relies on efficient rooting in regenerated shoot and their subsequent acclimatization. Once the sufficient numbers of shoots have been generated, portion of explants that contains one or more shoots could be transferred to a medium that contains higher concentration of auxin, resulting in root formation. The initiation of roots is easily achieved in some species by reducing the cytokinin level (Nawaz *et al*., 2013) or on MS medium with or without the addition of extra root promoting auxins (Singh *et al*., 2006). In sugarcane, auxins especially IBA from 0.5-3 mg/l (Singh, 2003; Khan *et al*., 2009) and NAA from 0.5-7mg/l (Pathak *et al*., 2009; Nawaz *et al*., 2013; Adilakshmi *et al*., 2014) alone or in combination are the most common used auxins for rooting.

Most of researchers reported *in vitro* regenerated roots of sugarcane shoots on MS basal medium fortified with auxins (Pathak *et al*., 2009, Sahoo *et al*., 2011, Tawar *et al*., 2008). Khan *et al*. (2009) reported vigorous root development on MS medium containing 6% table sucrose + 1 mg/l IBA among the combinations used. However, the MS medium without growth regulators promoted rooting in more than 90% of two cultivars after 30 days of culture (Dibax *et al*., 2011; Singh *et al*., 2006). In addition to the presence and absence of growth regulators, rooting was greatly dependent on the strength of MS medium in various plant species. Jagadeesh *et al*. (2011) reported that half MS media were more responsive than full MS medium for rooting of sugarcane. This resulted in 77.78% of root inducing shoots from 14.3 days of shoots inoculated on ½ MS + 6 mg/l NAA medium. Sahoo *et al*. (2009) reported that rooting of shoots was achieved on half MS basal medium with 2 mg/l NAA plus 6% sucrose. Tiwari *et al*. (2011) obtained 100% rooted shoots on ½ MS medium supplemented with 50 g/l sucrose and 5.0 mg/l NAA at pH 6.0 within two weeks. Rooting (85-92%) was induced by transferring shoots on 1/2 MS medium supplemented with 2 mg/l NAA and 1.0 mg/l IBA (Pawar *et al*., 2002).

Rooting was highly influenced by the different types and concentrations of auxin used. Even if there also results reported on the IBA and IAA, NAA was the most efficient auxin for root initiation of sugarcane *in vitro* propagation (Singh, 2003; Khan *et al*., 2009). Jagadeesh *et al*. (2011) reported NAA was better than IBA either alone or in combination with other hormones for rooting of sugarcane. In general, many researchers reported that 5 mg/l NAA was good for rooting (Karim *et al*.,2002; Pathak *et al*., 2009; Sandu *et al*., 2009; Yadav *et*

al., 2012), but more than 5 mg/l NAA inhibits rooting (Biradar *et al*., 2009). In contrary, many researchers obtained best rooting at lower concentration of NAA from 0.5-3 mg/l (Behera and Sahoo, 2009; Sahoo *et al*., 2009; Nawaz *et al*., 2013; Yadav *et al*., 2013)

2.1.2.5. Acclimatization of *in vitro* regenerants

Acclimatization of *in vitro* propagated plants to the *ex vitro* environment is a critical step for successful propagation. It is ultimately depending on their ability to withstand the conditions transferring from *in vitro* to *ex vitro* because the *in vitro* environments are highly conducive than *ex vitro environment.* In sugarcane, successful acclimatization can be possible by taking *in vitro* shoots at two different stages of the plantlets. Either this is when *in vitro* regenerated plantlets have an optimum shoot/root ratio (Jagadeesh *et al*., 2011; Jahangir *et al*., 2014) or after optimum shoot formation but before rooting on *in vitro* medium what is called *ex-vitro* rooting (Pandey *et al*., 2011). Furthermore, the acclimatization of *in vitro* regenerated shoots can be achieved efficiently if the plants are initially maintained with high humidity conditions. Tiwari *et al*. (2011) reported that over 6000 rooted shoots were transferred to greenhouse for hardening, of which 94% of the plantlets survived. Yadav *et al*. (2012) reported that 90% survival rate was recorded in the greenhouse condition. Similarly, Snyman *et al*. (2006) and Sengar *et al*. (2009) demonstrated easily acclimatized sugarcane plantlets using soil as substrate and the initial plantlets cultured in mist chamber condition by adding fertilization weekly.

The media compositions with its ratio also play a vital role to increase survivality of plantlets. Ather *et al*. (2009) reported that in *vitro* grown plantlets survived successfully with the rate of 96% after four weeks when farmyard manure was used with garden soil in the ratio of 1:4 as a potting mixture. Jagadeesh *et al*. (2011) reported that the treatment combination of vermicompost: soil: sand (1:1:1) gave the highest survival percentage of 75%, followed by the treatment combination of press mud: soil: sand (1:1:1) which produced 50% survival. Dibax *et al*. (2013) also reported that use of composed substrate of vermiculite + MS salts was effective for acclimatization. In addition, the type of media used such as liquid and semisolid are also a highly important detrimental factor. Snyman *et al*. (2011) obtained approximately 18,000 plants/leaf roll by using temporary immersion *in vitro* culture in 12 weeks when compared with approximately 2000 plants/leaf roll produced on semi-solid

medium. However, due to hyperhydricity, only ~ 34% of the plants produced in RITA® were survived in acclimatization.

2.1.2.6. Genetic stability of *in vitro* plantlets

Plants that clonally propagated by *in vitro* tissue culture have a chance exposed to exhibit a wide array of genetic variation; this is termed as somaclonal variation. For those primarily interested in clonal fidelity, this can be a serious problem, and strategies have been developed in order to reduce the variation to manageable levels (James *et al*., 2004). Plants regenerated from relatively undifferentiated callus cultures possess a vast array of genetic changes, and sometimes, adventitious regeneration also induced variation, this may be from the genotype sensitivity or source of explants (Hoy *et al*., 2003).

Somaclonal variations can result in useful agricultural and horticultural products, however, variations in traits other than those of interest may be undesirable; for instance, using cultured cells for genetic engineering and commercial micropropagation (Gill *et al*., 2006). Such any steps made toward understanding the basis of tissue culture induced genetic variation should be helpful in developing a more stable and manipulatable somatic cell system (Phillips *et al*., 1994). The source of this variation may derive from variation pre-existing in the mother plant or it may be induced *in vitro* due to many factors, which are known to influence *in vitro* induced variation. However, it has been proposed that hypo- or hypermethylation of DNA, which may trigger genome-wide changes (James *et al*., 2004). Hence, *in vitro* culture induced variability, although infrequently beneficial, is undesirable for both commercial propagation and germplasm storage (Rakesh *et al*., 2011).

Genetic instability has been frequently reported in tissue culture derived sugarcane plantlets. Assessing the extent of variability arising from *in vitro* regeneration and its transmission into successive generations via vegetative propagation was reported (Burner and Grisham, 1995). Thus, Song *et al*. *(*2010) compared sugarcane *in vitro* multiple shoots quality, which are produced from embryogenesis, shoot tips and apical buds. Their findings showed that some abnormal plantlets were occurred with the incidence of 1.77%, 1.56% and 0.31% from embryogenesis, shoot tips and apical bud, respectively. Therefore, the reliable option in order to get true to type plants presently available is to secure the enhanced release of axillary shoots through apical and axillary bud meristem culture (Biradar *et al*., 2009). So far, in order

to ensure highest possible yield and quality of field crops, the disease free and genetically uniform stock plant could be extended to the growers by using this technique.

2.1.2.7. Media composition

One of the most important factors governing the growth and morphogenesis of plant tissues on *in vitro* culture is the composition of the culture medium besides physical environment. Plant tissue culture provides major (macro), minor (micro), carbon source (sucrose) and trace amounts of organic additives (vitamins, amino acids and others), gelling agents (Agar), and plant growth regulators (George *et al*., 2008).

Several media formulations are commonly used for the majority of all cell and tissue culture work. However, a better understanding of the nutritional requirements of cultured cells and tissues can help to choose the most appropriate culture medium for the explants used because each variety, even explants at different parts requires different types of nutrition (Loyola-Vargas, 2012). Among the media formulations, MS medium (Murashige and Skoog, 1962) and B-5 (Gamborg *et al*., 1976) medium are both commonly used for most plant species and have high macronutrients. However, the other media formulations contain considerably less macronutrients. In general, MS media were used in all early works of *in vitro* multiplication of sugarcane (Snyman *et al*., 2006 & 2007; Jalaja *et al*., 2008; Pathak *et al*., 2009; Belay *et al*., 2014; Dereje *et al*., 2014).

