

***IN VITRO* PROPAGATION OF SELECTED SUGARCANE (*Saccharum officinarum* L.) VARIETIES (C 86-12 and C 86-165) THROUGH APICAL MERISTEM**

**M.Sc. THESIS**

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**HARAMAYA UNIVERSITY, HARAMAYA**

***IN VITRO* PROPAGATION OF SELECTED SUGARCANE (*Saccharum officinarum* L.) VARIETIES (C 86-12 and C 86-165) THROUGH APICAL MERISTEM**

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MASTER OF SCIENCE IN BIOTECHNOLOGY**

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**JUNE 2017**

**Haramaya University, Haramaya**



## **DEDICATION**

This work is dedicated to my mother, Menahlesh Gebru, and my father, Hailu Redae.

## STATEMENT OF THE AUTHOR

First, I declare that this thesis is my genuine work and that all sources of materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for M.Sc. degree in Biotechnology at Haramaya University. The Thesis is deposited in the University Library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or Certificate.

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## **BIOGRAPHICAL SKETCH**

The author was born on 02 January 1990, from his father, Hailu Redae and his mother Menahlesh Gebru at Enba-Alaje, Maichew, Southern Tigray, Ethiopia. He attended his primary and secondary educations at Lemlem Borra from 1999-2008, and preparatory school at Addishehu preparatory school from 2009-2010, Addishehu town that is located in Southern Tigray Zone. After completing his preparatory school, Mr.Mulugeta joined Mekelle University in 2010/11 Academic Year, and graduated on July, 2013 with a Bachelor of Science Degree in Biotechnology. He was then employed at Mekelle Institute of Technology-Plant Tissue Culture Laboratory for three months in the year 2013 from September to November and served as transfer room supervisor. After that, in December 2013, he was employed at Mekelle University, Department of Biotechnology, and served as Graduate Assistant and Assistant Lecturer for two years. Mr.Mulugeta joined the School of Graduate Studies at Haramaya University in 2015/16 Academic Year to study for Master of Science degree in Biotechnology.

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## ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
2, 4-D	2, 4- Dichlorophenoxy acetic acid
6-BAP	6-benzylaminopurine
EDTA	Ethylene diamine tetra acetic acid
ESDA	Ethiopian Sugar Development Agency
FAO	Food and Agricultural Organization
FeEDTA	Iron ethylene diamine tetra acetic acid
GA <sub>3</sub>	Gibberellic acid
GDP	Growth and development plan
IAA	Indole acetic acid
IBA	1H-indole-3-butyric acid
ISO	International sugar Organization
Kn	kinetin
MS	Murashige and Skoog medium
NAA	1-Naphtalene acetic acid
PGR	Plant growth regulator
PVP	N-polyvinylpyrrolidone
RH	Relative humidity
SAS	Statistical analysis system
TDZ	Thidiazuron
Var.	Variety

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## ***In Vitro Propagation of Selected Sugarcane (*Saccharum officinarum* L.) Varieties (C 86-12 and C 86-165) Through Apical Meristem***

### **ABSTRACT**

*Sugarcane (*Saccharum officinarum* L.) is monocotyledonous crop plant that belongs to the Poaceae family that mostly propagates through conventional methods. However, conventional propagation lacks rapid multiplication procedures to commercialize newly released varieties within a short period of time. Hence, the objective of this work was to optimize in vitro micro propagation protocol for two sugarcane varieties (C 86-165 and C 86-12) using shoot apical meristem explants. The two varieties were cultured on MS medium supplemented with different concentrations of growth regulators on shoot initiation, multiplication and root induction stages. Finally, rooted plantlets were transplanted to three types of acclimatization medium in greenhouse, namely: coco peat alone, mixture of coco peat soil, sand, and composite; and mixture of garden soil, manure, and were kept under different lighting conditions. Analysis of variance (ANOVA) revealed that the two varieties showed statistically significant difference in their response to the various hormonal treatments with regard to the parameters measured. For initiation stage vars.C 86-165 and C 86-12 performed best on 1.0 mg/l and 0.5 mg/l of BAP, respectively. On the other hand, multiplication stage was best in MS media enriched with 2.0 mg/l BAP + 0.5 mg/l NAA and 1.5 mg/l BAP + 0.5 NAA as manifested in terms of number of shoots, shoot length, mean fresh weight and mean dry weight per explants after four weeks of culture for vars.C 86-165 and C 86-12, respectively. With regard to root induction, best rooting response in terms of mean root number, mean root length, mean fresh weight and mean dry weight was achieved in 1/2 MS media enriched with 2.0 mg/l NAA and 1.0 mg/l NAA after four weeks of culture for vars.C 86-165 and C 86-12, respectively. Survival rate during acclimatization was best on coco peat media alone for both varieties of C 86-165 and C 86-12 survived 76.7% and 84%, respectively. Lastly, factors causing low acclimatization, tissue dying, contamination and phenol exudation in the study should be further investigated.*

**Keywords:** *acclimatization, apical meristem, explants, growth medium, in vitro*

## 1. INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a monocotyledonous crop plant that belongs to the family Poaceae. It has tillers or stems bunched in to primary shoots, with a sucrose content of 10-18% and fiber content of 10-15% at harvest. It is the main crop that supplies sugar, and the second for ethanol production, grown in tropical and subtropical areas, which provides around 80% of the world production of sugar and 35% of ethanol (FAO, 2012). It is a tropical grass native to Asia where it has been grown in gardens for over 4,000 years. It is typically a plant that reaches a height of around three to four meters and has a diameter of about 5 centimeters (Magness, 2014).

Sugarcane is a clonally propagated crop from which multiple annual cuttings of stalks are typically obtained from each planting. This crop is especially vulnerable to diseases and propagation from cuttings facilitates the spread of pathogens and may results in epidemics. Sugarcane stalks can be infected by various pathogens without exhibiting any symptoms, and therefore there is a high risk of disease transfer during exchange and transport of sugarcane cuttings. Its growth is closely related to temperature (Schenck and Lehrer, 2000).

In certain countries, sugarcane is eaten raw in minor quantities, while it is also used in the preparation of juices, animal feed and Mulch for gardens and farms. The main byproducts of the sugar industry are bagasse and molasses. Molasses, the chief by-product, is the main raw material for alcohol and for alcohol-based industries. Excess bagasse is also used as raw material in the paper industry. In addition, bagasse is effectively used for co-generation of power in most sugar mills (Woodheap *et al.*, 2006).

Sugarcane was introduced to Ethiopia during the Italian occupation of and commercial sugar production was started in 1954 from cane cultivated on a large scale by Dutch Company at Wonji (Mukerji, 2000). Sugar industry plays a great role in the Ethiopian socio-economy and provides employment opportunity for the people.

Ethiopia's sugarcane is recently declining rapidly as a result of which lower sugar yield, close to 10 t/ha, has been recorded in Ethiopia (ESDA, 2009). Among others lower sugar yield is one of the major reasons for lower per capita annual consumption of sugar in Ethiopia, 5.1kg/year as compared to 16.3 kg/year of African average as estimated by International Sugar

Organization (ISO, 2009). There are many reasons for lower sugar yield, the most important ones are the non availability of disease free elite seeding stock and incapability to make use of advanced technologies in sugarcane propagation (ESDA, 2009).

The government of Ethiopia, in its growth and development plan (GTP), has launched sugar development program to undertake new and expansion projects across the country with a clear objective of boosting sugar production to satisfy the domestic sugar demand as well as for any possible export (Anonymous, 2013).

However, propagation of sugarcane conventionally fails to produce adequate quantity because of susceptibility to disease. On the other hand, availability of adequate amount of quality and disease free planting materials within a short time is the major limiting factor to attain large scale sugarcane production using the conventional method of propagation and the yield of the existing few and old commercial sugar cane varieties is declining and some productive sugarcane varieties are also obsolete due to lack of alternative technologies for disease cleansing and rejuvenation (Tolera *et al.*, 2014).

To solve the multitude problems of the conventional propagation method, the sugar industry utilizes the advantage of micropropagation technology, which is characterized by rapid multiplication to obtain disease free sugarcane varieties. The nutritional requirement for *in vitro* propagation protocol of sugarcane should be according to genotype and explants used (Soodi *et al.*, 2006); varieties (genotypes) of the same species respond differently to media (Roy, 2000). Besides rapid clonal propagation, sugarcane planting materials depend on the genotype and the plant growth regulators combinations used and needs to develop plant growth regulators combinations for each genotype. Similarly, plant growth regulators requirements for *in vitro* propagation responses vary from cultivar to cultivar in sugarcane (Raman *et al.*, 2005). The nutritional requirement for every sugarcane variety is specific and exact (Geetha *et al.*, 2001). In addition, an efficient protocol is needed for any new variety or clone to get rapid shoot initiation, shoot multiplication, root induction & elongation (Jai *et al.*, 2014). So it is recommended that an efficient protocol is needed for every new variety or clone of sugarcane to get rapid callus induction, shoot initiation, shoot multiplication and root induction and elongation (Behera and Sahoo, 2009). Therefore, this study was carried out to

develop or optimize *in vitro* protocol for mass propagation of two sugarcane varieties (C 86-165 and C 86-12) through apical meristem.

### **Objective of the study**

The general objective of this study was to develop optimization protocol for *in vitro* regeneration of two sugarcane varieties namely C 86-12 and C 86-165 from shoot apical meristem explants.

