IN VITRO PROPAGATION OF ELITE SUGARCANE (*Saccharum officinarum* L.) GENOTYPES IN LIQUID MEDIA USING SHOOT TIPS

MSc. Thesis

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APRIL, 2015
JIMMA, ETHIOPIA

IN VITRO PROPAGATION OF ELITE SUGARCANE (*Saccharum officinarum* L.) GENOTYPES IN LIQUID MEDIA USING SHOOT TIPS

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BY

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APPROVAL SHEET
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As *Thesis* research advisor, we hereby certify that we have read and evaluated this thesis prepared, under our guidance, by Melaku Tesfa entitled "***In vitro*** **Propagation of Elite Sugarcane (*Saccharum officinarum* L.) Genotypes in Liquid Media Using Shoot tips**". We recommend that it be submitted as fulfilling the thesis requirement.

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DEDICATION

I dedicate this thesis to my father Tesfa Oljira and my mother Bogalech Niguse for their commitment in the success of my life.

STATEMENT OF THE AUTHOR

I, Melaku Tesfa, hereby declare that the work presented in the thesis manuscript entitled, "***In vitro*** **Propagation of Elite Sugarcane (*Saccharum officinarum* L.) Genotypes in Liquid Media Using Shoot Tips**" for partial fulfillment of the requirements for the award of the Degree of Master of Science in Plant Biotechnology at Jimma University is an authentic record of my own work, under the supervision of my advisor Dr. Belayneh Admassu and my co-advisor, Dr. Kassahun Bantte.

Duly acknowledging all source material I used in this thesis, I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. It can be deposited at the University Library to be made without special permission, provided that accurate acknowledgment of source is made. Request for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the School of Graduate Studies when in his or her judgment the proposed use of material is in the interest of scholarship. In all other instances, however, permission must be obtained from the author.

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BIOGRAPHICAL SKETCH

The author, Melaku Tesfa, was born from his father Ato Tesfa Oljira and his mother W/ro Bogalech Niguse in April 1976 in Gindeberete, West Shoa Zone, Oromia Regional State. He attended his elementary and secondary high school education at Mukedima and Gindeberete schools, respectively in Gindeberete. After taking the Ethiopian Schools Leaving Certificate Examination (E.S.L.C.E.) he joined the then Ambo College of Agriculture in 1995 and graduated with Diploma in General Agriculture in 1997. After graduation, he was employed by the then Ethiopian Agricultural Research organization (EARO) and served as technical assistant from 1998 - 2002 and then he was employed by the then Ethiopian Sugar Industry Support Center Share Company, now Ethiopian sugar corporation, as technical assistant from 2002-2010. Then he joined Haramaya University in 2006, to pursue his first degree and graduated with BSc. in Plant Science in 2010. After serving for two years as junior researcher, he joined the School of Graduate Studies of Jimma University to pursue graduate studies in Plant Biotechnology in September 2012/13.

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ABBREVIATIONS and ACRONOMS

2 iP	2-isopentenyl adenine
2, 4 - D	2, 4- dichlorophenoxy acetic acid
ANOVA	Analysis of Variance
BAP	6-Benzyl Amino Purine
CRD	Completely Randomized Design
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
ISO	International Sugar Organization
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
PGR	Plant Growth Regulator
PVC	Polyvinyl Chloride
PVP	Polyvinyl Pyrolidone
REGWQ	Ryan-Einot-Gabriel-Welsch Q
SAS	Statistical Analysis System
TDZ	Thidiazuron

IN VITRO PROPAGATION OF ELITE SUGARCANE (*Saccharum officinarum* L.) GENOTYPES IN LIQUID MEDIA USING SHOOT TIPS

ABSTRACT

Conventional vegetative propagation of sugarcane generally has low multiplication rate and allows dissemination of diseases. This results in shortage of quality planting materials. In vitro propagation is emerging as powerful technique to alleviate such limitations. To date, there is no protocol developed for in vitro propagation of commercial sugarcane genotypes through liquid culture in Ethiopia. Therefore, the present study was conducted with the aim of optimizing the protocol for in vitro propagation of two sugarcane genotypes (N52 and N53) in liquid culture through shoot tip culture. Experiments on shoot multiplication, in vitro rooting, ex vitro rooting and acclimatization were laid out in completely randomized design with factorial treatment arrangements. For in vitro multiplication, different concentrations and combinations of BAP (0, 0.5, 1, 1.5 and 2 mg/l) and Kinetin (0, 0.5, 1 and 1.5 mg/l) were used. For in vitro root induction, ½ strength MS liquid medium supplemented with different concentrations and combination of Sucrose (0, 40, 50, 60 and 70 g/l) and NAA (0,3,5 and 7 mg/l) were used. In ex vitro rooting, uniform micro-shoots were dipped in different concentrations of NAA (0, 10 20, 30 and 40 mg/l) at their basal ends and transferred onto tray filled with sand and soil substrate mixture in 2:1 ratio. For acclimatization, substrate mixtures of sand + soil + farmyard manure were used in six different ratios. Data was subjected to analysis of variance (ANOVA) and means were separated using REGWQ (Ryan-Einot-Gabriel-Welsch). With regard to shoot multiplication, genotype N52 showed a maximum of 6.95 ± 0.19 shoots per explant with 4.75 ± 0.06 cm shoot length on a medium fortified with 3% sucrose and 2 mg/l BAP + 0.5mg/l kinetin while genotype N53 produced a maximum of 6.30 ± 0.26 shoots per explant with 3.94 ± 0.03 average shoot length on a medium supplied with 3% sucrose and 1.5 mg/l BAP and 0.5 mg/l kinetin. Half MS liquid medium + 50 g/l sucrose + 3 mg/l NAA induced the highest rooting (100%) with an average root number per shoot of 23.5 ± 1.29 for N52. For N53, ½ MS liquid medium supplemented with 5 mg/l NAA + 50 g/l sucrose induced the highest rooting response of 100% with an average root number per shoot of 21.76 ± 0.57. In ex vitro rooting, 20 mg/l NAA was found optimal concentration with the highest (76%) rooting frequency with an average of 8.06 ± 0.13 root number per shoot for N52 whereas 30 mg/l NAA gave a maximum of 70% rooting frequency with 4.52 ± 0.19 average root number per shoot for N53. In acclimatization, best survival rate with vigorous growth was achieved on substrate mixtures containing sand + soil in 1:1: ratios in both N52 and N53. From the present results we can conclude that Ms + 2 mg/l BAP with 0.5 mg/l Kinetin was the best combination for shoot multiplication of N52, while MS + 1.5 mg/l BAP with 0.5 mg/l Kinetin was optimum for best multiplication of N53. For in vitro rooting of genotype N52 and N53, ½ MS liquid medium supplemented with 3 mg/l NAA + 50 g/l sucrose and 5 mg/l NAA + 50 g/l sucrose were the optimal combination, respectively. For ex vitro rooting of N52 and N53, 20 mg/l and 30 mg/l NAA were the best concentrations, respectively. Substrate mixture composed of sand + soil in 1:1 ratio found to be the best acclimatization media for both genotypes. Finally, it could be suggested that this protocol can be used for rapid in vitro propagation of these genotypes. Developing protocol for these genotypes using bioreactor and other PGRs types and combinations are the future line of work suggested.

Key words: In vitro propagation, Liquid medium, *Ex vitro* rooting, Acclimatization

1. INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a monocotyledonous perennial plant that belongs to the family *Gramineae* (Jahangir and Nasir, 2010). The six species comprised in the genus *Saccharum* are *S. spontaneum, S. officinarum, S. robustum, S. edule, S. barberi, and S. sinense* (Yadav *et al*., 2012). Sugarcane originated in New Guinea (Henry, 2010). S. *officinarum* is the primary sugar producing species and also called "noble cane" as characterized by its stout and thick stalks, soft rind, high tonnage, and high sucrose content and low proportion of fiber (Sengar *et al*., 2011).

The crop tillers 4-12 stalks, which can grow three to five meters in height and its sucrose content fluctuates between 11 to 16% (Anonymous, 2000). Sugarcane is octoploid (2n=80) having x=10 basic chromosome number (Ming *et al*., 2006; Aitken *et al*., 2010). It is C_4 plant, and is the most efficient converter of solar energy into sugars and other renewable forms of energy (Jalaja *et al*., 2008). Most of the sugarcane varieties currently cultivated are derived from the interspecific hybridization of *Saccharum officinarum* and *Saccharum spontaneum* (Ming *et al*., 2006; Henry, 2010; Snyman *et al*., 2011).

Sugarcane is a commercial crop that plays an important role in the economy of several tropical and sub-tropical countries due to its major end byproducts and labour intensive production operations. Sugar is the principal product of sugarcane and it accounts for nearly 70% of sugar produced worldwide (Jalaja *et al*., 2008; Sengar *et al*., 2010); the rest of which is made from sugar beet. Besides sugar, several byproducts are produced from crushing sugarcane. Among these, molasses, filter cake and bagasse are the most important ones. Molasses is used in alcohol factories, road construction and as an additive in livestock feed; filter cake is used as organic fertilizer; and bagasse is used to produce paper, particleboard and generate steam engine. It is also an important input for the production of other industrial valuable products such as, beverages, pharmaceuticals, pastries, and other food industries. Apart from these, cane tops and leaves generally left in the field, supply nutrients through nutrient recycling for further add to the economic value of the crop. Furthermore, the sugar industry is one of the key industries, which contributes in providing jobs opportunity.

Sugarcane grows in all tropical and subtropical regions of the world and it is cultivated in over 100 countries situated between 37°N and 31°S (Hunsigi, 2001). According to FAOSTAT (2014), in 2012 on worldwide base, sugarcane was cultivated on 26.1 million hectares producing 1.83 billion tons of sugarcane with an average cane productivity of 70.4 tons/ha. In Africa, it was cultivated on 1.5 million hectares and produced 94 million tons of sugarcane with an average productivity of 62.90 tons/ha. Similarly, in Ethiopia, it was cultivated on 22389 hectares producing 2.7 million tons of cane with an average productivity of 120.6 tons/ha. Brazil and India account about 50% of the world sugarcane production (FAO, 2008). Other major sugarcane producing countries are China, Thailand, Mexico, Pakistan, Cuba, Philippines, Australia and Colombia (Ming *et al*., 2006).

Although, sugarcane is known to be cultivated on small scale in Ethiopia even before 1950's, there is no well-documented reference on how, where and when it was introduced into the country (Aregaw, 2000). However, sugarcane cultivation on a commercial scale in Ethiopia was started in 1952 and the first production of sugar was commenced in 1954 by Dutch Company, Handles-Vereening Amsterdam (HVA) (Girma and Awulachew, 2007). Currently there are three operating sugar factories in Ethiopia at three different locations namely: Wonji/Shoa, Metahara, and Finchaa, in sequence of their periods of establishment. These three factories together produced about 300,000 tons of sugar and 11.1 million liters of ethanol annually (Sugar Corporation, 2013).

According to ISO (2012), sugar consumption per capita per year in World, Africa and Ethiopia was 23.3, 16 and 5.5 kg, respectively. Although per capita sugar consumption in Ethiopia is one of the lowest in the world, the volume of consumption has been growing steadily from time to time. As a result, the current production covers only 60% of the annual demand for domestic consumption. The gap between demand and supply necessitated the importation of substantial amount of sugar from abroad. Accordingly, in 2011 alone, 88,082 tons of sugar was

imported (ISO, 2012). This indicates that Ethiopia needs to establish additional sugar factories and expand the area and augment productivity of the existing plantation in order to satisfy the existing sugar demand.

As a result of this fact, the government of Ethiopian has launched an expansion programme on existing sugar estates, and the development of huge new plantations with the aim of producing 2.5 million tons of sugar and 181.6 million liters of ethanol in 2015. It was planned to increase the sugarcane plantation area to more than 350,000 ha (Sugar Corporation, 2013). However, to realize this huge expansion programme and to achieve the desired yield improvement, the availability of disease free, true to type and adequate amount of quality planting material within short period of time is important.

Commercially, sugarcane is propagated vegetatively by stem cuttings with each cutting or set having two or three buds. This conventional propagation method has a low seed multiplication rate usually one to ten in one year period of time. Hence, once a desired genotype is selected, it usually takes 8-10 years to produce sufficient quantity of improved seed material. This long duration poses a major bottleneck in commercializing newly released sugarcane genotypes. In addition, there are also chances of perpetuation of sett-borne diseases from generation to generation, thus the growing demand of newly released genotypes could not be fulfilled in time only by conventional propagation methods. Therefore, efficient propagation system is required for mass multiplication of sugarcane in a short time period.

Plant tissue culture is now emerging as a powerful tool for fast multiplication (Ramanand and Lal, 2004; Sengar *et al*., 2011). It is currently the only realistic means of achieving rapid, large-scale production of disease-free seed canes of newly developed genotypes in order to speed up the commercialization process in sugarcane (Lorenzo *et al*., 2001). In contrast to conventional methods where one bud produces 4-5 shoots, tissue culture can produce around 260,000 identical plants from a single shoot tip in 3-4 months (Hendre *et al.,* 1983). Hence, tissue culture can increase the propagation potential by 20-35 times (Geijskes *et al.,* 2003; Synman *et al*., 2006).

In line with this, protocols have been developed for rapid multiplication of newly released and commercially important genotypes of sugarcane through shoot tip, callus, and axillary bud cultures (Baksha *et al*., 2002; Cheema and Hussain, 2004; Ali *et al*, 2008). However,

slow rate of bud proliferation, high cost of agar and manual handling makes conventional micro-propagation costly (Prasad and Gupta, 2006). Besides, agar creates potential problems such as non-uniform dispersal of nutrients and growth regulators in the medium (Scholten and Pierik, 1998).

To overcome these problems, the use of shake culture with a liquid medium has been promoted. Accordingly, several investigators suggested the use of this approach for fast and efficient micropropagation of sugarcane genotypes (Kenganal *et al*., 2009; Khan *et al.,* 2009; Pathak *et al*., 2009).

Genotype specific protocols are needed as the hormonal requirements for *in vitro* morphogenetic responses vary from genotype to genotype in sugarcane (Singh *et al.,* 2001; Ramanad and Lal, 2004; Sood et *al*., 2006).