2.1.2.7.1. Essential inorganic salts

Growth and morphogenesis of *in vitro* cultures of plant cells, tissues and organs are greatly influenced by the composition of the culture medium, which has been modified to stimulate the growth of particular plant material. Mineral nutrients are one of the compositions and necessary for growth and development of plants in the culture medium. According to the International Association for Plant Physiology, the essential elements in concentrations greater than 0.5 mM are defined as macro elements and those required in concentrations less than 0.5 mM called microelements (Bhojwani and Razdan,1996). Unlike *in vivo* seedling, for healthy and vigorous growth, intact plants need to take up ions of N, K, Ca, P and Mg in large amount as macronutrient, and small quantities of other elements like Fe, Cl, Mn, Zn, B, Cu, and Mo as micronutrient from the *in vitro* media (George *et al*., 2008).

However, the optimal range of medium nutrients investigation is required for *in vitro* culture of diverse species and commercial cultivars. In sugarcane, Cheong *et al*. (2009) reported that macronutrients notably nitrogen (N), phosphorous (P) and potassium (K), are essential for growth of meristems. Thom *et al.* (1981) observed a strong preference to uptake organic nitrogen over inorganic nitrogen from the medium in first seven days of culture, while it also increased Sodium uptake during the time when K^+ was becoming deficient in the medium. Further, an *in vitro* system was established for the characterization of inorganic nitrogen uptake by sugarcane plantlets of variety NCo376. The results showed that *in vitro* plants always had a higher uptake for ammonium than ammonia (Hajari *et al*., 2014).

2.1.2.7.2. Plant growth regulators

Plant growth regulators are the critical media components in determining the developmental pathway of the plant cells. The hormone substances used most commonly are plant hormones or their synthetic analogues called plant growth regulators. There are five classes of plant growth regulator: Auxins, cytokinins, Gibberellins, Abscisic acid and ethylene (Gaspar *et al*., 1996). Currently, there are so many kinds of auxins and cytokinins. Among those 2,4-D, IAA, IBA, NAA, picloram etc. are grouped under the former and BAP, BA, KIN, Zeatin and 2ip are in the later one.

Auxins, cytokinins and gibberellins are the most widely used plant growth regulator in plant tissue culture, which determines the type of culture and regeneration of explants. Auxin in high ratio generally induces the roots, whereas high cytokinin will induce the shoots and the intermediate ratio favors on callus formation (Gaspar *et al*., 1996). Auxin promotes both cell division and cell growth and cytokinins promote cell division (Slater *et al*., 2003). Cytokinins have clearly played an important role in shoot induction including the promotion of cell division, the counteraction of senescence and the regulation of apical dominance (Sakikabara, 2004). Hence, the combination of high cytokinin with low auxin was better to promote both cell division and cell growth, DNA synthesis ability and to control morphogenesis (George *et al*., 2008).

The endogenous hormones that are naturally synthesized such as IAA and Zeatin are sensitive to both heat and light. However, currently more stable form of synthetic auxins and cytokinins have been widely used in plant cell culture (Slater *et al*., 2003). These also found

in young plant tissue than old, and hence the possible variable quantity of exogenous cytokinin and auxin play the major role in the adjustment of hormone ratio to get sufficient shoots in sugarcane. Cheong *et al.* (2009) reported that cytokinins (BA and kinetin), GA3 and auxin (NAA) in combination, were critical for maintaining viability and growth of meristems. Uzma *et al.* (2012) reported that culture initiation was dependent upon plant growth regulators, genotype and type of explants. The highest shoot initiation frequency of 96% was obtained by combination of 0.1 mg/l BAP, 0.1 mg/l NAA, 0.1 mg/l Kn and 0.1 mg/l GA3.

Auxins promote cell division and elongation when applied in low concentrations to plant tissue segments (Jennifer *et al*., 2004). There is evidence of the cell elongation and growth of tobacco young shoot, where the presence of auxins on the lower side of the stem resulting in elongation of one side of the leaves and causing the leaf to bend. Endogenous auxin IAA, is synthesized in young apical meristem, and transported to the growing zones of stem and more distantly to the root via polar transport system (Davies, 2004). Hence, the ease of root formation on auxins free medium may be due to the availability of endogenous auxins in the *in vitro* shoots (Minocha, 1987). The root induction capacity and differential response of classical auxins evaluated and demonstrated. NAA, IBA and IAA stimulated adventitious rooting on the stem segment (Biradar *et al*., 2009; Pathak *et al*., 2009; Sandu *et al*., 2009; Jahangir *et al*., 2014) while 2, 4 D and picloram did not produce root organogenesis. This happens probably by light conditions, which profoundly influenced the root induction capacity of the auxins (Verstraeten *et al*., 2013).

2.1.2.7.3. Gelling agents

Media for plant tissue culture can be used in either solid or liquid forms, depending on the type of culture being grown. For any plant cells or tissues culture to be grown on the surface of the medium, it has to be gelled with agar. The firmness of an agar gel is controlled by the concentration, brand of agar and the pH of the medium. The agar concentration commonly used in plant cell culture media range between 0.5 and 1.0 % (w/v). Another gelling agent used for commercial as well as research purposes is gelrite. It is synthetic and used at 1.25-2.5 g/liter, resulting in a clear gel that aids in detecting contamination (Manchanda and Gosal, 2012)

Currently there is also another highly effective alternative gelling agent to reduce the production cost of plant tissue culture through increasing multiplication rate. Because a higher proportion of media cost comes from agar besides sucrose. Puchooa *et al*. (1999) reported there were no significant differences between the gelling agents in terms of fresh weight, dry weight and the number of shoots produced after 32 days in culture. Aggrawal *et al.* (2010) reported that the total cost of medium used for *in vitro* conservation was decreased by 59% by using isabgol as an alternative gelling agent to agar and phytagel. Ayenew *et al*. (2012) reported Enset flour 'Bulla' at 80 g/l as alternative gelling agent, and showed no significant difference in shoot number, root number, and shoot height, of the pineapple plantlets besides the good gelling ability and can also save up to 76 % of cost than 8 g/l Agar.

2.1.2.7.4. Carbon source

Sugars play important role for *in vitro* cultures as an energy and carbon source as well as an osmotic agent. In Plant cell culture media, sucrose is the most common carbohydrate used as carbon source at a concentration of 2-6%. Other carbohydrates are also used, however, they were less effective than sucrose and glucose (George and Manuel, 2013). Bahmani *et al*. (2009) studied the influence of fructose, sucrose, glucose, sorbitol and maltose carbon sources at various concentrations on rooting and hyperhydricity of apple. The authors obtained type and concentration of sugars had a significant effect on rooting percentage, mean root number, mean root length, hyperhydricity, as well as survival rate.

Glucose was more effective than fructose considering that it is utilized by the cells in the beginning followed by fructose (Saad and Elshahed*, 2012*). Flower *et al*. (1982) showed glucose as being the most suitable carbon source, principally on the grounds of biomass yield and growth rate. However, Manchanda and Gosal (2012) observed maximum percent regeneration of 94.17% in CoJ 83 and 89.67% in CoH 119 on MS media fortified by NAA (5 mg/l) and Kin (0.5 mg/l) medium supplemented with 25 g/l maltose as compared with sucrose. Moreover, the autoclaved sucrose is better for growth than filter sterilized sucrose, was frequently demonstrated because autoclaving seems to hydrolyze sucrose into more efficiently utilizable sugars for plants such as glucose and fructose. It was acting as a morphogenetic trigger in the formation of auxiliary buds and branching of adventitious roots (Zahed, 2000).

In Ethiopia, *in vitro* multiplication of plants started before years ago in the research centers and universities, but still now, it does not extend to reach up the commercial level and the required coverage due to the cost of media and other constraints. Supplements of sugar cane molasses, sugarcane juice, banana extract, and coconut water to basal media can be found a good alternative for reducing medium costs, because media chemicals account for less than 15%, while the carbon sources such as laboratory grade sucrose contributes about 34% -51% of the production cost (Demo *et al*., 2008). So far, these substrates in addition to carbon sources, they are sources of vitamins and inorganic ions required for growth (Zahed, 2000). Buah *et al*. (2011) reported that plants that were cultured on 5% sugar cane juice were better in terms of shoot length and number of shoots per plant than those cultured on 30g/l sucrose. Thus, 5% sugarcane juice was found to be a better substitute for laboratory grade sucrose for the *in vitro* propagation of *Musa sp.* among the treatments.