### **Specific objectives were**

- To determine optimum concentrations of BAP hormone for initiation culture of apical meristem
- To determine optimum concentration of BAP and NAA hormones for shoot induction and multiplication.
- To determine optimum concentration of NAA hormones for root growth
- To evaluate survival rate of plantlets under greenhouse condition on different substrates

## 2. LITERATURE REVIEW

### 2.1. Botanical description of sugarcane

Sugarcane is a perennial tropical grass (Poaceae) that originated in South East Asia. In commercial production, sugarcane is propagated from stem cuttings (seed-pieces), each having two or more buds (Gianesis *et al.*, 2002)

All varieties of sugarcane are hybrids of the species genus *Saccharum*. It is a tall perennial tropical grass that tillers at the base to produce unbranched stems, 2.5 – 4.0 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 inter node. A node consists of a lateral bud in leaf axils (Wrigley, 1982)

### 2.2. Importance of sugarcane

Sugarcane is a cash crop, but it is also used as livestock fodder (Magness, 2014). It is one of the most efficient converters of solar energy into organic substances, such as sugars, and many other forms of renewable energy. The first people to use the plant and domesticate it due to the presence of its sweet stem were the people in Polynesia (Jalaja *et al.*, 2008).

Nowadays, sugarcane plant has emerged as a multipurpose crop that is used not only to produce sugar but also used in a series of value added products such as paper, ethanol and other alcohol derived chemicals, animal feed, antibiotics, particle board, bio-fertilizer and raw material for generating electricity. Sugarcane is a crop with high economic significance as it accounts for more than 70% of the world's sugar production (Jalaja *et al.*, 2008).

### 2.3. The concept of plant tissue culture

Plant tissue culture (PTC) is a generic description which embraces that the culture and maintenance of plant cell, tissue and organ, growing in microbe-free plant material in an aseptic (sterile) environment, such as sterilized nutrient medium in a test tube. (Bhojwani and Razdan, 1992). Plant tissue culture can also be defined as the regeneration of whole plants *in vitro* using the culture of plant cells or plant tissues in a synthetic culture medium under controlled aseptic conditions (Garg, 2011). It is clear from literatures that the general

phenomenon of tissue culture is the ability to produce embryoids, organs, and tissues from different explants like leaves, stem, cotyledons, and microsporocyte and shoot tips. *In vitro* propagation via organogenesis usually involves four stages including initiation of cultures, multiplication of shoots, rooting of shoots, and acclimatization of plants (Cardoza, 2008).

The historical report regarding tissue culture dates back to the beginning of the 20<sup>th</sup> century and developed to maintain mesophyll cells in culture, but did not have much success (Bhojwani and Razdan, 1996). Since then, results and reports regarding the application of tissue culture techniques and its development have been made. Tissue culture is useful for plant propagation (Illg, 1991; Singh *et al.*, 2013) and the study of plant hormones (Gaspar *et al.*, 1996). Elite varieties can be clonally propagated (Illg, 1991; Engelmann, 2010), endangered plants can be conserved. (Paunescu, 2009; Pasqual *et al.*, 2014), virus-free plants can be propagated by meristem culture (Illg, 1991; Sim, 2006), germplasm can be conserved (Mathur, 2013), and secondary metabolites can be produced by cell culture (Vanisree *et al.*, 2004; Mathur, 2013). Plant tissue culture now has direct commercial applications as well as value in basic research into cell biology, genetics and biochemistry (Garg, 2011).

### **2.3.1. Types of plant tissue culture**

George *et al.* (2008) classified plant tissue culture into two; namely: cultures of unorganized tissues and cultures of organized tissues. Cultures of unorganized tissues with their respective explants in the bracket are; callus cultures (any plant tissue or organ), cell-suspension cultures (friable callus), protoplast culture (protoplast) and microspore culture (anthers). Root cultures (tip of either primary or lateral roots), shoot culture (tips of shoots), meristem culture (meristem), nodal culture, embryo culture (immature and mature embryos), leaf or leaf sheath cultures (leaf or leaf sheath), fruit culture and flower cultures are classified as cultures of organized tissues, with their respective explants indicated in brackets. The first four of this group are called cultures of indeterminate organs (growth is potentially unlimited) and the last four are termed culture of determinate organ (destined to have only a defined size and shape).

### **2.3.2. Micro propagation**

The objective of plant propagation via tissue culture is to propagate plants true-to-type (clones), which is termed micro propagation. It is the process in which explants placed in a

suitable culture media within aseptic environment results in multiple shoots available for rooting and become ready for transferring to soil after acclimatization (Singh, 2003).

According to George *et al.* (2008) there are five stages of micro propagation. Namely,

Stage 1. Selection of the plant tissue (explants) from a healthy vigorous 'mother plant'. This tissue must be sterilized to remove microbial contaminants.

Stage 2. Establishment of the explants in a culture medium. The medium sustains the plant cells and encourages cell division. It can be solid or liquid.

Stage 3. Multiplication. The explant gives rise to a callus or shoots which is manipulated by varying the auxin: cytokinin to form multiple shoots.

Stage 4. Root formation. The shoots are transferred to a growth medium with relatively higher auxin: cytokinin ratios.

Stage 5. The rooted shoots are potted and 'hardened off' by gradually decreasing the humidity by process called acclimatization.

### **2.3.3. Culture medium of Tissue culture**

One of the most important factors governing the growth and morphogenesis of plant tissues in culture is the composition of the culture medium (Rai, 2007). The appropriate composition of the medium largely determines the success of cultures. Plant material does vary in their nutritional requirements and therefore it is often necessary to modify the medium to suit a particular tissue. The basal medium employed for the culture of sugarcane is MS medium (Murashige and Skoog, 1962). A variety of growth regulators such as 6- Benzyl amino purine (BAP), alpha-Naphthalene acetic acid (NAA), 3-Indole Butyric acid (IBA) and 2,4- dichloro phenoxy acetic acid (2,4-D) will be added to the medium singly or in combinations at various concentrations and used for initiating different experiments (Saad and Elshahed, 2012).

Plant tissue culture media should generally contain some or all of the following components: macronutrients, micronutrients, vitamins, amino acids or nitrogen supplements, source(s) of carbon, undefined organic supplements, growth regulators and solidifying agents (Saad and Elshahed, 2012).

**Macronutrients:** The basic elements in plant cell or tissue culture media include, besides C, H and O, macro elements, nitrogen (N), phosphorus (P), potassium (K), calcium (Ca),

magnesium (Mg) and sulphur (S) for satisfactory growth and morphogenesis. Culture media should contain at least 25-60 mM of inorganic nitrogen for satisfactory plant cell growth. Potassium is required for cell growth of most plant species. Most media contain K in the form of nitrate chloride salts (Steiner and Winden, 1970).

**Micronutrients:** The essential micronutrients (minor elements) for plant cell and tissue growth include iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo). Iron is usually the most critical of all the micronutrients. The element is used as either citrate salts in culture media, however, there exist some problems with these compounds for their difficulty to dissolve and precipitate after media preparation. There have been trials to solve this problem by using ethylene diamine tetra acetic acid (EDTA)-iron chelate (FeEDTA). A procedure for preparing an iron chelate solution that does not precipitate has been also developed. Cobalt (Co) and iodine (I) may be added to certain media, but their requirements for cell growth has not been precisely established (Steiner and Winden, 1970).

**Carbon and energy sources:** In plant cell culture media, besides the sucrose, frequently used as carbon source at a concentration of 2-5%, other carbohydrates are also used. Other mono or disaccharide and sugar alcohols like glucose, maltose or sorbitol may be used depending upon plant species (Rahman *et al.*, 2010). These include lactose, galactose, maltose and starch and they were reported to be less effective than either sucrose or glucose, the latter was similarly more effective than fructose considering that glucose is utilized by the cells in the beginning, followed by fructose. It was frequently demonstrated that autoclaved sucrose was better for growth than filter sterilized sucrose. Autoclaving seems to hydrolyze sucrose into more efficiently utilizable sugars such as fructose. Sucrose was reported to act as morphogenetic trigger in the formation of auxiliary buds and branching of adventitious roots (Vinterhalter, 1997).

**Vitamins and myo-inositol:** Some plants are able to synthesize the essential requirements of vitamins for their growth. Some vitamins are required for normal growth and development of plants they are required by plants as catalysts in various metabolic processes. They may act as limiting factors for cell growth and differentiation when plant cells and tissues are grown *in vitro*. The vitamins most used in the cell and tissue culture media include thiamin (B1),

nicotinic acid and pyridoxine (B6). Thiamin is necessarily required by all cells for growth (Torres, 1989)

**Plant growth regulators:** Hormones are organic compounds naturally synthesized in higher plant, which affect the growth and development of plants by exerting a profound influence on physiological processes (Hopkins and Huner, 2009). They are usually active at a different site in the plant from where they are produced and are only present and active in very small quantities (George *et al.*, 2008). Apart from natural hormones, synthetic hormones have been developed which act-like the natural ones. These synthetically produced hormones are commonly called plant growth regulators and have biological activity which equals or exceeds that of the equivalent endogenous hormones (Gaspar *et al.*, 1996).

The synthetic plant growth regulators are used in commercial applications largely than natural ones because they are more effective, low cost, and have greater chemical. In addition to the nutrients, it is generally necessary to add one or more growth substances, such as auxins, cytokinins, and gibberellins, to support good *in vitro* growth of tissues and organs (Hopkins and Huner, 2009)

The requirement for growth substance varies depending on the type and source of the explants and on their endogenous level (Bhojwani and Razdan, 1996). The balance composition of the medium, particularly the plant hormones and the nitrogen source (nitrate versus ammonium salts) have profound effects on the morphology of the tissues that grow from the initial explants. Culture plant growth regulators act alone and in interaction to produce the final effect (George *et al.*, 2008).