However, there is no protocol developed for commercial sugarcane genotypes through liquid culture in Ethiopia so far. Therefore, it is necessary to optimize protocol for all commercial, newly introduced and released sugarcane genotypes for their subsequent *in vitro* multiplication through liquid culture. Hence, this research work was conducted with the following objectives:

General objective

To develop *in vitro* multiplication protocol for two elite sugarcane genotypes using liquid culture

Specific objectives:-

- To determine the effect of various concentrations and combinations of BAP and Kinetin on shoot multiplication of selected sugarcane genotypes
- To determine the effect of various concentrations and combinations of NAA and Sucrose on *in vitro* rooting of regenerated plantlets
- To identify a suitable *ex vitro* rooting methods protocol for *in vitro* generated plantlets
- To identify best acclimatization procedure for *in vitro* derived plantlets

2. LITERATURE REVIEW

2.1. Origin and Distribution of Sugarcane

The origin of *Saccharum officinarum* is intimately associated with the activities of humans, as *S.officinarum* a purely cultivated or garden species which is not found in the wild. Commercial sugarcane hybrid cultivars have arisen through intensive selective breeding of species within the *Saccharum* genus, primarily involving crosses between *Saccharum officinarum* and *Saccharum spontaneum. S. officinarum* accumulates very high levels of sucrose in the stem but is highly susceptible to diseases (Lakshmanan *et al*., 2005), whereas *S. spontaneum* accumulates little sucrose, has thinner stalks and higher fiber content but is a highly polymorphic species with resistance or tolerance to many pests and diseases (Jackson, 2005).

The center of origin of *S. officinarum* is thought to be New Guinea (Daniels and Roach, 1987) where it has been grown as a garden crop since 8000 B.C. (Fauconnier, 1993). From New Guinea, its cultivation spread to Indonesia, Malaysia, china, India and Polynesia during prehistoric times. Its distribution from Polynesia to Hawaii took place with native migrations around 500-1000 A.D and from Indonesia to Southern Arabia and East Africa probably before 500 A.D (Ming, 2006). Currently sugarcane is grown in over 100 countries on all contents worldwide situated between 37°N and 31°S (Hunsigi, 2001).

2.2. Economic Importance of Sugarcane

Normally, sugarcane crop is primarily grown as source of sugar. It accounts about three fourth of the world's sugar production (Verma, 2004). Sugar has become an important item of human diet currently. Sugarcane has lately gained increased attention because of ethanol (biofuel) which is derived from cane and has contribution in mitigating climate change (Zuurbier and Vooren, 2008). In addition, there are several by-products from crushing sugarcane. Sugarcane bagasse, the fibrous plant residue that remains after sugarcane crushing burned to provide heat and electricity. It also serves as raw material for paper, cardboards, particle board (Anonymous, 2004). Molasses is used for alcohol fermentation, as an additive in livestock feed and as a fertilizer for cane fields (Mackintosh, 2000). Furthermore, sugarcane wax used in cosmetics and pharmaceutical products, such as in products used to lower cholesterol. Sugarcane ash and filter cake are often used as fertilizer in sugarcane farm.

Moreover, green tops left after harvesting in the field used as a mulch and also used as a low-grade cattle feed (Sundara, 2000).

2. 3. Sugarcane Propagation

2.3.1. Conventional propagation

Commercially, sugarcane is propagated vegetatively; either using whole stalks (Willcox *et al*., 2000) or stalk's cut up in shorter segments called setts as planting material (Srivastava, 2006). The stem cuttings or setts generally used for planting are section with two to three buds (Abbas *et al*., 2013). In Ethiopia, two to three bud setts are used for commercial cane planting and the whole stalk is used solely for initial seedcane propagation purpose. The age of the cane used for setts depends on the length of the growing seasons. In case of Ethiopian sugar estates, cane having age of 6-10 months is preferred both for good yield and quality (Ayele *et al*., 2014).

Ethiopian sugar estates have been utilizing three-tier seed production system comprising initial seedcane, seedcane and commercial cane production. The initial seedcane is used as source for planting materials to establish seedcane fields and in turn seedcane fields are harvested to plant commercial cane in large area of the plantation. However, this conventional method of seedcane production has certain limitations in that the multiplication rate is too slow (1:10) which makes the spread of newly introduced and released varieties slow, hence, it usually takes over 10 years to scale up to the commercial level (Cheema and Hussain, 2004; Sengar *et al.,* 2010) and it also requires large nursery space, one hectare seedcane nursery for ten hectares field planting (Khan *et al*., 2008; Behera and Sahoo, 2009). In addition, this method uses substantial number of seedcane (40 - 60,000/ha) (Sundara, 2000; Hunisgi, 2001) that otherwise used for sugar and by product production. Hence, production and transportation of such large numbers of setts during the planting season is laborious and time consuming (Pandey *et al*., 2011), consequently, increase cost of production

The efficiency of conventional propagation is further constrained by systemic pathogen transfer from seedcane to the successive crops. This method has a potential to spread virus diseases namely Fuji and mosaic virus diseases; bacterial disease such as red rot and ratoon

stunting disease (RSD), and the main fungal disease like smut and pineapple disease. As sugarcane is a ratooning monoculture; once the planting materials are infected, the pathogens keep on accumulating generation after generation, which ultimately reduces the yield and quality of sugarcane (Naz *et al*., 2009; Yadav *et al*., 2012).

In Ethiopian sugar estates, during seedcane preparation the cane knife used for chopping is disinfected by immersing either in Lysol (120 ml lt^{-1} water), Ethanol 98.8% (1lt lt^{-1} water) or Dettol (10 ml lt^{-1} water) solution for 5 minutes after every single stalk chopped into pieces, so as to prevent the transmission of pathogens from one cane to the other. The chopped setts are also dipped either in Tilt (propiconazole), Bumper (propiconazole) Topzole (propiconazole), Noble (triadimefon) or Bayleton (triadimefon) fungicide solution at recommended rate before planting in order to avoid infection of planting material (Firehun *et al*., 2009). Moreover, the seedcane used to establish initial seedcane field is also subjected to hot water treatment at 50°C for 2 hours to eliminate seed-borne diseases like grassy shoot disease, ratoon stunting disease and smut (Sundara, 2000; Hunsigi, 2001).

Even though, all these disease preventive measures have been taken, reports have revealed a greater cane yield losses due to disease and high disease incidence throughout the plantation fields of Ethiopian sugar estates. For instance, a yield loss assessment study carried out at Metahara revealed 19 to 43% cane yield loss due to smut (Tafesse and Huluka, 1992) and annually about ten million birr is spent due to smut disease in this sugar estate (Firehun *et al*., 2009). Although yield loss due to ratoon stunting disease (RSD) is not yet quantified in the Ethiopian sugarcane plantations, it can cause up to 50 % losses of both cane and sugar yields on susceptible varieties (Firehun *et al*., 2009). However, disease survey made at Finchaa, Metahara and Wonji Shoa sugar estates has revealed 31, 46 and 74 % incidence of RSD, respectively (Tafesse and Baiyssa, 2005). Furthermore, Gemechu (2006) reported that sugarcane mosaic virus (SCMV) is found almost in all sugarcane cultivars. In line with this, it was suggested that plant tissue culture techniques for propagation of sugarcane help to circumvent the drawbacks associated with conventional propagation methods (Khan *et al*., 2006).

2.3.2. *In vitro* propagation

In vitro propagation offers many advantages over conventional methods of plant propagation such as rapid large scale multiplication of true to type and uniformly growing plants, continuous and reliable sources of plants and products round the year irrespective of the seasonal variation; planting materials can be made free from viral and bacterial disease; and large number of plants can be produced in short time and space (Parihar, 2007).

The sugarcane tissue culture initiated in 1961 at Hawaii (Yadav *et al.*, 2012). Then, the first sugarcane plant regeneration from callus culture was reported by Heinz and Mee (1969). This important finding showed the way to the utilization of *in vitro* cell and tissue culture for various application such as micropropagation for seedcane multiplication; rejuvenation of older varieties, breeding, *in vitro* germplasm conservation, the elimination of systemic pathogens and genetic engineering of sugarcane (Sengar *et al.*, 2011*)*.

Regeneration of sugarcane plants *in vitro* can take place through two main ways, organogenesis and somatic embryogenesis. Direct organogenesis involves the regeneration of shoots directly (adventitiously) from either apical meristem (Singh *et al.*, 2006; Pathak *et al.*, 2009) shoot tip (Khan, 2009; Biradar *et al.*, 2009), axillary bud (Shankar *et al.*, 2011) or immature leaf discs (Lakshmanan *et al.*, 2006; Ali *et al.*, 2010) after exposure to at least one cytokinins and an auxin at a high cytokinins: auxin ratio. Somatic embryogenesis (SE), the production of embryos from somatic cells, is induced in sugarcane explants in response to auxins, particularly 2, 4-dichlorophenoxyacetic acid (2, 4- D) (Behera and Sahoo, 2009).

As with other plant species, sugarcane *in vitro* multiplication rate is high in somatic embryogenesis. However, this method did not receive much importance in plant propagation because of problems associated with somaclonal variation (Ali *et al.*, 2012). Hence, direct organogenesis from either meristem, shoot tip or axillary bud remain the best method for propagation as it produces plants phenotypically similar to the mother plant and gives much more rapid multiplication rate (Biradar *et al.*, 2009; Sandhu *et al.*, 2009). Accordingly, large numbers of identical clone by *in vitro* culture were reported by many authors (Khan *et al.*, 2006; Ali *et al.*, 2008; Pathak *et al.*, 2009). For instance, Ramgareeb *et al.* (2010) obtained approximately, 1,300 shoots from a single shoot meristem within 11 weeks. Jalaja *et al.*

(2008) also reported that 180,000 plantlets can be produced from a single shoot tip within 372 days.

Meristem and shoot tip culture are also suitable for rejuvenating and prolonging the life span of outstanding varieties under cultivation. According to Sood *et al*. (2006) report, 13.02% increase in productivity of sugarcane and 11.03% sugar recovery recorded as a result of tissue culture plantlets. Similar results have been reported by some earlier workers (Geetha and Padmana, 2002). Ramanand *et al*. (2005) also pointed out a significant increase in cane height, number of millable cane, and cane yield in tissue cultured sugarcane varieties as compared to the conventional propagation of the crop.

In addition, micropropagation has potential for disease elimination in vegetatively propagated crop including sugarcane. In many cases, meristem cultures are found to be the best way to remove the pathogens from the sugarcane plant. For instance, successful elimination of sugarcane mosaic virus (Naz *et al.*, 2009), sugarcane leaf yellows (Parmessur *et al*., 2002), and sugarcane grassy shoot disease (Tiwari *et al*., 2011) chlorotic streak disease, ratoon stunting disease (Sandhu *et al*., 2009) and white leaf disease (Wongkaew and Fletcher, 2004) through meristem culture have been reported. Cha-um *et al*. (2006) have pointed out that the size of sugarcane meristems and genotypes plays a key role in disease-free production. Accordingly, meristems having a size 0.2-1.5 mm in length were recommended for pathogenic virus disease free production of sugarcane (Parmessur *et al*., 2002; Cha-um *et al*., 2006).

2.3.2.1. Media composition

One of the most important factors determining the growth and morphogenesis of plant tissue culture is the composition of the culture medium (Saad and Elshahed, 2012)**.** The basic nutrient requirements of plant cells and tissue grown *in vitro* are very similar in general to those intact plants grown in nature. The amount of nutrients required for sugarcane tissue culture varies with the species and genotype; even tissues from different part of a sugarcane plant have different requirements for satisfactory growth (George *et al*, 2008). Therefore, it is necessary to find out the exact composition of a medium suitable for a particular sugarcane genotype. Generally, sugarcane tissue culture media are made up of some or all of the following components: macronutrient, micronutrient, vitamins, amino acids or other nitrogen

supplements, sugar, water, other undefined organic supplements, solidifying agents and growth regulators (Smith, 2013). All these compounds full fill one or more functions in the *in vitro* growth of sugarcane plant (Murashige and Skoog 1962).

Several media formulations are commonly used for the different cell and tissue culture works. Among them Murashige and Skoog's (MS) medium, Schenk and Hilderbrandt's (SH) medium and Gamborgs's (B-5) medium are greatly elevated in mineral salts (Molnar *et al.*, 2011).These high-salt media are excellent for supporting callus growth and morphogenesis. Murashige and Skoog's (1962), MS basal salt medium becomes the most commonly used medium for sugarcane tissue culture (Hunsigi, 2001).

Inorganic nutrients

In vitro propagation of sugarcane plant requires combination of macro and micronutrients. Macronutrients are those elements that are required in concentration greater than 0.5 mmol/l. The macronutrients provide the six major elements: nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S) in the form of salt in the media for satisfactory growth and morphogenesis. Micronutrients are those elements that are required in concentrations less than 0.05 mmol/l. The essential micronutrients include the minor elements such as iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo) Cobalt (Co) and iodine (I). The optimum concentration of each nutrient for achieving maximum growth rates varies among species (Saad and Elshahed, 2012).

Carbon and energy source

Sucrose is a major component of most tissue culture media. It functions as both a carbon or energy source and osmotic agent (Bhojwani and Razadn, 1996). Since sugarcane tissue culture are unable to photosynthesis effectively owing to inadequate developed cellular and tissue development, lack of chlorophyll, limited gas exchange and carbon dioxide in tissue culture vessels required the supplementation of exogenous sucrose. The most preferred energy source is sucrose at a concentration of 20-60 g/l (Rai, 2007). Other carbohydrates such as maltose, starch, galactose and lactose are also used as carbon source at a concentration of 2-5%, however; they are less effective than either sucrose or glucose (Saad and Elshahed, 2012). Sucrose is cheap, easily available, readily assimilated and relatively stable and is

therefore the most commonly source of energy. Sugar plays a significant role in sugarcane shoot multiplication under *in vitro* condition.

Organic supplement

Plants synthesize vitamins endogenously and these are used as catalyst in various metabolic processes. However, it is necessary to supplement the medium with the required amount of vitamins to achieve the best growth of the tissue. The vitamins most frequently used in cell and tissue culture media of sugarcane include thiamine (B_1), nicotinic acid, pyridoxine (B_6) and Myo-inositol as they may enhance cellular responses (Smith, 2013). Similarly, addition of amino acid to media is important in stimulating cell growth in protoplast cultures and in inducing and maintaining somatic embryogenesis. Furthermore, complex organic or groups of undefined supplements such as casein hydrolysate, coconut milk, yeast-extract, orange juice and tomato juice often used when no other combinations of known define components produce the desired growth. However, these natural extracts have to be avoided, as their composition is unknown and vary from the lot to lot and with age affecting reproducibility of result (Sathyanarayana and Varghese, 2007).

