

**ADDIS ABABA UNIVERSITY**  
**SCHOOL OF GRADUATE STUDIES**  
**INSTITUTE OF BIOTECHNOLOGY**



**Effect of Growth Regulators Concentration on *in vitro* Propagation of Three Elite Sugarcane (*Saccharum officinarum* L.) Cultivars Using Shoot Tip Culture**



A Thesis Submitted to the Institute of Biotechnology, School of Graduate Studies, Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

**BY**  
**Gerema Amente Dhisata**

**Addis Ababa Ethiopia**

**March 2017**

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**SIGNATURE PAGE**

This is to certify that the thesis prepared by, **Gerema Amente** entitled **Effect of Growth Regulators Concentrations on *in vitro* Propagation of three Elite Sugarcane (*Saccharum officinarum* L.) Cultivars using Shoot tip Culture** and submitted in partial fulfillment of the requirements for the degree of Masters of Science in Biotechnology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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# Effect of Growth Regulators Concentrations on *In vitro* Propagation of three Elite Sugarcane (*Saccharum officinarum* L.) Cultivars using Shoot Tip Culture

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## ABSTRACT

*Conventional vegetative propagation of sugarcane generally has low multiplication rate and allows distribution of diseases. This results in shortage of quality planting materials. Micropropagation is currently the only practical means of achieving rapid, large-scale production of disease-free quality planting material. The present study was conducted with the objective of studying the in vitro propagation of elite sugarcane genotypes (Q200, Q217 and Co-0238) using shoot tip explants. Experiments on shoot tip culture initiation, shoot multiplication and in vitro rooting were laid out in completely randomized design with 2x3x3, 4x5x3 and 3x5x3 factorial treatment arrangements respectively. Data was subjected to analysis of variance (ANOVA) and significant means were separated using Duncan's multiple range tests. With regard to shoot multiplication, genotype Q200 showed a maximum of 13.59 shoots per explant with 5.83cm shoot length on a medium fortified with 2 mg/l BAP alone, while genotype Q217 produced a maximum of 15.28 shoots per explant with 5.37cm mean shoot length on a medium supplied with 2.0 mg/l BAP and 0.25 mg/l kinetin. Likewise, Co-0238 produced maximum of 13.56 shoots per explant with mean shoot length 6.50 cm on medium fortified with 1.5 mg/l BAP + 0.5 mg/l kinetin. Half MS semi solid medium containing 3.0 mg/l NAA induced the highest rooting with mean root number per shoot of 14.93 for Q200. For Co-0238, half MS medium supplemented with 5.0 mg/l NAA induced the highest rooting response with mean root number per shoot of 18.40. Similarly, ½ MS medium supplemented with 1.5 mg/l IBA in combination with 3.0 mg/l NAA induced highest rooting response with 16.50 mean root number per shoot. In conclusion, this study can be used for rapid in vitro propagation of these three elite sugarcane genotypes.*

**Keywords:** - Genotypes, 6-Benzylaminopurine, Naphthalene acetic acid, Indole butyric acid, Kinetin

## STATEMENT OF THE AUTHOR

I, Gerema Amente, hereby affirm that the work presented in the thesis entitled, “**Effect of Growth Regulators Concentrations on *In vitro* Propagation of three Elite Sugarcane (*Saccharum officinarum* L.) Cultivars using Shoot tip Culture**” for partial fulfillment of the requirements for the award of the Degree of Master of Science in Biotechnology at Addis Ababa University is a real record of my own work, under the supervision of my major advisor Dr. Tileye Feyissa and my co-advisor, Dr. Mulugeta Diro.

Appropriately acknowledging all source materials I used in this thesis, I sincerely state 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 thesis in whole or in part may be granted by the Director of Institute of Biotechnology, Addis Ababa University when in his/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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## LIST OF ABBREVIATIONS AND ACRONYMS

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
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
PGR	Plant Growth Regulator
PVC	Polyvinyl Chloride
PVP	Polyvinyl Pyrolidone
DMRT	Duncan's Multiple Range Test
Kinetin	N-(2-furanylmethyl)-1H-puring-6-amine
SEM	standard Error of Means
FAO	Food and Agricultural Organization

# 1. INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a monocotyledonous crop plant that belongs to family *Poaceae* and tribe *Andropogonae*. The family *Poaceae* includes grasses consisting of about 620 genera and 10,000 species, occupying maximum land and water bodies across the globe (Sharma, 2005; Cha-um *et al.*, 2006). This family constitutes a natural and homogeneous family and is among the most successful of all angiosperms in their spread (Godheja *et al.*, 2014). Modern sugarcane cultivars are derived from the combination of the species *S. officinarum*, the domesticated sugar-producing species with  $x=10$  and  $2n=8x=80$ , and *S. spontaneum*, a vigorous wild species with  $x=8$  and  $2n=5x=40$  to  $16x=128$  (Jalaja *et al.*, 2008). It is a tall-growing crop that is cultivated in the tropical and subtropical regions of the world, primarily for its ability to store high concentrations of sucrose, or sugar in the stem (Hunsigi, 2001).

Sugarcane is a commercial crop that plays an important role in the economy of several tropical and sub-tropical countries due to its major byproducts and labor intensive production operations and accounts for about 70% of the world's total sugar production (Sandeep *et al.*, 2009), 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, particle board 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 which is an addition to the economic value of the crop.

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 20.6 tons/ha. Brazil and India account for 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; Melaku *et al.*, 2016).

Currently there are three operating sugar factories in Ethiopia at three different locations, namely: Wonji/Shoa, Metahara, and Fincha, in order of their periods of establishment. These three factories together produced about 300,000 tons of sugar and 11.1 million liters of ethanol annually (Ethiopia Sugar Corporation, 2013). The sugar industry has great contributions to the socio-economy of the country, given its agricultural and industrial investments, foreign exchange earnings, its high employment, and its linkages with major suppliers, support industries and customers (Belay *et al.*, 2014). Hence, much research has been focused on sugarcane crop improvement through conventional breeding and quite recently, extensive attention has been paid to biotechnological approaches (Baksha *et al.*, 2003; Hoy *et al.*, 2003).

The conventional breeding method takes 10 to 15 years of work to complete a selection cycle program (Ali *et al.*, 2008, and Biradar *et al.* 2009). Once a desired variety is developed or clone is identified, it usually takes six to seven years to produce sufficient quantity and quality planting materials via conventional propagation method (Khan *et al.*, 2006). As varieties of sugarcane are highly heterogeneous, multiplication for commercial purpose is usually carried out vegetatively by stem cuttings (Bahera and Sahoo, 2009).

Conventional propagation method, where nodal sections with two or three nodes or sets are used as planting material, has a wide-range of limitations. A bud produces 4 to 5 shoots and the rate of propagation is 1:10 in a year. Propagation from stem cuttings also facilitates spread of pathogens with accumulation of disease like red rot, leaf scald, ratoon stunting, grassy shoot and mosaic over vegetative cycles leading to reduction in yield and quality. Conventional propagation of sugarcane generally suffered from low propagation rates, expensive labor, large land areas demand, time consuming and potential transmission of pathogens from the seed cane to the subsequent crop limits the efficiency of this method (Sandeep *et al.*, 2009). Therefore, efficient propagation system is required for mass multiplication of sugarcane in a short period of time.

Micropropagation is currently the only practical means of achieving rapid, large-scale production of disease-free quality planting material as seed canes of newly developed varieties in order to speed up the breeding and commercialization process in sugarcane (Lee, 1987; Feldman *et al.*, 1994 and Lal and Krishna, 1994). Through micropropagation, 10,000 identical plants can be produced from a single bud in about 3 to 4 months and the rate of propagation is 1:22 to 1:25 in 8 to 10 months (Belay *et al.*, 2014; Tilahun *et al.*, 2014). Anita *et al.* (2000) reported that by micropropagation over 1.5 million plants could

be produced from a single shoot tip of early maturing varieties of sugarcane in six months. In addition, Lal *et al.* (1996) demonstrated that micropropagated system showed a potential *in vitro* production of 75600 shoots from a single shoot apex explants in a period of about 5.5 months. By this technology, nearly 1000,000 healthier, virus-free, type sugarcane plantlets can be produced from single plant in one year (Lee, 1987). Plants raised through micropropagation are of uniform quality, pathogen free, can be produced much more rapidly as new cultivars could become commercially available within 2 to 3 years from development rather than 10 to 15 years needed using conventional propagation. Shoot tip culture is the best method for propagation as it produces plants that are the same to the mother plant and gives much more rapid multiplication rate compared to other procedures (Lee, 1987). Shaw (1990) also indicated that shoot-tip culture is being used in most sugarcane industries for the development of disease-free clones mostly to facilitate their safe and speedy movement through quarantine.

The success of *in vitro* culture depends largely on the growth conditions of the source material (Caswell *et al.*, 2000; Delporte *et al.*, 2001), medium composition and culture conditions (Saharan *et al.*, 2004) and on the genotypes of donor plants. Mullegadoo and Dookun, (1999), studied the explant source of three sugarcane varieties and its effect on multiplication and found that shoot apex performed better growth response as compared to axillary bud and apical bud. Hoy *et al.* (2003) studied the effect of tissue culture explant source on sugarcane yield components. Kalpana *et al.* (2011) reported effect of light intensity, photoperiod and growth room temperature on *in vitro* morphogenetic responses of leaf sheath explants of sugarcane varieties. Pawar *et al.* (2002) reported effect of growth regulators on *in vitro* multiplication of sugarcane cultivars and studied the effect

of different levels of kinetin, BAP and coconut water (CW) on shoot multiplication, length of main shoot and number of leaves on main shoot. Among these all factors, the genotype appears to be important factor influencing the effectiveness of sugarcane *in vitro* culture.

Rapid clonal propagation of sugarcane planting materials depends on the genotype and the plant growth regulators combinations used and needs to develop plant growth regulators combinations for each genotype (Singh *et al.*, 2006). Plant growth regulators requirements for *in vitro* propagation responses differ from cultivar to cultivar in sugarcane (Pathak *et al.*, 2009). In view of the fact that in plant tissue culture different varieties (genotypes) of the same species respond differently to media (Roy, 2000). Growth of cultured tissues or organs, and regeneration of *in vitro* are very much influenced by the genotype. It is probably true to say that effects of genotypes impose the greatest constraints to the tissue culture and micropropagation (Edwin, 2008). Therefore, it is necessary that an efficient protocol is needed for every new variety or clone of sugarcane to get rapid shoot initiation, shoot multiplication and root induction. Thus, this thesis research work was designed with the following general and specific objectives.

## **2. OBJECTIVES OF THE STUDY**

### **2.1. General Objective**

To study the response of three genotypes (Q200, Q217 and Co-0238) to *in vitro* shoot multiplication, root induction and acclimatization of sugarcane cultivars using shoot tip explants

### **2.2. Specific Objectives**

- ✓ To evaluate the response of the genotypes to shoot initiation on different concentrations and combination of BAP+IBA hormones.
- ✓ To evaluate the response of the genotypes to *in vitro* shoot multiplications on different concentrations and combinations of BAP and Kinetin.
- ✓ To evaluate the response of the genotypes to root induction on different concentrations and combination of auxins (IBA and NAA).
- ✓ To evaluate survival percentage of the three genotypes during acclimatization..

### 3. LITERATURE REVIEW

#### 3.1 Origin and Distribution of Sugarcane

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 continents worldwide situated between 37°N and 31°S (Hunsigi, 2001).

All varieties of sugarcane are species or hybrids of the genus *Saccharum*. Species included are *S. officinarum*, *S. spontaneum*, *S. barberi*, *S. sinense*, *S. robustum*, and *S. edule*. *S. spontaneum* and *S. robustum* are wild species whereas *S. officinarum*, *S. barberi*, *S. sinense*, and *S. edule* are cultivated. The four cultivated species are complicated hybrids, and all intercross readily. 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).

