IN VITRO PROPAGATION OF SUGAR CANE (*Saccharum Officinarum L. var. (Sp*-70) THROUGH APICAL MERISTEM

MSc. THESIS

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JUNE 2017
HARAMAYA UNIVERSITY, HARAMAYA

IN VITRO PROPAGATION OF SUGAR CANE (*Saccharum Officinarum L. VAR. (SP*-70) THROUGH APICAL MERISTEM

A Thesis Submitted to the, Department of Biology, College of Natural and Computational Sciences, Postgraduate Program Directorate
HARAMAYA UNIVERSITY

In Partial Fulfillment of the Requirements for the Degree of
MASTER OF SCIENCE IN BIOTECHNOLOGY

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JUNE 2017
Haramaya University, Haramaya

POSTGRADUATE PROGRAM DIRECTORATE

HARAMAYA UNIVERSITY

As research advisors, we hereby certify that we have read and evaluated this thesis prepared under our guidance by Guesh Desta entitled: "***In Vitro* Propagation of Sugar cane (*Saccharum Officinarum L var. (*Sp-70)**". We recommend that it be submitted as fulfilling the thesis requirement.

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DEDICATION

I dedicated this thesis to my mother, Abrhet Asemehegn, and my lovely aunt, Mtslal Asemehegn, who always pray and support me to complete this thesis work.

STATEMENT OF THE AUTHOR

I declare that this thesis entitled **'*In Vitro* Propagation of Sugar cane (*Saccharum Officinarum L. var. (Sp*-70)'** is my own 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 and is deposited at the University Library to be made available to borrowers under the rules of the Library. I solemnly declare that this thesis is not submitted to any other institutions anywhere for the award of any academic degree, diploma or certificate.

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my special gratitude and heartfelt thanks to the Almighty God for blessing my efforts, giving me the strength, determination and discipline to complete my study.

I would like to extend my sincere appreciation to my major advisor Dr. Meseret Chimdessa for his encouragement, constructive suggestions and moral support from the very beginning in executing the research work and in the write-up of the thesis. I take this opportunity to extend my special gratitude and indebtedness to my co-advisor Dr. Beyene Dumtsu for his inspiration, thoughtful guidance and suggestions which led me to the successful completion of this research work.

My sincere gratitude also goes to all laboratory staff of Tigray Biotechnology Research Center Laboratory in particular, Mr. Ykunoamlak Tesfahun, Mr. Mola Geremew, Miss. Selam Tewelde and Mr. Tsehaye who shared their research experience with me and hosted me in the Laboratory.

I also wish to express my special thanks to my friends, Kidane Welemariam, Haregot Fissha, Binyam Fissha, and Girmay Mekonen, who helped and shared information with me in finishing my thesis.

Last but not least, I acknowledge the financial assistance of Ministry of Education for my post graduate study and Haramaya University School of Graduate Studies and Department of Biology for providing me all of the required support.

ABBREVIATIONS AND ACRONYMS

AC	Activated charcoal
ANOVA	Analysis of Variance
BAP	6-benzyl aminopurine
CM	Coconut milk
FAO	Food and Agricultural organization
IBA	Indole -3-butyric acid
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
PGR	Plant growth regulator

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ABSTRACT

Ethiopia's sugar industry is increasing more than ever before and becoming one of the major pillars of the country's economy. Conventional propagation of sugar cane has very low rate of multiplication and harbors diseases. This study was conducted to develop micro-propagation techniques for rapid multiplication of disease-free sugarcane planting material through apical meristem explant. Sterile sugarcane variety (sp-70) was cultured on MS basal medium supplemented with BAP (0, 0.25. 0.5, 1.0, 2.0 mg/l), in combination with, IBA (0, 0.5, mg/l). For shoot initiation, explants were incubated for a week in dark. Thereafter, the initiated shoots were transferred to MS basal medium supplemented with 0, 0.5, 1, 2, 3mg/l of BAP combined with 0, 0.5, 1mg/L, IBA and incubated for a month in a growth room. For rooting, plantlets were transferred to half-strength MS medium with 0.5,1,2,3,4, mg/l of NAA. Acclimatization was done in greenhouse on substrate mix of autoclaved garden soil, farmyard manure and sand in 2:1:1 ratio. Analysis of variance showed that 0.5mg/l BAP resulted in higher shooting response than other treatments. Shoot multiplication was significantly higher when cultured on MS basal medium supplemented with 1mg/l BAP and 0.5mg/l IBA. In rooting better result was obtained in 1/2MS medium supplemented with 1mg/l NAA. The plantlets transferred to greenhouse showed a survival rate of 98% after 30 days. From the result of the study it can be concluded that for establishment of culture 0.5mg/l BAP, for shoot multiplication 1mg/l BAP and 0.5mg/l IBA, whereas, for rooting 1mg/l NAA respectively was the best hormonal concentration and combinations. Plantlets were survived 98% in green house in soil, farmyard manure and sand in 2:1:1ratio. Though this protocol can be used for efficient propagation of this sugar cane variety and further investigation on some factors that may hinder propagation effort should be done to enhance the success.