Plant Growth regulators

Plant growth regulators are the critical media components in determining the developmental pathway of the plant cells. The plant growth regulators that used most commonly are plant hormones or their synthetic analogues. The type and the concentration of hormones used depend mainly on the species of the plant, the tissue or organ cultured. Auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture and usually used together where the ratio of the auxin to the cytokinin determining the type of culture established or regenerated. Other hormones, in particular gibberellins, ethylene, and abscisic acid are used occasionally (Smith, 2013). A high auxin to cytokinin ratio generally favour root formation, where as a high cytokinin to auxin ratio favours shoot formation. An intermediate ratio favours callus production.

The common Auxins used in plant tissue culture media include IAA, NAA, 2,4-D and IBA promote both cell division and cell growth. The most important naturally occurring auxin is IAA (indole-3-acetic acid) but its use is limited because it is unstable to both heat and light. Auxins differ in their physiological activity and in the extent to which they translocate through tissue and are metabolized. In tissue cultures, they are usually used to stimulate

callus production and cell growth, to initiate shoots and rooting, to induce somatic embryogenesis, to stimulate growth from shoot apices and shoot stem culture (Bhojwani and Razadn, 1996).

Cytokinins commonly used in culture media include Kinetin, BAP, BA, Zeatin, 2ip and TDZ. They stimulate protein synthesis and participate in cell cycle control. In culture media, cytokinins proved to stimulate cell division, induce shoot formation and axillary shoot proliferation and to retard root formation. However; the synthetic analogues, kinetin and BAP are used more frequently (George *et al*., 2008). Gibberellins used in culture media is GA3 type which enhance growth of callus and help elongation of dwarf plantlets (Bhojwani and Razadn, 1996)

2.3.2.2. Physical state of culture medium

In addition to chemical composition of culture medium, its physical form also strongly influence the growth and multiplication rate of cultured explants. Hussein (2014) categorized *in-vitro* culture media as solid, semi-solid, semi-liquid and liquid based on physical form. Solid media is prepared from adding a solidifying agent into the liquid media to increase viscosity and ensure that the explants remain in an upright position. Agar is the most common gelling agent and important ingredient of plant tissue culture media (Ozel *et al*., 2008). Gelled or liquid medium can significantly change the *in vitro* performance of the explants, even when using the same medium formulation, because of difference in micro-culture humidity and nutrient availability.

2.3.2.2.1. Liquid culture

The use of liquid media is considered as one of the ideal solution for reducing plantlet production costs to certain extent, without compromising quality of plant and enabling automation (Berthouly and Etienne, 2005; Raghu *et al*., 2007). Accordingly, several protocols have been developed for *in vitro* propagation of sugarcane using liquid medium (Ali *et al*., 2008; Khan *et al.,* 2009; Pathak *et al*., 2009). In liquid medium, the close contact of the tissue with the medium may stimulate and facilitate the uptake of nutrients and phytohromones, leading to better shoot and root growth (Suthar *et al*., 2011). Indeed, liquid culture system offer a number of technical advantages over semi-solid culture in that the media can easily be renewed without changing the container, exuded growth inhibitors such as oxidized phenols

are rapidly diluted to mild levels. In addition to that, media sterilization is possible by microfiltration, and container cleaning after a culture period is much easier. Furthermore, with liquid culture, much larger containers can be used and transfer times can be reduced since explants are no longer positioned, but in many cases simply placed in contact with the liquid medium (Etienne and Berthouly, 2002).

Use of liquid culture media has been reported by a number of authors to show better shoot and root growth in many plant species as compared to semi-solid media. For instance, Prasad and Gupta (2006) reported 33.46% increases in shoot multiplication of *Gladiolus* in liquid shake culture system than semi-solid media. Chong *et al.* (2012) also revealed that *Curcuma zedoaria* cultured in the liquid shake culture system produced a significantly higher number of shoots and a heavier biomass (bigger shoot) as compared to that cultured on the solid medium. In pineapple genotypes, shoot production in liquid medium was 9-fold higher than with agar-gelled medium (Zuraida *et al*., 2011). Similarly, Qureshi *et al*. (2014) revealed significantly greater shoot and root length in potato plantlets of liquid media with a mean value of 11.34 cm and 1.72 cm respectively, while in solid medium, it was 6.04 cm and 1.59 cm respectively. Moreover, Ali *et al*. (2008) achieved a superior multiple shoot formation in sugarcane genotypes cultured in liquid medium than those cultured on solid medium.

The major disadvantage encountered when plants are cultured in liquid media is the problem of shoot malformation. Plants tend to accumulate excess of water in their tissue resulting to abnormal morphogenesis, a phenomenon known as hyperhydricity. The plants that develop in liquid media are fragile, have a glassy appearance, with succulent leaves or shoots and a poor root system (Fauguel *et al*., 2008). The leaves are the organs affected most severely in liquid cultures. However, in order to avoid or minimize these problems and improve the efficiency, the use of shake culture techniques has been promoted for mass propagation of plants in addition to inert support materials used on stationary liquid media such as membrane raft (Prasad and Gupta, 2006), autoclaved cotton (Ali *et al*., 2008), filter paper bridge (Shankar *et al*., 2011) and glass beads (Suthar *et al*., 2011); temporary immersion (Takayama and Akita, 2008) and thin film cultures (Adelberg, 2008).

In shake culture conditions, the growth and multiplication rate of the shoots is enhanced by forced aeration, since continuous shaking of the medium provides sufficient oxygen supply to the tissue, which ultimately leads to their faster growth. The lesser expression of apical

dominance due to continuous shaking of the tissues in the medium is another important feature of liquid cultures, which generally leads to the induction and proliferation of numerous axillary buds (Mehrotra *et al*., 2007).

2.3.2.3. In vitro initiation

Establishment of *in vitro* culture is the first stage in any micropropagation programme. The success of the initial stage of micropropagation is influenced by several factors such as, genotype (Gandonou *et al*., 2005), source of explants (Neumann *et al*., 2009), PGRs, type of media, and *in vitro* conditions (Saharan *et al*., 2004) before and after the regeneration process. Moreover, the effective *in vitro* culture establishment is also associated with the effectiveness of sterilization of collected explants and the inhibition of phenol-induced browning of explants.

Explants of various organs vary in their rates of growth and regeneration due to differences in the stage of the cells in the cell cycle, the availability of or ability to transport endogenous growth regulators and the metabolic capabilities of the cells. The correct choice of explant material can have an important effect on the success of tissue culture (George *et al*., 2008).

Ali and Afghan (2001) worked with micropropagation of 8 sugarcane clones using meristem tip culture method and found that 4 mm size of meristems were the most suitable for establishment of culture. Taylor (1994) reported faster shoot development from shoot tip than apical meristem and shoot growth more rapid from shoot tip than apical meristem (Hendre *et al*., 1983). Similarly, Mulleegadoo and Dookun (1999) studied the effect of explant source and genotype on growth of sugarcane *in vitro* and found that growth responses were better with apical buds than axillary buds. There was also growth response variation among the genotypes under study. Similar experiment was conducted and the same result reported by Yadav *et al.* (2012). Higher responses regarding the frequency of shoot initiation in shoot tip than in meristem explants suggested that large size of explant have endogenous growth regulators (cytokinins) and nutrients, which help in survival of explant while meristem explant is comparatively smaller in size (Smith, 2013).

The initiation of adventitious shoots is also dependent on plant growth regulators regime to which the explant is subjected. Auxins and cytokinins are most widely used plant growth

regulators in sugarcane plant tissue culture. The proportion of auxins to cytokinins determines the type of culture established or regenerated. High concentration of cytokinins to auxin generally promotes shoot regeneration. Cytokinins are found to be very effective for both direct and indirect shoot bud initiation. Some investigators used only single cytokinins for culture initiation (Ali *et al*., 2008; Biradar *et al*., 2009; Meena *et al*., 2014), while others used mixture of cytokinins (Cheema and Hussain, 2004; Bisht, 2011; Tolera *et al*., 2014a). A combination of cytokinin and auxin was also used for sugarcane initiation by a number of researchers (Baksha *et al*., 2002; Bhor and Mungse, 2005; Tolera *et al*., 2014b).

Khan *et al*. (2009) reported 70-85% shoot initiation for sugarcane genotypes HSF-240, CP-77-400 and CPF-237 at 1.0 mg/l kinetin in combination with 0.1 mg/l GA3. Maximum culture establishment (62.7 ± 5.3%) was recorded in presence of BAP and Kinetin (0.5 mg/1 each) and minimum culture establishment (7.3 ± 1.3%) was recorded on medium containing no cytokinin (Ramanand and Lal, 2004). Tolera *et al*. (2014b) reported 83.33% establishment of shoot tip culture in MS medium fortified with 0.5 mg/l GA_3 and 1 mg/l Kinetin for sugarcane genotype B41-227 and 70% culture establishment for genotype N14 on MS supplemented with 1 mg/l GA_3 and 1.5 mg/l Kinetin.

2.3.2.4. In vitro shoot multiplication

Once the aseptic cultures are established, next step is to develop methods for continuous multiplication. Plant growth regulators are, perhaps, one of the most prominent factors that influence rate of multiplication. For instance, application of cytokinin eliminates apical dominance, thereby stimulating the growth of lateral buds (George *et al.*, 2008). The most commonly used cytokinins are: benzylaminopurine (BAP), isopentenyl-adenine (2-ip), furfurylaminopurine (kinetin), thidiazuron (TDZ) and zeatin (Saad and Elshahed, 2012).

The optimal cytokinin or growth regulators required for specific morphogenic response varies with the sugarcane genotype under investigation. This is because; the endogenous levels of growth regulators vary with the genotype of the species and largely influence the requirement of the exogenous hormones in the plant system (Fatima *et al*., 2009). For instance, in study carried out for rapid multiplication of three elite sugarcane genotype through liquid culture in MS medium, optimum shoot proliferation was obtained at 1.5 mg/l BAP and 0.5 mg/l Kin for genotype HSF-240, at 1.0 mg/l BAP and 0.5 mg/l Kin for genotype CP-77-400 and at 1.0

mg/l BAP and 0.1 mg/l kin for genotype CPF-237 (Khan *et al*., 2009). Singh *et al*. (2006) also reported difference in hormonal preference of individual genotypes under study.

In many cases the synergistic effect of two or more cytokinins is known to give the most optimal multiplication rate. In sugarcane a combination of cytokinins (BAP and Kin) was found essential for healthy shoot development in many studies (Sood *et al*., 2006; Khan *et al*., 2009; Sandhu *et al*., 2009). Adilakshmi *et al*. (2014) reported superior shoot multiplication at MS liquid medium supplemented with 0.25 mg/l BAP and 0.1 mg/l Kinetin in genotype 96A3 (7.74 shoot /explant) and Co 6907 (6.39 shoot/explant). Moreover, Pathak *et al*. (2009) pointed out that BAP or Kinetin alone induced only 6-10 shoots per culture. However, a significant increase in number of shoots has been obtained when both the cytokinins used simultaneously. Ali *et al*. (2008) also found best results for shoot multiplication in liquid medium with 0.25 mg/l BAP + 0.25 mg/l Kin in sugarcane genotypes BL-4.

Similarly, synergistic effect of cytokinins and auxins is reported to have a profound impact on the growth of the cultures as well as multiplication rate. In the presence of a cytokinin (usually BAP), with little or no auxin, explants proliferate to produce shoot mass with new shoots arising largely from axillary buds. Mamun *et al*. (2004) reported that *in vitro* propagation for sugarcane variety viz., Isd-28 and Isd-29 showed best shoot proliferation when media were supplemented with BA 1.5 mg /l + 0.5 mg /l NAA. Maximum numbers of shoots (26.7±3.1) were obtained in liquid medium containing 0.5 mg/l BAP, 0.5 mg/l Kinetin and 0.5 mg/l NAA (Ramanand and Lal, 2004). Best response in terms of multiplication was observed on MS medium with BAP, Kinetin and NAA (0.5 mg/l each) (Yadav *et al*., 2012). Similarly, Pawar *et al*. (2002) have revealed that addition of an auxin (IAA) to the medium containing cytokinin enhance the number of shoots per culture. Furthermore, Bhor and Mungse (2005) achieved a maximum number of shoots (9.8 shoots) in Co-86032 and 8.1 shoot in CoM-88121 in medium MS + 1.0 mg/I BAP + 0.5 mg/l NAA.

2.3.2.5. In vitro rooting

Rooting can be achieved either by transferring the shoots to medium lacking cytokinin with or without a rooting hormone (Auxin). The most frequently incorporated auxins in rooting medium are NAA, IAA and IBA. However, due to its instability to both heat and light the use of IAA is limited (Smith, 2013). Reducing the concentration of macro and micronutrients to

half of their normal concentrations during the rooting phase is beneficial for root induction. This practice has been applied for sugarcane plant (Bhor and Mungse, 2005; Khan *et al*., 2008). Ali *et al.* (2012) reported a profuse and healthy rooting of sugarcane shoots on half strength of MS medium.

Sugarcane rooting is highly influenced by the different types and concentrations of auxin used. Although auxin is essential for sugarcane root induction, appropriate amounts of auxin are crucial in the rooting medium. High concentration has been reported to inhibit sugarcane rooting. For instance, Biradar *et al*. (2009) investigated and found 80% and 70% rooting frequency with NAA at 2 mg/l and 3 mg/l, respectively for genotype CoC-671. Maximum, 88.9±6.9% shoots developed roots in presence of 5 mg/l NAA in sugarcane variety CoSe01235. When the NAA concentration was raised up to 7.0 mg/l, the rooting frequency was slightly reduced to 61.2±5.8 % (Yadav *et al*., 2012). Many workers also reported that 5 mg/l NAA was good for sugarcane rooting (Ramanand *et al*., 2007; Pathak *et al*., 2009; Adilakshmi *et al*., 2014) and more than 5 mg/l NAA inhibits rooting.

Indole Butyric Acid (IBA) is another auxin used frequently at lower concentration for root induction of *in vitro* raised sugarcane shoots. Baksha *et al*. (2002) achieved rooting on MS medium containing 5 mg/l IBA. Karim *et al*. (2002) also reported optimum root induction at 3 mg/l IBA with 11 roots per shoot along with 3.8 cm root length in 12 days. Earlier findings indicate that the *in vitro* response of rooting in micropropagated shoots varied from genotype to genotype. Ramanand *et al*. (2007) demonstrated a maximum of 75.4 % rooting in genotype CoS96268 and 81% in CoS95255 on half strength MS medium fortified with NAA (5 mg/l) along with sucrose (50 g/l). Similarly, Singh *et al*. (2001) reported 75% rooting in sugarcane genotype CoJ85 and 95% in CoJ86 on half strength liquid rooting medium supplemented with NAA (5 mg/l) and elevated level of sucrose (60 mg/l).