Moreover, commercial table sugar is also the best alternative rather than using pure grade sucrose to reduce the cost of *in vitro* plant production especially for commercial micropropagation. Khan *et al*. (2006) reported that the rates of sugarcane micro shoots obtained from micropropagule were greatly influenced by the concentration of sugar in the medium. A maximum of 11.50 ±0.57 in AEC82-223 and 12.00 ±0.81 in NIA-2004 shoots/explant were obtained at 4% and 6% table sugar among four sugarcane clones respectively. Of the two concentrations tested, 4% commercial sugar appeared to be optimum for shoot regeneration and multiplication, whereas 6% commercial sugar was recommended for rooting. However, many researchers used 3% grade sucrose as usual. Thus, Swamy *et al*. (2010) obtained the highest shoot length (4.87±0.41cm) and of multiple shoots (61.43±0.l9) on MS media fortified with 20% sugarcane juice, followed by 2% table sugar in patchouli. Rukundo *et al*. (2013) also stated a possibility of replacing the laboratory grade sucrose by the table sugar without significant loss in quality and growth to reduce the production cost of *in vitro* plantlets. Tilahun *et al*. (2014) reported the possibility of utilizing the locally available (in each shop and super market), relatively cheap (currently $0.75-1.5 per kg) 30g/l table sugar as carbon source in place of graded sucrose which is imported and expensive ($147 per kg) product in sugarcane tissue culture. The authors obtained no significant difference regarding shoot number, shoot length and leaf number by comparing analytical grade and table sucrose at 30g/l. Furthermore, Gamborg (2002), and Kodym and Zapata (2001) also reported superior performances of *in vitro* plantlets of banana, chrysanthemum,

peanut, and chickpea in medium supplemented with carbohydrates such as glucose, maltose, and table sugar.

2.1.2.7.5. Vitamins

Vitamins are one of the critical organic supplements in plant cell culture media, and frequently benefits have been obtained from supplements of amino acids. Thiamine has been the only vitamin that has consistent importance in plant cell and organ culture. Others like nicotinic acid, pyridoxine HCl and glycine are utilized due to the ability to stimulate specific growth process. Moreover, there are also many additives (coconut water, Adenine hemisulfate, methylene blue, and casein hydrolysate) which are used for different purposes such as embryo induction, shoot growth and multiplication enhancement (Lage & Esquibel, 1997; Visessuwan *et al*., 1999; Gill *et al*., 2004; Ramgareeb *et al*., 2010). Proline also significantly increased the shoot induction frequency (Gill *et al*., 2004).

Asad *et al*. (2009) tested five levels of different amino acids (glutamine, asparagine, glycine, cysteine and arginine) to compare their ability to induce somatic embryogenesis and shoot regeneration from callus. Glycine (0.75 mM), arginine (0.5 mM) and cysteine (0.25 mM) had significant effect on somatic embryogenesis (94%) and shoot production compared to non amino acid medium. Glycine was most effective to promote somatic embryogenesis and shoot regeneration. Lee (1987) also used arginine (60 mg/l) containing medium for indirect somatic embryogenesis of sugarcane.

2.1.2.8. Stock plant establishment

A pre-propagation stage requires proper maintenance of the mother plants in the greenhouse under disease and insect free conditions with minimal dust. Collection of plant materials for *in vitro* propagation should be done after appropriate pretreatment of the mother plants with fungicides and bactericides to minimize contamination in the *in vitro* cultures (Ahloowalia *et al*., 2004). To enhance the probability of success, the mother plant should be grown under optimal conditions in the greenhouse to minimize contamination *in vitro* (Cassells, 2005).

Explants taken from field plants have problem of microbial contamination, as total sterilization of these explants is generally difficult. Moreover, physiological status of donor plant also influences the response of explants (Rakesh *et al*., 2011). As a result, the explants

are then brought to the production facility, surface sterilized and introduced in to culture explants from greenhouse grown stock plants give rise to better results for *in vitro* propagation study as the load of contaminants is minimal compared to the ones grown in field conditions (Tiwari *et al*., 2012a). There are huge variations regarding tissue culture response of explants excised from plants grown in field condition depending on weather conditions during the year, hence, the best results obtained from explants excised from *in vitro* grown seedlings gave best results (Rakesh *et al*., 2011). About 85-95% shoots tip cultures reached successfully to the shoot proliferation.

2.1.2.9. Explants surface sterilization

Contamination in tissue culture can originate from two sources, both on the surface and in the tissues of explants or through faulty procedures in the laboratory. Establishing of tissue culture depends on the explants used, surface and endophytic microorganisms. In meristem culture, most organisms would be eliminated due to its small size whereas in large explants (leaf, stem etc), most if not all microorganisms in the tissues may be carriedover (Cassells, 2005). To avoid contamination, the explant has to be washed and cleaned up prior to surface sterilization by using liquid soap, commercial detergent, kocide, tween 20 or 80 etc with tap water.

Although different sterilization agents such as Ca $(OCl)_2$, H_2O_2, NaOCl, $HgCl_2$ and ethanol can be used for surface sterilization, ethanol, NaOCl and $HgCl_2$ are the most common frequently used agents. Tiwari *et al*. (2012b) studied four sterilization agents (EtOH, NaOCl, $HgCl_2$ and H_2O_2) in combination and alone using leaf sheath explants of two field grown sugarcane varieties. The authors showed that the use of only one sterilizing agent is not successful, and $HgCl_2$(0.1%) for 5min and EtOH (90%) for 10 min was the best along with prior washing and surface sterilization with tween 20 and bavistin. Benisheikh *et al*. (2012) used 70% ethanol for 30 second to one-minute using shoot tip explants, followed by 0.1% $HgCl_2$ for another five minutes.

However, $HgCl_2$ is highly carcinogenic, and toxic to the plant cells entering through the xylem during sterilization. Hence, other safer alternative sterilization methods are developed to avoid this risk through replacing $HgCl_2$ by NaOCl and ethanol (Tiwari *et al*., 2008). Cheong *et al*. (2012) used 70% ethanol for surface sterilization of apical meristem and

auxiliary bud. Khan *et al*. (2009) reported 50% Clorox (Berekina, 5.25% active chlorine) for 30 minutes then put in 70% ethanol for 45 minutes. Tilahun *et al*. (2013) reported surface sterilization with 25% Berekina (5% active chlorine) for 25 min exposure time is optimal for sugarcane shoot tip decontamination, and this treatment could replace 0.1% mercury chloride for 10 minutes. Generally, shoot regeneration in several tropical plant species has been studied and found satisfactory efficiency (82-93%) for maize, citrus species, Brassica spp. and winged been except sugarcane (53%) which was thought to be due to the presence of surface hairs on the leaves and stalks (Kumari and Verma, 2001).

2.1.2.10. Culture environment

Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions. The controlled physical environment provides the culture environment conducive for tissue morphogenesis response (Sengar *et al*., 2011). These physical environments include adequate light, temperature, and proper gaseous and moisture environment. A maximum shoot regeneration and number of shoots per culture were obtained at 16hr photoperiod of 4000 lux light intensity at room temperature for CoS 96258 and CoS 99259 sugarcane varieties. Ali *et al*. (2008) showed the fluorescent light having 2500-lux light intensity and 26◦C ± 1◦C incubation temperature with 16/8 hour light/dark period was optimal. Benisheikh *et al*. (2012) reported that a 16/8 hours light/dark photoperiod at 27±2^{0}C temperature with 2000 – 3000 Lux light intensity were optimal for cultures placing at 25 –30 cm blow fluorescent light. Jahangir *et al*. (2014) used the photoperiod approximately at 16 hrs of 2000 lux and 8hrs of dark at 24 ± 2°C.