It was at Wonji in 1951, some 110 km east of the capital Addis Ababa, modern sugar industry started in Ethiopia as a share company established by Dutch Company investors & Ethiopian government. Then followed by Shewa Sugar Factory in 1962 with 187.4 tons

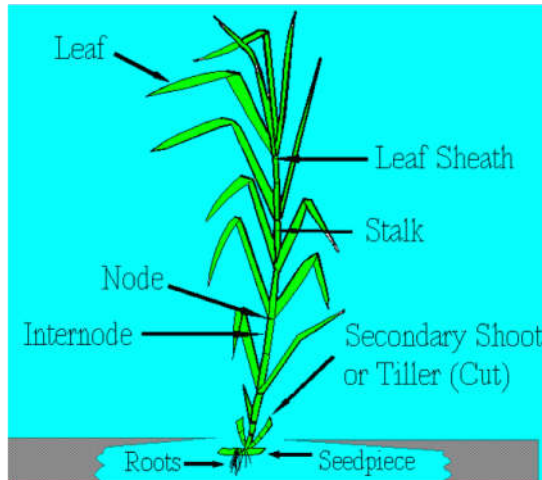
of sugar production capacity a day and Metahara in 1969, currently having a capacity of production more than 143,300.5 tons sugar and 12.5 million liters ethanol a year on average. Fincha Sugar Factory, located 300 Km west of Addis Ababa. Started its regular sugar production in 1998 with its initial sugar production capacity of 55115.6 tons per annum, though its establishment process and other activities dated back to 1975 ([www.etsugar.gov.et](http://www.etsugar.gov.et); Melaku *et al.*,2016 ; Belay *et al.*, 2014 ).

### **3.2 Botanical Descriptions of Sugarcane**

Sugarcane is a tall perennial tropical grass that tillers at the base to produce unbranched stems, 2.5-4 m or taller and about 3-5 cm in diameter. The solid unbranched stems, roughly circular or oval in cross section are clearly differentiated into joints each comprising a node and an internode. The node consists of a lateral bud in leaf axils. The nodes are spaced at an interval of 0.15-0.25m., but are much closer at the top of the stems (canes) of which the internodes vary in length (15-25cm.),girth (1.5-6 cm in diameter) shape (cylindrical, conical, barrel and circular or oval in cross section) and colour (yellow, green, red, striped, variegated). Each stem has a hard, wax covered rind (epidermis), surrounding a mass of softer tissue (parenchyma), which is interspersed with fibres (Wrigley, 1982; Sharma 2005, Hardev *et al*, 2016).

### 3.2.1 Main parts of the sugarcane plant

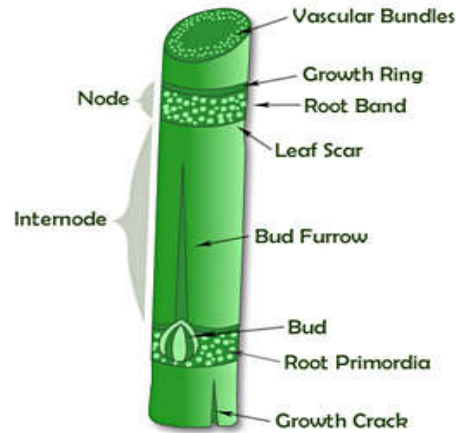
The main parts of the sugarcane plant are the stalk, leaf, and root system



**Figure 1. Parts of the sugarcane plant.** Source: (Hardev *et al*, 2016)

#### 3.2.1.1 The stalk

The stalk consists of segments called joints. Each joint is made up of a node and an internode (Figure 2). The node is where the leaf attaches to the stalk and where the buds and root primordial are found. A leaf scar can be found at the node when the leaf drops off the plant. The length and diameter of the joints vary widely with different varieties and growing conditions. Generally the nodes are placed at an interval of 15-25 cm, but are much shorter at the top of the stem, where elongation and rapid growth takes place, than at the bottom where they form part of the rootstock and are essential to the formation of tillers (Sharma, 2005, Hardev *et al*, 2016).



**Figure 2. Parts of the stalk (stripped of leaves)** Source: (Hardev *et al*, 2016)

The buds, located in the root band of the node, are embryonic shoot consisting of a miniature stalk with small leaves. The outer small leaves are in the form of scales. The outermost bud scale has the form of a hood in commercial production. (Sharma, 2005).

#### 3.2.1.2 Leaf

The sugarcane leaves, like those of grasses, are made up of the leaf blade (lamina) and the leaf sheath, separated by a blade joint (Figure 3). The leaves are usually attached alternately to the nodes, thus forming two ranks on opposite sides. The mature sugarcane plant has an average total upper leaf surface of about 0.5 square meters and the number of green leaves per stalk is around ten, depending on variety and growing conditions.



**Figure 3. Parts of the leaf (peeled from the stalk)**

**Source:** (Hardev *et al*, 2016)

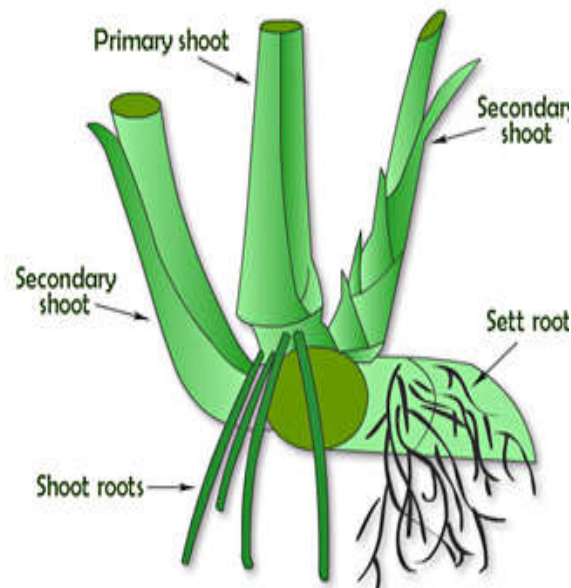
The leaf has a strong midrib, white and concave on the upper surface, convex and green. The blade joint is where two wedge shaped areas called "dewlaps" are found (Figure 3). Leaf pubescence, or the covering of the various leaf parts with short hairs, is also variety and age dependent. Pubescence is not found on the leaf blade of commercial varieties. Sheath pubescence can be used to identify plants (Sharma, 2005, Hardev *et al*, 2016)

### **3.2.1.3 Inflorescence**

The inflorescence of sugarcane, generally known as the arrow, is an open panicle. It is long (30 cm or more) and tapering. The arrangement of the spikelet is acemose, that is, the oldest flowers are at the bottom and the youngest at the top. The flowers open in succession over a number of days. Flowers have both male and female organs but not all produce fertile pollen. Some of the cultivars have fertile pollens, but they are usually small and of low vitality. Sugarcane usually flowers at the age of 10-12 months, but some cultivars do not flower at all. Due to this fact, cane has long been propagated vegetatively by cuttings of sugarcane. Cane produced from seed is not so vigorous, but it is important input for sugarcane breeders (Hardev *et al*, 2016).

### 3.2.1.4 The Root System

Two kinds of roots will develop from a planted seed piece. The set roots, which arise from the root band, are thin and highly branched; the shoot roots, originating from the lower root bands of the shoots, are thick, fleshy and less branched. New roots are continually produced from tillers (Figure 4).



**Figure 4. Set and shoot roots Source: (Hardev *et al*, 2016)**

### 3.3 *Economic importance of sugarcane*

The primary use of sugarcane is to produce sugar, which can then be used in an infinite number of products. It accounts for about three fourth of the world's sugar production (Verma, 2004). The type of sugar produced by sugarcane is called sucrose. This is the most important of all the sugars. Sucrose is used as a sweetening agent for foods and in the manufacture of cakes, candies, preservatives, soft drinks, alcohol, and numerous other foods. Today, sugarcane has many industrial uses and is one of the most widely used and cheapest domestic products (Jenkins, 1966). Important industrial products of sugar-

cane include bagasse, molasses, and filter cake. Bagasse, the residual woody fiber of the cane, is used for several purposes: fuel for the boilers and lime ovens, production of numerous paper and paperboard products and reconstituted panel board, agricultural mulch, and as a raw material for production of chemicals. Bagasse and bagasse residues are primarily used as a fuel source for the boilers in the generation of process steam. Thus, bagasse is a renewable resource. Dried filter cake is used as an animal feed supplement, fertilizer, and source of sugarcane wax. Molasses is produced in two forms: inedible for humans (blackstrap) or as edible syrup. Blackstrap molasses is used primarily as an animal feed additive but also is used to produce ethanol, compressed yeast, citric acid, and rum. Edible molasses syrups are often blends with maple syrup, invert sugars, or corn syrup (Mackintosh, 2000).

### **3.4 Sugarcane Nutrition Facts**

Sugarcane contains various essential minerals like calcium, phosphorus, iron, magnesium and potassium. Sugarcane juice is rich in flavonoid and phenolic compounds which are powerful antioxidants that prevent cell damage by free radicals and reduce risk of cancer and heart disease. It has a low glycemic index between 30-40, about half that of refined sugar. Sugarcane juice is easily digestible, provides instant energy and hydrates the body. It is alkaline, contains soluble fiber, proteins and vitamins A, C and B complex

(<http://nutrition.indobase.com/articles/sugarcane-juice-nutrition.php>)

Table 1. Nutritional Value & Calories in Sugarcane Juice

Nutrients	Amount(1 Ounce Sugarcane Juice)
Proteins	0.20 g
Water	0.19 g
Ash	0.66 g
Fat	0.09 g
<b>Calories</b>	
Total calories	111. 43
Calories From Fats	0.03
<b>Carbohydrates</b>	
Total Carbohydrates	27.40 g
Sugar	25.71 g
<b>Vitamins</b>	
Riboflavin	0.16 mg
Niacin	0.20 mg
Pantothenic Acid	0.09 mg
<b>Minerals</b>	
Calcium	32.57 mg
Iron	0.57 mg
Magnesium	2.49 mg
Phosphorus	0.01 mg
Potassium	162.86 mg
Copper	0.09 mg
Manganese	0.09 mg

(Amount 1 ounce weight 28.35g)

Source; <http://nutrition.indobase.com/articles/sugarcane-juice-nutrition.php>)

*Ounce means a unit of weight equal to approximately 28 grams*

### **3.5 Conventional Sugarcane 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 seed cane 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 seed cane, seed cane and commercial cane production. The initial seed cane is used as source for planting materials to establish seed cane fields and in turn seed cane fields are harvested to plant commercial cane in large area of the plantation (Melaku *et al.*, 2016).

#### **3.5.1 Seed Sett Preparation**

Seed setts are prepared manually. Seed canes are harvested and dry leaves removed manually to avoid any damage to the buds. Canes are cut with a sharp knife into setts containing two or three buds each. Sett-cutting machines are now available making the process more efficient. The cut ends of seed setts become easy entry points of many disease causing microbes, leading to Sett rotting and damage to the buds and root primordial. Soaking the setts for 5 to 10 minutes in 0.1% solution of a systemic fungicide such as methyl benzimidazole-2-yl-carbamate (MBC) just before planting is recommended to ensure protection (Jalaja *et al.*, 2008).

### 3.5.2 Heat Treatment of Setts

Sugarcane setts may harbor a host of diseases such as sugarcane smut, red rot, grassy shoot, ratoon stunting, sugarcane mosaic and yellow leaf. Scale insects and borers present on the setts can also cause heavy damage to the new crop. Heat treatment of setts helps in getting rid of several diseases and pests. There are four types of heat therapies:

**Hot water:** setts are immersed in water maintained at 50°C for two to two and a half hours. Often, fungicides are mixed in hot water to eliminate smut disease.

**Hot air:** dry heat produced by electric heaters placed at different points in the heating chamber is circulated with a fan. Temperature is maintained at 56°C and the seed is treated for eight hours.