Sucrose is another important media component that serves as energy source and osmoticum and regulates *in vitro* shoot rooting in sugarcane tissue culture. Sucrose at 50 g/l gave better rooting responses, however at 70 g/l; it inhibited the frequency of rooting as well as number of roots per shoots (Ramanand *et al.,* 2007). Similarly, Gopitha *et al*. (2010) found maximum percentage (96%) of microshoot rooted in the medium supplemented with sucrose concentration of 50 g/l, however, medium containing no sucrose or very high level concentration (70 g/l) of sucrose were showing 20% and 42% rooting in them respectively.

Many workers have also reported the same result (Pathak *et al*., 2009; Yadav *et al*., 2012). In contrary, Ramanand and Lal, (2004) reported best rooting at 70 g/l concentration of sucrose in liquid rooting medium. Other researchers induced rooting in medium supplemented with sucrose at 60 g/l concentration (Khan *et al.*, 2008; Khan *et al*., 2009).

2.3.2.6. Ex vitro rooting

Micropropagation involves three steps: initiation, multiplication of established cultures and rooting of microshoots. However, the last step, *in vitro* rooting process is expensive and can even double the final price of micropropagated plants. An analysis of production cost has shown that 60-80% of the total cost due to the intensive manipulation needed, 40% of which is contributed by *in vitro* rooting (Leva, 2011). *Ex vitro* rooting is a promising method in that there is a reduction in cost by avoiding the *in vitro* rooting, reduction in labour and the time of establishment from laboratory to soil and also gives the plants better developed root system (Borkowska, 2001; Shekafandeh, 2007; Yan *et al*., 2010). Pandey *et al*. (2011) revealed that *ex vitro* rooting reduced more than 50% cost of sugarcane plantlet raised by conventional micropropagation. Similarly, Ranaweera *et al*. (2013) reported 71% cost reduction using *ex vitro* rooting of tea (*Camellia sinensis* L.) compared to *in vitro* rooting step. Furthermore, *ex vitro* rooting and acclimatization phase can be done at the same time, hence it is more efficient.

Ex vitro rooting involves exogenous auxin such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthalene-acetic acid (NAA) (Shekafandeh, 2007). Auxin is usually applied as a solution by dipping the basal end of the shoots. Auxin is applied singly or in a combination at different concentrations to improve rooting frequency of plantlets in the acclimatization period. *In vitro* shoots, treated overnight with 20 mg/l NAA, led to formation of complete plantlets with more than 90% root induction (Pandey *et al*., 2011). These plantlets possessed more than 6 roots of 4 cm average length per plantlet and exhibited 95% survival when transferred to polybags containing soil. Thus *ex vitro* rooting can be applied to sugarcane micropropagation to reduce cost of plant production.

2.3.2.7. Acclimatization of plantlets

In vitro regenerated plantlets are finally acclimatized in a greenhouse prior to their transfer to natural habitat or fields. The process of acclimatization is a critical step because it finally determines the success of the micropropagation protocol. *In vitro* developed plantlets are delicate plants because they are produced in closed, sterile environment and grown on nutrient - rich artificial media under controlled conditions with high humidity and low intensity. As a result, plantlets developed have small juvenile leaves, with reduced photosynthetic capacity, malfunctioning stomata, root systems devoid of, or with very few root hairs, and poor cuticle development and low wax deposits on leaves (Mathur *et al*., 2008; Chandra *et al*., 2010). Thus acclimatization of tissue cultured plantlets under controlled conditions of temperature and humidity prior to their transfer to extreme environmental conditions in the field becomes essential (Lavanya *et al*., 2009; Deb and Imchen, 2010). When removed from the tissue culture environment, the plantlets must be allowed to the outside environment with its varying light levels, changing temperature, reduced humidity, lower nutrient availability, and pathogen presence (Hazarika, 2003; Chandra *et al*., 2010).

Different potting mixes also has significant role in determining survival percentage in sugarcane plantlets (Behera and Sahoo, 2009). Warakagoda *et al*. (2007) studied suitable acclimatization procedure with different combinations of sterilized sand and coir dust (1:0, 0:1, 1: 1, 2: 1 and 1: 2) as potting media for the acclimatization of *in vitro* derived sugarcane plantlets. The result showed that sand: coir dust at 1:2 ratios found to be most suitable potting medium in which 100% survival rate was observed. Best hardening response was obtained in sand + soil + Peat (1:1:1) after three week of transplantation in glass house (Ali *et al*., 2008). Baksha *et al*. (2003) also reported survival rate of 70% for plantlets transferred to polybags containing a mixture of soil and sand in 2:1 ratio for hardening. Moreover, 85% survival was obtained for sugarcane plantlets acclimatized on autoclaved garden soil, farmyard manure and sand in 2:1:1 ratio (Behera and Sahoo, 2009).

3. MATERIALS AND METHODS

3.1. Plant Materials

The study was conducted using two pipe line sugarcane genotypes N-52/219 and N-53/216. The genotypes were selected based on their agronomic performance and sugar quality. N52/219 yields 206.3 ton cane/ha with 15.36 % sucrose content while N53/216 gives 166.9 ton cane /ha with 13.36% sucrose content. The materials were obtained from the Ethiopian Sugar Corporation, Wonji.

3.2. Stock Solution and Media Preparation

The Murashige and Skoog (1962) MS nutrient with its macro, micro and vitamin compositions grouped as MS1, MS2, MS3, MS4, MS5 and MS6 were used as the basic components of the medium (Appendix 1). Full strength stock solutions of these individual MS components were prepared in six separate volumes of stock solutions. To do so, appropriate amount of each nutrient was weighed in grams per liter and dissolved in double distilled water sequentially in such a way that the next nutrient was added after the first one was completely dissolved. After, all the components were dissolved completely using magnetic stirrer, the solution was poured in to plastic bottles, labeled and stored in refrigerator at a temperature of +4°C for maximum of a month till used. Exceptionally, iron stock solution (MS5) preparation procedure was varied from other MS stock solution in that FeSO4.7H2O and Na_2.EDTA were dissolved in hot double distilled water separately. After both components were dissolved completely, FeSO4.7H2O solution was added upon Na_2.EDTA solution, and then poured into the bottle wrapped with aluminum foil so as to protect it from direct light contact.

Different growth regulators were used in this research, 6-benzyl aminopurine (BAP, Sigma), Kinetin (UNI-CHEM) and α-naphthalene acetic acid (NAA, Himedia). The plant growth regulators (PGRs) were prepared in 1mg/ml concentration. The powder crystal of the PGRs was first weighed and dissolved in 3-4 drops of 1N NaOH. Then, the volume was adjusted to required level by adding double distilled water and gently stirred till the PGRs were dissolved completely. Finally, the PGRs solution was poured in to separate labeled bottles and stored in refrigerator at a temperature of +4°C, not more than four weeks.

The culture medium for shoot initiation and multiplication contained full strength MS basal medium with and without (control) PGRs plus sucrose. For micro-shoot rooting half strength liquid MS basal medium was used with and without PGR (NAA) and sucrose. During preparing individual working medium, the required amount of macronutrient, micronutrient, vitamins from respective stock solution were added along with sucrose into plastic beakers containing double distilled water, and later thoroughly stirred on magnetic stirrer until the sugar was completely dissolved. After adding the respective type and amount of PGRs from stock solution, the final volume was made up to the required level with double distilled water. The pH was adjusted at 5.8 using 1N HCl and/or 1N NaOH before gelling with agar. For semi solid medium, agar (Agar- Agar, type I) was added to the nutrient solution at a rate of 4.5 g/l and heated on magnetic hot plate till it was completely melted for semi-solid media preparation while no agar was added for liquid media preparation.

Then after, for culture initiation, molten medium of 20 ml was dispensed into each culture tube (145 mm long and 25 mm diameter) and plugged with non-absorbent cotton. For shoot multiplication experiment, 15 ml liquid media (devoid of agar) was dispensed into each screw-capped jars having a size of 120 mm long and 60 mm diameter. For rooting experiment, 10 ml of liquid medium was poured into 25 mm diameter test tubes and plugged with non-absorbent cotton. The dispensed medium was autoclaved at 121°C and 105 Kpa pressure for 20 minutes. Finally, the autoclaved medium was kept in a shelf for about a week to check some indication of microbial contamination before it was used.

3.3. Explant Sterilization and Preparation

The seedcane (setts) with two buds were treated with hot water at 50°C for 2 hours followed by immersing in fungicide (Bayleton® DF 50%) solution at rate of 1 g/l for 5 minutes. The treated setts were planted in plastic pots containing mixture of autoclaved forest soil, farm yard manure and river sand in the ratio of 1:1:1 and allowed to grow in a screen house of Holetta National Agricultural Biotechnology Laboratory for five months. Actively growing shoot tops were used as source of shoot tip explant.

The method of explant preparation and surface sterilization was adopted from Mekonnen *et al*. (2013) and Singh *et al*. (2006) with some modifications. Actively growing shoot tops were taken from 5-months-old screen house grown healthy mother plants and used as explants. Shoot tops were cut at the base of the mother plants above soil surface with some nodes. The

entire leaves were removed, and then taken to the laboratory for surface sterilization and explant preparation. Trimmed shoot tops were washed thoroughly under running tap water for 30 minutes, then the outer leaf sheath was removed until yellowish white sheath appeared and the tops were sized to 10 cm length by cutting off at the two ends.

After that, the explants were further washed for 30 minutes with tap water containing a drop of liquid detergent solution plus two drops of tween-20 with continuous shaking and rinsed three times with double distilled water. Later, the explant was taken to a laminar air flow cabinet and immersed in 0.1% (w/v) Bavistin® DF 50% (Carbendizem) fungicide solution, ascorbic acid (0.2% w/v) and citric acid (0.4% w/v) for 30 minutes followed by three times rinsing each for five minutes with sterile double distilled water. The shoot tips were washed again with 70% ethanol for one minute and rinsed with sterile double distilled water three times each for five minute to remove residual ethanol from the shoot tip surface. Finally, 10 cm sized explants were surface sterilized with 50% (v/v) aqueous solution of Sodium hypochlorite (5.25% w/v active chlorine) containing a few drops of a wetting agent (tween-20) with gentle shaking for 25 minutes. After pouring out sodium hypochlorite solution, the explants were rinsed with sterile double distilled water three times each for five minutes to remove all the trace of the sterilant. Subsequently, about 1.5 cm long shoot tip explants comprising apical meristem and two to three leaf primordia were aseptically excised from sterilized segments and immediately cultured on agar gelled MS medium (Murashige and Skoog,1962).

3.4. Culture Initiation

Shoot tip explants having 1.5 cm long size were cultured in test tube containing 20 ml of sterilized and agar (4.5 g/l) solidified MS medium (Murashige and Skoog, 1962) fortified with BAP, Kinetin and NAA (0.5 mg/l each) plant growth regulators (Pathak *et al.*, 2009) and 20 g/l sucrose as carbon source. The test tubes with cultured explants were properly plugged with non-absorbent cotton, sealed with PVC film and labeled with pertinent information. Afterwards, the cultures were transferred and maintained on shelves in growth room adjusted at temperature of 25±2°C under 16 hours photoperiod with photo flux density of 30 μmol m^2/s provided by cool white fluorescent light and 70-80% relative humidity.

3.5. *In vitro* Shoot Multiplication

In this experiment, healthy micro-shoots having the same shoot length obtained from the initiation stage were used for shoot multiplication after they were subcultured for two cycles on semi-solid media. Healthy uniform micro-shoots were cut and transferred to full MS liquid media supplemented with 3% sucrose under aseptic. The experiment was laid out in CRD with three factor factorial combination of five levels of BAP (0, 0.5, 1.0, 1.5 & 2 mg/l); four levels of kinetin (0, 0.5, 1.0 & 1.5 mg/l) and two levels of sugarcane genotypes resulting in 2x5x4 factorial treatment combinations arrangement. Five shoots per culture jar and four replications for each treatment were used. The culture jars were properly sealed with PVC film, labeled and randomly placed on a rotary shakers that revolved at a speed of 80 rpm and the shakers were maintained for 30 days in environmentally controlled growth room shelves with the same culture conditions (temperature, photoperiod and light intensity) as that of the initiation culture. Sub-culturing was carried out at fortnightly interval by transferring the newly multiplied micro-shoots to fresh medium of the same composition as the previous one. The responses of microshoots to different treatments such as number of shoot per explants, length of shoots and number of leaves per shoot were carefully recorded after four weeks.

3.6. *In vitro* Rooting of Microshoots

Microshoots comprising uniform shoot length derived from the multiplication experiment were used for rooting studies. The shoots were maintained on PGR free MS medium with 2 g/l activated charcoal for two weeks before transferring to a liquid rooting media in order to avoid the carry over effect of hormones from the multiplication media on rooting. In this experiment, the rooting response of *in vitro* regenerated shoots was determined on half strength liquid medium supplemented with different concentrations of NAA (0, 3, 5 & 7 mg/l) and sucrose (0, 40, 50, 60 & 70 g/l) with two level of genotype resulting in 2x4x5 three factor factorial treatment combinations. For each treatment 3 test tubes, each with two shoots supported by filter paper bridge (whatman filter paper) were lined up randomly in CRD with four replications. All shoots were incubated on rooting medium for 3 weeks with the same culture condition to that of initiation and multiplication culture. After 3 weeks of culture growth, data on number of roots per shoot, length of roots (cm) and number of rooted shoots were recorded.

3.7. *Ex vitro* Rooting of Microshoots

The effects of auxin, NAA treatments on efficiency of e*x vitro* rooting were examined. Healthy micro-shoots (3–4 cm in height) from multiplication medium were transferred to PGR free medium containing 2 g/l activated charcoal for 15 days. Clumps of *in vitro* shoots were separated to obtain single micro shoot. The basal end of these microshoots were dipped in distilled aqueous solution containing auxin i.e. NAA at different concentrations i.e. 0, 10, 20, 30, & 40 mg/l overnight to induce rooting under *ex vitro* condition. The experiment was arranged in completely randomized design (CRD) with five replications and each treatment had 50 micro shoots. After treated with auxins, the shoots were transferred to polystyrene trays containing autoclaved mixture of river sand and forest soil in 2:1 ratio. Subsequently, maintained in greenhouse which uses Fan-Pad evaporative cooling system providing 25–30°C temperature. During experimenting, high humidity level (80-85%) was maintained by covering the tray with moisten polyethylene sheet and red shade cloth and then sprinkled with water three times a week as necessary.