Furthermore, the strength of light and temperature in the growth room has an important role to get more survival plantlets during acclimatization. Aggrawal *et al*. (2012) reported that the number of shoots proliferated, elongated, rooting frequency, and subsequent survival of plants after acclimatization were higher in cultures incubated under photosynthetically active radiation (PAR) compared to those incubated under cool fluorescent lights (CFL). Subsequently, osmotic potential of the sap and chlorophyll content of cultures incubated under PAR were also higher than incubated under CFL.

3. MATERIALS AND METHODS

3.1 Plant Material

The genotypes (Pr1013 and B4906) were introduced in 2006 from India and Barbados, which are equatorial zones similar to Ethiopia. They were released to commercial propagation in Wonji and Metehara Sugar Estates since 2013. They have high cane and sugar yield and are among a few productive and adaptive ones (Abiy *et al*., 2013).The stem cuttings of genotypes were collected from Fincha Sugar State 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 after which actively growing shoot tops were collected and prepared as source of explants.

3.2. Explants Sterilization and Preparation

Apical meristem of sugarcane clones were used as explants. 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, removed outer leaf sheath and cut into 5 cm length. Thereafter, 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 rinsed in sterile distilled water three times each for 5min, and then sterilized using 0.3% (w/v) mancozuim (fungicide) solution for 30 min, and shaked gently to assure proper submerging. 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. Finally, the cultures were inoculated on jelled MS basal medium

prepared for initiation. To minimize the exposure contamination, all steps were done aseptically in the laminar airflow cabinet. The cultures were maintained at room temperature with 16/8h light and dark photoperiod respectively and used cool white fluorescent lamps in the growth room.

3.3. Media Preparation

MS media (Murashige and Skoog, 1962) supplemented with various plant growth regulators were used. Stock solutions of the macro salts, micro salts, vitamins, iron source and plant growth regulators (1mg: 1ml) were prepared and stored at $4^{o}C$ in refrigerator. Plant growth regulator, NAA was dissolved using a drop of ethanol and cytokinins (BAP and KIN) by 2N NaOH before making up the final volume with distilled water. Iron EDTA stock solution was stored in brown colored bottle.

The culture medium was prepared from their respective stock solutions, and the appropriate amount of sucrose (3% w/v), Myo-inositol (0.1%w/v), plant growth regulators (NAA, kin and BAP) were added to the medium as required at various concentrations and combination. The final volume was adjusted using distilled water. In addition, 1mg/l methylene blue and 0.08mg/l adenine hemisulfate were used in all initiated experiments for shoot growth and multiplication enhancement respectively. The pH was adjusted in all cases to 5.8 before autoclaving using a drop of 1N KOH and 1N HCL. Agar at 0.8 % (w/v) was added for solid medium throughout the experiment. Before autoclaving, the media were dispensed into washed and sterilized culture jars (40ml-50ml), capped and labeled properly. These media were steam sterilized using autoclave at a temperature of $121^{o}C$ with a pressure of 0.15 Kpa for 15 minutes and transferred to the culture room and stored under aseptic conditions until their use (3-4 days).

3.4. Shoot Initiation and Establishment of Aseptic Cultures

The prepared apical meristems were cultured on the MS medium supplemented with combination of BAP+KIN+NAA, 0.5mg/l each (Pathak *et al*., 2009) for shoot initiation to produce healthy and best elongation shoots; 1mg/l methylene blue (1mg:1ml) and 0.08 mg/l adenine hemisulfate were also added in the medium. Streptomycin (1mg: 1ml) and gentamycine (1mg: 1ml) 0.5ml of each, were used for one liter of media to reduce the

systemic bacterial contamination. For the establishment of aseptic culture, the cultures were placed in the growth room, under white fluorescent light and photoperiod of 16/8 hrs light and dark conditions.

3.5. Experiment 1: The Effect of BAP and NAA on Shoot Multiplication

For shoot multiplication, the initiated shoots were taken after 30 days of first culture 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, various concentrations and combinations of BAP (0.5, 1.0, 1.5, 2.0, and 2.5 mg/l) and NAA (0, 0.2, 0.3 0.4, and 0.5 mg/l) were used. The experiment was arranged in completely randomized design factorial arrangement (2x5x5- two genotypes and two PGRs) with nine regenerated shoots per treatment. The cultures were placed in white florescent light room adjusted at 16/8 hrs light/dark regimes at room temperature. Data on number of shoots, shoot length and number of leaves were recorded 30 days after culturing for multiplication.

3.6. Experiment 2: The Effect of Table Sugar on Shoot Multiplication

In this experiment, the *in vitro* multiplied shoots were cultured on the MS media supplemented with 0.5 mg/l each of BAP, KIN and NAA (Pathak *et al*., 2009), as initiation media, were used to test table sugar concentration effects on multiplication. Different levels of table sugar (20, 30, 40, 50 and 60 g/l) were used. In addition, 30g/l grade sucrose was used as a control for both genotypes because researchers used 30g/l sucrose by default. Completely randomized design (CRD) in 2X6 (two genotype and six levels of sucrose concentration) in factorial arrangement with nine explants per treatments were used. Data on number of shoots, shoot length and number of leaves were recorded after 30 days of culture.

3.7. Experiment 3: The Effect of NAA on Rooting of Micro Shoots

After multiplication, shoots that have above 3 cm length were separated and cultured on hormone free MS medium to avoid carryover effect. After two weeks, the healthy looking and conditioned shoots were transferred to the experimental media, and the leaves that became yellow at the bottom of the shoot were removed before placing them on the

medium. Different levels of NAA (2.0, 3.0, 4.0, 5.0 and 6.0 mg/l) with elevated amount of table sugar (60g/l) were used. The completely randomized design (CRD) in 2x5 (two genotypes and five levels of NAA) factorial arrangement with 12 shoots per treatment were used. Data on the number of roots, length of root and percentage of rooted plantlets were recorded after 30 days of culture.

3.8. Acclimatization

For acclimatization, the rooted plantlets were washed thoroughly and gently using spray to remove agar and sucrose attached on the roots of plantlets. Then thirty plantlets from each genotype were transferred to plastic pots containing hardening medium composed of soil, compost and sand (1:1:1), covered by perforated white plastics to maintain the moisture for plantlets. Thereafter, plantlets were kept under box in the greenhouse for one week. Then they were exposed to direct sunlight in the acclimatization room. The plantlets were watered twice a day, and 0.2% potassium phosphorus (KH_2PO4) was given for a day by using watercane. Finally, numbers of dead and survived plantlets were counted after 15 days.

3.9. Data analysis

SAS software (SAS, 2008 version 9.2) was used for the analysis of variance and Duncan multiple range test (DMRT) was used for mean separation at 5 % probability.

4. RESULTS AND DISCUSSION

4.1 Shoot Initiation and Establishment of Aseptic Cultures

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*. (2014) 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.

4.2. Effects of BAP and NAA on Shoot Multiplication of Two Genotypes

Analysis of variance revealed that the interaction effects of genotype, BAP and NAA were very highly significant ($p< 0.001$) for number of shoots/explant, shoot length and leaves/shoot (Appendix 1). 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 (Lage& Esquibel, 1997; Ramgareeb *et al*., 2010), and adenine hemisulfate enhanced shoot multiplication (Visessuwan *et al*., 1999).

The genotype "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 1.5mg/l BAP and 0.4mg/l NAA (Table 1; Fig. 1). 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 2mg/l BAP and 0.5 mg/l NAA (Table1; Fig.2). This multiplication rate difference might be due to genotypic difference, which affects the frequency of shoot organogenesis (Jahangir *et al*., 2014), and also endogenous cytokinin and auxin concentration differences (Viet, 2009; George *et al*., 2008). 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 Ogero *et al*. (2012). 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 0.4 mg/l at 1.5 mg/l 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 mg/l, significantly reduced the 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. In addition, proliferation increased (from 4.83±0.38 to 8.17±0.44) with increasing of BAP from 0.5 to 1.5 mg/L at 0.0 mg/l NAA, but further increase of BAP to 2 mg/l led to decrease of shoot length and aggregation of shoots in B4906 (Table 1). 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 (Gopitha *et al*., 2010; Abdu *et al*., 2012; Belay *et al*., 2014b).