**Moist hot air:** steam is injected into the treatment chamber for four hours maintaining the temperature at 54°C.

**Aerated steam:** steam is mixed with air in 1:4 proportions and forced into the treatment chamber through small holes. The treatment is given for one hour at 50°C ((Jalaja *et al.*, 2008).

### 3.6 Plant Tissue Culture

Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions often to produce the clones of plants. The resulting clones are true-to type of the selected genotype. The controlled conditions provide the culture an environment conducive for their growth and multiplication. These conditions include proper supply of nutrients, pH, adequate temperature and proper gaseous and liquid environment.

Plant tissue culture technology is being widely used for large scale plant multiplication. Apart from their use as a tool of research, plant tissue culture techniques have in recent years, become of major industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites. Small pieces of tissue (named explants) can be used to produce hundreds and thousands of plants in a continuous process. A single explant can be multiplied into several thousand plants in relatively short time period and space under controlled conditions, regardless of the season and weather on a year round basis. This process is known as micropropagation. Endangered, threatened and rare species have successfully been grown and conserved by micropropagation because of high coefficient of multiplication and small demands on number of initial plants and space (Hussain *et al.*, 2012; Bhanwar *et al.*, 2016).

### **3.6.1 Micropropagation**

The objective of plant propagation via tissue culture is to propagate plants true-to-type (clones), which is termed micropropagation. It is the process in which an explant placed in a suitable culture media under aseptic environment to produce multiple shoots available for rooting and become ready for transferring to soil after acclimatization. According to George *et al.* (2008), there are five stages of micropropagation. Namely,

Stage 0. Selection of the plant tissue (explant) from a healthy vigorous ‘mother plant’. This tissue must be sterilized to remove microbial contaminants.

Stage 1. Establishment of the explant in a culture medium. The medium sustains the plant cells and encourages cell division. It can be solid or liquid.

Stage 2. Multiplication. The explant gives rise to a callus or shoots which is manipulated by varying the auxin: Cytokinin to form multiple shoots.

Stage 3. Root formation. The shoots are transferred to a growth medium with relatively higher auxin: Cytokinin ratios.

Stage 4. The rooted shoots are potted up and ‘hardened off’ by gradually decreasing the humidity by process called acclimatization

Micropropagation offers several distinct advantages that are not possible with conventional propagation techniques. (I) Through micropropagation, it is possible to produce over a million clonally uniform plants, within one year *in vitro* from a single explant (shoot tip, nodal segment, leaf pieces etc.); (II) newly developed cultivars can also be released for commercial planting within a short period of time. (III) It can also be used for production of disease/pathogen free stock material (widely used in horticulture industry) - as through meristem culture. (IV) Apart from these conventional benefits, research on various aspects has widened the scope of plant tissue culture. (V) Secondary metabolite production through tissue culture technology has been proved more efficient as compared to conventional extraction from field grown/naturally cultivated plants (Bhanwar *et al.*, 2016).

### **3.7 Tissue culture of *Poaceae***

Tissue culture of *Poaceae* has attracted considerable attention because of its importance for grain and sugar crop. Many of the cereals are annual crops and grow slowly from seed. Others, such as sugarcane are propagated vegetatively by cuttings. Conventional grass breeding takes years to evolve and to select a suitable cultivar, if supplemented by techniques of cell and tissue culture could hopefully shorten the time taken to evolve a new variety and to introduce genetic variability in progenies for better selection. The species of *Poaceae* for a long time were considered recalcitrant to growth in culture and were

a neglected group. Tissue culture of monocots came to be realized only a decade and a half after the establishment of dicot tissues *in vitro*. The first successful culture on a long term basis was reported by La Rue (1949) while working on maize endosperm. This was followed by Straus and La Rue (1954) and Starus (1958) in standardizing media for culture of corn endosperm and later a number of species of Poaceae. Presently tissue culture techniques are useful systems for *in vitro* clonal propagation and for generating genetic variability in Poaceae (Vasil *et al.*, 1984). A brief sum up on micropropagation in sugarcane is given below.

### **3.8 *In vitro* propagation studies in sugarcane**

Sugarcane tissue culture was initiated in Hawaii in 1961 (Liu, 1974) and first report of plant differentiation from callus tissue of *Saccharum* species was documented by Heinz and Mee (1969). These authors reported callus formation using parenchyma tissue of shoot apices and leaves of *Saccharum sp.* on basal medium containing coconut water and 2, 4-D. Withdrawal of 2, 4-D from the medium induced organ differentiation. Subsequently every organ of the plant has been exploited for *in vitro* culture including reproductive parts with the ultimate objective of producing high yielding, high sucrose content and disease resistant clones of sugarcane (Liu, 1981). Genetic variability has been induced (Liu and Chen, 1974) through physical and chemical mutagens by treating callus and suspension cells to produce resistant and environmentally stable mutant lines of sugarcane.

Sugarcane was also cultured *in vitro* by Nickell (1964) using mature parenchyma of internodal tissues for some physiological studies. Later, after demonstration of totipotency in callus cultures of sugarcane (Barba and Nickell, 1969), a rapid progress

was made in cell and tissue cultures of this crop and it was found that the cultures could be raised from any part of the plant that the highly regenerable callus could be produced from immature leaves and inflorescences. It is now well established that the morphogenetic responses of different tissues are under the influence of hormones (plant growth regulators) and are cultivar dependent (Taylor *et al.*, 1995).

In the past few years many, laboratories around the world, where sugarcane research programmes are in progress, have utilized tissue culture techniques in sugarcane improvement. The major potential areas identified so far include: I) somaclonal variation for crop improvement, II) micropropagation for seed cane multiplication of new varieties, IV) development of interspecific and intergeneric hybrids, V) disease resistance, VI) selection for biotic and abiotic stresses, VII) protoplast culture, VIII) molecular biology, IX) genetic transformation, X) *in vitro* germplasm conservation, XI) artificial seed etc.

Barba and Nickell (1969) reported that sugarcane tissues sub cultured for over 4 years had lost the capability to differentiate shoots. Freshly isolated tissues from 3 cultivars, readily obtained shoot differentiation on Murashige-Skoog medium, but shoot differentiation was lost, while tissues from a fourth cultivar produced only roots. The tissue cultures of all cultivars studied have the capability to form roots regardless of the length of time in culture; root formation was enhanced by treatment with dalapon (2, 2-dichloropropionic acid: herbicide and plant growth regulator). Heinz and Mee (1971) studied two populations derived from callus tissue of sugarcane clones, (H 37-1933) and (H 50-7209), for differences in morphology, chromosome number and four enzyme systems. Variations observed in morphological characteristics were not directly correlated with the differences in enzyme system. Shenk and Hildebrandt (1972) have reported requirement of

high concentration of auxins for rooting in sugarcane. Barba *et al.* (1977) also reported that root development requires higher sugar levels and auxins in nutrition media.

### **3.8.1 Explant**

In sugarcane, plants have been produced by direct regeneration from both apical and axillary meristem (Taylor and Dukie, 1993) and from immature leaf tissues (Lakshmanan *et al.*, 2002; Geijskes *et al.*, 2003). As with other plant species, sugarcane plants propagated *in vitro* from meristem are considered to be more genetically and phenotypically stable than those produced from callus (Lee, 1987; Sengar *et al.*, 2009). Thus, considerable effort has been expended to investigate the adaptability of meristem culture to commercially grown elite sugarcane cultivars (Taylor and Dukie, 1993; Burner and Grisham, 1995). Some recent reports indicate that direct regeneration can be obtained from a number of sugarcane genotypes using thin cell layer culture of immature leaf or inflorescence tissue (Geijskes *et al.*, 2003; Lakshmanan *et al.*, 2002), which would also reduce the time taken for *in vitro* propagation.

Hendre *et al.* (1983) reported that about 50,000 plants could be produced in six months from a single shoot tip. *In vitro* propagation provides rapid multiplication of sugarcane. Micropropagation of sugarcane was studied using two procedures (1) shoot tip culture, (2) indirect somatic embryogenesis from callus and reported that shoot tip culture produced plants phenotypically similar to the mother plant and gave a much more rapid multiplication rate compared to the other procedure (Lee *et al.*, 1987). Chen *et al.* (1988) reported sugarcane spindles of 6-12-month-old plant were successful for shoot regeneration. Dhumale *et al.* (1994) demonstrated that 2-3 mm long shoot tips of sugarcane plant

resulted in significant shoot regeneration. Burner *et al* (1995) propagated sugarcane *in vitro* from shoot tip.

By shoot tip micropropagation, over 1.5 million plants can be produced from a single shoot tip in six months (Anita *et al.*, 2000). Ali and Afghan (2001) reported micropropagation of sugarcane using meristem culture method and found that micropropagated plants were phenotypically similar to the mother plants. Hoy *et al.* (2003) studied the effect of tissue culture explant source on sugarcane yield components. Reports of this study showed that apical meristem culture was suitable for production of seed-cane, as sugarcane derived by meristem culture of three cultivars did not differ significantly from the original germplasm for any measured yield trait.

### **3.8.2 Surface sterilization**

Belay *et al.* (2014) surface sterilized shoot tip explants of sugarcane with 0.3% (w/v) Kocide solution for 30 minutes followed by three times washing for five minutes with sterile distilled water. The shoot tips were again rinsed in 70% ethanol for one minute and washed with sterile distilled water three times each for five minutes. Melaku *et al.* (2016) reported surface sterilization of shoot tip explant by 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. The authors also surface sterilized the shoot tips with 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. Tilahun *et al.* (2014) reported that sterilization of shoot tip explant with Berekina (5% active ingredient of chlorine) for 25 minutes gave satisfactory results with explants being contamination free. Shimelis *et al.* (2014) has illustrated the surface sterilization of shoot tip explant with

0.1% HgCl<sub>2</sub> (Mercuric Chloride) for 10 minutes. Godheja *et al.* (2014) has illustrated the surface sterilization of shoot tip with Bavistin solution (1 gm/liter) for 5 minutes followed by washing in running tap water to remove all traces of detergent followed by treatment with 10 % sodium hypo chloride solution for 15 minutes.

### **3.8.3 Shoot Multiplication**

The optimal cytokinins 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)