After 4 weeks the plantlets were carefully removed from the soil mix and data on number of rooted shoots, total number of primary roots and root length were recorded. All microshoots that remain green were considered living and used in calculating rooting percentage. Successfully rooted plantlets were subsequently transferred in medium polyethene bags (15 x 20 cm) containing mixture of river sand, farm yard and forest soil in 1:1:1 ratio for further hardening. Data on post rooting survival of plantlets was also recorded.

3.8. Acclimatization Procedure for *In vitro* Derived Plantlets

In vitro rooted plantlets were taken out of the culture test tubes and thoroughly washed with water to remove all the traces of the rooting medium. After trimming out excess leaves and roots, the plantlets were transferred to trays containing autoclaved mixture of river sand, forest soil and well decomposed farmyard manure in different proportion or ratio i.e. 1:1:0, 1:1:1, 1:2:1, 2:1:1, 1:1:2 and 1:2:0. Before planting the trays filled with the soil mixtures were properly irrigated with water. For acclimatization, the plantlets were maintained in the greenhouse for 4 weeks at about 25-30°c under high humidity (>85%) by covering the trays with transparent polyethylene sheets and red cheese cloth and then sprayed with water intermittently. The cover was removed from the tray after 10 days. Starting from the 1st

weeks of acclimatization, number of surviving and dead plantlets was recorded on weekly basis until the 4th week of acclimatization and ultimately survival percentage was calculated. The acclimatized plantlets were further transferred to medium polyethene bags (15 x 20 cm) containing forest soil, farm yard manure and river sand in 1:1:1, ratio and allowed to grow in the green house for further hardening. The experiment was carried out in five replications with 50 explants for each treatment in a completely randomized design (CRD).

3.9. Data Analysis

The analysis of variance for different variables was performed by SAS version 9.2 (SAS Institute Inc., 2009) and for significantly different treatments, mean separation was done with REGWQ (Ryan-Einot-Gabriel-Welsch) at or below the probability level of 0.05.

4. RESULTS AND DISCUSSION

4.1. The Effect of BAP and Kinetin on Shoot Multiplication

The result showed that shoot multiplication was influenced by the effect of both genotypes and growth regulators. Analysis of Variance (ANOVA) revealed that interaction effect of genotype, BAP and kinetin was highly significant ($P<0.001$) on the number of shoots per explant, shoot length, and number of leaves per shoot (Appendix Table 2). The interaction of genotype, BAP and kinetin indicated that all the three factors are reliant on each other for *in vitro* shoot proliferation of sugarcane.

The result of the different combination and concentrations of BAP (0, 0.5, 1, 1.5, 2 mg/l) and Kinetin (0, 0.5, 1, 1.5 mg/l) on the shoot multiplication is presented in Table 1. Shoot multiplication was not observed within 4 weeks when explant cultured on MS medium devoid of plant growth regulators (BAP and kinetin) in both genotypes (Table 1). However, increasing the concentration of kinetin alone from 0 to 0.5 mg/l resulted in 3.35 and 3.15 shoots per explant for N52 and N53, respectively. In the same way, increasing BAP concentration alone from 0 to 0.5 mg/l yielded 4.80 and 3.25 shoot per explant in genotype N52 and N53, respectively. This result indicates that the supplementation of exogenous plant growth regulators to MS medium is imperative to develop multiple shoots. In fact, cytokinins are capable of overcoming apical dominance and release lateral buds from dormancy thereby enhance shoot multiplication (George *et al.*, 2008).

In the present study, it was observed that the two genotypes responded differently to the same media for all parameters studied. The highest average number of shoots per explant (6.95) was observed in genotype N52 on MS medium fortified with 2 mg/l BAP in combination with 0.5 mg/l kinetin (Table 1 and Figure 1a) while N53 gave only 3.20 shoot per explant on the same medium composition. Similarly, N53 produced maximum of 6.30 shoots per explant on MS medium fortified with 1.5 mg/l BAP + 0.5 mg/l kinetin (Table 1 and Figure 1b) while the same medium composition resulted in only 5.15 shoots per explant in N52.

Table 1**:** The effect of BAP and Kinetin on number of shoots per explant, shoot length and number of leaves per shoot

PGRs(mg/l)		Genotypes					
		N52			**N53**		
BAP	Kinetin	Number of shoots per explant ±SD	Shoot length (cm) ±SD	Number of leaves per shoot ±SD	Number of shoots per explant	Shoot length (cm) ±SD	Number of leaves per shoot
0	0	0.00^{s} ± 0.00	0.00^{s} ± 0.00	0.00^{q} ± 0.00	0.00^{s} ± 0.00	0.00^{s} ± 0.00	0.00^{q} ± 0.00
	0.5	3.35^{m-q} ± 0.25	3.67^{b-h} ± 0.23	3.83^{l-o} ± 0.24	3.15^{pq} ± 0.19	2.87^{n-q} ± 0.03	4.83^{cf} ± 0.10
	1	4.15^{jk} ± 0.19	3.63^{b-i} ± 0.04	4.58^{e-j} ± 0.24	3.45^{l-q} ± 0.19	3.08^{l-p} ± 0.09	4.93^{cf} ± 0.10
	1.5	3.80^{l-n} ± 0.16	3.20^{i-p} ± 0.10	4.55^{e-j} ± 0.17	3.75^{k-o} ± 0.19	2.79^{o-q} ± 0.13	5.18^{c-d} ± 0.22
0.5	0	4.80^{e-h} ± 0.16	3.87^{b} ± 0.17	3.45^{op} ± 0.13	3.25^{n-q} ± 0.25	2.99^{l-p} ± 0.15	5.67^{ab} ± 0.17
	0.5	3.95^{j-l} ± 0.19	3.91^{b} ± 0.22	3.70^{m-p} ± 0.18	5.15^{d-f} ± 0.19	3.31^{e-n} ± 0.08	4.75^{d-f} ± 0.06
	1	3.45^{l-q} ± 0.19	4.55^{a} ± 0.27	3.95^{k-n} ± 0.19	4.50^{g-j} ± 0.26	3.86^{b} ± 0.03	4.48^{f-j} ± 0.10`
	1.5	3.25^{n-q} ± 0.10	3.66^{b-i} ± 0.59	3.83^{l-o} ± 0.17	4.40^{i-j} ± 0.33	3.50^{b-i} ± 0.25	3.95^{k-n} ± 0.06
1	0	5.00^{e-g} ± 0.28	3.64^{b-i} ± 0.22	5.25^{bc} ± 0.27	3.50^{l-q} ± 0.26	2.77^{q} ± 0.14	4.70^{ef} ± 0.18
	0.5	6.05^{bc} ± 0.19	3.71^{b-e} ± 0.16	5.63^{abc} ± 0.19	3.60^{k-p} ± 0.28	2.88^{n-q} ± 0.35	4.98^{c-e} ± 0.17
	1	3.75^{k-o} ± 0.25	3.60^{b-j} ± 0.22	4.00^{k-n} ± 0.18	5.60^{cd} ± 0.16	3.35^{e-l} ± 0.01	4.65^{e-h} ± 0.06
	1.5	3.50^{l-q} ± 0.12	3.28^{g-n} ± 0.08	3.28^{p} ± 0.25	3.05^{pq} ± 0.10	3.24^{g-o} ± 0.18	4.58^{e-j} ± 0.28
1.5	0	5.15^{d-f} ± 0.19	3.45^{d-l} ± 0.02	4.38^{g-k} ± 0.29	4.45^{g-j} ± 0.19	2.50^{q} ± 0.03	4.70^{ef} ± 0.22
	0.5	5.15^{d-f} ± 0.25	3.39^{d-l} ± 0.11	4.93^{c-f} ± 0.26	6.30^{b} ± 0.26	3.94^{b} ± 0.03	5.83^{a} ± 0.10
	1	6.35^{b} ± 0.19	3.27^{g-n} ± 0.24	4.60^{e-I} ± 0.14	3.60^{k-p} ± 0.43	2.98^{m-p} ± 0.06	5.20^{c-d} ± 0.22
	1.5	3.85^{k-m} ± 0.34	3.24^{g-o} ± 0.03	3.93^{k-n} ± 0.22	2.15^{r} ± 0.19	2.46^{n} ± 0.19	4.60^{e-I} ± 0.25
2	0	5.35^{de} ± 0.19	3.20^{h-p} ± 0.08	3.60^{n-p} ± 0.18	4.65^{f-i} ± 0.25	2.30^{r} ± 0.01	4.23^{h-l} ± 0.22
	0.5	6.95^{a} ± 0.19	4.75^{a} ± 0.06	5.65^{ab} ± 0.21	3.20^{oq} ± 0.28	3.82^{b-d} ± 0.03	5.75^{a} ± 0.10
	1	4.50^{g-j} ± 0.26	4.66^{a} ± 0.20	4.13^{j-m} ± 0.17	3.05^{pq} ± 0.19	3.74^{b-e} ± 0.19	4.58^{e-j} ± 0.15
	1.5	3.40^{l-q} ± 0.16	3.68^{b-g} ± 0.17	3.93^{k-n} ± 0.10	3.00^{q} ± 0.16	3.16^{j-p} ± 0.11	4.18^{i-l} ± 0.05
CV%		5.55	5.48	4.23	5.55	5.48	4.23

Values in the same column and variables with different letters are significantly different from each other according to REGWQ at $P<0.05$

Regarding shoot length, N52 produced the highest shoot length (4.75 cm) with the maximum number of leaves per shoot (5.65) on MS medium supplemented with 2 mg/l BAP + 0.5 mg/l Kinetin whereas only 3.07 shoot length with 4.23 leaves per shoot produced in N53. This different growth response to the same media composition might be as a result of difference in inherent endogenous growth hormone level among genotypes (George *et al*, 2008). Earlier research reports also confirmed that different genotypes respond differently to PGR and other media components (Khan and Rashid, 2003; Khan *et al*., 2006).

Increasing kinetin concentration from 0 to 0.5 mg/l at a constant level of BAP (2 mg/1) showed an increase in the number of shoots per explant, shoot length and number of leaves per shoot in N52. Similarly, in genotype N53, an increase in kinetin from 0 to 0.5 mg/l at 1.5 mg/l BAP, showed a significant increase in number of shoots per explant, shoot length and number of leaves per shoot. However, further increase in kinetin to 1.5 mg/1, significantly reduced the number of shoots per explant, shoot length and number of leaves per shoot to 2.15, 2.46 cm and 4.60, respectively (Table 1). This is an indication that higher concentrations of cytokinins inhibit cell division and hence multiplication while lower concentrations are suitable for cell division in sugarcane. These result in accordance with the findings of Tolera *et al*. (2014c) during multiplication of sugarcane genotype B41-227. They observed increasing in numbers of shoots per explant from 21.5 to 34 when kinetin concentration was increased from 0.25 mg/l to 0.5 mg/l in the culture medium. However, further increase in the concentration of Kinetin to 1 mg/l significantly reduced the number of shoot per explant to 27.21. Siddiqui *et al*. (1994) reported the positive effect of using lower concentration of BAP and Kinetin on shoot proliferation of sugarcane.

It is apparent from Table 1 that the use of 2 mg/l BAP alone produced only 5.35 shoots per explant, which was increased to 6.95 shoots by addition of 0.5 mg/l kinetin in genotype N52. In the same way, increased shoot number per explant was observed in genotype N53 from 4.45 to 6.30 when 0.5 mg/l kinetin was added to MS medium containing 1.5 mg/l BAP. This positive effect indicates the significance of adding the two growth regulators in combination rather than alone in shoot multiplication medium. In similar experiments, proliferation of higher number of shoots per explant due to the synergistic effect of the two cytokinins (BAP and Kinetin) was

reported (Geetha and Padmanadhan, 2001; Khan *et al*., 2009; Adilakshmi *et al*., 2014). Ali and Afghan (2001) also reported that medium supplemented with BAP and Kinetin resulted in rapid multiplication of shoots.

The best result achieved in genotype N53 is consistent with the result obtained by Khan *et al*. (2009). They reported a maximum multiplication from HSF-240 produced 11 shoots per explant; 16.5 cm mean shoot length, and 32 leaves per shoot on MS medium amended with 1.5 mg/l BAP + 0.5 mg/l kinetin. The current result in N52 is also in harmony with the results reported by Mekonen *et al*. (2014), who obtained best result from Co 678 genotype on MS medium fortified with 2 mg/l BAP + 0.5 mg/l Kinetin with 9.1 number of shoots 6.83cm shoot length and 5.67 leaves per shoot. In both cases, the observed difference in number of shoots per explant, number of leaves and shoot length could be due to genotypic difference. Adilakshmi *et al*. (2014) also obtained optimum multiplication of 7.74 and 6.39 shoots per explant at MS medium augmented with lower concentration of BAP and Kinetin (0.25 mg/l BAP + 0.1 mg/l Kinetin) in genotype 96A3 and Co 6907, respectively.

Similar results were also reported by Singh (2003) who observed an average of 12.33 shoots on MS medium fortified with 1.5 mg/l BAP + 0.5 mg/l Kinetin. Khan *et al*. (2009) observed maximum shoot multiplication on MS medium augmented with 1 mg/l BAP + 0.1 Kinetin and 1 mg/l BAP + 0.5 Kinetin in sugarcane genotype CPF-237 and HSF-240, respectively. Ali *et al*. (2008) achieved best shoot multiplication for sugarcane genotype BL-4 on MS medium amended with 0.50 mg/l BAP + 0.25 mg/l kinetin. However, there are also reports (Sughra *et al*., 2014) that indicated higher multiplication rate of sugarcane at lower concentration of BAP than obtained in this study. Result of the present study indicated that 2 mg/l BAP + 0.50 mg/l Kinetin was the optimum and best hormone concentration and combination for maximum shoot multiplication of sugar cane genotype N52.While 1.5 mg/l BAP + 0.5 mg/l kinetin was found to be the best for sugarcane genotype N53. Comparison of the two genotypes showed that N52 was a better responsive than N53 for *in vitro* multiplication in a liquid culture.