Cytokinin is similar to adenine and was first discovered in 1941 as the active component in coconut milk that promoted growth of plant cells in tissue culture (Cardoza, 2008), and it is added to culture medium to promote cell division, stimulation of DNA synthesis to yield shoot formation and auxiliary shoot proliferation (George *et al.*, 2008).

The irreversible degradation of cytokinins, catalyzed by cytokinin oxidase, is an important mechanism by which plants modulate their cytokinin levels (Brugie *et al.*, 2003). Cytokinins like BAP, furfuryl amino purine (kinetin), thidiazuron (TDZ) and zeatin have been commonly employed alone and/or in combination with other cytokinins or/and auxins for proliferation

and multiplication of different plant species (Gaspar *et al.*, 1996). Of these BAP is the most active, cheapest and easily autoclaved without exhaust. Therefore, it is the one most frequently used, particularly in commercial micro propagation establishments where cost and ease of handling are major considerations (George *et al.*, 2008).

Auxins induce cell division, cell elongation, apical dominance, adventitious root formation, and somatic embryogenesis (Rai, 2007). Auxins like NAA, 2, 4 dichlorophenoxyacetic acid (2, 4-D), indole-3 acetic acid (IAA) and IBA are the most frequently used in plant tissue culture alone and in combination. IAA (indole-3-acetic acid) is the most important naturally occurring auxin, but its use in plant tissue culture media is limited because it is unstable in the medium (Doods and Roberts, 1985). It is also destroyed rapidly by many tissues and is often not very effective in supporting the growth of cultured plant tissues (Hangarter *et al.*, 1980). IBA advantage over some other synthetic auxins, i.e., IBA is metabolized to IAA, which is the natural auxin (Strader and Bartel, 2011). Generally; the type of morphogenesis that occurs in a plant tissue culture largely depends on the ratio and concentration of auxins and cytokinins in the medium (George *et al.*, 2008).

**Amino acids:** The required amino acids for optimal growth are usually synthesized by most plants, however, the addition of certain amino acids or amino acid mixtures is particularly important for establishing cultures of cells and protoplasts. Amino acids provide plant cells with a source of nitrogen that is easily assimilated by tissues and cells faster than inorganic nitrogen sources. Amino acid mixtures such as casein hydrolysate, L-glutamine, Lasparagine and adenine are frequently used as sources of organic nitrogen in culture media (Torres, 1989).

**Undefined organic supplements:** Some media supplemented with natural substances or extracts such as protein hydrolysates, coconut milk, yeast extract, malt extract, ground banana, orange juice and tomato juice, to test their effect on growth enhancement. A wide variety of organic extracts are now commonly added to culture media. The addition of activated charcoal is sometimes added to culture media where it may have either a beneficial or deleterious effect (Pan and Van Staden, 1998).

**pH and solidifying agents:** The hydrogen ion concentration of the tissue culture medium is important since it influences the uptake of various components of the medium as well as regulating a wide range of biochemical reactions occurring in plant tissue cultures by altering

pH of the cells (Owen *et al.*, 1991; Singh *et al.*, 2013). Most media are adjusted to a pH of 5.2–5.8. Values of pH lower than 4.5 or higher than 7.0 greatly inhibit growth and development *in vitro* (Rai, 2007). Cultures can be grown in either liquid or solid medium. Solidifying agents are used for preparing solid and semisolid tissue culture media to enable explants to be placed in right contact with nutrient media (not submerged but on surface or slightly embedded) to provide aeration (Puchooa *et al.*, 1999).

The most frequently used solidifying agent is Agar. Agar is high molecular weight polysaccharide obtained from sea weeds and can bind water. It is added to the medium in concentration ranging from 0.5 % to 1 % (w/v). Agar is preferred over other gelling agents because it is inert, Neither does it neither react with media constituents nor digested by plant enzymes (Cameron, 2006). Hardness of the culture medium greatly influences the growth of cultured tissues. There are a number of gelling agents such as agar, agarose and gellan gum (Prakash *et al.*, 2002).

**Light and temperature:** The average constant growth room temperature employed in a large sample of experimental reports was found to be 25°C ranging from 17 to 32°C. In many plants, *in vitro* root induction on the shoot micro-cuttings produced requires a slightly lower temperature than is necessary for shoot multiplication and growth. Fluorescent tubes are used almost universally to provide light for cultures (George *et al.*, 2008). For sugarcane, different light and temperature combination are recommended by different authorities. Concomitantly, Singh (2003) reported *in vitro* cultures are incubated in a growth room with a 16h photoperiod (2000-3000 Lux) and the temperature is maintained at  $25 \pm 3^\circ\text{C}$ .

#### **2.3.4. Aseptic techniques**

Most tissue culture procedures are conducted in sterile operations, such as laminar flow cabinet. Besides the special design of gentle flow of sterile air in cabinet, aseptic cabinet is also equipped with germicidal lamp emitting ultraviolet light. This type of radiation is useful in eliminating airborne contaminants and for surface disinfection. Glassware and all the tools used for tissue culture process can also cause contamination. It is extremely necessary to autoclave all the material before using it, so that all the microbial contaminants are destroyed (Reed and Tanprasert, 1995). Living plant materials from the environment are naturally

contaminated on their surfaces (and sometimes interiors) with microorganisms, so surface sterilization of starting materials (explants) in chemical solutions (disinfectants) such as ethanol, sodium hypochlorite (NaOCl), and Tween-20 is required (Mineo, 1990; Aishwarya and Robinson, 2013) to control the growth of fungi and bacteria on the growth media (Singh *et al.*, 2011).

### **2.3.5. Stages of micro propagation**

**Stage I - Establishment of culture:** The purpose of stage-I is to initiate axenic culture. This stage begins with the excision of meristem tissue from an identified stock plant. These explants are treated with anti microbial chemicals to remove contaminating organisms. Using aseptic techniques the explants are cultured into appropriate nutrient medium. A rapidly proliferating culture is established under optimum levels of light and temperature (Franclet *et al.*, 1987).

The major constraints in establishing sterile cultures of woody tissues are microbial contamination and interference of phenolic exudates. Use of fungicides and antibiotics limits the microbial infection to some extent. Use of antioxidants like ascorbic acid, PVP and charcoal helps in eliminating the interfering phenolic exudates. Contamination in tissue culture can originate from two sources through carryover of microorganisms on the surface and/or in the tissues of explants or through faulty procedures in the laboratory. For most micro propagation work the explants of choice is an apical or auxiliary bud. The developmental stage of the explants is an important factor. The explants must be physiologically competent to survive the initial culture and elicit the appropriate response. In general, younger tissues such as terminal or auxiliary shoot buds regenerate better than older and mature tissues of the same stem. The age of the stock plant, physiological age of the explants and its developmental stage as well as its size can determine the success of a procedure (Franclet *et al.*, 1987).

In general the ingredients of culture medium in this stage are determined by the kind of response desired e.g. auxiliary shoot formation or adventitious shoot formation or callusing etc. Supplementation of cytokinins like BAP, TDZ in media is desired for auxiliary shoot formation whereas auxins like NAA, IAA and 2, 4-D is preferred for callusing. Carbohydrate as carbon source is one of the important components of the plant tissue culture medium.

Sucrose is the most commonly used carbon source in the media. Most of the earlier studies strongly suggested the use of sucrose as carbon source. Depending on the mineral requirements of different plant species, several media compositions have been formulated. The most commonly used basal medium is Murashige and Skoog's medium (Franclet *et al.*, 1987).

**Stage II - Proliferation of shoots in culture:** In this stage, shoots are proliferated in culture, which then serves as the source of shoots for subsequent propagation as well as the material that is required to maintain the stock. Multiplication of shoots is achieved by sub culturing the shoot cultures at intervals in appropriate medium. Variant plants may arise from the cultures maintained *in vitro* for a long period. For the purpose of micro propagation, usage of shoots from the cultures maintained for several passages and having higher subculture numbers is avoided to ensure genetic fidelity (Pasqual *et al.*, 2014)

Multiplication may be proceeding with several times sub culturing to increase the supply of material to a predetermined level for subsequent rooting and transplanting. Sometimes micro shoots deteriorate with time, lose leaves, and fail to grow, develop tip-brown, and lose potential to regenerate due to the lack of stabilization. The difficulty in stabilization appears to be associated with the forced changes in the differentiated state of plant cells during the culture and regeneration process and the particular conditions of the culture environment generate conditions of stress for the plant genome. Frequency of shoot formation and further development were greatly influenced by the presence of auxins and cytokinin in the medium (Gopitha *et al.*, 2010).

Shoot proliferation is a prerequisite for micro propagation of plants and cytokinins especially BAP is effective for inducing shoot proliferation (Arya *et al.*, 1999; Tuan *et al.*, 2012); and (Arya *et al.*, 2012); alone or in combination with auxins and/or cytokinins.

**Stage III – Rooting:** In this stage the shoots obtained from multiplication media are separated and shifted to conditions that favor root initiation and shoot elongation. For *ex vitro* rooting, shoots are excised as small cuttings (micro cuttings), treated with commercial rooting mixture and planted in soil (Debergh and Read, 1991).