Chen *et al.* (1987) reported that at lower concentration of BAP (0.5 mg/L) and Kinetin (0.5 mg/L), there was loss of proliferation and vigor. Pawar *et al.* (2002) reported effect of growth regulators on *in vitro* multiplication of sugarcane cultivars (Co-86032, Co-740 and Co-8014) and studied the effect of different levels of kinetin, BAP and coconut water (CW) on shoot multiplication, length of main shoot and number of leaves on main shoot using MS medium supplemented with three levels of kinetin (0.5, 1.0 and 1.5 mg/L), four levels of BAP (0.5, 1.0, 1.5 and 2.0 mg/L) and two levels of CW (10 and 20%). The treatment MS + 1.5 mg/L kinetin + 1.0 mg/L BAP + 20% CW gave the highest values for length of main shoot in all cultivars. Baksha *et al.* (2002) reported multiple shoots from shoot tip explant of sugar-cane (*Saccharum officinarum*) by culturing on MS medium supplemented with BAP (0.5-2.0 mg/L), Kn (0.1-0.5 mg/L). Mamun *et al.* (2004) reported that *in vitro* propagation for sugarcane variety Isd-28 and Isd-29 showed best shooting when media were supplemented with BA 1.5 mg/L + 0.5 mg/L NAA. Khan *et al.* (2008) revealed rapid micropropagation of three elite sugarcane (*Saccharum officinarum*) varie-

ties by shoot tip culture. The study showed that shoot initiation from explant of all three varieties was achieved at 1.0 mg/L Kin and 0.1 mg/L GA<sub>3</sub>. Best response of multiplication for variety CP-77-400 was observed at 0.5 mg/L BAP with 0.1 mg/L GA<sub>3</sub> while variety CPF-237 was multiplied at 1.0 mg/L BAP with 0.5 mg/L GA<sub>3</sub>. Dereje *et al.* (2014) study revealed that MS medium supplemented with 1.5 mg/L BAP and 0.5 mg/L of Kin for B86-12; and 1.5 mg/L of BAP and 1.0 mg/L of Kin for C86-56 were found to be the optimum media for shoot multiplication. Tilahun *et al.* (2014) studied the protocol optimization for *in vitro* mass propagation of two sugarcane (*Saccharum officinarum*) clones grown in Ethiopia. The study showed that genotype Co -449 showed maximum regeneration frequency of 80% with 7.87±1.08 shoots per explant on MS medium with 3% sucrose and 2.0 mg/L BAP and 0.25 mg/L Kinetin. On the same carbon source, genotype Co-678 showed highest multiplication frequency of 90% with 9.10±0.10 shoots per explant on medium supplied with 2.0 mg/L BAP and 0.5 mg/L Kinetin. Belay *et al.* (2014) Study the effects of 6-Benzyl amino purine and Kinetin on *in vitro* shoot multiplication of sugarcane (*Saccharum officinarum*) varieties. MS medium fortified with 1.0 mg/L BAP and 0.5 mg/L kinetin for B41-227; and 2.0 mg/L BAP without kinetin for N14 were found to give optimum shoot proliferation results. On these medium, B41-227 gave 34 ± 1.54 shoots per explant with 6.95 ± 0.01cm average shoot length and 12 ± 0.17 leaves per shoot while N14 produced 21± 0.58 shoots per explant with 5.63 ± 0.01 cm average shoot length and 5.4 ± 00 leaves per shoot after 30 days of culture transfer to multiplication medium. Belay *et al.* (2014) studied response of sugarcane (*Saccharum officinarum*) varieties to BAP and IAA on *in vitro* shoot multiplication. The study confirmed that medium fortified with 1.5 mg/L BAP and 0.5 mg/L IAA

for B41-227 and 2.0 mg/LBAP and 0.25 mg/L IAA for N14 resulted in optimum multiplication response. Godheja *et al.* (2014) study revealed that shoot proliferation & multiplication of MS medium supplemented with 0.3 mg/L BAP, was found to be the best compared to other media.

#### **3.8.4 Rooting**

Shenk and Hildebrandt (1972) have reported requirement of high concentration of auxins for rooting in sugarcane. Barba *et al.* (1977) reported that root development requires higher sugar levels in nutrition media. Nadar and Heinz (1977) reported that preferred auxin for root initiation was NAA. Naritoom *et al.* (1993) initiated rooting on MS medium with 1.0 mg/L IBA/l. Dhumale *et al.* (1994), induced roots on half strength MS medium containing 2.0 mg/L IBA + 1.0 mg/L IAA. Cheema *et al.* (1995) demonstrated rooting on MS medium supplemented with 7% sugar and 5.0 mg/L NAA. Singh *et al.* (2001) transferred clumps with 5-10 well grown shoots on 1/2 strength MS liquid medium supplemented with NAA (5.0 mg/L) and elevated sucrose level (60 g/L). The development of fine roots, which began after 7-15 days, ranged from 75% (CoJ 85) to 95% (CoJ 86). Profuse rooting was achieved within 30-40 days. Pawar *et al.* (2002) induced rooting (85-92%) by transferring shoot clumps on 1/2 MS medium containing 2 mg/l NAA and 1.0 mg/ IBA. Baksha *et al.* (2002) achieved rooting on MS medium containing 5.0 mg/L IBA. Karim *et al.* (2002) also reported optimum root induction at 3.0 mg/L IBA with 11 roots per shoot along with 3.8 cm root length in 12 days. Mamun *et al.* (2004) reported that best results of rooting were observed on modified MS with auxins. Eighty to ninety percent regenerated plantlets were viable at normal temperature with 85% humidity. Belay (2014) reported that culture medium containing 2.0 mg/L NAA and 1.0 mg/L IBA for

B41-227 and 1.0 mg/L NAA alone for N14 was optimum. On these medium, B41-227 gave  $33 \pm 0.15$  roots per shoot with  $2.92 \pm 0.18$  cm root length and N14 produced  $35 \pm 0.20$  roots per shoot with  $3.2 \pm 0.25$  cm root length. Godheja *et al.* (2014) research showed that  $\frac{1}{2}$  strength MS media supplemented with 3.0 mg/L NAA & 3.0 mg/L IBA is best for root induction. Shimelis *et al.* (2015) reported  $\frac{1}{2}$  MS medium supplemented with 2 mg/L IBA+1 mg/L NAA for C86-56 has gave best number of roots (14.88), but best root length (3.14 cm) on  $\frac{1}{2}$  MS+3 mg/L IBA+4 mg/L NAA and C86-12 genotype has gave best number of roots (17.8) and root length (3.2 cm) at 5 mg/L NAA alone. Belete *et al.* (2016) found that half MS medium with 2.0 mg/l NAA resulted in 91.67% rooted shoots with  $12.58 \pm 0.23$  roots and  $2.54 \pm 0.04$  cm root length in B4906 whereas 4 mg/L resulted in 66.67% rooted shoots with  $7.83 \pm 0.70$  roots and  $2.60 \pm 0.05$  cm root length in Pr1013. These all findings indicate that the *in vitro* response of rooting in micropropagated shoots varied from genotype to genotype.

### **3.8.5 Hardening/Acclimatization**

Pawar *et al.* (2002) reported plantlets kept for 6-7 days in trays for hardening containing coconut peat showed higher survival percentage (86%) than plantlets hardened on sand, soil rite, sand + soil rite (1:1), or sand + soil rite + soil (1:1:1). Melaku *et al.* (2016) have demonstrated that best survival rate (94% in N52 and 100% in N53) was achieved on substrate mixtures containing sand + soil in 1:1: ratios. Belay (2016) reported that rooted plantlets showed 100% survival after four weeks of acclimatization in greenhouse on farmyard manure and soil at 2:8 ratios. Belete *et al.* (2016) also reported that plantlets acclimatized in greenhouse showed 96.1% of survival using soil, compost and sand (1:1:1) as potting mixture.

## **4. MATERIALS AND METHODS**

The study was conducted at Holetta National Agricultural Biotechnology Laboratory of the Ethiopian Institute of Agricultural Research. It is located 28 km west of Addis Ababa, in the Oromia Special Zone Surrounding Finfinne of the Oromia Region. It has a latitude and longitude of 9°3'N, 38°30'E respectively and an altitude of 2391 meters above sea level.

### **4.1 Plant Materials**

Three elite sugarcane genotypes (Q-200, Q-217 and Co-0238) were used for the study. They were obtained from the Ethiopian Sugar Corporation, Research and Training Division. The Sugar Corporation imported Q-200 and Q-217 varieties from Australia and Co-0238 variety from India. These genotypes are among the selected ones to be commercialized by the Sugar Corporation for their cane yield performance and sugar quality.

#### **4.1.1 Genotype Q-200**

The variety was originated in Australia. It is early maturing variety, erect, medium thick, solid, medium hard stalk, light yellow in colour and medium long light pith (Figure 5). Internodes are lightly conical, bud grooves and spines are absent in its stalk. The bud is small and round. The yield potential of this variety ranges from 75-86 metric ton per hectare (<http://www.sugarresearch.com.au/>).

#### **4.1.2 Genotype Q-217**

This variety was also originated in Australia. It is mid late maturing variety characterized by erect medium thick, solid and medium hard stalks bearing spines at the leaf lamina margins. Eyes are slightly triangular and small in size. Internodes are long and cylindrical. The yield potential of this variety ranges from 77-89 mt ton per hectare (figure 6).

#### **4.1.3 Genotype Co-0238**

The variety Co 0238 was originated in Coimbatore (India). It is a high yielding and high sugar content variety, derived from the cross of Co LK 8102 x Co 775. Notification and release of varieties was made in 2009 as an early maturing variety for commercial cultivation in North-West Zone (NWZ). It showed 19.96, 15.83 and 0.50% improvement in cane yield, sugar yield and sucrose % respectively. It performs best on heavy to moderately heavy and moderately light soil and has moderate flowering potential. No germination problem, high tillering capacity, relatively soft rind and excellent ratooning ability (<http://www.sugarcane.res.in> ) (figure .7)

**Figure 5; Planting material (sugarcane setts) of three genotypes**



**A) Q200 genotype**



**B) Q-217 genotype**



**C) Co-0238 genotype**

## 4.2 Mother plant propagation

The planting material (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 10 minutes. The treated setts were planted in plastic pots containing mixture of autoclaved garden soil, sand soil, and farmyard manure in the ratio of 3:2:2 and allowed to grow in a screen house for four months.

## 4.3 MS stock preparation

The Murashige and Skoog (1962) full basal stock solution was prepared by weighing and dissolving the powder in single distilled water and stored in refrigerator at -20°C. MS nutrient with its macronutrient, micronutrient 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 FeSO<sub>4</sub>.7H<sub>2</sub>O and Na<sub>2</sub>.EDTA were dissolved in hot double distilled water separately. After both components were dissolved completely, FeSO<sub>4</sub>.7H<sub>2</sub>O solution was added upon Na<sub>2</sub>.EDTA solution, and then poured into the bottle wrapped with aluminum foil so as to protect it from direct light contact.

#### **4.4 Plant Growth regulators (PGRs) stock solution preparation**

Plant growth regulators (PGRs) stock solutions were prepared in the ratio of 1:1(1mg/1ml). Different growth regulators were used in this research, 6-benzyl aminopurine (BAP, Sigma), Kinetin (UNI-CHEM) and  $\alpha$ -naphthalene acetic acid (NAA, Himedia). Volumes of solvent depend on the amount of hormone powder going to be prepared (Appendix 2). 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.

#### **4.5 Culture Media Preparation and Sterilization**

The basal medium employed for the culture of shoot tip of sugarcane is MS medium (Murashige and Skoog, 1962). During preparing the medium, the required amount of macronutrient, micronutrient, vitamins from respective stock solution were dissolved in double distilled water along with 30 g/L sucrose ( for initiation and multiplication) and 50g/L sucrose(for rooting) and carefully stirred on magnetic stirrer until the sugar and Phytigel (for multiplication and rooting) was completely dissolved. Finally, the final volume was made up to the required level with double distilled water and then divided into required volume of treatments, to which amount of PGRs from stock solution were added in combinations at different concentrations.

The pH was adjusted in all cases to 5.8 by using 1N NaOH and/or 1N HCL before gelling with agar. For semi solid medium, Agar-Agar type I (4.5 g/l for culture initiation) and

Phytigel (2.0 g/l for multiplication and also for rooting induction) were added to the nutrient and heated on magnetic hot plate till it was completely melted.

Before autoclaving, the media was poured into washed and dried test tubes of 30 ml volume (50ml capacity test tube) for initiation and 50 ml volume of culture jars(200ml capacity culture jars) for multiplication, root induction and then, capped and labeled properly. The dispensed medium was then autoclaved at 121° C for 15 minutes at 15-psi pressure. The autoclaved medium was kept in a shelf for about a week to make sure that there is no any microbial contamination.

#### **4.6 Explant collection and sterilization**

Actively growing shoot tips were used as source of explant. The method of explant preparation and surface sterilization was adopted from Melaku Tesfa (2016) and Belay Tolera (2014 with some modifications. Actively growing shoot tips were taken from 4-months-old greenhouse grown healthy mother plants and used as explants. The intact leaves were removed, and the segments with leave sheath were taken to the laboratory for surface sterilization and explant preparation. Trimmed shoot tips were washed thoroughly under running tap water for 20 minutes, and resized to just about 10 cm length by cutting off at the two ends. Then the explants were further washed for 30 minutes with tap water containing a drop of liquid detergent (Largo liquid soap) 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 0.3% (w/v) Kocide 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 autoclaved double distilled water. The shoot tips were washed again with 70% ethanol for one minute and 30 seconds and

rinsed with sterile double distilled water three times each for five minute to remove residual ethanol from the shoot tip surface. Finally, 2.5cm sized explants were surface sterilized with 50% (v/v) aqueous solution of Sodium hypochlorite bleach (5% v/v active chlorine) containing two 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 for five minutes to remove all traces of the sterilant. All the steps of sterilization were carried out under aseptic condition.