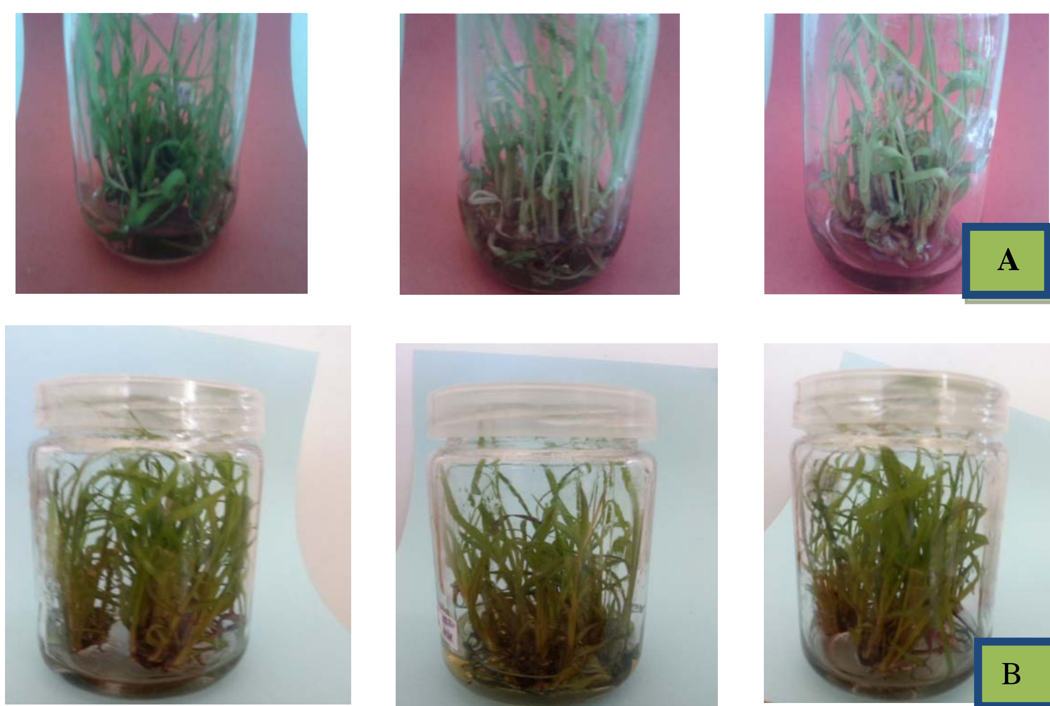


Figure1. *In vitro* shoots multiplication: A) N52 at 2 mg/l BAP and 0.5 mg/l Kinetin B) N53 at 1.5 mg/l and 0.5 mg/l Kinetin

4.2. Effect of NAA and Sucrose on Rooting of *in vitro* Regenerated Shoots

The analysis of variance (ANOVA) showed that main effect of genotype, interaction effect of genotype and sucrose and interaction effect of genotype, sucrose and growth regulator (NAA) were non-significant ($p>0.05$) on rooting frequency of sugarcane micro-shoots. On the other hand, the main effects of NAA and sucrose; interaction effect of genotype and NAA, and the interaction effect of NAA and sucrose were statistically highly significant ($p<0.0001$) on rooting frequency of sugarcane micro-shoots (Appendix table 3). In both genotypes, no rooting response was observed on half strength liquid MS medium devoid of NAA and sucrose (control). Again, there was no rooting of microshoot recorded on half strength liquid MS medium supplemented with different level of NAA (3, 5 and 7 mg/l) without the presence of sucrose in both genotypes (Table 2). These results indicated that significance of sucrose as a source of energy in rooting medium for sugarcane microshoot root induction.

On the contrary, root induction occurred in both genotypes when the microshoots were cultured in half strength liquid MS medium supplemented with various concentrations of sucrose without NAA (Table 2). This result showed that the two genotypes are rich in

endogenous auxins that at least enable them to induce root. Both genotypes showed significant increase in rooting frequency because of the increase in the concentration of sucrose from 0.0 mg/l to 40 mg/l in every level of NAA. There was significant level of reduction of rooting frequency from 100% to 87.84% as the concentration of NAA and sucrose increased from 3 to 7 mg/l and 50 to 70 g/l, respectively (Table 2).This might be due to inhibition of rooting at higher concentration of NAA. Higher concentrations of NAA reduce root induction as it promotes the biosynthesis of ethylene which has inhibitory effect in sugarcane rooting (Biradar *et al*, 2009). Ramanand *et al*. (2007) had also reported that higher concentration of NAA (7 mg/l) and sucrose (70 g/l) inhibit root induction frequency.

ANOVA showed highly significant ($p<0.0001$) effect of all main and interaction effect of genotype, NAA and sucrose on root length and number of roots per shoot of the two sugarcane genotypes studied (appendix table 3).

No root was initiated on 1/2 MS liquid medium that lack NAA and sucrose in both genotypes, N52 and N53 (Table 3). It was observed that there was differential response of the two genotypes used. N52 was responsive to lower concentration of NAA than N53. Genotype N52 gave a maximum of 4.95 cm and 23.5 average root length and average root number per shoots, respectively on 1/2 MS liquid medium fortified with 3 mg/l NAA and 50 g/l sucrose while only 1.68 cm average root length with 16.43 average roots number per shoot were recorded in N53 in the same medium composition (Table 2). On the other hand, in genotype N53, the highest of 4.54 average root length and 21.76 average roots number per shoot were produced on 1/2 MS liquid medium supplemented with 5 mg/l NAA and 50 g/l sucrose; with this medium composition, N52 resulted in 4.58 average root length and 18.00 average roots number per shoot (Table 2).

The result also showed that an increase in the concentration of NAA from 0.0 mg/l to 3 mg/l at fixed quantity (50 g/l) of sucrose increased the average root length and roots number of N52 significantly to 4.95 cm and 23.5, respectively. In the same trend increasing the concentration of NAA from 0 mg/l to 5 mg/l maintaining the concentration of sucrose at 50 g/l increased the average root length and number of roots to 4.54 cm and 21.76, respectively in genotype N53. Conversely, further increasing the concentration of NAA and sucrose to 7 mg/l reduced the shoot length and number of root noticeably to 3.05

cm and 16.1 in N52 and to 2.34 cm and 19.05 in N53, respectively. This was due to the fact that high auxin concentration inhibits sugarcane root elongation through production of ethylene in the culture jar (Biradar, 2009). Similarly, increase in sucrose concentration from 0 g/l to 50 g/l along with a definite concentration of NAA (5 mg/l), significantly increased the average root length and root number to 4.58 cm and 18.00 in genotype N52, and to 4.54 cm and 21.76 in genotype N53, respectively. However, further increase in sucrose concentration to 70 g/l resulted in reduction in average root length and number of roots in both sugarcane genotypes (Table 3). Earlier reports also confirmed that higher concentrations of sucrose in the medium have a negative impact on overall rooting due to accumulation of rooting inhibitors, the reduction of rooting promoters in the medium, and the transformation of added sugars in to insoluble and storage form (Ahmed *et al*., 2004).

The current result obtained in genotype N53 was in agreement with earlier results reported by Ramanand *et al*. (2007).They obtained the longest root of 6.7 cm and the highest average roots number of 6.8 in sugarcane genotypes CoS 96268 and CoS 95255, respectively on 1/2 MS liquid medium containing 5 mg/l NAA in combination with 50 g/l sucrose; whereas NAA at 7 mg/l reduced the root length. The result obtained in N53 was also in harmony with the previous findings of Yadav *et al*. (2012). They reported best root growth with 5.8 and 5.7 average number of roots per shoot in sugarcane genotypes CoSe 01235 and CoS 99259, respectively. Bakash *et al*. (2003) also obtained a maximum average root length of 4.5 cm and average root number of 17 per shoot in sugarcane genotype lsd 31 on ½ MS medium with 5 mg/l of NAA.

The results recorded in the present investigation in genotype N52 was consistent with findings of Gopitha *et al*. (2010), who obtained the highest average root length (4.9 cm) and average root number per shoot (15.1) on ½ MS liquid medium fortified with 3 mg/l NAA with 50 g/l sucrose in genotype Co671. Sughra *et al*. (2014) revealed a maximum of 2.50 cm average root length and 6.8 average numbers of roots at 3.0 mg/l NAA for genotype BL-4. Behera and Sahoo (2009) found a maximum of 13.4 average numbers of roots per shoot with 4.0 cm average root length on ½ MS medium containing 3 mg/l NAA. Khan *et al*. (2006) reported the highest root induction with best root growth at ½ MS medium containing 1 mg/l NAA and 60 g/l sucrose. The current result also disagrees with the report of Khan *et al*. (2009). They obtained a maximum roots (35) and average root

length (3.05cm) from genotype HSF-240, at 0.5 mg/l IBA with 60 g/l of sucrose. The same author also reported a maximum of 34 roots with 1.8 cm average root length from genotype CPF-237 at 1.5 mg/l IBA with 60 g/l of sucrose. However, there were other research reports (Khan *et al*., 2009) that showed a different result, where low IBA concentration (0.5 mg/l) combined with 60 g/l sucrose gave the best results in average root number 35 per plant and root length of 3.05 cm.

Therefore, 1/2 MS liquid medium fortified with 3 mg/l NAA and 50 g/l sucrose was the optimal combination for *in vitro* rooting of *in vitro* generated shoots of sugarcane genotype N52.While ½ strength MS supplemented with 5 mg/l NAA and 50 g/l sucrose was the best combination for *in vitro* rooting of shoots of sugarcane genotype N53.

Table 2: Effect of genotype, NAA and sucrose on rooting percentage, number of roots per shoot and root length

Treatments		Genotypes					
		N52			N53		
NAA mg/l	Sucrose g/l	Rooting percentage	Root length (cm)	Number of root per shoot	Rooting percentage	Root length (cm)	Number of root per shoot
0	0	0.00^{e} ± 0.00	0.00^{o} ± 0.00	0.00^{p} ± 0.00	0.00^{e} ± 0.00	0.00^{o} ± 0.00	0.00^{p} ± 0.00
	40	83.31^{d} ± 8.90	4.24^{cd} ± 0.17	6.50^{mn} ± 0.58	83.31^{d} ± 8.90	3.45^{h} ± 0.31	4.00^{o} ± 0.23
	50	85.41^{cd} ± 13.90	4.36^{b-d} ± 0.13	6.25^{mn} ± 0.50	85.41^{cd} ± 13.90	4.36^{b-d} ± 0.24	5.45^{no} ± 0.17
	60	89.56^{a-d} ± 8.64	4.91^{a} ± 0.07	5.75^{n} ± 0.96	89.56^{a-d} ± 8.64	4.49^{bc} ± 0.08	8.28^{l} ± 0.10
	70	83.31^{d} ± 8.90	4.64^{ab} ± 0.16	5.75^{n} ± 0.50	83.31^{d} ± 8.90	3.38^{h} ± 0.21	7.45^{lm} ± 0.24
3	0	0.00^{e} ± 0.00	0.00^{o} ± 0.00	0.00^{p} ± 0.00	0.00^{e} ± 0.00	0.00^{o} ± 0.00	0.00^{p} ± 0.00
	40	100^{a} ± 0.00	3.81^{fg} ± 0.39	16.25^{gh} ± 0.96	100^{a} ± 0.00	1.52^{m} ± 0.10	11.35^{k} ± 1.22
	50	100^{a} ± 0.00	4.95^{a} ± 0.06	23.50^{a} ± 1.29	100^{a} ± 0.00	1.68^{m} ± 0.03	16.43^{f-h} ± 0.39
	60	93.74^{a-d} ± 8.64	4.14^{de} ± 0.21	17.25^{e-g} ± 0.96	93.74^{a-d} ± 8.64	2.74^{jk} ± 0.20	18.05^{d-f} ± 0.97
	70	89.56^{a-d} ± 8.64	3.55^{gh} ± 0.19	15.25^{hi} ± 0.96	89.56^{a-d} ± 8.64	1.82^{m} ± 0.14	14.13^{ij} ± 0.75
5	0	0.00^{e} ± 0.00	0.00^{o} ± 0.00	0.00^{p} ± 0.00	0.00^{e} ± 0.00	0.00^{o} ± 0.00	0.00^{p} ± 0.00
	40	97.91^{ab} ± 5.90	3.91^{ef} ± 0.14	16.25^{gh} ± 0.96	97.91^{ab} ± 5.90	3.77^{fg} ± 0.05	19.28^{cd} ± 0.69
	50	100^{a} ± 0.00	4.58^{bc} ± 0.13	18.00^{d-f} ± 0.82	100^{a} ± 0.00	4.54^{bc} ± 0.06	21.76^{b} ± 0.57
	60	93.74^{a-d} ± 8.64	2.90^{j} ± 0.08	22.75^{ab} ± 0.50	93.74^{a-d} ± 8.64	2.28^{l} ± 0.05	13.95^{ij} ± 0.58
	70	91.65^{a-d} ± 8.93	2.78^{jk} ± 0.05	18.25^{d-e} ± 0.50	91.65^{a-d} ± 8.93	1.77^{m} ± 0.04	13.48^{j} ± 0.34
7	0	0.00^{e} ± 0.00	0.00^{o} ± 0.00	0.00^{p} ± 0.00	0.00^{e} ± 0.00	0.00^{o} ± 0.00	0.00^{p} ± 0.00
	40	95.83^{a-c} ± 7.73	3.26^{hi} ± 0.09	18.25^{de} ± 0.96	95.83^{a-c} ± 7.73	1.04^{n} ± 0.14	12.73^{kj} ± 0.73
	50	95.83^{a-c} ± 7.73	3.05^{ij} ± 0.13	18.50^{c-e} ± 1.00	95.83^{a-c} ± 7.73	2.34^{l} ± 0.07	15.43^{hi} ± 0.57
	60	95.83^{a-c} ± 7.73	2.75^{kj} ± 0.13	19.00^{c-d} ± 1.41	95.83^{a-c} ± 7.73	1.73^{m} ± 0.14	20.02^{c} ± 0.96
	70	87.48^{b-d} ± 7.73	2.55^{kl} ± 0.06	16.10^{gh} ± 0.60	87.48^{b-d} ± 7.73	1.62^{m} ± 0.13	19.05^{cd} ± 0.30
CV %		8.88	5.77	6.32	8.88	5.77	6.32

*Values in the same column and variables with different letters(s) are significantly different from each other according to REGWQ at $P<0.05$.