Keywords: auxin, cytokinins, in vitro, micro propagation sugar cane, tissue culture

1. INTRODUCTION

Sugar cane (*Saccharum officinarum.*) is a tall monocotyledonous perennial grass 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. Sugarcane is considered the world's most valuable crop estimated to worth US $ 143 billion (Mendoza, 2000). Sugarcane accounts for approximately 70% of the world's sugar and is an economically important cash crop in the tropical and sub-tropical regions of many countries (Chengalrayan and Gallomeagher, 2001).

At present sugarcane is grown as a commercial crop primarily in South America, North/Central America, Asia, Africa, Australia and the Pacific islands. Brazil was the largest producer with 719 million tons (FAO, 2013). Other countries which produce sugar from sugar cane include Guatemala, Vietnam, South Africa, Cuba, Egypt, El Salvador, Peru and Myanmar (FAO, 2013). *Saccharum* is an adaptable species and grows in a wide range of habitats and at various altitudes in the tropics through to temperate regions from latitude 8˚S to 40˚N extending across three geographical zones including in Kenya.

Sugarcane is one of the most widely grown crops in Ethiopia, even though the history is not well documented when it was introduced (Assefa, 2006). According to Tafesse and Haile Michael (2001), the Dutch company Handles Vereeniging Amsterdam (HVA) pioneered commercial cultivation of sugarcane in Wonji, Ethiopia in 1954/55.

Though Ethiopia started large scale commercial sugarcane production in 1954 at Wonji and in 1962 at Shoa (together named Wonji-Shoa), in 1969 at Metahara, and in 1998 at Finchaa (Tadesse and Ambachew, 2009), source of improved varieties has been totally dependent on importing of varieties from other countries to date. However, this option has not fully bridged the need of modern varieties in Ethiopia. As a result of lack of modern improved varieties and some other short comings, analysis of long years production data revealed that, cane productivity on two estates was declining by 0.18 t /ha at Metahara, and 0.6 t/ha at Wonji-Shoa (Nayamuth, 2010).

varieties and some other short comings, analysis of long years production data revealed that, cane productivity on two estates was declining by 0.18 t /ha at Metahara, and 0.6 t/ha at Wonji-Shoa (Nayamuth, 2010). Moreover, current sugar production of Ethiopia is below the national demand and this resulted in importing of over 150,000 tons of sugar per annum. On the contrary, in some countries the increase in cane yield is achieved using modern varieties (Nayamuth, 2010). In order to alleviate shortage of sugar in Ethiopia, the country is expanding the existing sugarcane plantations and establishing new sugar projects. Moreover, it has been importing modern varieties from different sources. In the latter case, increasing wide genetic variability will contribute towards providing option of baskets of improved varieties for specific or wide adaptation (Kakde, 1985).

Accordingly, ten modern sugarcane varieties were introduced from Cuba, passed through quarantine procedures at Worer Research Center, and tested against smut at Metahara Sugarcane Plantation and all of them exhibited resistance to smut (Abera and Leul, 2009). As with other plant species, sugarcane plants are propagated *in vitro* from meristems are considered to be more genetically and phenotypically stable than those produced from callus (Hendre *et al.*, 1983). Thus, considerable effort has been expended to investigate the adaptability of meristem culture to commercially grow elite sugarcane cultivars (Hendre *et al.*, 1983; Burner and Grisham, 1995). Rapid multiplication of disease-free sugarcane planting material through *in vitro* culture technology has been an important step towards quality seed production.