In addition, Pr1013 showed continuously increased proliferation from 2.42±0.09 to 8.53±0.36 when BAP was increased from 0.5mg/l to 2.5mg/l at 0.0 mg/l NAA. This also indicates that it needs further increase of BAP to get the optimum proliferation. Jalaja *et al*. (2008) obtained maximum shoot multiplication on MS medium with high levels of BAP (6 mg/l) and 0.5 mg/l NAA for several genotypes. Khan *et al*. (2006) obtained a higher shoot multiplication on a medium containing high concentration of BAP (4.5mg/l) 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 (Gopitha *et al*., 2010). Hence, the presence of auxin with cytokinin stimulates cell division and control morphogenesis thereby influences adventitious shoot production.

The present results from B4906 are different from the reports of Roy and Kabir (2007) and Ali *et al*. (2012) who obtained 15.5-17.2 shoots/explant on MS supplemented with 1.5mg/l BAP and 0.5mg/l 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 Behara and Sahoo (2009) who obtained the highest (8.2) shoots/explant on MS with 2mg/l BAP and 0.5mg/l 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 $p \leq 0.05$.

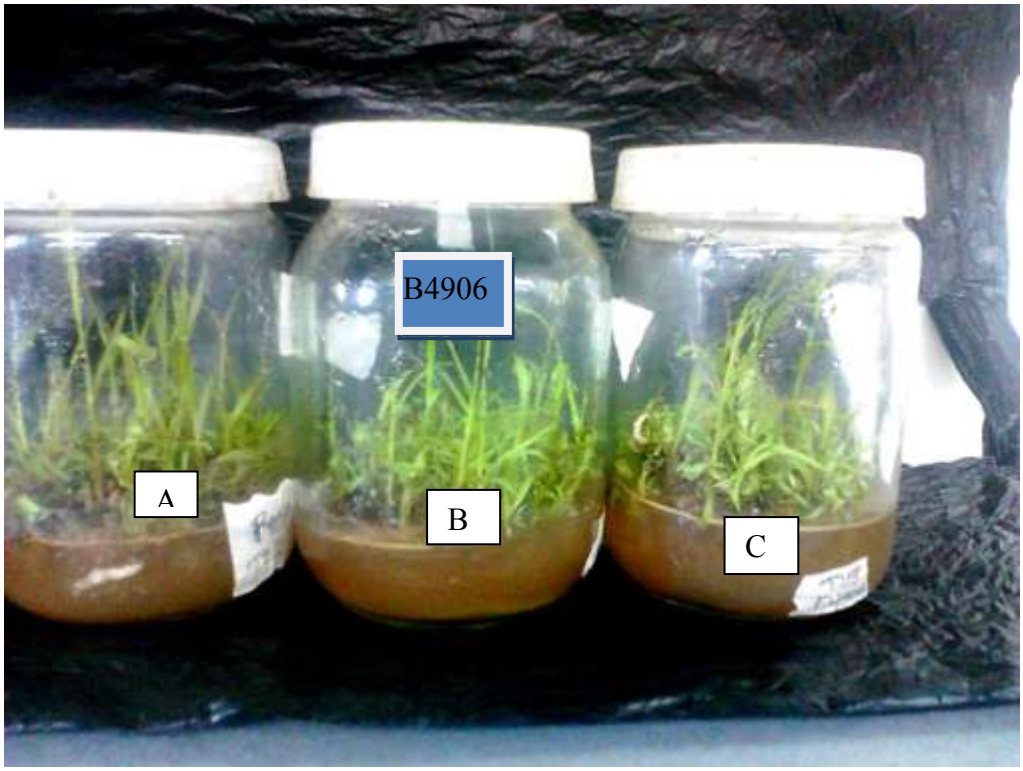

Figure 1. *In vitro* shoot multiplication of B4906 on MS medium containing 1.5mg/l BAP and 0.4mg/l NAA combination after 30 days of culture A, B and C are replicated treatment that performed best for shoot multiplication.

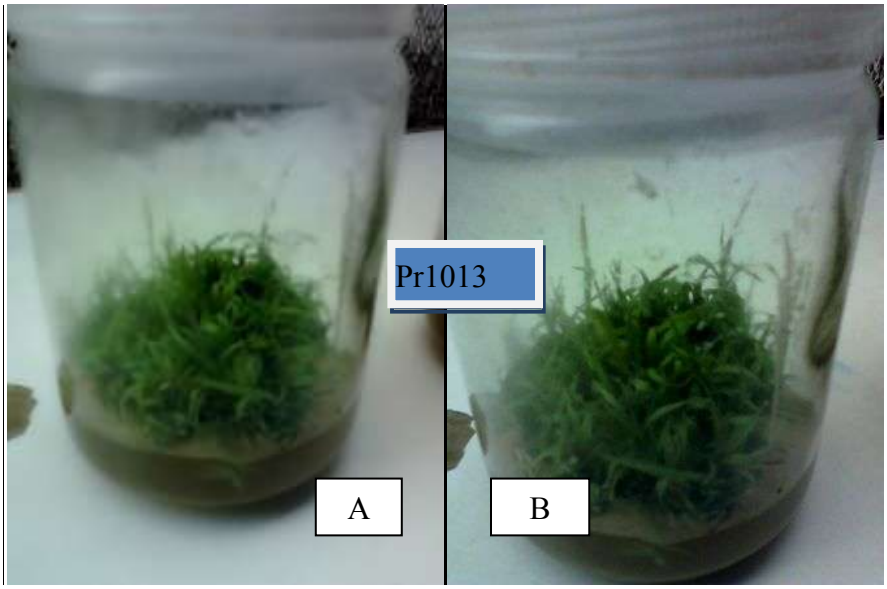

Figure 2. *In vitro* shoot multiplication of Pr1013 on MS medium containing 2mg/l BAP and 0.5mg/l NAA combination after 30 days of culture.

4.2. Effects of Table Sugar Concentration on Shoot Multiplication

Analysis of variance showed that the interaction effects of genotypes and different table sugar concentration was very highly significant ($P<0.001$) for number of shoots/explant, shoot length and number of leaves/shoot (Appendix 2). In B4906, MS medium with 30g/l grade sucrose (control) was statistically different from 20, 30, 40, 50 and 60g/l table sugar for shoot number per explant, shoot length and number of leaves per shoot (Table 2). Except 20g/l, all concentrations of table sugar gave much numbers of shoot than 30g/l pure sucrose. B4906 gave 6.22±0.05 shoot number with 5.39±0.10 cm shoot length and 5.33±0.14 leaves/shoot at 30g/l pure grade sucrose while 30g/l table sugar resulted in 7.17±0.14, 3.05±0.05 cm and 7.42±0.10 shoot number, shoot length, and leaf number per shoot respectively (Table 2). Whereas, 30g/l pure sucrose was statistically different from all treatments for shoot number, shoot length and leaf number in Pr1013, however, only 50g/l and 60g/l table sugar gave better multiplication than 30g/l pure sucrose (Table 2). Pr1013 gave 4.00±0.14 shoot number with 2.67±0.06 cm shoot length and 6.89±0.02 leaves/shoot on MS medium with 30g/l table sugar, while 5.04±0.12, 3.23±0.15cm and 7.75±0.25 for shoot number, shoot length and leaf number per shoot on grade sucrose respectively (Table 2).

This indicates that table sugar was better than grade sucrose to get more multiple shoots and can be an alternative to reduce the cost of plant tissue culture media. It is reported that table sugar enhanced micropropagation and extensively reduced costs by 34% to 51% compared with pure sucrose (Demo *et al*., 2008). According to the current exchange rate, table sugar is much cheaper (USD 0.75-1.5$/kg) than sucrose (USD 31.2$/kg) besides its ease of availability compared to sucrose which needs to be imported. In addition, the difference in terms of shoot number may be due to the impurities of table sugar that contained other elements like iron, phosphorus, potassium and sodium, which are important to promote shoot development when compared with grade sucrose (Demo *et al*., 2008; Buah *et al*., 2011). In addition, table sugar has impurities of glucose, which is easily and highly assimilated by plant tissue primarily than sucrose. Buah *et al*. (2011) and Ogero *et al*. (2011) also confirmed this by using table sugar as carbon source for the *in vitro* culture of sweet potato and banana. The authors found table sugar to be superior to grade sucrose in terms of shoot number, but there is contradiction in terms of shoot length, which this may be due to genotypic difference of used in the experiment.