#### **4.7 Shoot initiation from shoot tip explant**

Shoot tip containing apical meristem of genotypes Q-200, Q-217 and Co-0238 were cultured on MS medium containing different concentrations and combinations of BAP (0.0, 1.5 and 2.0 mg/l) and IBA (0.0 and 0.5 mg/l), 30 g/l sucrose and solidified with 4.5g/l agar. The factorial treatment was **3x2x3** combinations arrangements. The experiment was carried out at growth room temperature of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$  under 16 hours photoperiod with light intensity of  $30 \mu\text{mol m}^{-2}\text{s}^{-1}$  provided by cool white fluorescent light and 70% to 80% relative humidity. Thirty test tubes with each having one shoot tip explant were cultured for each three genotypes (30 shoot tips per treatment). Sub culturing was done frequently (7 days). Data of shoot initiation were recorded three weeks after culture.

#### **4.8 *In vitro* Shoot Multiplication**

In this experiment, healthy micro-shoots having approximately same size obtained from the subculture stage were used for shoot multiplication experiment after maintaining on plant growth regulator free semi-solid medium for two weeks to minimize the carry over effect of the initiation medium. These micro-shoots were transferred to full MS semi-solid media containing BAP (0, 0.5, 1.0, 1.5 & 2 mg/l) in combination with kinetin (0,

0.25, 0.5 & 1.0 mg/l) and supplemented with 3% sucrose. 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, 0.25, 0.5 & 1.0 mg/l) and three levels of sugarcane genotypes (Q200, Q217 and Co-0238) resulting in 3x5x4 factorial treatment combinations arrangement. The pH of the media was adjusted to 5.8 by 1N HCl or 1N NaOH and autoclaved at 121°C and 15-psi pressure for 15 minutes. Each treatment was replicated three times and four shoot bunches (12 shoot bunches per treatment) were cultured in each culture jar. The culture jars were properly sealed with PVC film and placed in environmentally controlled growth room shelves at 25°C ± 2°C and 16 and 8 h light and light intensity of 30  $\mu\text{mol m}^{-2}\text{s}^{-1}$  provided by cool white fluorescent light and 70% to 80% relative humidity. Sub-culturing was carried out at 15 days interval to obtain shoot multiplication. Data on number of shoots per explants, length of shoots and number of leaves per shoot were carefully recorded during each sub culturing.

#### **4.9 *In vitro* Rooting of Micro shoots**

Micro shoots derived from the multiplication experiment were used for rooting studies. The shoots were maintained on PGR free MS medium for two weeks before transferring to a half MS semi solid medium 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 semi solid MS medium supplemented with different concentrations and combination of three levels of IBA (0.0, 1.5, and 2.0) in combination with five levels of NAA (0.0, 2.0, 3.0, 4.0 and 5.0) with three level of genotype resulting in **3x5x3** factor factorial treatment combinations. For each treatment, five shoots in each jar (15 shoots per treatment) were lined up randomly in CRD with three

replications. All shoots were incubated on rooting medium for 3 weeks with the same culture condition to that of multiplication culture. After 3 weeks of culture, root growth, data on number of roots per shoot and length of roots (cm) were recorded.

#### **4.10 Acclimatization of *in vitro* Derived Plantlets**

*In vitro* rooted plantlets were taken out of the culture jars and the roots were carefully 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 1:1:1 ratio. 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 red cheese cloth and then sprayed with water from time to time. The cover was removed from the tray after 14 days. Starting from the 1<sup>st</sup> week of acclimatization, number of surviving and dead plantlets was recorded on weekly basis until the 4th week of acclimatization and eventually survival percentage was calculated.

#### **4.11 Data Analysis**

The analysis of variance for different variables was performed by **GenStat Version 15.1**, (GenStat15.1 (64-bit Edition) 2012, VSN International Ltd.) and for significantly different treatments, mean separation was done with Duncan's multiple range tests at or below the probability level of 5%.

## 5. RESULTS AND DISCUSSION

### 5.1 Culture Initiation

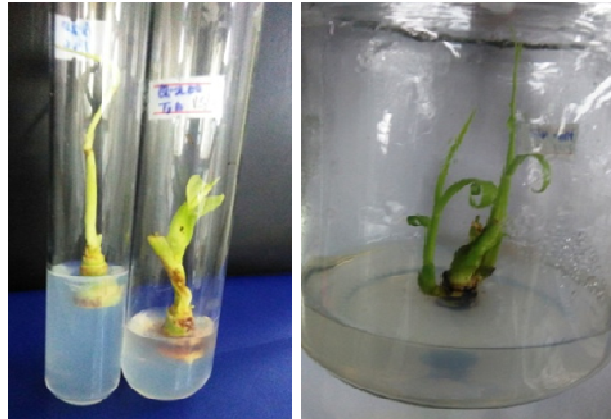
The shoot tip explants on full MS media devoid of plant growth regulator (control) failed to grow. However, shoot initiation was observed in MS media supplemented with PGR. Healthy shoots with succulent and green leaves started appearing within 6-8 days in many of the culture tubes containing 2.0 mg/L BAP in combination with 0.5 mg/L IBA, in Q200 and Q217 varieties, while 8-10 days in Co-0238 variety on medium containing 1.5 mg/L BAP in combination with 0.5 mg/L IBA in the culture tubes. Initially, there was problem of tissue browning due to release of phenols from the base of shoot tip, which slightly slowed down the shoot growth. Sub culturing was done frequently (7 days) during the early stages of establishment to reduce the adverse effect of browning of tissues and the release of pigments in the medium. The percent establishment varied between different combinational levels of BAP and IBA. Frequency of establishment was maximum for Q217 (86.6%) and Q200 (90%) genotypes at 2.0 mg/L of BAP and 0.5 mg/L of IBA hormone combination (Table.2 and Figure 8 and Figure 9), while it was maximum in case of Co-0238(93.3%) variety at 1.5 mg/L of BAP and 0.5 mg/L of IBA, on which shoots were active and healthy (Table.2 and Figure 10). The study of Tilahun *et al.* (2014) indicated that normal shoot initiation and establishment was obtained in two cultivars Co449 and Co678 with 2.0 mg/L of BAP and 0.5mg/L of IBA, which was similar result with Q200 and Q217 variety. Godheja *et al.* (2014) reported that initiation of shoot tip explant of Co-86032 variety was maximum at 1.0 mg/L BAP. The study of Sawant and Meti (2016) showed that variety Co86032 shoot formation (96.67%) on MS medium supplemented with BAP (0.2 mg/L) and Kinetin (0.1 mg/L), on the other hand variety

CoC671 showed maximum shoot formation (93.33%) on MS medium supplemented with only Kinetin( 0.5mg/L). The study of Sandeep *et al.* (2008) also indicated shoot initiation and establishment was obtained in cultivars of CoC-671 at 2.0 mg/L of BAP. These observations showed that the concentration of BAP and IBA required for shoot initiation and establishment varies with genotypes. The amount of cytokinin and auxin applied depends on the genotype used and the micropropagation strategy employed.

**Table 2. Effect of BAP and IBA on establishment of shoots**

PGR(mg/L)		Sugarcane Varieties					
IBA	BAP	Q200		Q217		Co-0238	
		shoots established	% of establishment	shoots established	% of establishment	shoots established	% of establishment
0	0	0	0%	0	0%	0	0%
0	1.5	12	40%	13	43.3%	14	46.6%
0	2	17	56.6%	15	50%	11	36.6%
0.5	0	3	10%	4	13.3%	2	6.6%
0.5	1.5	16	53.3%	18	60%	28	93.3%
0.5	2	26	86.6%	27	90%	17	56.6%

**Figure 6 Shoot tip culture initiation and establishment of three genotypes**



**A) Q200 on MS medium containing 0.5 mg/L IBA +2.0 mg/LBAP**



**B) Q217 on MS medium containing 0.5 mg/L IBA +2.0 mg/LBAP**



**C) Co-0238 on MS medium containing 0.5 mg/l IBA+1.5 mg/l BAP**

## 5.2 Shoot Multiplication

Analysis of variance revealed that the interaction effects of genotype, BAP and kinetin was very highly significant (Genotype \* BAP \* Kinetin =  $p < 0.001$ ) on the number of shoots per explant, average shoot length (cm) and number of leaves per shoot. 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. Shoot multiplication was not observed within 4 weeks when shoot explants were cultured on MS medium devoid of plant growth regulators in all the three genotypes. However, increasing the concentration of kinetin alone from 0.0mg/L to 0.25 mg/L resulted in 5.22, 6.51 and 5.39 shoots per explant for Q-200, Q-217 and Co-0238, respectively. In the same way, increasing BAP concentration alone from 0.0mg/L to 0.5 mg/L gave 5.50, 5.49 and 3.57 shoot per explant in genotype Q-200, Q-217 and Co-0238, respectively. This result indicates that the supplementation of exogenous plant growth regulators to MS medium is fundamental to develop multiple shoots. Certainly, cytokinins are capable of overcoming apical dominance and release lateral buds from dormancy thereby boost shoot multiplication (George *et al.*, 2008).



Figure 7. *In vitro* shoot multiplication of Q200 on 2.0 mg/l BAP



Figure 8. *In vitro* shoot multiplication of Q217 on 2.0 mg/l BAP and 0.25 mg/l kinetin



Figure 9. *In vitro* shoot multiplication of Co-0238 on 1.5 mg/l BAP and 0.5 mg/l kinetin.

In the present study, it was observed that the three genotypes responded differently to the same media for all parameters studied. A significantly highest average number of shoots per explant (13.59), with average shoot length 6.97cm and 5.83 number of leaves per shoot was observed in genotype Q200 on MS medium fortified with 2.0 mg/L BAP alone (Table 3 and Figure 11), while Q217 and Co-0238 gave only 5.16 and 6.43 shoots per explant, respectively on the same medium composition. Among different combinations of BAP and Kin, genotype Q217 produced the highest number of shoots per explant (15.28) with mean shoot length of 6.30 cm and average number of leaves per shoot of 5.37 on MS medium fortified with 2.0 mg/L BAP and 0.25 mg/L Kinetin (Table 3 and Figure 12) while the same medium composition resulted in only 5.47 and 6.76 shoots per explant in Q200 and Co-0238 genotypes respectively. Similarly, Co-0238 produced maximum of 13.56 shoots per explant with average shoot length of 6.67cm and mean number of leaves per shoot of 5.50 on MS medium fortified with 1.5 mg/L BAP + 0.5 mg/L kinetin (Table 3 and Figure 13) while the same medium composition resulted in only 5.26 and 6.74 shoots per explant in Q217 and Q200, respectively.

In three genotypes, maintaining Kin at 0.25 mg/L while increasing BAP levels from 0.5 to 2.0 mg/L, showed a marked increase in the number of shoots per explant from 5.50 to 13.59, 5.16 to 5.4 and 3.57 to 3.80, average shoot length from 2.93 cm to 5.80 cm, 2.87 cm to 4.13 cm, 2.30 cm to 3.43 cm and number of leaves per explant from 3.10 to 6.97, 3.63 to 4.87, 3.60 to 4.47 in Q200, Q217 and Co-0238 genotypes, respectively. Increase of kinetin from 0.25 to 1.0 mg/L without BAP showed a significant increase in the number of shoots per explant from 6.51 to 7.34 but, decrease the average shoot length from 4.37 to 3.60cm and number of leaves per shoot from 5.03 to 4.13 in Q217 genotypes. On

the other hand, significant reduction of the same parameter with the above genotype was observed in Q200 and Co-0238 varieties.