Induction of roots in excised shoots and subsequent survival of plantlets in the soil is the most crucial step for success of any micro propagation protocol. Root induction is the stage prior to

acclimatization in which, individual shoots or shoots in clumps are transferred to a nutrient medium supplemented with auxins and ingredients that do not encourage further shoot proliferation and which promote rooting (George *et al.*, 2008). Whilst root induction of any plant tissue is dependent on the proper amount and combination of rooting hormones (Doods and Roberts, 1985) there is considerable evidence that other factors, including carbohydrate supply, are important in determining ultimate success (Thompson and Thorpe, 1987).

Generally adventitious root formation varies and depends on the genotype and on cultural methods. Even in few species root formation occur during multiplication process; usually it is necessary to develop a separate rooting procedure using special media, or methods, to induce root formation (George *et al.*, 2008). Because, different auxins differ in their physiological activities depending upon the extent to which they move through tissues, remains bound inside the cells, or get metabolized.

**Stage IV – Hardening of tissue culture raised plants:** This stage involves transfer of plantlets from aseptic condition to greenhouse and ultimately to the final location (environment). Plantlets develop within culture vessel under aseptic condition, on a medium containing sugar and nutrients to allow heterotrophic growth and in an atmosphere with high relative humidity and low levels of light, these all contribute to a phenotype that cannot survive the environmental conditions when directly placed in green house or field. Thus it is necessary to acclimatize plantlets gradually to ensure survival until they develop new leaves that are more adapted to ambient conditions under which plants are normally grown. High relative humidity has to be maintained during hardening process to protect the plants from desiccation and enable them to initiate (Seelye *et al.*, 2003).

During *in vitro* culture, plantlets grow under very special conditions in relatively higher air humidity, limited inflow of CO<sub>2</sub> and outflow of gaseous, higher saccharides as carbon and energy sources (Seelye *et al.*, 2003). These special conditions during *in vitro* culture result in the formation of plantlets of abnormal morphology, anatomy and physiology. After *ex vitro* transfer, these plantlets might easily be impaired by sudden changes in environmental conditions. Therefore, after *ex vitro* transplantation plants usually need some period of acclimatization with gradual lowering in air humidity (Bolar *et al.*, 1998).

## 2.4. Factors affecting *in vitro* propagation of sugarcane

**Explants:** Explants are sterile pieces of a whole plant and may consist of pieces of organs, such as shoot tips, leaves, seeds, flowers or roots, or may be specific cell types, such as pollen or endosperm (George *et al.*, 2008). The major requirements for effective explants tissue are a high cell division potential and morphogenic plasticity. These criteria are usually satisfied by immature, more rapidly growing tissue (or tissue at an early stage of development). The growth conditions of the parent plant can have a large influence on the efficiency of regeneration from explants tissue. These conditions possibly exert their effects by modifying the hormonal status of the parent plant (George *et al.*, 2008). Tissue polarity (position in the explant), the orientation of the explants in culture, size and developmental phase of explants play a significant role in determining the organogenic potential of sugarcane leaf tissue in culture (Lakshman *et al.*, 2006).

**Genotype:** The major influence on tissue-culture response appears to be genetic, with culture requirements varying between species and varieties are probably true to say those effects of genotype impose one of the greatest constraints to the tissue culture and micro propagation of plants (George *et al.*, 2008). Many genotype-dependent effects are caused by interactions between the plant's genotype, media and the cultural environment and endogenous growth regulators have a significant influence on the effects of applied regulators (George *et al.*, 2008).

## 2.5. Contaminants of *in vitro* culture

There are several pathogens (microbial contaminants) which have been a major threat to *in vitro* cultures due to their rapid proliferation characteristics (Enjalric *et al.*, 1998). Axenic cultures are often mandatory throughout the entire *in vitro* culture process. The contaminated cultures are usually discarded at any stage of sub-culturing because many *in vitro* culture contaminants eventually cause plant death by exuding their phytotoxins (George, 1993). Many fungal contaminants are visible right from the primary *in vitro* plant culture initiation except for the cryptic contaminants. Generally, these contaminants overgrow plant cultures and often compete with them for resources in the nutrient media (Pierik, 1987). Consequently, *in vitro* contaminants often adversely interfere with culture growth and survival. Therefore, freedom

from pathogens (asepsis) in plant cultures has been the ultimate goal in many *in vitro* protocols (Pierik, 1987).

**Fungal contaminants:** Fungal contaminants have posed a major threat at every stage of the *in vitro* plant culture process. These fungal contaminants are characterized by the presence of hyphae (thread like) appearing on the explants and culture media. Colonization of fungi progresses with time and the plant cultures are eventually covered in fungal mycelia. Unlike other contaminants, fungal contaminants are visible in the culture media. Furthermore, the development and growth of fungal contaminants are faster than the growth of the plant cultures. However, the rapid proliferation of fungi in the plant cultures depends on the type of fungi colonizing the culture media (Herman, 1990).

The fungi colonizing the plants could be endogenous, cryptic or entophytic. Herman (1990) reported that endogenous or endophytic microbes are often difficult to decontaminate. It is known that many endophytes are beneficial for the growth of plants in the wild, but they also become contaminants in culture media. Helander *et al.* (1996) reported that mutualism between endophytes and the host plant depends on the prevailing plant condition. Endophytes can be harmful to plant cultures although they are useful outside the plant culture laboratories. In the tropics, fungal contaminants are very common due to the prevailing favorable environmental conditions (especially the high temperatures and humidity) for the proliferation of fungal pathogens on the plants. Furthermore, the conditions in the plant culture incubation chambers are favorable for the rapid growth of fungi and other *in vitro* contaminants. For instance, the range of temperatures (usually between 23 – 26°C), high humidity and plant nutrients (available inside the culture vials or test-tubes) can not deter or inhibit the proliferation of many fungi in the tubes placed in the culture incubation chambers. Moreover, some plants live and survive by mutual association with fungi in the wild. However, such fungi are likely to become ‘*in vitro* pathogenic’ in the plant cultures.

**Exogenous fungal contaminants:** Generally, exogenous fungal pathogens are easy to eliminate from the stock plants using many fungicides. This is because such pathogens are found on the outer surface of the stock plants. However, insufficient surface disinfection might not dislodge exogenous pathogens and spores from the stock plants. It is always difficult to detect the presence of either exogenous, endogenous or both pathogens on the stock plants. So

it might be advisable to use systemic fungicides (e.g. Benomyl) before other disinfectants such as sodium hypochlorite are used. It is also a good practice to ensure that all possible culture contaminants are dealt with at the initial decontamination stage instead of re-disinfecting the explants at later stages (sub-culturing stages). Re-decontamination of explants might weaken the ensuing explants due to cell injury. This can easily cause death of explants (Herman, 1990)

**Endogenous fungal contaminants:** Symbiotic microbes which are beneficial to many plants may become contaminants in the culture media (Herman, 1990). It has been reported that endogenous or endophytic fungi become pathogenic to the host plants when the plants are stressed, for example, when the cell walls are weakened or under other unfavorable *in vitro* conditions (Darworth and Callan, 1996). According to Cassells (1991), culture asepsis is important in all plant culture protocols. However, many trees in the tropics live and survive in association with endogenous or cryptic microbes (fungi) in the wild (Darworth and Callan, 1996). However; it remains unclear whether this association may continue to be mutual or lethal to the growth of plant cultures. This is because some fungi may not be '*in vitro* pathogenic', despite being prolific in the cultures (Herman, 1990).

### 3. MATERIALS AND METHODS

#### 3.1. Description of the study area

The study was conducted at Tigray Biotechnology center; Plant Tissue Culture Laboratory which is located in Mekelle town, specifically at Ellala near to Tigray Agricultural Research Center that is located at latitude of 13°29'N, longitude of 39°28'E and altitude of 2076 meters above sea level (MARC, 2012).

#### 3.2. Plant material and explant preparation

Mother plants of the two varieties namely C 86-12 and C 86-165 that were used as a source of explants were raised from stem cuttings (setts) obtained from Welkayt Sugar Factory. According to Dereje (2014) those two Cuban varieties were imported in 2006 and passed through agronomic performance evaluation. They were among the selected ones to be commercialized. Before planting, the setts were treated with hot water at 50°C for 2 hours. Explant preparation were made following the method employed by Belay *et al.* (2014), first the actively growing shoot tips with apical meristem were collected from three months old mother plants to serve as explants. Shoot tips were cut from mother plants at the base with some nodes. After trimming of the leaves, the shoot tips were taken to the laboratory for surface sterilization and explant preparation.

Trimmed shoot tips were washed thoroughly under running tap water, outer leaf sheath were removed and cut into about 10 cm length. Thereafter, the shoot tips further washed three times each for 15 minutes with tap water containing liquid soap solution and three drops of Tween-20. Then, explants were taken to laminar airflow chamber, immersed in 0.3 % (w/v) Kocide solution for 30 minutes followed by three times washing each for five minutes with sterile distilled water. The shoot tips were then rinsed in 70% alcohol for one minute and washed with sterile distilled water three times each for five minutes. Finally, the explants were treated with 10% (v/v) sodium hypochlorite solution (4% active chlorine) for 20 minutes. After discarding the sodium hypochlorite solution, the explants were washed with sterile distilled water three times each for five minutes and the surface sterilized explants were excised and sized to 2.5 cm long for culturing (Belay *et al.*, 2014).