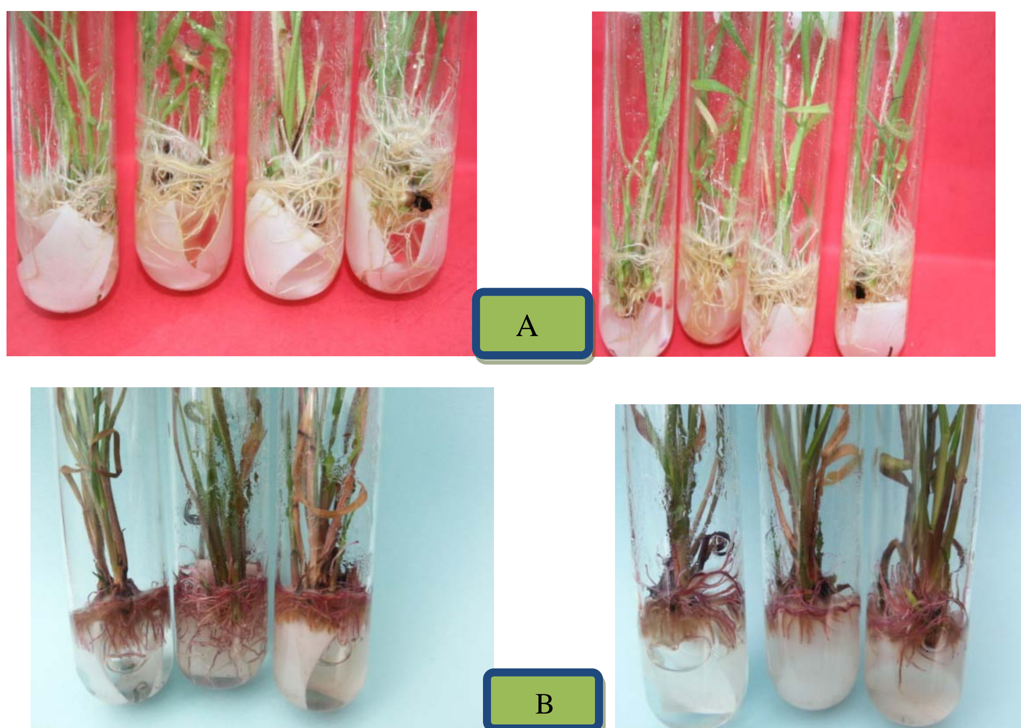


Figure 2. *In vitro* rooting of sugarcane microshoots. A) Genotype N52 at 3 mg/l NAA + 50 g/l Sucrose. B) Genotype N53 at 5 mg/l NAA + 50 g/l sucrose

4.3. Effect of NAA on e*x vitro* Rooting of *in vitro* Generated Sugarcane Microshoots

Statistical analysis of variance showed highly significant (p<0.0001) the interaction effect of genotype and NAA on rooting percentage, number of roots per shoot, root length of the two sugarcane genotype (Appendix table 4). The present result showed that rooting was induced *ex vitro* over the entire range of NAA concentration tested including the control shoots in both sugarcane genotypes (Table 3).

In the control treatment reduced rooting frequency of 36% and 28% were obtained in genotypes N52 and N53, respectively. However, in NAA treated microshoots than 50% of the shoot developed roots regardless of the NAA concentration (Table 3). Shekafandeh (2007) also observed increased rooting frequency and number of roots from zero percent in untreated shoots to 91.7% and 3.3 roots per shoot, respectively, when the basal end of the shoots were dipped in a solution of 1.5 mg/l IAA and 0.3 mg/l IBA for 24 h before culturing in soil mixture in Myrtle (*Myrtus communis* L.) plant. Similar results were also reported by Sumaryono and Riyadh (2011) in oil palm (*Elaeis guineensis* Jacq.). These

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

There was a significant response variation in rooting between the two genotypes. Genotype N52 had the highest (76%) rooting frequency with a maximum (5.88 cm) average root length and 8.06 average number of roots per shoot on microshoots dipped in 20 mg/l concentration of NAA (Table 3). At the same concentration of NAA, N53 had only 60% rooting frequency with 4.34 cm average root length and 4.08 ± 0.08 average roots number per shoot. On the other hand, genotype N53 showed a maximum rooting frequency (70%) with 5.42 cm and 4.52 numbers of roots per shoot on microshoots treated with 30 mg/l concentration of NAA (Table 3). At this concentration of NAA, genotype N52 gave almost equal rooting frequency (70%) with comparable root length (5.04 cm) and higher (6.36) root number per shoot than N53.The result of this experiment revealed that genotype N52 was more responsive than N53 for different NAA concentrations.

The rate of rooting frequency increased from 36% in control shoots to 76% when the basal ends of shoots were dipped in a solution of 20 mg/l NAA overnight. Similarly, average root length and average number of roots increased from 4.44 cm and 2.14 to 5.88 cm and 8.06, respectively, in genotype N52. However, when NAA concentration was further elevated to higher concentration (40 mg/l), rooting frequency, average root length and average number of roots per shoot, reduced significantly to 56%, 4.68 cm and 5.68, respectively. The same trend was observed in genotype N53, in that rooting frequency increased from 28% in control shoots to 70% in treated shoots with a solution of NAA at 30 mg/l. The average root length and average roots number also increased from 2.58 cm and 1.74 to 5.42 cm and 4.52 respectively, as the concentration of NAA increased from 0.0 mg/l to 30 mg/l but as the concentration of NAA was increased to 40 mg/l the rooting frequency, root length and root number reduced markedly to 54%, 4.08 cm and 3.88' respectively. This reduction in rooting response could be due to the fact that higher concentrations of NAA promote the biosynthesis and build up of ethylene at the basal end of the shoot, which have inhibitory effect on the overall rooting response of sugarcane microshoots (Biradar, 2009).

The result of the present study on genotype N52 were in agreement with earlier results by Pandey *et al*. (2011), who obtained the highest rooting frequency, root length and number of roots per shoot from shoots treated in 20 mg/l of NAA concentration in sugarcane genotype CoS96268. Similarly, Martin (2003a) obtained an average of 5.6 roots per shoot after the microshoots of *Rotula aquatica* Lour were dipped in 0.5 mg/l NAA for 25 days. Sumaryono and Riyadh (2011) found best root formation on shoot treated with 2 mM NAA with 80% rooting frequency in oil palm. However, other authors (Chinnu *et al*., 2012; Martin, 2003b) obtained best result of *ex vitro* rooting by using IBA.

Table 3: The effect of NAA on rooting percentage, root length and number of roots per shoot

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

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

Figure 3. *Ex vitro* rooting of sugarcane micro-shoots. A). genotype N52 at 20 mg/l NAA. B). genotype N53 at 30 mg/l NAA

Figure 4. Acclimatized plantlets. A) Genotype N52 B) genotype N53

4.4. Effect of Different Substrate Mixture on *ex vitro* Acclimatization of *in vitro* Regenerated Sugarcane Plantlets

Statistical analysis of variance showed that the main effect of substrates mixture and interaction effect of genotype and substrates mixture were highly significant (P<0.0001) on survival rate of *ex vitro* acclimatized shoots (Appendix Table 5).

There was a significance difference observed between the two genotypes at substrate mixture composed of sand + soil + FYM in 1:1:1 ratio. Genotype, N52 exhibited 90% survival while genotype N53 showed only 70%. Similar trend was observed at substrate mixture of sand + soil + FYM at 1:2:1 ratio, where, N52 showed 90% survival rate whereas N53 exhibited significantly low (78%) survival rate. The highest survival rates 100% and 94%, were observed in substrates mixture of sand + soil + FYM at 1:2:0 ratio, in genotype N53 and N52, respectively; however, there was no significance difference between them. Similarly, no significance difference was observed between genotype N53 (94%) and N52 (86%) on substrate mixture made up of sand + soil + FYM at 2:1:1 ratio. The lowest (76%) survival was obtained in substrate mixture with 1:1:2 ratio for genotype N52 while, the lowest (70%) survival rate was observed in substrate mixture with 1:1:1 ratio for genotype N53. Substrate mixtures comprised of sand + soil + FYM at 1:1:0 and 1:2:0 ratios had no significance difference in survival rate of plantlets for both genotypes. They had 94 and 92% in genotype N52 and 100 and 96% in genotype N53, respectively.

The current result also revealed that there was no significance difference in survival rate of N53 among substrates mixtures containing different proportion of sand + soil + farmyard manure (FYM) in 1:1:0 (T1), 2:1:1(T4) and 1:2:0 (T6) ratios (Table 5). Likewise, in genotype N52, the survival rate difference was not significant among substrates mixtures composed of sand + soil + FYM in 1:1:0 (T1), 1:1:1(T2), 1:2:1(T3) and 1:2:0 (T6) ratios (Table 4). However, plantlets grown on substrate mixture devoid of FYM (T1 and T6) exhibited vigorous growth and deep green leaves while those plantlets grown on substrate mixture containing FYM (T2 and T3 in genotype N52 and T4 in genotype N53) had weak growth and yellowish leaves. This could be due to the fact that farmyard manure increases the pH of the substrate mixture to 7.4 to 7.5 (Khan *et al*., 2006), while the optimum substrate mixture pH value for container grown plants should

range from 5.5 to 6.5 (Fitzpatrick *et al*., 1998). Similar result was reported by Yasmeen *et al*. (2012).

The number of surviving and dead plants was counted every week for 4 weeks. The first data were collected after a week of acclimatization. During that period no plantlets were dead in all treatments of both sugarcane genotypes (Figures 5 & 6). In the 2nd weeks of acclimatization, few dead plantlets were observed and recorded from treatment 2 (1:1:1), 3 (1:2:1) and 5 (1:1:2) in genotype N53 (Figure 6) and from treatment 4 (2:1:1) and 5 (1:1:2) in genotype N52 (Figure 5). However, in the third weeks of acclimatization, mortality of plantlet was progressively increased in most treatments especially in treatment 5 (1:1:2) in genotype N52 and the same trend was observed in treatment 2 (1:1:1), 3 (1:2:1) and 5 (1:1:2) in genotype N53. In the fourth week of the acclimatization period, highest mortality was observed in treatment 2 (1:1:1), 3 (1:2:1) and 5 (1:1:2) while few plantlets were dead in treatment 1(1:1:0) and 4 (2:1:1). On the contrary, there was no any mortality recorded (100% survived) in treatment 6 (1:2:0) of genotype N53 (Figure 6). Similarly, in genotype N52, the highest plantlet mortality was observed in treatment 5 (1:1:2) followed by treatment 4 (2:1:1) while, in remaining treatments only few plantlets were dead (Fig 6).

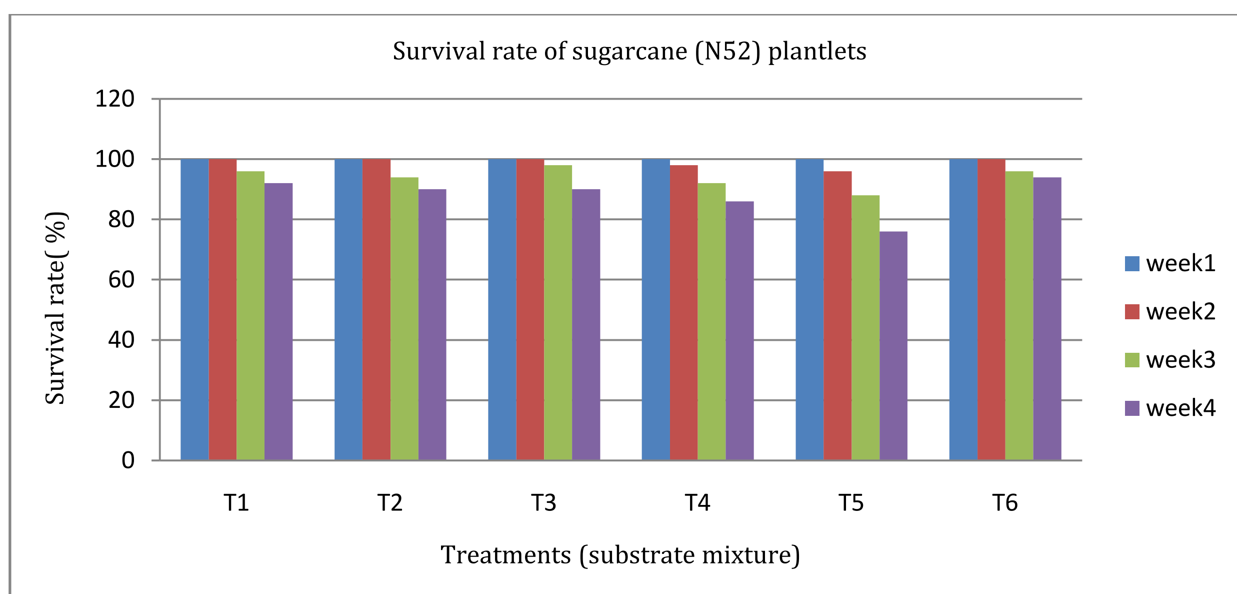
The result obtained in the present study in genotype N53 was in agreement with previous findings of Warakagoda *et al.* (2007) who reported 100% survival rate of sugarcane plantlets acclimatized on substrates mixtures containing sand + coir dust in 1:2 ratio. Khan and Rashid (2003) reported more than 90% survival when plantlets were transplanted in vermiculite. Seventeen % plantlet survival was also reported under *ex vitro* condition by Bakash *et al*. (2002). Similarly, Bakash *et al*. (2003) found survival rate of 75% for sugarcane plantlets acclimatized on pots containing a mixture of soil + sand in 2:1 ratio. Best acclimatization response was obtained in a mixture of sand + soil + peat at 1:1:1 after three week of transplanting to greenhouse (Ali *et al*., 2008). Ather *et al*. (2009) also found 96% survival rate for sugarcane plantlets acclimatized on substrate mixture composed of FYM and soil in 2:8 ratio. Eighty five % survival was obtained from sugarcane plantlets acclimatized on potting mixture composed of soil + sand + compost in 1:1:1 ratio (Bisht *et al*., 2011). Biradar *et al*. (2009) also declared 72% survival rate of micropropagated plantlets. Therefore, in the present study, substrates mixture comprising sand and soil substrate in 1:1 ratio was found to be an ideal substrate mixture for best *ex vitro*

acclimatization with higher plantlets survival rate for both sugarcane genotype, N52 and N53.