**Table 3 ANOVA summary of Effect of BAP and Kinetin on invitro Shoot Multiplication**

Source of variation	DF	MS		
		No of shoots	Shoot length(cm)	No of leaves
Genotype	2	6.0768***	0.6282***	7.4362***
BAP	4	42.9885***	7.6049***	9.5074***
Kin	3	54.9209***	25.8728***	11.0867***
Genotype*BAP	8	9.5184***	1.3318***	1.0957***
Genotype*Kin	6	23.9311***	2.8170***	1.3338***
BAP*Kin	12	30.0544****	12.1818***	7.9424***
Genotype*BAP*Kin	24	14.4409***	1.6580***	1.0865***
CV %		8.2	16.5	14.8

\*\*\* = very highly significant at  $P \leq 0.001$ , DF =Degree of freedom, BAP = 6- Benzyl aminopurine, Kin = Kinetin, CV = Coefficient of variation, (\*= $p \leq 0.05$ , \*\*= $p \leq 0.01$ , \*\*\*= $p \leq 0.001$ )

Regarding shoot length, Q200 produced the highest shoot length (5.83 cm) with the maximum number of leaves per shoot (6.97) on MS medium supplemented with 2.0 mg/L BAP alone, whereas only 4.13 cm and 3.43 cm shoot length with 4.87 and 4.47 leaves per shoot production in Q217 and Co-0238, respectively. This different shoot length growth response to the similar media composition might be as a result of difference in inherent endogenous growth hormone level among genotypes (George *et al.*, 2008).

It is evident from Table 2 that the use of 2.0 mg/L BAP alone produced only 5.16 shoots per explant, which was increased to 15.28 shoots by addition of 0.25 mg/L kinetin in genotype Q217. In similar way, increased shoot number per explant was observed in

genotype Co-0238 from 3.80 to 13.56 when 0.5 mg/L kinetin was added to MS medium containing 1.5 mg/L BAP. This positive effect implies the significance of adding the two growth regulators in combination rather than alone in shoot multiplication medium. Ali and Afghan (2001) reported that medium supplemented with BAP and Kinetin resulted in rapid multiplication of shoots.

When the same concentration of Kinetin (0.5mg/L) and BAP (0.5mg/L) are used in combination for shoot multiplication, Q200 genotype produced more number of shoots (6.72) per explant than other genotypes. The same is also true when 1.0 mg/L Kinetin and 1.0 mg/L BAP were used in combination. This result also points out that different genotypes show different performance on the same hormone (cytokinins) concentration and combination.

**Table 4. The effect of BAP and Kinetin on number of shoots per explant, shoot length and number of leaves per shoot**

PGRs(mg/L)		Sugarcane Varieties								
Kin	BAP	Q200			Q217			Co-0238		
		#of shoots per explant	#of leaves per shoot	Shoot length (cm)	#of shoots per explant	#of leaves per shoot	Shoot length (cm)	#of shoots per explant	#of leaves per shoot	Shoot length (cm)
0	0	0.00 <sup>A</sup>	0.00 <sup>r</sup>	0.00 <sup>s</sup>	0.00 <sup>A</sup>	0.00 <sup>r</sup>	0.00 <sup>s</sup>	0.00 <sup>A</sup>	0.00 <sup>r</sup>	0.00 <sup>s</sup>
0	0.5	5.50 <sup>j-o</sup>	3.10 <sup>q</sup>	2.93 <sup>k-r</sup>	5.49 <sup>j-o</sup>	3.63 <sup>pq</sup>	2.87 <sup>l-r</sup>	3.567 <sup>xy</sup>	3.60 <sup>pq</sup>	2.30 <sup>r</sup>
0	1	7.05 <sup>f</sup>	4.10 <sup>j-q</sup>	3.90 <sup>d-m</sup>	5.35 <sup>l-q</sup>	3.57 <sup>pq</sup>	3.27 <sup>f-r</sup>	5.470 <sup>j-p</sup>	4.00 <sup>a-q</sup>	2.93 <sup>k-r</sup>
0	1.5	9.15 <sup>c</sup>	4.80 <sup>d-p</sup>	4.33 <sup>b-g</sup>	4.87 <sup>rs</sup>	4.17 <sup>j-q</sup>	3.83 <sup>d-n</sup>	5.647 <sup>j-l</sup>	4.13 <sup>j-q</sup>	3.73 <sup>d-n</sup>
<b>0</b>	<b>2</b>	<b>13.59<sup>b</sup></b>	<b>6.97<sup>a</sup></b>	<b>5.83<sup>a</sup></b>	5.16 <sup>pq</sup>	4.87 <sup>d-p</sup>	4.13 <sup>c-j</sup>	3.80 <sup>x</sup>	4.47 <sup>f-q</sup>	3.43 <sup>f-r</sup>
0.25	0	5.22 <sup>o-q</sup>	5.03 <sup>c-o</sup>	4.37 <sup>b-q</sup>	6.51 <sup>g-i</sup>	6.00 <sup>a-e</sup>	3.97 <sup>d-m</sup>	5.39 <sup>l-q</sup>	4.83 <sup>d-p</sup>	3.63 <sup>f-o</sup>
0.25	0.5	6.36 <sup>i</sup>	4.07 <sup>k-q</sup>	3.17 <sup>g-r</sup>	5.63 <sup>j-l</sup>	4.87 <sup>d-p</sup>	3.50 <sup>f-q</sup>	4.657 <sup>st</sup>	5.67 <sup>b-i</sup>	3.77 <sup>d-n</sup>
0.25	1	8.44 <sup>d</sup>	4.37 <sup>j-q</sup>	5.50 <sup>a</sup>	6.46 <sup>g-i</sup>	4.43 <sup>j-q</sup>	4.20 <sup>c-h</sup>	5.767 <sup>k</sup>	5.10 <sup>c-o</sup>	3.30 <sup>f-r</sup>
0.25	1.5	9.27 <sup>c</sup>	4.37 <sup>j-q</sup>	4.80 <sup>a-e</sup>	7.30 <sup>e</sup>	5.83 <sup>a-g</sup>	4.83 <sup>a-d</sup>	5.873 <sup>j</sup>	5.30 <sup>c-l</sup>	2.53 <sup>o-r</sup>
<b>0.25</b>	<b>2</b>	5.47 <sup>j-p</sup>	5.43 <sup>b-k</sup>	5.13 <sup>a-c</sup>	<b>15.28<sup>a</sup></b>	<b>6.30<sup>a-c</sup></b>	<b>5.37<sup>ab</sup></b>	6.763 <sup>B</sup>	4.13 <sup>j-q</sup>	3.63 <sup>f-o</sup>
0.5	0	4.60 <sup>s-u</sup>	4.40 <sup>j-q</sup>	3.80 <sup>d-n</sup>	8.45 <sup>d</sup>	5.47 <sup>b-j</sup>	3.40 <sup>f-r</sup>	5.600 <sup>j-m</sup>	3.80 <sup>n-q</sup>	2.30 <sup>r</sup>
0.5	0.5	6.72 <sup>gh</sup>	5.27 <sup>c-l</sup>	3.33 <sup>f-r</sup>	4.27 <sup>vw</sup>	4.50 <sup>f-p</sup>	3.30 <sup>f-r</sup>	6.43 <sup>hi</sup>	5.37 <sup>b-l</sup>	3.50 <sup>f-q</sup>
0.5	1	6.65 <sup>g-i</sup>	4.10 <sup>j-q</sup>	2.97 <sup>j-r</sup>	5.44 <sup>l-p</sup>	5.17 <sup>c-n</sup>	3.67 <sup>e-o</sup>	6.553 <sup>g-i</sup>	5.83 <sup>a-f</sup>	3.50 <sup>f-q</sup>
<b>0.5</b>	<b>1.5</b>	5.26 <sup>n-q</sup>	5.03 <sup>c-o</sup>	3.57 <sup>f-p</sup>	6.74 <sup>B</sup>	5.20 <sup>c-m</sup>	3.67 <sup>e-o</sup>	<b>13.56<sup>b</sup></b>	<b>6.67<sup>ab</sup></b>	<b>6.50<sup>a</sup></b>
0.5	2	4.327 <sup>u-w</sup>	3.73 <sup>o-q</sup>	4.17 <sup>c-i</sup>	7.13 <sup>ef</sup>	3.60 <sup>pq</sup>	2.67 <sup>n-r</sup>	8.623 <sup>d</sup>	6.27 <sup>a-c</sup>	2.80 <sup>m-r</sup>
1	0	4.28 <sup>vw</sup>	4.13 <sup>j-q</sup>	3.60 <sup>f-o</sup>	7.34 <sup>e</sup>	5.80 <sup>a-h</sup>	3.33 <sup>f-r</sup>	4.270 <sup>vw</sup>	5.47 <sup>b-j</sup>	3.00 <sup>i-r</sup>
1	0.5	6.35 <sup>i</sup>	6.13 <sup>a-d</sup>	4.40 <sup>b-f</sup>	5.30 <sup>m-q</sup>	5.03 <sup>c-o</sup>	4.10 <sup>c-k</sup>	5.557 <sup>j-n</sup>	5.27 <sup>c-l</sup>	4.00 <sup>d-h</sup>
1	1	5.08 <sup>qr</sup>	5.47 <sup>b-j</sup>	3.53 <sup>f-q</sup>	4.41 <sup>t-v</sup>	4.30 <sup>j-q</sup>	3.77 <sup>d-n</sup>	4.76 <sup>s</sup>	5.13 <sup>c-n</sup>	3.50 <sup>f-q</sup>
1	1.5	4.39 <sup>t-v</sup>	4.73 <sup>e-p</sup>	3.83 <sup>d-n</sup>	3.63 <sup>xy</sup>	4.00 <sup>l-q</sup>	2.97 <sup>j-r</sup>	3.480 <sup>y</sup>	4.30 <sup>l-q</sup>	3.03 <sup>h-r</sup>
1	2	4.06 <sup>w</sup>	3.87 <sup>m-q</sup>	3.70 <sup>d-o</sup>	3.18 <sup>z</sup>	4.00 <sup>l-q</sup>	2.47 <sup>p-r</sup>	3.593 <sup>xy</sup>	3.83 <sup>m-q</sup>	2.40 <sup>qr</sup>
	%Cv	<b>8.2</b>	<b>14.8</b>	<b>16.5</b>	<b>8.2</b>	<b>14.8</b>	<b>16.5</b>	<b>8.2</b>	<b>14.8</b>	<b>16.5</b>
	SE	<b>0.274</b>	<b>0.389</b>	<b>0.332</b>	<b>0.274</b>	<b>0.389</b>	<b>0.332</b>	<b>0.274</b>	<b>0.389</b>	<b>0.332</b>

Means in columns with the different letters are significantly different by Duncan's multiple range test at  $\alpha=5\%$  significant level

The best result achieved in genotype Co-0238 is consistent with the result obtained by Khan *et al.* (2009). They reported variety HSF-240 produced 11 shoots per explant; 16.5 cm mean shoot length and 32 leaves per shoot on MS medium fortified with 1.5 mg/L

BAP+ 0.5 mg/l kinetin. Dereje *et al.* (2014) reported maximum multiplication from C86-12 genotype with  $33.8 \pm 0.837$  shoots per explant,  $8.4 \pm 0.008$  cm mean shoot length and  $13.04 \pm 0.089$  leaves per shoot on MS medium fortified with 1.5 mg/L BAP+ 0.5 mg/l kinetin. The present result in Q217 is also in agreement with the results reported by Tilahun *et al.* (2014) who obtained best result from Co-449 genotype on MS medium fortified with 2.0 mg/L BAP + 0.25 mg/L Kinetin with  $7.87 \pm 1.06$  number of shoots,  $6.33 \pm 0.21$  cm shoot length and  $5.44 \pm 0.19$  leaves per shoot. The result in Q200 is similar with the report of Belay (2014) who found optimum result from N14 genotype on MS Medium fortified with 2.0 mg/L BAP without kinetin with  $21 \pm 0.58$  numbers of shoots,  $5.63 \pm 0.01$  cm shoot length and  $5.4 \pm 00$  leaves per shoot.