Plant growth regulators (PGR's) play an essential role in determining the development pathway of plant cells and tissues in culture medium. The auxins, cytokinins and recently gibberellins are most commonly used plant growth regulators for *in vitro* plant propagation. Micro propagation of sugarcane was not practiced in Ethiopia for commercial and breeding purposes. However, recently, there are some encouraging activities with regard to micro propagation of sugarcane. Ethiopian Sugar Corporation has given due attention to this technology and is expected to put in use in the coming years. It is also worth mentioning that, in Ethiopia, sugar industry is increasing at higher rate due to favorable government policy for sugar industry. This can be verified by the expansion of projects of the previous sugar estates

and/or factories, the establishment of other new sugar factories and participation of investors in the industry.

Therefore, developing an efficient propagation system for mass multiplication of sterile sugarcane planting material of selected variety is of paramount importance. It often requires testing of various types, concentrations and mixtures of the growth regulators during the development of a tissue culture protocol for a new plant tissue. Previously, there were attempts to micro propagate different sugar cane varieties using different concentrations and combinations of PGRs (Pawar *et al*., 2002; Baksha *et al*., 2002). However, there was no such trial done on variety sp-70. As there is variation between varieties in PGRs requirement for optimal micro- propagation, this study was designed to optimize *in vitro* micro- propagation protocol for mass propagation of sugarcane variety (sp-70).

General Objectives

- To optimize a standard protocol for *in vitro* micro propagation of sugar cane variety of SP -70

Specific Objectives:

- To determine optimum hormone combination and concentration for culture initiation
- To determine optimum hormone combination and concentration for shoot proliferation
- To determine optimum plant growth regulator concentration for rooting
- To evaluate degree of acclimatization of plantlets under greenhouse conditions

2. LITERATURE REVIEW

2.1. Origin Taxonomy and Distribution of Sugarcane

Commercial sugarcane hybrid cultivars have arisen through intensive selective breeding of species within the *Saccharum* genus, primarily involving crosses between *S. officinarum* and *S. spontaneum. Saccharum officinarum* accumulates very high levels of sucrose in the stem but is highly susceptible to diseases (Cox *et al*., 2000; 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 (Bull and Glasziou, 1979; Jackson, 2005).The origins of *S. officinarum* are intimately associated with the activities of humans, as *S. officinarum* is a purely cultivated or garden species which is not found in the wild (Sreenivasan *et al*., 1987).

The center of origin of *S. officinarum* is thought to be in Indonesia/New Guinea area (Daniels and Roach, 1987) where it has been grown as a garden crop since 8000 B.C. (Fauconnier, 1993). It has been proposed that *S. officinarum* evolved from the selection of sweet forms of *S. robustum*. Its cultivation spread along the human migration routes to Southeast Asia, India and the Pacific, hybridising with wild canes. It reached the Mediterranean around 500 B.C. (Fauconnier, 1993).From there it spread to Morocco, Egypt, Syria, Crete, Greece and Sicily, the main producers until the 15th Century, followed by introduction to West Africa and subsequently Central and South America and the West Indies (Fauconnier, 1993). It is thought to have reached Australia in 1788 on the First Fleet, but did not become established until after it was reintroduced in 1817 from Tahiti (Bull and Glasziou, 1979).

2.2. Morphological Characteristics of Sugar Cane

Sugarcane is a large tropical grass that produces multiple stems or culms each of which consists of a series of nodes separated by internodes. Following germination (sprouting of sett), the terminal vegetative bud of each shoot lays down a series of nodes. Each node consists of a growth ring or intercalary meristem, the root band (containing root primordia) and a bud above the leaf scar where the leaf sheath attaches, which delimits the node from the

internode below. The stem of sugarcane is similar to maize (corn) and sorghum in that it is filled with parenchyma cells and is not hollow like many grasses (Griffee, 2000). The stem is the major storage area for photosynthetic (sucrose) within the sugarcane plant, rather than fruit or seed structures. Leaves and internodes develop in a basipodial direction in that the leaf blade expands at the base then the internode elongates. As the stem develops, the leaves emerge, one leaf per node, attached at the base of the node, forming two alternate ranks on either side of the stem. Mature stems consist of a number of immature leaves still enclosed in the leaf spindle, a dozen or so green leaves and a number of senescent leaves, increasing in number with increasing age of the plant.

2.3. Conventional Propagation Methods and Its Limitations

Commercial sugarcane is propagated vegetatively and germination refers to the initiation of growth from buds present on the stems of the stools that remain in the soil after harvest of the previous crops. Either whole stalks or stalk's cut up in shorter segments called sets are used as planting material (Willcox *et al.,* 2000). The conventional seed cane multiplication method where stem cuttings with two to three buds are used as planting material has various limitations. The multiplication rate of sugarcane planting material is very slow (1:6 to 1:8). In addition, it requires large quantity of seed and land (Willcox *et al*., 2000).