Table 4: The effect of different substrates mixtures on *ex vitro* acclimatization of *in vitro* generated plantlet of sugarcane

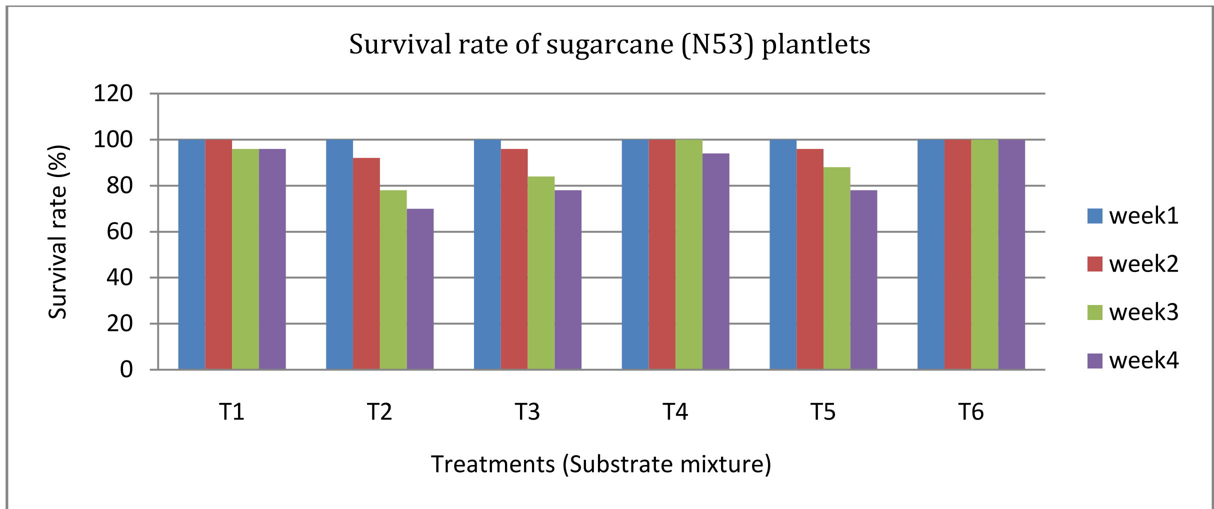
Substrates mixture (Sand + Soil + FYM)	Survival Rate (%)	
	Genotype	
	N52	N53
1:1:0	$92^{ab} \pm 4.47$	$96^{ab} \pm 5.48$
1:1:1	$90^{ab} \pm 0.00$	$70^{d} \pm 0.00$
1:2:1	$90^{ab} \pm 0.00$	$78^{cd} \pm 13.04$
2:1:1	$86^{bc} \pm 5.48$	$94^{ab} \pm 5.48$
1:1:2	$76^{cd} \pm 5.48$	$78^{cd} \pm 4.47$
1:2:0	$94^{ab} \pm 5.48$	$100^{a} \pm 0.00$
CV%	5.91	5.91

FYM= farmyard manure, value in the same column with different letters are significantly different from each other according to REGWQ at P<0.05.



T1= sand +soil + FYM (1:1:0) T2 = Sand +soil + FYM (1:1:1) T3 = sand +soil + FYM (1:2:1) T4= Sand +soil + FYM (2:1:1) T5 = sand +soil + FYM (1:1:2) T6 = sand +soil + FYM (1:2:0)

Figure 5. Survival rate of plantlets of sugarcane genotype N52 in different substrate mixture



T1= sand +soil + FYM (1:1:0) T2 = Sand +soil + FYM (1:1:1) T3 = sand +soil + FYM (1:2:1) T4= Sand +soil + FYM (2:1:1) T5 = sand +soil + FYM (1:1:2) T6 = sand + soil + FYM (1:2:0)

Figure 6. Survival rate of plantlets of sugarcane genotype N53 in different substrate mixture

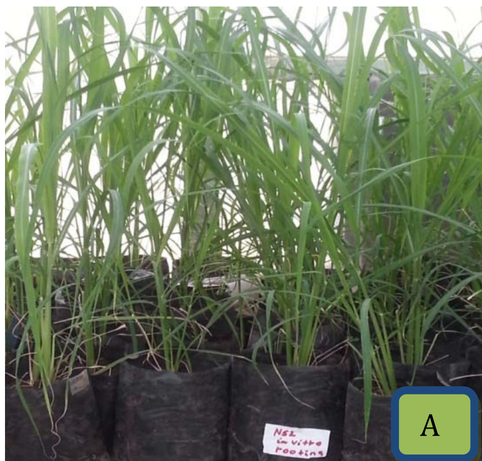


Figure 7. *In vitro* rooted & *ex vitro* acclimatized plantlets. A) Survived genotype N52 B) Survived genotype N53

5. SUMMARY AND CONCLUSION

5.1. Summary

Commercially, sugarcane is propagated vegetatively by stem cuttings with each cutting having two to three buds. This method generally has low seed multiplication rate, usually one to ten in one year. In addition there is also a chance of perpetuation of sett-borne disease from generation to generation. Hence a new effective and efficient technique is required for mass multiplication of sugarcane. Plant tissue culture is the best alternative and powerful tool for rapid, mass multiplication of disease free and true to type planting material. However, conventional micropropagation technique that uses agar as solidifying agent becomes uneconomical owing to slow rate of bud proliferation and high cost of agar. Consequently, use of shake culture with liquid media was invented so as to alleviate the limitation of agar solidified medium in mass multiplication of shoots.

Since the multiplication response of genotypes differs for various plant growth regulators, *in vitro* propagation protocol should be optimized for each genotype in liquid medium. Furthermore, there is no protocol developed for commercial sugarcane genotypes through liquid culture in Ethiopia so far. Thus, present study was aimed to optimize protocol for *in vitro* propagation of two elite sugarcane genotypes (N52 and N53) in a liquid culture using shoot tip culture.

The study consisted of four series of experiments: shoot multiplication, *in vitro* root induction, *ex vitro* root induction and acclimatization of plantlets. The experiments were carried out in CRD with four, four, five and five replications respectively. Five months old and healthy shoot tips were excised and collected using sterile surgical blade and used as explants. The explants were carefully washed with tap water, liquid detergent with 2-3 drops of tween 20, then they were treated with ascorbic acid (0.2%) and citric acid (0.4%), antioxidants and 0.1% (w/v) Bavistin fungicide (Carbendizem) and finally surface sterilized with 70% alcohol and 50% sodium hypochlorite (5.25% w/v active chlorine) in the laminar airflow cabinet.

Aseptically initiated uniform microshoots were transferred to full strength liquid MS media fortified with 3% sucrose and various concentrations of BAP (0, 0.5, 1, 1.5, 2) and

kinetin (0, 0.5, 1, 1.5) plant growth hormone after two cycle of subculturing on semi-solid media. Microshoots with uniform length obtained from shoot multiplication medium were transferred to ½ strength liquid MS rooting medium supplemented with various concentrations of sucrose (0, 40, 50, 60, and 70 g/l) and NAA (0, 3, 5,and 7 g/l). Similarly, uniform length microshoots obtained from shoot multiplication medium were directly transferred onto tray containing substrates mixture composed of soil + sand in 1: 2 ratio after dipping the basal end of the shoots in various concentrations of NAA (0, 10, 20, 30 and 40 mg/l). Uniform *in vitro* rooted plantlets were transplanted onto tray containing different substrates mixture composed of sand + soil + FYM in different proportions and then transferred to greenhouse for hardening.

Among different combination of BAP and kinetin tested in multiplication stage, 2 mg/l BAP + 0.5mg/l kinetin was found to be optimal for maximum (6.95 shoots per explant) shoot multiplication of genotype N52. While for genotype N53, best shoot proliferation (6.30 shoots per explant) was achieved on liquid MS containing 1.5mg/l BAP + 0.5 mg/l kinetin. In rooting stage, 3 mg/l NAA + 50 g/l sucrose was found best combination for root induction in genotype N52, which resulted in 100 % rooting frequency with a mean of 4.95 cm root length and 23.5 number of roots per shoot. While, 5 mg/l NAA and 50 g/l sucrose was found to be the best combination for rooting genotype N53 and gave 100% rooting frequency with 4.54 cm root length and 21.76 roots per shoot.

From the five concentrations of NAA tested for *ex vitro* rooting, 20 mg/l NAA was found to be the optimal concentrations for ex *vitro* rooting of genotype N52. It produced the highest rooting frequency (76%) with an average of 8.06 roots per shoot while in genotype N53, 30 mg/l NAA gave a maximum of 70% rooting frequency with 4.52 average root numbers per shoot. In *ex vitro* acclimatization of *in vitro* rooted plantlets, best survival rate with vigorous growth was achieved on a substrates mixtures containing sand + soil + farmyard manure in 1:1:0 ratios in both N52 and N53 genotypes.

5.2. Conclusion

From the result obtained in the present study, it is concluded that the optimized protocol is helpful for rapid *in vitro* propagation of the sugarcane planting materials and hence enhance the availability of healthy and true to type planting materials in Ethiopian sugarcane plantations. Moreover, it is reasonable to deduce that the developed protocol is cost effective in that it uses liquid media at multiplication and rooting stages as an alternative to agar gelled medium; locally available table sugar instead of costly graded sucrose and include *ex virto* acclimatization as cost reduction strategy. Accordingly,

- A combination of 2 mg/l BAP + 0.5 mg/l Kinetin was the best combination for shoot multiplication of genotype N52 while 1.5 mg/l BAP + 0.5 mg/l kinetin was the optimum combination for genotype N53.
- For *in vitro* rooting, half strength MS liquid medium fortified with 3 mg/l NAA + 50 g/l sucrose was best combination for genotype N52 while half MS liquid medium supplemented with 5 mg/l NAA + 50 g/l sucrose was best combination for genotype N53.
- For *ex vitro* rooting 20 mg/l NAA and 30 mg/l were optimal for genotype N52 and N53, respectively.
- For *ex vitro* acclimatization, a substrate mixture composed of sand + soil in 1:1 ratio was best mixture for both genotypes (N52 and N53).

5.3. Recommendation

Based on the results of the present study, the following recommendations were made:

- In the future it will be better to determine optimum volume of liquid medium per a given jar or flasks so as to improve the quality of shoots multiplied on liquid shake culture.
- It is also recommended to optimize protocols for these genotypes using other type and plant growth hormone combination so as to get best multiplication and reduce cost.
- To improve the efficiency of liquid medium and quality of shoots and further reduce cost of laborer, it is desirable to develop protocol using bioreactor.

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7. APPENDICES

Table 1: List of Components in MS Medium and the Concentration in Stock Solution

Constituents	Amount (g)	For one liter media (ml)
MS 1	**For 1000 ml**	**50**
Ammonium Nitrate (NH_4NO_3)	33	
Potassium Nitrate (KNO_3)	38	
MS 2	**For 500ml**	**5**
Magnesium Sulphate ($MgSO_4.7H_2O$)	18.07	
Manganese Sulphate ($MnSO_4.H_2O$)	1.69	
Zink Sulphate ($ZnSO_4.7H_2O$)	0.86	
Copper Sulphate ($CuSO_4.5H_2O$)	0.0025	
MS 3	**For 500 ml**	**5**
Calcium Chloride ($CaCl_2.2H_2O$)	33.22	
Potassium Iodide (KI)	0.083	
Cobalt Chloride ($CoCl.6H_2O$)	0.0025	
MS 4	**For 500 ml**	**5**
Potassium dibasic Phosphate (KH_2PO_4)	17	
Boric Acid (H_3BO_3)	0.62	
Sodium Molbdate ($Na_2MoO_4.2H_2O$)	0.025	
MS 5	**For 500 ml**	**5**
Na_2EDTA	3.72	
Iron Sulphate ($FeSO_4.7H_2O$)	2.78	
MS 6	**For 500 ml**	**5**
Myoinositol	10	
Glycine	0.2	
Thiamine HCl	0.01	
Pyridoxine HCl	0.05	
Nicotinic acid	0.05	

Table 2. ANOVA Summary of Effect of BAP and Kinetin on Shoot Multiplication

Source of variation	DF	Number of shoots per explant	Shoot length (cm)	Number of leaves per shoot
		MS	MS	MS
Genotype	1	14.28***	11.70***	11.18***
BAP	4	17.66***	8.85***	8.32***
Kinetin	3	13.56***	12.30***	13.53***
Genotype*BAP	4	4.74***	0.24***	0.73***
Genotype*Kinetin	3	0.88***	0.19***	0.023*
BAP*Kinetin	12	8.81***	3.66***	10.24***
Genotype*BAP*Kinetin	12	4.97***	0.38***	1.00***
CV %		5.55	5.43	4.20

*** = very highly significant at $P \leq 0.0001$, * = significant at $P \leq 0.05$, NS = Non-significant at $P \geq 0.05$, DF = Degree of freedom, BAP = 6- Benzyl aminopurine, MS = mean square CV = Coefficient of variation

Appendix Table 3: ANOVA Summary for Effect of NAA and Sucrose on *In vitro* Rooting

Source of variation	DF	Rooting percentage	Root length (cm)	Number of roots per shoot
		MS	MS	MS
Genotype	1	27.56^{ns}	31.83***	52.10***
NAA	3	630.59***	16.40 ***	799.74***
Sucrose	4	5246.20***	70.01***	1390.98***
Genotype*NAA	3	194.44**	3.87***	11.95***
Genotype*Sucrose	4	88.72^{ns}	2.29***	8.35***
NAA*Sucrose	12	88.69^{*}	2.81***	64.03***
Genotype*NAA*Sucrose	12	52.95^{ns}	0.96***	34.09***
CV %		8.88	5.35	5.93

*** = very highly significant at $P \leq 0.0001$, ** = highly significant at $P \leq 0.01$, * = significant at $P \leq 0.05$, NS = Non-significant at $P \geq 0.05$, DF = Degree of freedom, NAA = α-naphthalene acetic acid, MS = mean square, CV = Coefficient of variation

Appendix Table 4: ANOVA Summary for Effect of NAA on *Ex vitro* Rooting

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

*** = very highly significant at P≤0.0001, DF = Degree of freedom, NAA = α-naphthalene acetic acid, MS = mean square CV= Coefficient of variation

Appendix Table 5. ANOVA Summary of Effect of Different Substrate Mixture on Survival Rate of *Ex vitro* Acclimatized Sugarcane Plantlets

Source of variation	DF	Survival rate (%)
		MS
Genotype	1	60.00^{NS}
Substrate mixture	5	632.00***
Genotype*Substrate mixture	5	320.00***
CV %		5.91

*** = very highly significant at P ≤ 0.0001, NS = Non-significant at P ≥ 0.05, DF = Degree of freedom, MS = mean square, CV = Coefficient of variation