In the three cases, the observed difference in number of shoots per explant, number of leaves and shoot length could be due to genotypic difference. Similar results were also reported by Sandeep *et al.* (2008) who observed an average of 19.91 shots on MS medium fortified with 2.0 mg/L BAP alone. However, there are also reports (Sawant and Meti, 2016, Yadav *et al.*, 2012) that indicated higher multiplication rate of sugarcane varieties at lower concentration of BAP and kinetin than obtained in this study. Result of the present study indicated that 1.5 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 Co-0238. The present result showed that 2.0 mg/L BAP + 0.25 mg/L kinetin, and 2.0 mg/L BAP alone was found to be the best for sugarcane genotype Q217 and Q200, respectively. Comparison of the three genotypes showed that Q217 was a better responsive than Q200 and Co-0238 for *in vitro* multiplication in full MS medium.

### **5.3 *In vitro* Rooting of Micro shoots**

ANOVA showed very highly significant ( $p \leq 0.001$ ) effect of all main and interaction effect of genotypes, IBA and NAA (Table. 4) on average roots number per shoot and shoot length in three genotypes indicating the interdependence of these factors on *in vitro* root induction of sugarcane.

The very highly significant effect of genotypes indicates existence of genotype difference among the three genotypes on their rooting potential for the three levels of IBA and five levels of NAA combination. Genotype Co-0238 responded better in mean root number per shoot than Q200 and Q217.

Fine roots began to be induced from the basal portion of the shoots after 10-12 days in Co-0238 genotypes, while 12-15 days in Q200 and Q217 cultivars on control treatment and  $\frac{1}{2}$  MS media fortified with combination of IBA and NAA at different concentration, but the roots in the control were not well elongated and became thin and brownish in color. This is due to the presence of elevated sucrose, which has increased the cell metabolism and stress to induce rooting. Sucrose was reported to act as morphogenetic activator in the formation of auxiliary buds and branching of adventitious roots (Vinterhalter, 1997).

**Table 5. ANOVA Summary for effect of IBA and NAA on *in vitro* rooting and root growth on half MS media**

PGRs(mg/L)		Sugarcane Genotypes					
IBA	NAA	Q200		Q217		Co-0238	
		# of roots per shoot	Root length (cm)	# of roots per shoot	Root length (cm)	# of roots per shoot	Root length(cm)
0	0	2.30 <sup>q-t</sup>	0.90 <sup>n</sup>	1.80 <sup>st</sup>	1.23 <sup>mn</sup>	1.77 <sup>st</sup>	1.67 <sup>j-n</sup>
	2	6.33 <sup>d-f</sup>	1.80 <sup>j-n</sup>	4.57 <sup>h-o</sup>	1.83 <sup>j-n</sup>	3.27 <sup>n-s</sup>	1.90 <sup>j-n</sup>
	3	<b>14.93<sup>c</sup></b>	<b>5.83<sup>a</sup></b>	6.43 <sup>d-f</sup>	2.60 <sup>e-l</sup>	3.73 <sup>k-q</sup>	3.20 <sup>d-i</sup>
	4	7.23 <sup>d</sup>	2.27 <sup>g-m</sup>	5.20 <sup>e-k</sup>	2.20 <sup>h-m</sup>	7.63 <sup>d</sup>	3.63 <sup>c-f</sup>
	5	4.53 <sup>h-o</sup>	1.83 <sup>j-n</sup>	6.23 <sup>d<sup>e-g</sup></sup>	3.50 <sup>c-f</sup>	<b>18.40<sup>a</sup></b>	<b>4.97<sup>b</sup></b>
1.5	0	3.40 <sup>m-r</sup>	1.47 <sup>l-n</sup>	5.17 <sup>e-k</sup>	4.07 <sup>cd</sup>	3.77 <sup>k-q</sup>	2.87 <sup>e-j</sup>
	2	2.53 <sup>p-t</sup>	2.67 <sup>e-k</sup>	6.10 <sup>d-h</sup>	3.73 <sup>c-e</sup>	4.67 <sup>g-n</sup>	1.87 <sup>j-n</sup>
	3	3.67 <sup>k-r</sup>	2.20 <sup>h-m</sup>	<b>16.50<sup>b</sup></b>	<b>4.50<sup>c</sup></b>	5.63 <sup>e-i</sup>	2.70 <sup>e-k</sup>
	4	4.50 <sup>h-o</sup>	2.57 <sup>e-l</sup>	7.17 <sup>d</sup>	2.63 <sup>e-k</sup>	4.93 <sup>f-m</sup>	3.27 <sup>d-h</sup>
	5	5.47 <sup>e-i</sup>	3.53 <sup>c-f</sup>	5.03 <sup>f-l</sup>	1.60 <sup>k-n</sup>	3.67 <sup>k-r</sup>	4.23 <sup>cd</sup>
2	0	5.43 <sup>e-j</sup>	2.70 <sup>e-k</sup>	2.37 <sup>q-t</sup>	2.13 <sup>h-m</sup>	7.47 <sup>d</sup>	3.40 <sup>d-g</sup>
	2	3.67 <sup>k-r</sup>	3.30 <sup>d-h</sup>	2.67 <sup>p-t</sup>	1.73 <sup>j-n</sup>	3.50 <sup>l-r</sup>	2.70 <sup>e-k</sup>
	3	4.57 <sup>h-o</sup>	2.73 <sup>e-k</sup>	3.87 <sup>j-p</sup>	3.57 <sup>c-f</sup>	2.97 <sup>o-t</sup>	2.47 <sup>f-l</sup>
	4	5.53 <sup>e-i</sup>	1.97 <sup>j-n</sup>	3.83 <sup>k-q</sup>	2.83 <sup>e-j</sup>	2.13 <sup>r-t</sup>	2.07 <sup>l-n</sup>
	5	6.70 <sup>de</sup>	1.80 <sup>j-n</sup>	4.13 <sup>i-p</sup>	1.96 <sup>j-n</sup>	1.70 <sup>t</sup>	1.53 <sup>k-n</sup>
<b>%CV</b>		<b>18.4</b>	<b>25.1</b>	<b>18.4</b>	<b>25.1</b>	<b>18.4</b>	<b>25.1</b>
<b>SEM</b>		<b>0.5014</b>	<b>0.387</b>	<b>0.5014</b>	<b>0.387</b>	<b>0.5014</b>	<b>0.387</b>

Singh *et al.* (2006) obtained 100% root induction from sugarcane plant growth regulator frees ½ MS media fortified by elevated 60 g/L sugar. Sugarcane shoots regenerated under *in vitro* conditions develop roots without supplementary addition of exogenous plant growth regulators. The present result of root induction on MS medium devoid of auxins was in contrast to the report of Belay (2016) and Shimelis *et al.* (2015). They reported no root induction occurred on ½ MS medium without auxins. Vigorous rooting *of vitro*

multiplied shoots of sugarcane is of interest to plant biotechnologists for mass propagation and adaptation of plants in the environment (Mustafa and Khan, 2016).

**Table 6 ANOVA summary for Effect of IBA and NAA on In vitro Rooting and root growth on half MS media**

Source of variation	DF	MS	
		Number of roots per shoot	Root length(cm)
Genotypes	2	2.1696***	1.1143***
IBA mg/l	2	46.0056***	2.5765***
NAA mg/l	4	37.5334***	5.9107***
Genotypes*IBA mg/l	4	43.2804***	2.5676***
Genotypes*NAA mg/l	8	14.0471***	3.2027***
IBAmg/l*NAA mg/l	8	28.4439***	5.1499***
Genotypes *IBAmg/l*NAA mg/l	16	28.6456***	4.4638***
%CV		15.9	21.9

\*\*\* = very highly significant at  $P \leq 0.001$ , \*\* = 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 =  $\alpha$ -naphthalene acetic acid, MS = mean square, CV = Coefficient of variation, IBA=Indol-3-Butyric Acid

Vital rooting occurred in media fortified with IBA and/or NAA, indicating the implication of adding auxin/s in rooting induction media for rooting of *in vitro* generated sugarcane micro shoots. It was observed that an increase of IBA from 0.0 mg/L to 1.5 mg/L while maintaining concentration of NAA at 0.0 mg/L increased the average root number per shoot and average root length of Q200 genotype from 2.30 to 3.40 and 0.90 cm to 1.47cm.

In the same trend, increased mean root number per shoot and mean root length of Q217 cultures from 1.80 to 5.17 and 1.23 cm to 4.07 cm, and of Co-0238 genotype cultures

from 1.77 to 5.77 and 1.67 to 2.87cm. In the absence of IBA, an increase of concentration of NAA from 0.0 mg/l to 5.0 mg/L increased mean root numbers per shoot and mean root length from 4.53 and 1.83 cm in Q200, 6.23 and 3.50 cm in Q217 and 16.40 and 5.83cm in Co-0238, respectively. Phytohormones NAA and IBA are chosen because of their rhizogenic efficacy, which results from their high stability in plant tissues. They are often combined, because the potassium salt form of IBA and NAA have the advantage of being water soluble and not need a solvent. This, in turn, also makes them the preferred choice for varieties that are difficult to root and do not respond well to IAA application (Gaspar *et al.*, 1997).

Among all combination of IBA and NAA, genotype Q200 showed the highest rooting with mean root number per shoot of 14.93 and mean root length of 5.83 cm on half MS medium supplemented with 3.0 mg/L NAA only (Table 4 and figure 15). When higher concentration of NAA (5.0 mg/L) was used for the same genotype on the same media composition, decreased mean root number per shoot (4.84) and root length of 1.82 cm was recorded. On the other hand, genotype Q217 and Co-0238 showed maximum rooting with mean root number per shoot and mean root length of 16.50 cm and 4.50 cm, and 18.40 and 4.97 cm on half MS medium supplemented with 1.5 mg/L IBA +3.0 mg/L NAA, and 5.0 mg/L NAA+ 0.0 mg/L IBA respectively (Table 4, figure 14 and figure 16). When lower concentration of NAA (2.0 mg/L) was used for the Co-0238 genotype on the same media composition, reduced the average roots number per shoot (3.27) and average root length (1.90 cm) were observed. Similarly when higher concentration of IBA (2.0 mg/L) and NAA (5.0 mg/L) were used, mean root number per shoot and mean root length were reduced to 4.13 and 1.96 cm.

The current result obtained in genotype Q200 was in agreement with other *in vitro* root induction reports of sugarcane cultures. Gophita (2010) reported maximum average root number per shoot of 15 and average root length of 4.9 cm on half MS medium supplemented with 3.0 mg/l NAA from CO 671 sugarcane cultivar. Khan *et al.* (2008) observed maximum *in vitro* rooting on half MS media supplemented with 3.0 mg/L NAA with average roots number per shoot 37.00 and average root length of 3.05cm. Melaku (2016) reported 23.5 root numbers per shoot and 4.95 mean root length on half MS medium supplemented with 3.0 mg/L NAA. However, the current result is not in agreement with the report of Godheja *et al.* (2014) who reported 9.5 roots per shoot on MS media supplemented with 3.0 mg/L IBA +3.0 mg/L NAA.