Furthermore, the implements used for cutting seed cane preparation play a significant role in disease transmission. Besides the costly transport of the bulky cane cuttings, it harbors many pests and diseases with accumulation of diseases over vegetative cycles leading to further yield and quality decline over the years (Jalaja *et al*., 2008, Biradar *et al*., 2009) .On the other hand, decline in cane and sugar yield, increased cost of production, large plantation area under very few sugarcane varieties used over many years, lack of methods for fast commercialization of improved and adapted varieties, obsolation of productive commercial varieties due to disease, lack of alternative techniques for rejuvenation and disease cleansing of the old contaminated sugarcane varieties are the other challenges to attain the planed objectives using the conventional route of propagation (Tolera *et al.,*2014) . Therefore, application of plant tissue culture techniques provides an alternative method for the crop improvement (Sengar *et al.,* 2011). Plant tissue culture offers the best methodology through

micro propagation of sugarcane for quality and phytosanitary planting material at a faster rate in a shorter period of time.

2.4. Plant Regeneration by Tissue Culture Techniques

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 resultant 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 medium, 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, irrespective of the season and weather on a year round basis (Akin-Idowu *et al.*, 2009). Endangered, threatened and rare species have successfully been grown and conserved by micro propagation because of high coefficient of multiplication and small demands on number of initial plants and space.

2.5. Types of Plant Tissue Culture in Sugarcane

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).

2.5.1. Source of Explant

Hendre *et al*. (1983) reported that plants can be produced in six months from a single shoot tip. *In vitro* micro propagation provides for the rapid multiplication of sugarcane. By this technology nearly 10, 00,000 healthier, virus-free, sugarcane plantlets can be produced from single plant in one year. Micro propagation of sugarcane using two procedures (1) shoot tip culture, (2) indirect somatic embryogenesis from callus. 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). Explants from three sources, axillary bud, apical bud and shoot apex, were cultured. Severe bacterial contamination occurred in axillary buds resulting in necrosis and death of the explants.

By micro propagation over 1.5 million plants can be produced form a single shoot tip in six months (Anita *et al.*, 2000). Ali and Afghan (2001) reported micro propagation of sugarcane using meristem culture method and found that micro propagated plants were phenotypically similar to the mother plants. Noguera *et al*. (2003) described the micro propagation of sugarcane from shoot apex. Hoy *et al*. (2003) studied the effect of tissue culture explant source on sugarcane yield components.

2.5.2. Media sterilization

Prevention of contamination of tissue culture media is important for the whole process of plant propagation and helps to decrease the spread of plant parasites. Contamination of media could be controlled by adding antimicrobial agents, acidification or by filtration through microporous filters (Levin and Tanny, 2002). To reduce possibilities of contamination, it is recommended that sterilization rooms should have the least number of openings. Media preparation and sterilization are preferred to be performed in separate compartments. Sterilization area should also have walls and floor that withstand moisture, heat and steam (Ahloowali and Prakash, 2002). Sterilization of media is routinely achieved by autoclaving at

the temperature ranging from 115^{0} C– 135^{0} C. Advantages of autoclaving are: the method is quick and simple, whereas disadvantages are the media pH changes and some components may decompose and so lose their effectiveness. As example autoclaving mixtures of fructose, glucose and sucrose resulted in a drop in the agar gelling capacity and affecting pH of the culture medium through the formation of furfural derivatives due to sucrose hydrolysis (Torres, 1989). Filtration through microporous filters (0.22- 0.45 micrometer) is also used for thermos labile organic constituents such as vitamins, growth regulators and amino acids (Torres, 1989). Additives of antimicrobial agents are less commonly applied in plant tissue culture media. Limitation for their use was reported and attributed to harm imposed on plants as well (Savangikar, 2002).