### **3.3. Culture media preparation**

Full strength Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) were used as a culture medium. MS basal medium consisted of 30 g/l sucrose for initiation of apical meristem and shoot initiation, whereas 60 g/l sucrose for rooting. The pH of the medium was adjusted to 5.8 using 1 N KOH and 1 N HCl before being gelled with 5.0 g/l agar and autoclaved at 121°C, 15 psi for 20 minutes. While molten, the medium (40 ml) was dispensed into glass culture jar for culturing and stored under aseptic condition at + 4 °C until use for shoot initiation.

### **3.4. Initiation of apical meristem**

For shoot initiation, the sterilized shoot tips were aseptically transferred to MS-medium prepared as indicated above with supplementation of PGR (BAP) at a concentration of 0.5, 1.0, 1.5 and 2.0 mg/l. MS medium without PGR were used as control. The treatments of initiation for both varieties were as follows

T<sub>1</sub>=MS + 30 g/l sucrose + 5.0 g/l agar + 0.0 mg/l BAP

T<sub>2</sub>=MS + 30 g/l sucrose + 5.0 g/l agar + 0.5 mg/l BAP

T<sub>3</sub>=MS + 30 g/l sucrose + 5.0 g/l agar + 1.0 mg/l BAP

T<sub>4</sub>=MS + 30 g/l sucrose + 5.0 g/l agar + 1.5 mg/l BAP

T<sub>5</sub>=MS + 30 g/l sucrose + 5.0 g/l agar + 2.0 mg/l BAP

The explants were maintained in dark for 8 hours and light for 16 hours duration. The experiment was laid with three replicates.

### **3.5. Shoot Induction and Regeneration**

For shoot initiation, MS basal medium supplemented with BAP in a concentration of 1.0, 1.5, 2.0 and 2.5 mg/l combined with 0.5 mg/l of NAA was used. MS basal medium without PGR, i.e., BAP and NAA were used as a control. Details of the treatments for both varieties were as follows:

T<sub>1</sub>=MS + 30 g/l sucrose + 5.0 g/l agar + 0.0 mg/l BAP + 0.0 mg/l NAA

T<sub>2</sub>=MS + 30 g/l sucrose + 5.0 g/l agar + 1.0 mg/l BAP + 0.5 mg/l NAA

T<sub>3</sub>=MS + 30 g/l sucrose + 5.0 g/l agar + 1.5 mg/l BAP + 0.5 mg/l NAA

T<sub>4</sub>=MS + 30 g/l sucrose + 5.0 g/l agar + 2.0 mg/l BAP + 0.5 mg/l NAA

T<sub>5</sub>=MS + 30 g/l sucrose + 5.0 g/l agar + 2.5 mg/l BAP + 0.5 mg/l NAA

Then after, cultures were maintained in a growth room at a temperature of  $25 \pm 2^\circ\text{C}$  under 16/8 hours light/dark photoperiod adjusted with fluorescent light having 2500 lux light intensity. The incubation chamber had relative humidity of 75-80%. Shoots were allowed to grow 2 to 4 cm and then transferred to rooting media. The experiment was laid out with a two factors treatment combinations each with three replicates.

### 3.6. Root induction

Well grown 3-5 cm long shoots were aseptically transferred to 1/2 strength MS basal medium containing 0.0, 0.5, 1.0, 1.5, and 2.0 mg/l of NAA. The treatments of rooting for both varieties were as follows:

T<sub>1=1/2</sub> MS + 30 g/l sucrose + 5.0 g/l agar + 0.0 mg/l NAA

T<sub>2=1/2</sub> MS + 30 g/l sucrose + 5.0 g/l agar + 1.0 mg/l NAA

T<sub>3=1/2</sub> MS + 30 g/l sucrose + 5.0 g/l agar + 1.5 mg/l NAA

T<sub>4=1/2</sub> MS + 30 g/l sucrose + 5.0 g/l agar + 2.0 mg/l NAA

T<sub>5=1/2</sub> MS + 30 g/l sucrose + 5.0 g/l agar + 2.5 mg/l NAA

All the cultures were incubated at  $25 \pm 2^\circ\text{C}$  under 16/8 hours light/dark photoperiod adjusted with fluorescent light having 2500 lux light intensity. The relative humidity of the growth chamber was 75-80%. The experiment was laid out with NAA factor treatment each with three replicates.

### 3.7. Acclimatization

After four weeks of culture in a rooting media, well rooted *in vitro* plantlets were taken out gently from each PGR treatment bottle and washed under tap water to remove traces of agar that prevent the absorption of nutrients from the acclimatization culture substrates by roots. After this, plantlets were transferred to polystyrene tray that contains three different substrates, namely, coco peat, that is a multipurpose growing medium made up of coconut husk, garden soil: sand: compost in the ratio of 1:1:1 and garden soil and cow dung in the ratio of 1:1. Then, the polystyrene tray was arranged in completely randomized design in computerized green house with relative humidity (RH) gradually reduced from 90 to 60% and temperature of 30 to 31 °C for two weeks for primary acclimatization. All the plantlets that survived the primary acclimatization were put in a direct sunlight and nursery shades were provided with adequate amount of water for secondary acclimatization and their performance were monitored for three weeks. Finally, plantlets that survived secondary acclimatization were transplanted to open field.

### 3.8. Data collected

The following *in vitro* plant growth variables were recorded from this experiment and served as sources of quantitative data.

- 1. Percent of initiated culture:** Percent of culture formed from the apical meristem explants after three weeks.
- 2. Average number of days to shoot emergence:** number of days needed by the explants to induce shoots from the first day of culturing in the shooting medium.
- 3. Mean number of shoots:** is the average number of dissectible shoots regenerated and induced from each cultured explants in each type of treatment.
- 4. Shoot fresh and dry weights:** are average fresh and dry weights of regenerated shoots of each explant. Dry weights of shoots were measured after shoots were oven dried at 60 °C for 72 hours. Weights were taken using an electronic sensitive balance.
- 5. Average number of days to root emergence:** number of days needed by the shoots to induce roots from the first day of culturing in the rooting media.

**6. Mean number of roots:** is the average number of dissectible roots regenerated from each cultured shoot in each type of treatment.

**7. Mean length of shoot:** is the average length of shoots developed from the base of the shoot to the shoot apex before transferring to rooting media from each cultured explants. Lengths of the shoot were measured using sterilized ruler.

**8. Mean length of root:** is the average length of roots developed from each cultured explants. Length was measured on the 30<sup>th</sup> day of transferring the shoot to the rooting media. Root length measurement was taken from the point that the root attached to the shoot to root tip.

**9. Root fresh and dry weights:** are average fresh and dry weights of roots induced on each shoot. Dry weights of roots were measured after roots were oven dried at 60<sup>o</sup>C for 72 hours. Weights were measured using an electronic sensitive balance.

**10. Survival rate:** is the competence or the ability of the *in vitro* derived plantlets to endure in the *in vivo* condition for acclimatization. Data on this parameter was taken after one month after rooting transferred to greenhouse. Accordingly, the survival rate was calculated after three weeks as the ratio of plantlets survived to the total number of plantlets transferred to the greenhouse and expressed as percentage.

### **3.9. Data analysis**

Data were analyzed on the effect of treatments using SAS version 9.1 and means were compared using Fisher's Least Significant Difference (LSD) test at  $p < 0.05$ .

## 4. RESULTS AND DISCUSSION

### 4.1. Percent of initiated culture

Initiation culture from the apical meristem explants was observed within two weeks after inoculation of the explants on MS medium containing five different concentrations of BAP (0.0, 0.5, 1.0, 1.5, and 2.0 mg/l). The results showed that shoot apical meristem culture initiation or establishment responses in the sugarcane varieties was dependent on the effect of sugarcane varieties (genotype) and BAP. Among the different concentration BAP tested, sugarcane variety C 86-165 gave the highest initiation culture responses (73.33%) on MS medium containing 1.0 mg/l BAP while C 86-12 gave highest initiation culture responses (76.667 %) on MS medium supplemented with 0.5 mg/l BAP as shown in (Table 1).

Table 1: Effect of different concentrations of BAP on the percent of initiated culture from apical meristem explants of C 86-165 and C 86-12 sugar cane varieties.

Treatments	Varieties	
	C 86-165	C 86-12
T <sub>1</sub> (0.0 mg/l BAP)		
T <sub>2</sub> (0.5 mg/l BAP)	60.00 <sup>ab</sup>	76.667 <sup>a</sup>
T <sub>3</sub> (1.0 mg/l BAP)	73.33 <sup>a</sup>	66.667 <sup>ab</sup>
T <sub>4</sub> (1.5 mg/l BAP)	53.33 <sup>bc</sup>	60.000 <sup>c</sup>
T <sub>5</sub> (2.0 mg/l BAP)	43.33 <sup>c</sup>	50.000 <sup>c</sup>
Mean	4.600	5.0667
CV	19.4440	16.9016
LSD	1.6272	1.5579

Means followed within a column by the same letter are not significantly different at 5% significance level, CV=Coefficient of variance, LSD=Least significant different

This indicated that initiation response in these two varieties is different with respect to the concentration of BAP used. In line with this, variation of initiation culture response to different concentration of hormones with variety of sugarcane was reported by Dereje *et al.* (2014); Tilahun *et al.* (2014). Control showed no response for initiation in which all explants cultured on control (0.0 mg/l BAP) dried out after explantation.