The concentrations of table sugar affected the proliferation of shoot, also indicate that an optimum concentration was required for each genotype as evidenced in the results. B4906 gave the highest (13.42±0.29) shoots/explant with 4.09±0.08 cm shoot length and 8.92±0.14 leaves/shoot on MS media with 50g/l, followed by 8.78±0.05 shoots/explant with 2.94±0.04cm shoot length, 8.25±0.25 leaves/shoot at 40g/l. Pr1013 produced a maximum of 7.78 ± 0.19 shoots/explant with 4.61±0.04cm shoot length and 7.77±0.03 leaves/shoot at 60 g/l (Table 2 and Fig. 3), followed by 6.06±0.1, 4.77±0.11cm, and 7.45±0.03 shoot number, shoot length, and leaf number per shoot at 50 g/l respectively (Table 2). MS media with 30 g/l and 60 g/l were not statistically different in terms of shoot number in B4906 (Table 2). These results indicate that the concentration of sugar influenced the shoot multiplication besides the genotypic factor and PGRs for *in vitro* propagation as it facilitates metabolic rate and stress the genotypes to induce organogenesis. Khan *et al*. (2006) obtained different shoot number/explant from NIA-98, NIA-2004, BL4 and AEC82-223 genotypes tested using 40 and 60g/l table sugar.

By increasing the concentration from 40 to 50g/l, shoot number, shoot length, and leaf number per shoot were increased from 8.78±0.05 to 13.42±0.29, 2.94 ±0.04 to 4.09±0.08cm and 8.25±0.25 to 8.92±0.14 respectively in B4906, but further increase to 60g/l resulted in a decrease in shoot number, shoot length and leaf number per shoot (Table 2). Pr1013 also showed increased number of shoots and leaves from 6.06 ±0.10 to 7.78 ± 0.19 and 7.45 ±0.09 to 7.77 ±0.03 respectively when the concentration increased from 50 to 60 g/l, but decreased in shoot length from 4.77±0.11 to 4.61±0.04 (Table 2). This indicates that the concentration of sugar plays a vital role and it is critical besides plant growth regulators in sugarcane multiplication under *in vitro* conditions. Khan *et al*. (2006) reported that the presence of sugar was necessary for shoot proliferation, but its concentration in the medium is critical.

The present results for B4906 are in contrast to Khan *et al*. (2006) who obtained 11.50 ±0.57 shoots in AEC82-223 and 12.00 ±0.81shoots in NIA-2004 genotypes on MS media with 4% and 6% table sugar respectively. Whereas the result of Pr1013 is in line with Khan *et al*. (2006) who reported 12.00 ±0.81shoots in NIA-2004 at 6% table sugar, on which 7.78 ± 0.19 average shoots were produced in the current study. However, they did not use 50g/l rate in their experiment. Sorory & Hosien (2000) also confirmed this that the use of 6% sucrose concentration enhanced shoot regeneration in sugarcane.

Table 2.Mean values of shoot number, shoot length and leaf number of two genotypes under influence of table sugar concentrations

Genotype	Sucrose (gm/l)	No. of Shoot /explant (Mean±SD)	Shoot length /shoot (Mean±SD)	No. of Leaves /shoot (Mean±SD)
B4906	20	$4.67^{g} \pm 0.00$	$2.73^{ij}\pm0.04$	$6.78^{ef}\pm0.20$
	30	$7.17^{d} \pm0.14$	$3.05^{g}\pm0.05$	$7.42^{d}\pm0.10$
	30s	$6.22^{e} \pm0.05$	$5.39^{a}\pm0.10$	$5.33^{g}\pm0.14$
	40	$8.78^{b} \pm0.05$	$2.94^{gh}\pm0.04$	$8.25^{b}\pm0.25$
	50	$13.42^{a}\pm0.29$	$4.09^{d}\pm0.08$	$8.92^{a}\pm0.14$
	60	$7.39^{d} \pm0.10$	$3.48^{e}\pm0.10$	$7.00^{e}\pm0.17$
Pr1013	20	$3.31^{i}\pm0.17$	$2.84^{hi}\pm0.05$	$6.55^{f}\pm0.05$
	30	$4.00^{h} \pm0.14$	$2.67^{j}\pm0.06$	$6.89^{e}\pm0.02$
	30s	$5.04^{f} \pm0.12$	$3.23^{f}\pm0.15$	$7.75^{c}\pm0.25$
	40	$4.59^{g} \pm0.14$	$5.24^{a}\pm0.12$	$7.75^{c}\pm0.22$
	50	$6.06^{e} \pm0.10$	$4.77^{b}\pm0.11$	$7.45^{d}\pm0.09$
	60	$7.78^{c}\pm0.19$	$4.61^{c}\pm0.04$	$7.77^{c}\pm0.03$
CV		3.96	6.01	4.16

Note: 30s = 30g grade sucrose as a control. *Values for all parameters are given as mean ± SD. *Numbers within the same column with the same letter are not statistically different from each other at $p \leq 0.05$.

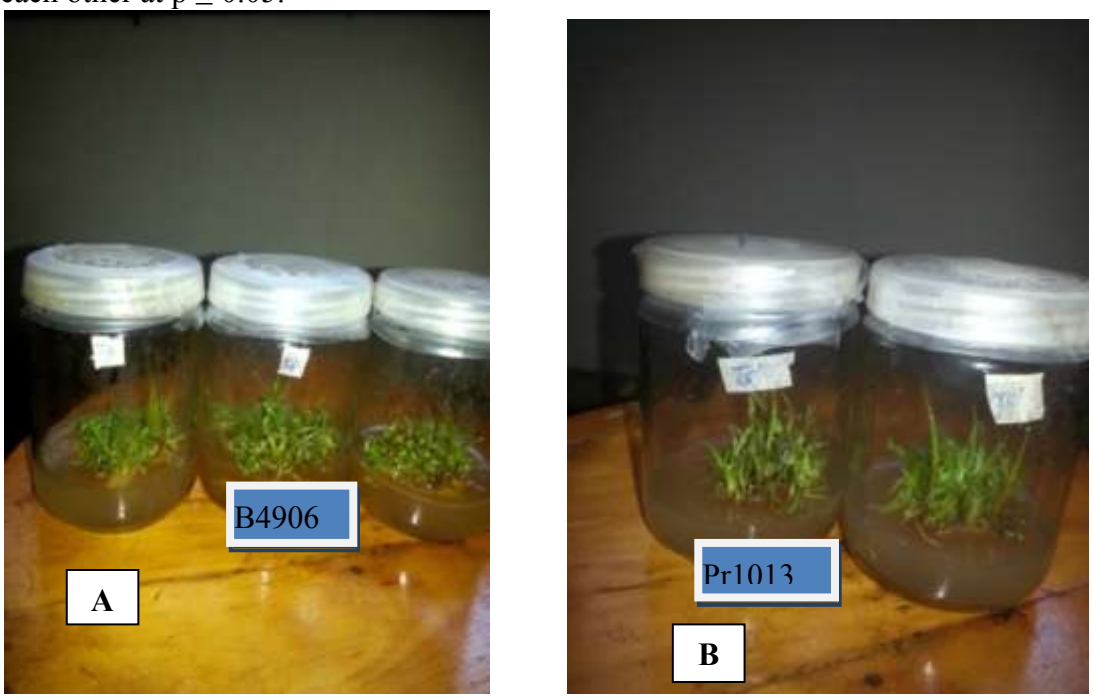

Figure 3. The best *in vitro* multiplication of A) B4906 and B) Pr1013 on MS medium containing with BAP+KIN+NAA(0.5mg/l each) with 50g/l and 60g/l table sugar respectively.

4.3. Effects of α -Naphthalene Acetic Acid (NAA) on Rooting

Analysis of variance indicated that the interaction effect of genotype and NAA was very highly significant ($p< 0.001$) for percentage of rooted shoots, number of roots per shoot and average root length of the two sugarcane genotypes tested (Appendix 3). Fine roots began to be induced from the basal portion of the shoots after 15 days in both genotypes on ½ MS media fortified by 60g/l with and without (control) of NAA, but the roots in the control were not well elongated. This is due to the presence of elevated table sugar, which has increased the cell metabolism and stress to induce rooting, and high source of energy is required to induce cells that are different from the source cell. Singh *et al*. (2006) obtained 100% root induction from plant growth regulator free ½ MS media fortified by elevated 60g/l sugar.