2.6. Factors Affecting *In Vitro* Regeneration of Sugarcane

The tissue culture study of sugarcane was first initiated at Hawaiian Sugar Planter Association Experimental Station (Nickel, 1964). Since then a number of works have been undertaken. Here, a few attempts are made to review achievements, especially with regard to protocols developed and factors influencing in vitro performance of sugarcane. The major influence on tissue-culture response appears to be genetic, with culture a requirement varying between species and varieties is probably true to say that effect 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 which have a significant influence on the effects of applied regulators (George *et al*., 2008). Gill *et al*. (2003); Singh, (2003); Gandonou *et al*. (2005); Khan *et al*. (2006); Behera and Sahoo (2009); Biradar *et al*. (2009); and Khan *et al*. (2009) have reported that somatic embryogenesis and plant regeneration ability in sugarcane is genotype dependent. According to Khan *et al*. (2006) multiplication rate was higher in early and mid-maturing sugarcane clones than late maturing clones. 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 explant 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 explant 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 explant in culture, size and developmental phase of explants play a significant role in determining the organogenic potential of sugarcane leaf tissue in culture (Lakshmanan *et al*., 2006). When explants of leaf rolls were cultured with their proximal end in direct contact with the medium, only about 8% of them produced shoots. In contrast, almost 80% of the explants cultured with their distal end in direct contact with the medium regenerated shoots. In these explants shoot production was almost always restricted to the proximal end of the explants. Increasing the explant size resulted in a marked reduction in shoot formation in leaf tissue.

In cultures where 5–6 mm long leaf roll segments were used, only 33% of those grown with their distal end in contact with the medium produced shoots as compared to 80% for 1-2mm long leaf roll segment (Lakshmanan *et al*., 2006). Ali *et al*. (2007) obtained 90, 70, and 40% direct somatic embryogenesis from leaf, shoot apical meristem and pith explants, and 80, 70 and 60% indirect somatic embryogenesis and 80, 64, 56% in regeneration from somatic embryos respectively from the three explants of sugarcane. Lakshmanan *et al*. (2006) reported the best response of young leaf explants for direct somatic embryogenesis in sugarcane. Ali *et al*. (2010) concluded that among different explants used for callus induction, leaf proved better explant source for all genotypes producing maximum callus masses from young whorled leaf discs. Shoot apical meristem and nodal explants excreted more phenolic compounds in the culture medium compared to leaf explants in all genotypes they tested.

2.7. Media Composition of the Tissue Cultured Plants

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 medium should be accurately defined of inorganic and organic chemical additives so as to provide(I) the nutrients for the survival of the plant cells, tissues and organs under culture and ii) the optimal

physical condition of pH, osmotic pressure (George *et al*., 2008). Optimal growth and morphogenesis of tissues may vary for different plants according to their nutritional requirements. Moreover, tissues from different parts of plants may also have different requirements for satisfactory growth (Murashige and Skoog, 1962).

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. Besides, C, H and O, macro elements: nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and Sulphur (S) are essential elements in tissue culture media for satisfactory growth and morphogenesis of plant cell. Potassium ions are transported quickly across cell membranes and two of their major roles are regulating the pH and osmotic environment within cells (George *et al*., 2008).

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) (George, *et al*., 2008). Because of its precipitate ability, iron (Fe) is seldom added directly to the medium chelated with EDTA (ethylene di amine tetra acetic acid) so that it is more stable in culture and can be absorbed by plants over a wide pH range. Sugar, as energy source, is a very important part of nutrient medium, since most plant cultures are unable to photosynthesize effectively owing to inadequately developed cellular and tissue development, lack of chlorophyll, limited gas exchange and carbon dioxide in tissue culture vessels etc. Hence they lack autotrophic ability and need external carbon for energy (Doods and Roberts, 1985). The most preferred carbon or energy source is sucrose at a concentration of 20-60g/l. While autoclaving the medium, sucrose is hydrolyzed to glucose and fructose which are then used up for growth (Rai, 2007). Other mono or disaccharide and sugar alcohols like glucose, maltose, sorbitol etc. may be used depending upon plant species. The preferred carbohydrate in plant cell culture media is sucrose. Glucose and fructose may be substituted in some cases, glucose being as effective as sucrose and fructose being somewhat less effective (George *et al*., 2008). It was observed that sucrose concentration affects the root induction in sugarcane. At low concentration, size and number of roots were very low as compared to high concentration of sucrose (Thrope, 1995). Vitamins are required by plants as catalysts in various metabolic

processes. When plant cells and tissues are grown in vitro, some vitamins may become limiting factors for cell growth (Doods and Roberts, 1985). Vitamins, in combination with other media constituents, have been shown to have direct and indirect effects on callus growth, somatic growth, rooting, and embryonic development (Abrahamian and Kantharajah, 2011). The vitamins most frequently used in cell and tissue culture media include thiamin (B1), nicotinic acid, and pyridoxine (B6). Thiamin is required by all cells for growth. Nicotinic acid and pyridoxine are often added to culture media but are not essential for cell growth in many species. Mayo-inositol, a carbohydrate not a vitamin, is commonly included in many vitamin stock solutions. Its presence in the culture medium is not essential, but in small quantities mayo-inositol stimulates cell growth in most species (George *et al*., 2008).