## **4.2. Shoot regeneration and multiplication**

### **4.2.1. Number of days to shoot emergence**

Shoot initiation was observed in PGRs treated cultures at all concentrations. However, no shoot initiation was observed in PGRs free (control) treatments. Previously, Dereje *et al.* (2014) reported that shoot-tip (apical meristem) explants of sugarcane variety C86-12 cultured on hormone free MS medium had slow shooting response. The fact that no shooting response/delayed shooting response observed in PGRs free medium show that the available endogenous hormones may not be sufficient to induce shooting. The result also showed that the number of days to shoot emergence was found to be influenced by different concentrations of BAP combined with constant concentration of NAA. Relatively, C 86-12 variety had faster response at all PGRs concentrations than C 86-165 variety in terms of shoot emergence (Table 2). In both varieties, shoot emergence was found to be earlier at lower concentration, and number of days to shoot emergence increased with increasing PGRs concentrations (Table 2). This finding was in line with that of Sowel *et al.* (2002) who reported the effectiveness of low concentration of BAP to result in rapid shoot multiplication.

Table 2: Different shoot parameters measured for apical meristem explants treated with different concentrations of BAP combined with 0.5 mg/l of NAA. Values are mean  $\pm$  SE, n=3.

Sugarcane varieties	Hormones (mg/l)		No of days to shoot emergence	No of shoots per expt.	Shoot length(cm)	Shoot fresh wt.per expt.	Shoot dry wt.per expt.
	BAP	NAA					
<b>C 86-165</b>	0.0	0.0	—	—	—	—	—
	1.0	0.5	13.333 <sup>c</sup>	8.333 <sup>b</sup>	3.4667 <sup>bc</sup>	0.633 <sup>a</sup>	0.250 <sup>b</sup>
	1.5	0.5	14.00 <sup>c</sup>	8.667 <sup>ab</sup>	5.0333 <sup>b</sup>	0.667 <sup>a</sup>	0.283 <sup>ab</sup>
	2.0	0.5	16.33 <sup>b</sup>	10.667 <sup>a</sup>	8.5333 <sup>a</sup>	0.700 <sup>a</sup>	0.350 <sup>a</sup>
	2.5	0.5	19.00 <sup>a</sup>	9.333 <sup>ab</sup>	5.8333 <sup>b</sup>	0.533 <sup>b</sup>	0.250 <sup>b</sup>
Mean			12.5333	7.4000	4.5733	0.5066	0.2267
CV			9.2130	16.3657	17.9602	17.6531	22.723
LSD			2.1007	2.2032	1.4943	0.1627	0.094
<b>C 86-12</b>	0.0	0.5	—	—	—	—	—
	1.0	0.5	12.333 <sup>b</sup>	7.333 <sup>b</sup>	3.2667 <sup>b</sup>	0.70 <sup>ab</sup>	0.333 <sup>a</sup>
	1.5	0.5	17.00 <sup>ab</sup>	9.333 <sup>a</sup>	5.6333 <sup>a</sup>	0.733 <sup>a</sup>	0.3667 <sup>a</sup>
	2.0	0.5	18.333 <sup>a</sup>	8.667 <sup>ab</sup>	4.4333 <sup>b</sup>	0.566 <sup>c</sup>	0.233 <sup>b</sup>
	2.5	0.5	18.000 <sup>a</sup>	8.333 <sup>b</sup>	3.1667 <sup>c</sup>	0.633 <sup>bc</sup>	0.317 <sup>ab</sup>
Mean			13.1333	6.7333	3.333	0.5267	0.250
CV			10.0246	15.3385	11.037	8.4914	13.663
LSD			2.3952	1.8789	0.663	0.0814	0.0621

Means followed by the same letter within each variety are not significantly different at 5% significance level, No=Number, Expt. =Explant, Wt. =Weight, CV=Coefficient of variance, LSD=Least significant different.

#### 4.2.2. Mean number of shoots

Number of shoot/explant was significantly higher in PGRs treated explants than PGRs free cultured explants. Number of shoot /explants was also significantly varied between PGRs treatments with highest number (10.667) counted at 2.0 mg/l of BAP combined with 0.5 mg/l of NAA for variety C 86-165 and 9.333 shoot/explants for variety C 86-12 at 1.5 mg/l of BAP combined with 0.5 mg/l of NAA (Table 2). Previously, Tarique *et al.* (2010) reported that 1.0 mg/l BAP + 0.5 mg/l NAA and 1.0 mg/l BAP + 0.5 mg/l IBA showed the best result for induction and multiplication of shoots for sugar cane varieties of B52-298 and NCO-334, respectively. This shows that different varieties of sugar cane respond differently to different types and concentrations of PGRs, suggesting unique optimization for better performance.

Moreover, Genotype specific response to number of shoot regeneration was reported by Gandonou *et al.*, (2005) and Behara *et al.*, (2009).

Shoot number /explant appeared to increase with increasing concentration of BAP up to 2.0 mg/l. It has been reported that a high level of cytokinin in combination with a low auxin level was essential for the differentiation of adventitious shoots in sugarcane (Belay *et al.*, 2014). However, it was observed that mean number of shoots per explants was found to decline with further increase in the concentration of BAP beyond optimum (2.0 mg/l BAP) for both varieties. This finding agrees with Khalafalla *et al.* (2007) who reported that BAP at the concentration of 5.0 mg/l gives low number of shoot per explants and concluded that shoot number decreases as BAP concentration increases beyond optimum. Increasing trend in shoot number per explants up to optimum level is due to the fact that cytokinin (BAP) stimulates protein synthesis and participates in cell cycle control in a cell division (George *et al.*, 2008).

#### **4.2.3. Effect of growth regulators on shoot length**

Average shoot length was significantly higher in PGRs treated explants than explants culture on PGRs free media. Shoot length also showed significant difference between PGRs treatments. Shoots cultured on MS media containing 2.0 mg/l BAP and 0.5 mg/l NAA showed significantly higher mean shoot length (8.533) compared to all other treatments for var. C 86-165. For var. C 86-12 highest mean shoot length (5.633) measured was on MS medium containing 1.5 mg/l BAP and 0.5 mg/l NAA (Table 2). Similar to this result, Dereje *et al.* (2014) reported maximum shoot length  $8.4 \pm 0.008$  for Cuban sugar cane variety C 86-12 when cultured on BAP (1.5 mg/l) + kin (0.5 mg/l ). Behera *et al.* (2009) also reported that maximum shoot length of  $6.2 \pm 0.37$  and  $4.0 \pm 0.61$  under BAP (2.0 mg/l) + IBA (0.5 mg/l) and BAP (2.0 mg/l) + IBA (1.0 mg/l) for two sugarcane varieties namely B52-298 and NCO-334, respectively. On the other hand, it was observed that shoot length was found to decline with the increase in the concentration of BAP beyond optimum (2.0 mg/l BAP). This findings agrees with that of Bhatia *et al.* (2005) who explained that increasing the concentration of the PGRs over optimum supplements may lead to negative effects on the morphology of the *in vitro* shoots.

#### **4.2.4. Fresh and dry weights of shoots**

Means of fresh and dry weights of shoots varied significantly with different treatments. The maximum fresh and dry weights of shoots 0.70 g and 0.35 g, respectively were measured in shoots cultured on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA for var.C 86-165, whereas for var. C 86-12 maximum fresh and dry weights of shoots 0.733 g and 0.366 g, respectively were measured in shoots cultured on MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA. Similar trend was also observed with regards to fresh and dry weights of shoots in the works of Oliveira *et al.* (2009) who reported that mean fresh and dry weights of shoots are the function of shoot number and length of the shoots in all treatments for sugarcane varieties NCO-334 and B52-298. Therefore, where higher mean shoot numbers and lengths were observed, the fresh and dry weights of the shoots are higher.

### **4.3. Effect of growth regulator (NAA) on root Induction**

#### **4.3.1. Number of days to root emergence**

Root initiation was observed in PGRs treated shoots at all concentrations. However, no root initiation was observed in PGRs free (control) treatment. Similar result has been reported by Rashid *et al.* (2009). The number of days to root emergence was also found to be influenced by different concentrations of NAA. The two varieties showed variation in responding to the different concentrations of NAA in terms of time taken for root emergence. Variety C 86-165 formed root earlier at 1mg/l NAA and root formation was delayed at the lowest (2.5 mg/l) of NAA. Whereas C 86-12 formed root earlier at the highest (2.5 mg/l) of NAA and root formation was delayed at the lowest (1.0 mg/l) of NAA.

#### **4.3.2. Number of roots per shoots**

Number of root/shoot was significantly higher in PGR treated shoot than PGR free cultured shoots. Number of roots was also significantly varied between PGR treatments with highest number (12.667) counted at 2.0 mg/l of NAA for variety C 86-165 and 9.0 for variety C 86-12 at ½ MS medium supplemented with 1.0 NAA (Table 3). This result can be complemented by a number of previous studies. For example, Behera *et al.* (2006) found highest number of roots per micro shoots ( $13.4 \pm 1.5$ ) on ½ MS medium supplemented 2.5 mg/l NAA for sugar cane

varieties B52-298. The above result contradicts to Dereje *et al.* (2015) reported that maximum root/shoot (17.8) on  $\frac{1}{2}$  MS medium supplemented with 5.0 mg/l NAA for C 86-12 sugar cane variety.