B4906 gave the highest (91.67%) rooted shoots on 1/2 MS medium with 2 mg/l NAA and the lowest 33.33% on 1/2 MS medium with 6mg/l NAA. Whereas Pr1013 produced a maximum of 75% at ½ MS with 2 mg/l and 3 mg/l of NAA, and minimum of 41.67% rooted shoots at 5 mg/l NAA (Table 3). This result indicates that each genotype responded differently due to their endogenous auxin amount. Each genotype requires different concentrations based on the amount of their endogenous auxin concentration (Singh *et al*., 2006). By increasing the concentration of NAA from 2mg/l to 6mg/l, percentage of rooted shoots decreased continuously from 91.67% to 33.33% in B4906, and discontinuously decreased from 75% to 41.67% in Pr1013. In Pr1013, root induction percentage increased from 41.67% at 5mg/l to 66.67% at 6mg/l NAA, but the roots were not well grown, and they were more stunted at 6mg/l than 5mg/l. Generally, this indicates that low concentration of NAA promotes more root induction and elongation than higher concentration that inhibited rooting in both genotypes. In contrary, many researchers reported that higher level of NAA was better for root induction (Pathak *et al*., 2009; Yadav *et al*., 2012).

B4906 gave the highest (12.58±0.23) roots/shoot with 2.54±0.04 cm average root length on ½ MS medium with 2 mg/l NAA (Table 3 and Fig.4). On the same media composition, only 5.67±0.27 roots/shoot with 1.49±0.12 cm average root length were observed for Pr1013. In Pr1013, a maximum of 8.17±0.31 and 7.83±0.70 roots/shoot with 1.49±0.06 and 2.60±0.05cm average root length were obtained on ½ MS supplemented with 3 and 4 mg/l NAA respectively (Table 3 and Fig.4). On the same media, B4906 produced only 9.50±0.15

and 5.75±0.32 roots/shoot with 2.08±0.10 and 1.87±0.06 cm average root length respectively. This indicates that rooting was highly influenced by the concentrations used. Hence, appropriate amounts of auxin in the rooting medium are crucial for root induction. Treatments with 3mg/l and 4mg/l NAA were not statistically significant in terms of root number, but 4mg/l was optimum to get better average root length in Pr1013.

The average number of roots produced per shoot ranged from 2.67 to 12.58. The highest and the lowest roots per shoot were recorded on B4906 at 2mg/l and 6mg/l NAA. This indicates that it needs further lower (< 2mg/l) levels of NAA to get more number of roots, as higher concentrations inhibit rooting in B4906. Nawaz *et al*. (2013) obtained maximum number of roots on MS plus 0.5mg/l NAA .The root length also ranged from 0.54 cm for B4906 at 6 mg/l NAA to 2.6 cm for Pr1013 at 4 mg/l. In general, the effect of NAA was different in two genotypes in terms of percentage, number and length of roots, B4906 showing better results in lower concentrations of NAA than Pr1013.

The results from B4906 are in line with Biradar *et al*. (2009) and Sahoo *et al*. (2011) who obtained the highest (8 and 8.8) number of roots on ½ MS+ 2mg/l NAA respectively, on which, 12.58 roots/shoot were obtained in this study. The present result from Pr1013 is also in line with Yadav and Ahmad (2013) and Gopitha *et al*. (2010) who obtained the highest roots number on ½ MS+3mg/l NAA, in which, 8.17±0.31 roots/shoot were produced in the current study. Yadav and Ahmad (2013) obtained 13.8 roots on MS +3mg/l NAA with 30g/l sucrose. Gopitha *et al*. (2010) obtained 15.1 roots/shoot on ½ MS media with 3mg/l and 50g/l sucrose.

Table 3.Mean values of rooting percentage, root number and root length of B4906 and Pr1013 genotypes under influence of NAA

Genotype	NAA(mg/l)	% of rooted shoots (Mean±SD)	No. of roots (Mean±SD)	Av. root length (Mean±SD)
B4906	2	91.67^{a}±2.36	12.58^{a}±0.23	2.54^{a}±0.04
	3	75.00^{b}±6.38	9.50^{b}±0.15	2.08^{b}±0.10
	4	58.33^{d}±2.36	5.75^{d}±0.32	1.87^{c}±0.06
	5	50.00^{e}±1.34	4.58^{e}±0.32	1.68^{d}±0.05
	6	33.33^{g}±0.00	2.67^{f}±0.00	0.54^{g}±0.00
Pr1013	2	75.00^{b}±4.08	5.67^{d}±0.27	1.49^{e}±0.12
	3	75.00^{b}±1.36	8.17^{c}±0.31	1.49^{e}±0.06
	4	66.67^{c}±2.72	7.83^{c}±0.70	2.60^{a}±0.05
	5	41.67^{f}±10.41	3.17^{f}±0.53	0.71^{f}±0.09
	6	66.67^{c}±2.72	4.75^{e}±0.65	0.62^{fg}±0.18
CV		7.09	6.7	5.44

Note:*Values given are as mean ± SD. *Means within the same column with different letter (s) are significantly different from each other at $p \leq 0.05$.

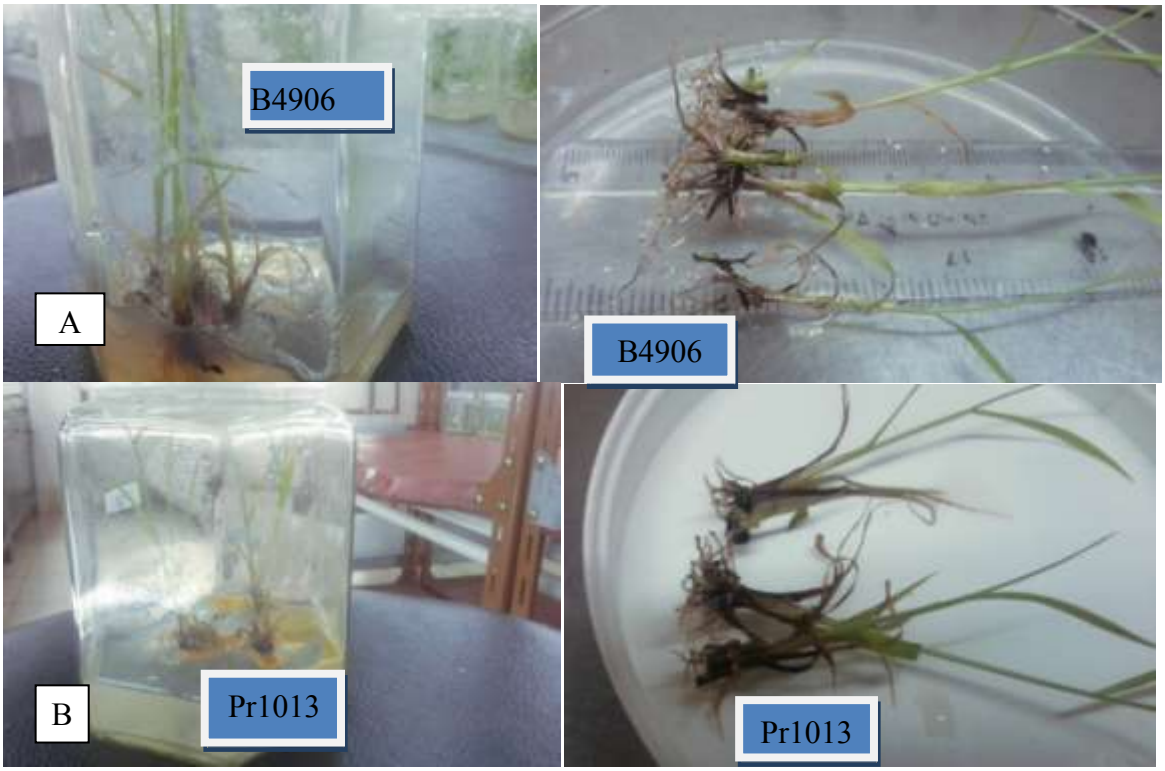

Figure 4. *In vitro* rooting of A) B4906 on ½ MS +2mg/l NAA and B) Pr1013 on ½ MS + 3mg/l NAA

4.4. Acclimatization of Plantlets

Among the acclimatized plantlets, 96.1% of plantlets survived and acclimatized successfully for both genotypes (Figure 4). This is inconsistent with the results of Ather *et al*. (2009) who found 96% successfully survived plantlets using farmyard manure with garden soil in the ratio of 1:4 as a potting mixture. However, this result is in contrast from the reports of Jagadeesh *et al*. (2011) who found the highest survival of 75% and 50% plantlets using the combination of vermicompost: soil: sand (1:1:1), and pressmud: soil: sand (1:1:1) respectively as mixture media.