On the other hand, the current result obtained in genotype Co-0238 was in agreement with the report by Yadav *et al.* (2012) who reported 5.6 roots per shoot on half MS media supplemented with 5.0 mg/L NAA. Ramanand *et al.* (2007) obtained the longest root of 6.7 cm and the highest mean root number of 6.8 in sugarcane genotypes CoS 96268 and CoS 95255, respectively on 1/2 MS liquid medium containing 5.0 mg/L NAA. Tilahun *et al.* (2014) obtained a maximum of 7.63 cm mean root length and 13.17 mean number of roots at 5.0 mg/l NAA for genotype Co-678. Melaku *et al.* (2016) also reported maximum mean root number per shoot of 21.76 and average root length of 4.5 cm on half MS medium supplemented with 5.0 mg/L NAA. As compared to Yadav *et al.* (2012) and Ramanand *et al.* (2007) studies, the current study produced more number of roots per shoot. But the report of Tilahun *et al.* (2014) and Melaku *et al.* (2016) found more numbers of roots per shoot than the current study. However, Baksha *et al.* (2002) used 5 mg/l IBA and obtained 12 + 0.2 average number of roots/shoot with 3 +0.1 cm average root

length in a period of 10 days. Karim *et al.* (2002) reported optimum root induction at 3 mg/l IBA with 11 roots per shoot along with 3.8 cm root length in 12 days. The Study reported optimum root induction at high concentration of IBA.

Therefore, 1/2 MS medium fortified with 3.0 mg/L NAA was the optimal combination for *in vitro* rooting of *in vitro* generated shoots of sugarcane genotype Q200. While ½ strength MS supplemented with 1.5 mg/L IBA +3.0 mg/L NAA was the best combination for *in vitro* rooting of shoots of sugarcane genotype Q217, and ½ strength MS supplemented with 5.0 mg/L NAA was optimal hormone concentration for *in vitro* rooting of *in vitro* generated shoots of sugarcane genotype Co-0238.



Figure 10. *In vitro* rooting of Genotype Q217 on 1.5 mg/l and 3.0 mg/l NAA



Figure 11. *In vitro* rooting of Genotype Q200 on 3.0 mg/l NAA



Figure 12. *In vitro* rooting of Genotype C0-0238 on 5.0 mg/l NAA

#### 5.4 *Ex vitro* acclimatization of plantlets

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 trays of three sugarcane genotypes (Figures 18). In the 2nd week of acclimatization, 10 plantlets died from tray of Q200 genotypes and 5 plantlets died from tray of Q217 and Co-0238 varieties. In the fourth weeks of acclimatization, there was no death of plantlets in pots of the three genotypes. For the three varieties, different acclimatization potential was observed. Genotype Q200 revealed 80% survival while genotype Q217 and Co-0238 showed 90 % (figure 19). This result was examined at substrate mixture of sand, forest soil and farmyard manure substrate in 1:1:1 ratio. Loss of some plantlets might be due to environmental factors: temperature and humidity and variation in method of propagation.

**Figure 13. Acclimatization of three sugarcane genotypes (Co-0238, Q217 and Q200)**

Genotype								
Q200			Q217			Co-0238		
No plantlets potted	No of plantlets survived	% of survival	No plantlets potted	No of plantlets survived	% of survival	No plantlets potted	No of plantlets survived	% of survival
50	40	80%	50	45	90%	50	45	90%

The result obtained in the current study in genotype Q200 is in agreement with previous findings of Ali *et al.* (2008) who reported 70-80% survival rate of micropropagated sugarcane plantlets. The result is also in agreement with Mekonnen *et al.* (2014) who reported 80% and 86.67% survival for C0449 and Co678 genotypes respectively. Similarly, the findings of the current result in Q217 and Co-0238 genotypes are consistent with those of Belete *et al.* (2014) and Belay (2016). They confirmed 96.1% and 100% survival of plantlets in greenhouse after four weeks of acclimatization

Therefore, in the present study, substrates mixture comprising sand, forest soil and farm-yard manure substrate in 1:1:1 ratio was an ideal substrate mixture for best *ex vitro* acclimatization with higher plantlets survival rate of the three sugarcane genotypes, Q200, Q217 and Co-0238.



Figure 14 *Ex vitro* cultivated plantlets of Co-0238, Q217 and Q200) during first day

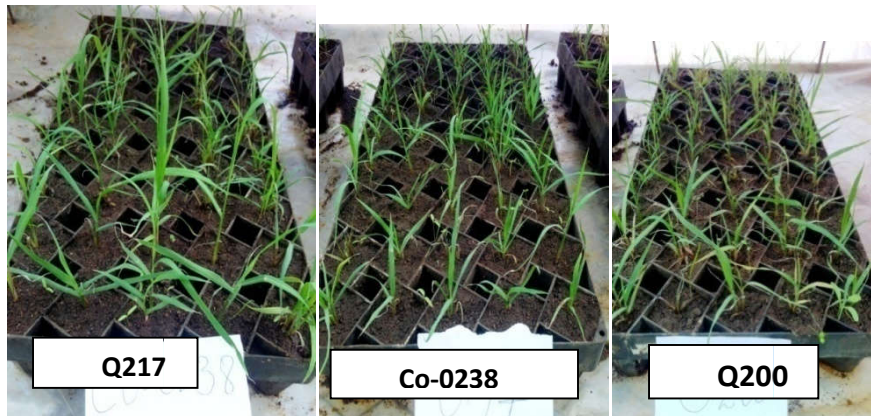


Figure 15. Acclimatized plantlets of Q217, Co-0238 and Q200 after 15 days

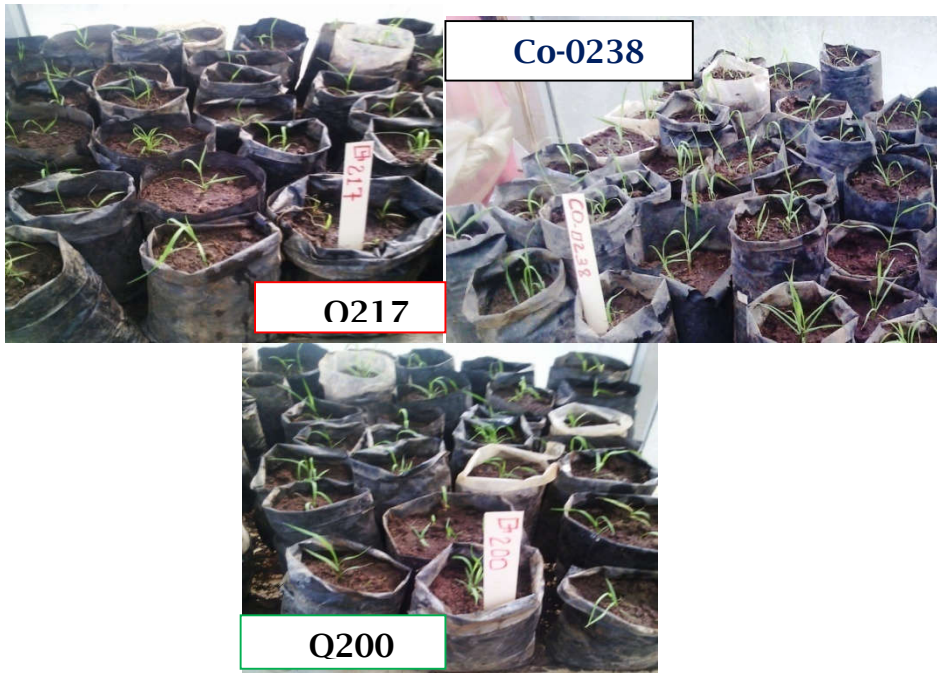


Figure 16. Acclimatized plantlets of Q217, Co-0238 and Q200 after 30 days

## 6. CONCLUSIONS AND RECOMMENDATIONS

### 6.1 Conclusion

From the result obtained in the present study, it is concluded that the developed 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. Accordingly, the information below was obtained

- For shoot tip culture initiation and establishment Q200 and Q217 varieties showed best performance on 2.0 mg/L BAP +0.5 mg/L IBA combination, while CO-0238 genotype best performed on 1.5 mg/L BAP +0.5 mg/L IBA.
- A combination of 2.0 mg/L BAP + 0.25 mg/L Kinetin was the best combination for shoot multiplication of genotype Q217 while 2.0 mg/L BAP without Kinetin and 1.5 mg/L BAP + 0.5 mg/l kinetin was the most selected combinations for genotypes Q200 and CO-0238 respectively.

For *in vitro* rooting, half strength MS semi-solid medium fortified with 3.0 mg/l NAA alone was best concentration for genotype Q200, while half MS semi-solid medium supplemented with 5.0 mg/L NAA was the best growth regulator concentration for genotype Co-0238, and half MS semi-solid medium supplemented with 1.5 mg/L IBA + 3.0 mg/L NAA was most outstanding combination for genotype Q217.

### 6.2 Recommendations

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 develop efficient protocols for the above mentioned varieties using Bioreactor system.
- It is also recommended to optimize protocols for these genotypes using other type of plant growth hormone concentration and combination so as to get best multiplication.

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# 1. APPENDICES

**Appendix Table 1. Full MS basal medium stock solution composition**

code	Nutrient	Stock solution(g)		Volume of Stock solution for 1L media
MS-1	Ammonium nitrate(NH <sub>4</sub> NO <sub>3</sub> )	33.0	In	50ml
	Potassium Nitrate(KNO <sub>3</sub> )	38.0	1000ml	
MS-2	Magnesium Sulphate (MgSO <sub>4</sub> .7H <sub>2</sub> O)	18.07		5ml
	Manganese sulphate (MnSO <sub>4</sub> .H <sub>2</sub> O)	1.69	In	
	Zinc sulphate (ZnSO <sub>4</sub> .7H <sub>2</sub> O)	0.86	500ml	
	Copper Sulphate (CuSO <sub>4</sub> .5H <sub>2</sub> O)	0.0025		
MS-3	Calcium Chloride(CaCl <sub>2</sub> .2H <sub>2</sub> O)	33.22	In 500ml	5ml
	Potassium Iodide(KI)	0.083		
	Cobalt Chloride(CoCl <sub>2</sub> .6H <sub>2</sub> O)	0.0025		
MS-4	Potassium dibasic phosphate(KH <sub>2</sub> PO <sub>4</sub> )	17.0	In 500ml	5ml
	Boric Acid (H <sub>3</sub> BO <sub>3</sub> )	0.62		
	sodium Molbdate (Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O)	0.025		
MS-5	Na <sub>2</sub> -EDTA	3.726	In 500ml	5ml
	Iron Sulphate(FeSO <sub>4</sub> .7H <sub>2</sub> O)	2.78		
MS-6	Myoinositol	10.0	In 500ml	5ml
	Glycine	0.2		
	Thiamine	0.01		
	Pyridoxine	0.05		
	Nicotinic Acid	0.05		

**Appendix table 2. Plant Growth Regulators stock solution preparation**

PGRs	Solvent
BAP,BA,2ip,TDZ, Kinetin, NAA, Zeatin	NaOH(1N)
GA3	EtOH
2,4-D	EtOH, NaOH(1N)

**Appendix table 3. Description of media formulation codes for invitro shoots multiplication treatment**

PGR(mg/l)	<i>Description of media formulation codes</i>																			
	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20
Kinetin	0	0	0	0	<b>0</b>	0.25	0.25	0.25	0.25	<b>0.25</b>	0.5	0.5	0.5	<b>0.5</b>	0.5	1	1	1	1	1
BAP	0	0.5	1	1.5	<b>2</b>	0	0.5	1	1.5	<b>2</b>	0	0.5	1	<b>1.5</b>	2	0	0.5	1	1.5	2

**Appendix table 4. Description of media formulation codes for invitro root induction**

PGR(mg/l)	<i>Details of media formulation codes for invitro rooting treatment</i>														
	R1	R2	<b>R3</b>	R4	<b>R5</b>	R6	R7	<b>R8</b>	R9	R10	R11	R12	R13	R14	R15
IBA	0	0	<b>0</b>	0	<b>0</b>	1.5	1.5	<b>1.5</b>	1.5	1.5	2	2	2	2	2
NAA	0	2	<b>3</b>	4	<b>5</b>	0	2	<b>3</b>	4	5	0	2	3	4	5