#### **4.3.3. Effect of growth hormones on root length**

Root length was significantly ( $p < 0.05$ ) affected by different concentrations of NAA supplemented to  $\frac{1}{2}$  MS medium for both varieties (Table 3). Variety C 86-165 produced maximum root length (5.0667 cm) on half strength MS media containing 2.0 NAA. But, variety C 86-12 produced maximum root length (4.7667 cm) on half strength MS media containing 1.0 mg/l NAA. In line with this, Belay (2016) reported root length of  $3.2 \pm 0.25$  cm when grown on  $\frac{1}{2}$  strength MS medium supplemented with 1.0 mg/l NAA alone for N14 sugarcane variety. Mangrio *et al.* (2009) obtained average root length of 2.5 cm on  $\frac{1}{2}$  MS media supplemented with 3.0 mg/l NAA for sugarcane Variety NCO-334. The effect of variations in the concentrations and combination of the same hormone in most of the cited literatures and in the present work is almost entirely due to variation in the varieties of sugarcane tested. That is why it is of paramount importance to optimize genotype specific *in vitro* propagation protocols for every variety.

Table 3: Effect of different concentrations of NAA on rooting responses of C 86-165 and C 86-12 sugarcane varieties. Values are means and n=3.

Sugarcane Varieties	Hormone(mg/l) NAA	No. of days to root emergence	No. of roots per shoot	Root Length(cm)	Root fresh Wt.per shoot	Root dry Wt.per shoot
<b>C 86-165</b>	0.0	—	—	—	—	—
	1.0	13.00 <sup>c</sup>	7.667 <sup>b</sup>	3.7667 <sup>b</sup>	0.8667 <sup>b</sup>	0.300 <sup>b</sup>
	1.5	15.333 <sup>b</sup>	9.000 <sup>b</sup>	3.833 <sup>b</sup>	1.00 <sup>ab</sup>	0.533 <sup>a</sup>
	2.0	18.000 <sup>a</sup>	12.667 <sup>a</sup>	5.0667 <sup>a</sup>	1.133 <sup>a</sup>	0.660 <sup>a</sup>
	2.5	18.333 <sup>a</sup>	12.000 <sup>a</sup>	3.700 <sup>b</sup>	1.122 <sup>a</sup>	0.5667 <sup>a</sup>
	Mean		12.8667	8.2667	3.1823	0.800
CV		9.4124	17.668	22.5452	15.4784	18.2574
LSD		2.2032	2.6572	1.3043	0.1627	0.1329
<b>C 86-12</b>	0.0	—	—	—	—	—
	1.0	16.000 <sup>a</sup>	9.000 <sup>a</sup>	4.7667 <sup>a</sup>	1.122 <sup>a</sup>	0.600 <sup>a</sup>
	1.5	17.333 <sup>a</sup>	5.000 <sup>c</sup>	4.500 <sup>ab</sup>	1.000 <sup>a</sup>	0.533 <sup>a</sup>
	2.0	16.333 <sup>a</sup>	7.000 <sup>b</sup>	3.333 <sup>b</sup>	1.000 <sup>a</sup>	0.333 <sup>ab</sup>
	2.5	13.667 <sup>a</sup>	5.667 <sup>bc</sup>	3.633 <sup>ab</sup>	1.000 <sup>b</sup>	0.5667 <sup>a</sup>
	Mean		12.667	5.333	3.2466	0.8267
CV		21.7643	15.3093	21.0710	19.7539	19.0475
LSD		5.0154	1.4854	1.2446	0.2271	0.1409

Means followed by the same letter within each variety are not significantly different at 5% significance level. No=number, Wt. =weight, CV=Coefficient of variance, LSD=Least significant different.

#### 4.3.4. Fresh and dry weights of roots

Means on fresh and dry weights of roots also varied significantly with different treatments where different concentrations of are supplemented to the ½MS shooting media (Table 3).The maximum fresh and dry weights of roots for var. C 86-12 were 1.133 g and 0.660 g, respectively on root culture containing ½ MS medium supplemented with 2.0 mg/l NAA. Whereas, for var. C 86-12 maximum fresh and dry weights of shoots were 1.122 g and 0.60 g, respectively when cultured on ½ MS medium supplemented with 1.0 mg/l NAA. Similar trend was also observed with regards to root fresh and dry weights in the works of Oliveira *et al.* (2009) who reported that mean fresh and dry weights of roots are the function of root number and length of the roots in all the treatments for NCO-334 and B52-298 varieties of sugarcane.

#### **4.4. Survival rate in green house during acclimatization**

*In vitro* induced shoots are very delicate and cannot resist sudden environmental changes that may damage the plantlets unless they are gradually adapted to the new environment. Thus, acclimatization is essential to enable the rooted plantlets to adapt the natural environment in *ex vitro* conditions at controlled temperature and humidity of greenhouse conditions. In the acclimatization stage of this experiment, a total of 90 and 75 well rooted plantlets for variety C 86-165 and C 86-12, respectively were transferred to greenhouse containing substrates namely, coco peat alone, that is a multipurpose growing medium made up of coconut husk; garden soil: sand: compost in the ration of 1:1:1 and garden soil and cow dung in the ration of 1:1. Then, the polystyrene tray was arranged in greenhouse with relative humidity (RH) gradually reducing from 90 to 60% and temperature of 30 to 31 °C for two weeks for primary acclimatization.

Generally the acclimatization phase of this experiment revealed that there was a difference in survival rate due to substrate nature and varietal difference. Both varieties had the highest survival value when grown on coco peat alone. On this media substrate, survival rate was 76.7% and 84% plantlet for C 86-165 and C 86-12 sugar cane varieties, respectively as shown (Table 4).

Table 4: Effect of different medium substrates on the survival of *in vitro* regenerated plantlets of the two varieties during acclimatization stage in green house.

<b>Types of sugarcane varieties</b>	<b>Types of culture medium</b>	<b>Total No of plantlet transferred</b>	<b>Survived plantlets</b>	<b>Percent of survived plantlets</b>
<b>C 86-165</b>	Coco peat only	30	23	76.7%
	Garden soil:sand:compost (1:1:1)	30	18	60%
	Garden soil: cow dung (1:1)	30	20	66.7%
<b>C 86-12</b>	Coco peat only	25	21	84%
	Garden soil:sand:compost (1:1:1)	25	15	60%
	Garden soil: cow dung(1:1)	25	16	64%

No=Number

## 5. SUMMARY, CONCLUSION AND RECOMMENDATION

### 5.1. Summary and Conclusion

Sugarcane, as a globally important industrial crop, is a main of sugar, ethanol, and other important by products. Hence, due consideration to the use of advanced technologies for sugarcane production is mandatory to obtain the unfolded benefits tapped from the crop. On the other hand, in Ethiopia, sugar industry is increasing at an alarming rate and is expected to play a significant role in poverty reduction. Thus, multiplication of sufficient quality of seeding material is needed more than ever before. However, in sugarcane seeding material multiplication usually takes up to 10 years following conventional method; besides the method allows continuation of diseases over vegetative cycles, which leads to drastic yield and quality reduction. To overcome the problem, *in vitro* propagation that enables rapid and large scale production of disease free planting material as being exercised with different crops is a prerequisite.

Based on this fact plant regeneration protocol was optimized in this study through direct organogenesis for two commercially important Cuban originated sugarcane varieties (C 86-165 and C 86-12) using apical meristem explants. Accordingly, the information below was obtained. For initiation stage of apical meristem explants and more initiated culture var. C 86-165 best perform on 1.0 mg/l BAP mg/l, while var. C 86-12 best perform on 0.5 mg/l of BAP. Shoot parameters were also highly influenced by varieties and the type and combinations of various growth regulators. The effect of varieties and hormones combinations was highly significant ( $p \leq 0.05$ ) on average values of shoot parameters. The highest number of initiated explants cultures regenerated more number of shoots and optimum shoot length were observed in var.C 86-165 on full MS media supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA, whereas maximum percentage of initiated explants cultures regenerated more number of shoots and optimum shoot length were recorded on 1.5 mg/l BAP + 0.5 mg/l NAA for C 86-12 variety. Roots regenerated 10 to 15 days after micro shoots were transferred to root induction media for both varieties. Highly significant ( $p \leq 0.05$ ) differences were observed among treatments and varieties were also highly significant ( $p \leq 0.05$ ). The highest percentage of shoots regenerated number of roots and root length were recorded for var.C 86-165 on half strength

MS media containing 2.0 mg/l NAA, whereas for *var.C 86-12* maximum percentage of number of root and root length was recorded on half strength MS media containing 1.0 mg/l NAA. As to the acclimatization response, relatively highest survivability percentages were recorded on coco peat media substrate as compared to the other medium substrates used. Besides from the two varieties C 86-12 survived higher percentage (84%) than C 86-165 (76.7%) on coco peat media.

The results clearly indicated the importance of evaluating individual variety to optimize a given tissue culture protocol. In other words, genotypic specificity was highly reflected in all of the parameters tested. Genotypic specificity has been reviewed in the literature review part of this paper and many researchers have imposed the evaluation of individual variety to recommend a tissue culture protocol.

## 5.2. Recommendation

Based on the above findings, the following recommendations are made for further investigation of *in vitro* culture of the two varieties.