Figure 5: Acclimatized plantlets of B4906 and Pr1013 after 15 days

5. SUMMARY AND CONCLUSION

Sugarcane (*Saccharum officinarum* L.) is one of the most important perennial crops widely cultivated in tropical and subtropical regions. Ethiopia produces 300, 000 tons sugar from 19000 ha area, which covers only 60% domestic consumption. The government of Ethiopia has a plan to establish sugarcane plantations on 538,343 ha area, which needs about 646,011.6 to 807,514.5 tons of planting material. However, only 37,000 hectares have been accomplished until now. One of the major reasons is shortage of planting material, which needs more rapid multiplication methods to produce sufficient and quality planting material supplementing conventional propagation. In addition, there is also a high risk of disease transmission, high cost of laborer and planting materials transportation. Plant tissue culture is the best alternative to the conventional methods, which also eradicates the risks of contamination by disease during seed production, ensures rapid multiplication, and reduces the time taken by half.

Micropropagation through 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. 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. However, there is no evidence on *in vitro* propagation using apical meristem, specifically B4906 and Pr1013 genotypes in Ethiopia. Therefore, the present study was initiated to optimize a suitable protocol for *in vitro* mass propagation of these sugarcane clones viz., B4906 and Pr1013 using apical meristem culture.

Three experiments; multiplication of propagules, effects of table sugar on propagules multiplication and *in vitro* rooting were carried out. All experiments were arranged in a completely randomized design with three-way and two way factorial arrangements for the first and the later two respectively. For shoot establishment, the explants were sterilized using 0.3% mancozuim, 70% ethanol and 25% commercial bleach (Berekina) through step by step by washing the explants subsequently between each steps using sterilized distilled water. Finally, they were inoculated on MS media supplemented with 0.5 mg/l each of BAP, Kin and NAA with 1mg/l methylene blue, 0.08 mg/l ADS and antibiotics (0.5 mg/l each). For

multiplication, the initiated shoots were cultured on MS media supplemented with a combination of BAP and NAA. Different concentrations of table sugar were also tested in both genotypes. Multiple clumps of shoots were separated and cultured on MS medium containing different concentrations of NAA for root induction. Shoots that have better number of roots and elongation were acclimatized.

The ANOVA revealed that the interaction effects of Gen*BAP*NAA, Gen*table sugar, and Gen*NAA were very highly significant for shoot number, shoot length and leaves number of the first two interaction, and percent of rooted shoot, root number and root length of the later one. On MS medium with 1.5 mg/l BAP and 0.4mg/l NAA, B4906 gave 16.88±0.54 shoots/explant with 5.94±0.17 cm shoot length and 6.33±0.29 leaves/shoot. Whereas, Pr1013 produced a maximum of 11.70±0.28 shoots with 4.48±0.08 cm shoot length and 4.95±0.11 leaves/shoot at 2mg/l BAP and 0.5 NAA. This multiplication difference might be contributed from either genotypic difference or endogenous hormone concentration difference between genotypes. In B4906, MS medium plus 1mg/l and 1.5 mg/l BAP with 0.4 mg/l NAA were the second best combinations, which produced 13.50±0.5 and 13.42±0.38 multiple shoots, respectively, but they were not statistically different. In addition, MS media supplemented by 2 mg/l BAP with 0.2 mg/l NAA, 2 mg/l with 0.3 mg/l NAA, and 2.5 mg/l BAP with 0.3 mg/l NAA were not statistically different in terms of shoot number in Pr1013.

On MS medium with 0.5mg/l each of BAP+Kin+NAA and 50g/l table sugar, B4906 gave 13.42±0.29 shoots with 4.09±0.08 cm shoot length and 8.92±0.14 leaves/shoot while Pr1013 produced 7.78 ± 0.19 shoots/explant with 4.61±0.04cm shoot length and 7.77±0.03 leaves/shoot. However, 40g/l sugar supplemented media was optimum to produce usable, morphologically good and separable shoots for successive subculture. On MS medium with 2 mg/l NAA, 91.67% B4906 shoots induced roots with 12.58±0.23 root number/shoot and 2.54±0.04 cm average root length. Whereas, MS medium with 4mg/l NAA produced 66.67% rooted plantlets, 7.83±0.70 roots and 2.60±0.05 cm root length in Pr1013. MS medium with 3 mg/l also produced similar number of roots like that of 4mg/l in Pr1013, but its root length were not equally good as they were stunted.

In general *in vitro* propagation, in which B4906 gave 16.88 shoots/explant and Pr1013 produced 11.7 shoots/explant within 30 days, was found to be better in producing high number of shoots compared with conventional propagation that produces 5-6 and 3-4 tillers

per stalk for B4906 and Pr1013 within 10-12 months respectively. For multiplication, combination of BAP and NAA was better and preferable to produce more adventitious shoots than BAP alone. Elevated sucrose concentrations produced much better shoot multiplication in sugarcane than using 30g/l. For rooting, half MS media with NAA produced much better developed and adventitious roots in both genotypes compared with the control. Thus, this optimized protocol could be useful for rapid *in vitro* propagation of sugarcane planting material and hence help to minimize the current limitations of sugarcane planting material in the new and expansion of Ethiopian sugar estates. In this study, 40g/l table sugar could be recommended considering its costs. For rooting, MS medium plus 2mg/l NAA and 4mg/l NAA could be recommended to produce profuse and elongated roots in B4906 and Pr1013 respectively.

In the future, further studies will be required to improve the optimized protocol using other types and combination of plant growth regulators with the aim of increasing efficiency of multiplication and reducing cost of production. In addition, protocol optimization for the remaining introduced promising clones should also be carried out.

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7. APPENDICES

Appendix 1. percentage of initiated and established explants

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

Appendix 2. Analysis of variance for the effect of 6-BAP and α-NAA on shoot multiplication of two genotypes

Source	DF	Shoot number	Shoot length	Leaves number
		MS	MS	MS
Gen	1	571.47***	14.64***	27.59***
BAP	4	99.83***	6.81***	2.76***
NAA	4	24.95***	1.88***	6.82***
Gen*NAA	4	30.99***	0.94***	19.57***
Gen*BAP	4	60.28***	2.78***	1.29***
BAP*NAA	16	8.75***	2.58***	1.93***
Gen*BAP*NAA	16	6.24***	2.08***	2.31***
CV		5.31	6.38	4.05

Note *** = Very highly significant at P ≤ 0.001 Gen = Genotype, MS = Mean square, DF = Degree of freedom, CV = Coefficient of variation

Appendix 3. Analysis of variance for the effect of table sugar concentrations on shoot multiplication of two genotypes

Source	DF	Shoot number	Shoot length	Leaves number
		MS	MS	MS
Gen	1	72.12***	1.02**	0.27^{ns}
Sucrose	4	23.15***	3.01***	2.44***
Gen*Sucrose	4	1.25***	3.26***	2.25***
CV		3.96	6.01	4.16

Note: ns= non significant, **=highly significant at P ≤ 0.01, *** = Very highly significant at P ≤ 0.001 Gen = Genotype, MS = Mean square, DF = Degree of freedom, CV = Coefficient of variation

Appendix 4. Analysis of variance for the effect of α - NAA on rooting of two genotypes

Source	DF	%of rooted seedling	root number	root length
		MS	MS	MS
Gen	1	111.16*	9.98***	1.41***
NAA	4	2041.65***	52.41***	3.73***
Gen*NAA	4	736.28***	26.37***	1.14***
CV		7.09	6.7	5.44

Note *= significant, *** = Very highly significant at P ≤ 0.001, Gen = Genotype, MS = Mean square.

Appendix 5. Components of modified Murashige and Skoog Medium (1962) with their concentrations

Constituents	Concentration(mg/l)
NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_20$	370
KH_2PO_4	170
CaCl2.2H2O	440
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.4H_2O$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na-EDTA	37.3
$FeSO_4.7H_2O$	22.3
Nicotinic acid	0.5
Thiamin HCl	0.5
Pyridoxine HCl	0.5
Glycine	2.0
Sucrose	30,000
Agar	8000
Myo-inositol	100