- Further studies are needed using other hormones such as Kn, 2,4-D, IAA, IBA with different concentration and interaction effects for observing their ability to induce shoots and roots for the reproducibility of the protocol optimization through direct or in direct organogenesis.
- Factors causing contamination, low acclimatization, tissue dying, and phenol exudation in the study should be further investigated.
- It is necessary to study the performance and genetic stability of the *in vitro* regenerated seedlings after transplanting in the field necessary.
- To enhance the acclimatization rate of *in vitro* developed plantlets in the glasshouse, various methods have to be manipulated.
- To sum up, the present study has developed protocol optimization for *in vitro* micro propagation of new Cuban origin *Saccharum officinarum* L. varieties (C 86-165 and C 86-12) using apical meristem explants through direct organogenesis. Hence, it is beneficial to use this developed *in vitro* micro propagation protocol as a best follow up to large scale propagation to generate large number of seedlings in short period of time.

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## 7. APPENDICES

### 7.1. Appendix table -1 Characteristics of the two varieties

Variety name	Agronomic Features and General information
<b>C 86-165</b>	<p>-It is originated from Cuba and introduced to Ethiopia in 2006 at wenji Sugar Factory.</p> <p>-No problem on growing both light and heavy soils.</p> <p>-moderately tolerant to water logging.</p> <p>-sensitive to stress</p> <p>-Excellent rationing ability not bulged bud.</p> <p>- Moderately affected by Iron</p>
<b>C 86-12</b>	<p>-The variety was originated in Cuba and introduced to Ethiopia in 2006 at Wenji Factory</p> <p>-Perform best on heavy to moderately heavy and Moderately light soils.</p> <p>-Highly resistant to smut</p>

Source: Manual from Tigray Biotechnology Centre, 2013

**Appendix Table 2: Compositions of Murashige and Skoog Medium (1960)**

Compounds	Concentration (mg/l)
<b>Macronutrients</b>	
NH <sub>4</sub> NO <sub>3</sub>	1650
CaCl <sub>2</sub> .2H <sub>2</sub> O	440
MgSO <sub>4</sub> .7H <sub>2</sub> O	370
KNO <sub>3</sub>	1900
KH <sub>2</sub> PO <sub>4</sub>	170
<b>Micronutrients</b>	
H <sub>3</sub> BO <sub>3</sub>	6.2
NaEDTA.2H <sub>2</sub> O	37.30
MnSO <sub>4</sub> .4H <sub>2</sub> O	16.9
FeSO <sub>4</sub> .7H <sub>2</sub> O	27.8
ZnSO <sub>4</sub> .7H <sub>2</sub> O	8.6
KI	0.83
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.25
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.025
CuSO <sub>4</sub> .7H <sub>2</sub> O	0.025
<b>Vitamins</b>	
Myoinositol	100
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	1.0
Sugar	30000/60000
Agar	6000

Source: Murashige and Skoog (1962)

**Appendix Table 3: computed mean square of initiation parameters**

	Mean square	
	Name of sugar cane variety	
	C 86-165	C 86-12
Survived mean square	23.40	26.90
Error R <sup>2</sup>	0.80	0.7333
CV	19.444	16.9016
LSD	1.6272	1.5579

Mean square is significant at P<0.05 ANOVA, Fisher's least significant difference (LSD), CV= coefficient of variance, R<sup>2</sup>=Error mean square, LSD=least significant difference

**Appendix Table 4: computed mean square of the two varieties on shooting parameters**

Sugarcane varieties	Mean squares				
	No of days to shoot emergence	N of shoots Per explant	Shoot Length(cm)	Shoot fresh Weight per explant	Shoot dry Weight per explants
<b>C86-165</b>	162.10	53.7333	29.7157	0.2523	0.05316
Error R <sup>2</sup>	1.333	1.4667	0.6746	0.0080	0.0026
Mean	12.533	7.400	4.5733	0.5066	0.2267
CV	9.2130	16.3657	17.9602	17.6531	22.7823
LSD	2.1007	2.2032	1.4943	0.1627	0.094
<b>C 86-12</b>	179.100	44.0667	13.2283	0.2723	0.0658
Error R <sup>2</sup>	1.3333	1.0667	0.1326	0.0020	0.0011
Mean	13.1333	6.7333	3.333	0.5267	0.250
CV	10.0246	15.3385	11.037	8.4914	13.6626
LSD	2.3952	1.8789	0.663	0.0814	0.0621

Mean square is significant at P<0.05 ANOVA, Fisher's least significant difference (LSD), CV= coefficient of variance, R<sup>2</sup>= Error mean square, LSD=least significant difference.

**Appendix Table 5: computed mean square of the two varieties on rooting parameters**

Sugarcane Varieties	Mean squares				
	No. of days to root emergence	No. of roots Per explants	Root Length(cm)	Root fresh Wt.per explant	Root dry Wt.per explant
<b>C 86-165</b>	168.2667	76.900	11.0023	0.62667	0.1916
Error R <sup>2</sup>	1.4667	2.1333	0.2080	0.0155	0.0053
Mean	12.8667	8.2667	3.2733	0.800	0.400
CV	9.4124	17.6685	13.9329	15.4784	18.2574
LSD	2.2032	2.6572	0.8297	0.1627	0.1329
<b>C 86-12</b>	155.8333	33.667	10.9343	0.6506	0.1673
Error R <sup>2</sup>	7.600	0.6677	0.4680	0.0266	0.006
Mean	12.667	5.333	3.2466	0.8267	0.4066
CV	21.7643	15.3093	21.0710	19.7539	19.0475
LSD	5.0154	1.4854	1.2446	0.2271	0.1409

Mean square is significant at P<0.05 ANOVA, Fisher's least significant difference (LSD), CV= coefficient of variance, R<sup>2</sup>=Error mean square, LSD=least significant difference

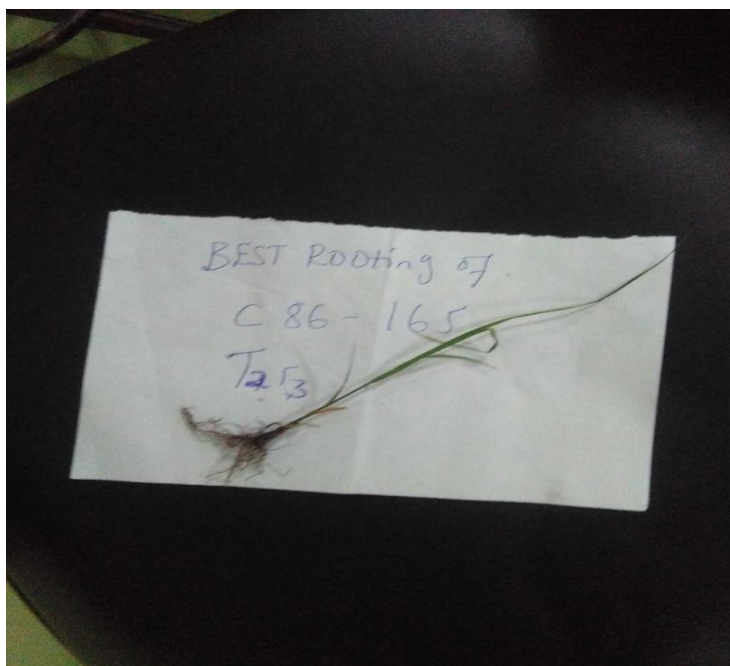
## APPENDIX OF FIGURES



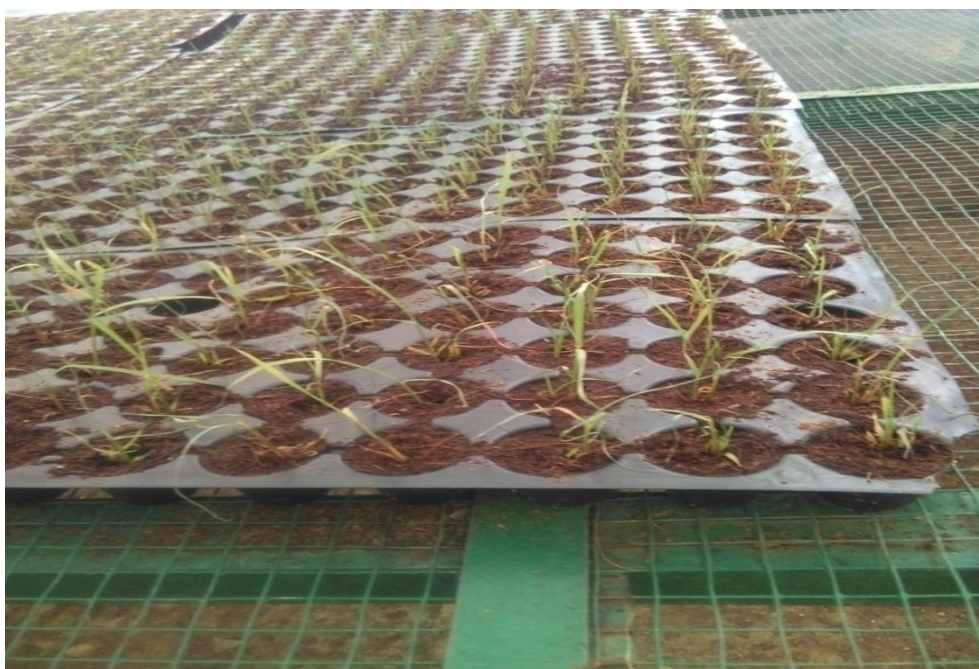
Appendix figure -1 Explant on initiation medium



Appendix figure -2 Best cultures of C 86-165 variety on shooting stage (MS +2.0 mg/l BAP + 0.5 NAA media)



Appendix-3 Best culture of C 86-165 variety on rooting stage (1/2 MS +2.0 NAA media)



Appendix-4 Acclimatization of plantlets on different types of medium.