Myoinositol has a diverse biological role and participates in several cellular processes, including signal transduction, stress response, cell wall biogenesis, growth regulation, osmo-tolerance, IAA metabolism, membrane trafficking. Addition of amino acids to media is important for stimulating cell growth in protoplast cultures and also in inducing and maintaining somatic embryogenesis. This reduced organic nitrogen is more readily taken up by plants than the inorganic nitrogen (Rai, 2007). L glutamine, L-asparagine, L-cysteine, L-glycine are commonly used amino acids which are added to the culture medium in form of mixtures as individually they inhibit cell growth (Molnár *et al*., 2011).

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 bio chemical reactions occurring in plant tissue cultures by altering pH of the medium. 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. 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 react with media constituents nor digested by plant enzymes (Cameron, 2006). Although the basic nutritional

requirement of *in vitro* cultured plant cells are very similar to those utilized by plants, the nutritional composition varies depending on the type of cells, tissues, organs, protoplasts and the plant species and the genotype of the same species. A nutritional medium is defined by its composition of mineral salts, carbon source, vitamins, PGR and other organic supplements. Several media formulations are commonly used for the majority of all cell and tissue culture work. These media formulations include those described by White, Murashige and Skoog, Schenk and Hilderbrandt, Nitsch and Nitsch, and Lloyd and McCown. MS medium, Schenk and Hildebrand's (SH) medium, and Gamborg's B-5 medium are all high in macronutrients, while the other media formulations contain considerably less of the macronutrients (Anonymous, 2003).

Among the above media, the MS medium is the most commonly used plant growth medium used in for cultivation of plant cell culture (Stafford and Warren, 1996). MS medium has been employed for shoot initiation in micro propagation with its full strength, which means mineral salts, carbon source, vitamins, and other organic supplements are added to the media with the amount indicated on MS media formulation. However, this amount is halved (half strength), in most of the cases, for root initiation. Gurel and Wren (1995) realized a direct relationship between the rate of cell division and energy (sucrose) consumption in the cultured tissues.

2.8. Use of Plant Growth Regulators in *In Vitro* Culture of Sugar Cane

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 Hüner, 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.

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 stability (Hopkins and Hüner, 2009). 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. 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 or amino acids) have profound effects on the morphology of the tissues that grow from the initial explants.

In tissue culture plant growth regulators act alone and in interaction to produce the final effect (George *et al*., 2008). Plant growth regulators (PGRs) are compounds which, at very low concentration, are capable of modifying growth or plant morphogenesis. Plant growth regulators (PGRs) which are most commonly used in plant tissue cultures are auxins, cytokinins and gibberellins (George *et al*.,2008).The auxins commonly used in plant tissue culture media are 1H-indole-3-acetic acid (IAA, naturally occurring), 1H-indole-3-butyric acid (IBA), 2,4-dichlorophenoxy acetic acid (2,4-D), and 1-napthalene acetic acid (NAA). 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 added to culture medium to promote cell division, stimulation of DNA synthesis to yield shoot formation and auxiliary shoot proliferation, can delay senesce and sometimes to inhibit root formation (George *et al*., 2008).

The irreversible degradation of cytokinins, catalyzed by cytokinin oxidase, is an important mechanism by which plants modulate their cytokinin levels (Brugiere *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), IBA etc. 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). During media preparation about 40% of IAA and 20% of IBA in MS was destroyed in 20 minute upon autoclaving (Nissen and Sutter, 1990). IBA has other advantage over some other synthetic auxins, i.e., IBA is metabolized to IAA, which is the natural auxin (Strader and Bartel, 2011).

The cytokines commonly used in the culture media include 6-benzylaminopurine (BAP), 6-γ-γ-dimethylaminopurine (2iP), N-(2-furanylmethyl)-1H-puring-6-amine(kinetin), and 6-(4-hydroxy-3-mehty-trans-2-butenylaminopurine (zeatin). Zeatin and 2iP are considered to be naturally occurring cytokinins; while BAP and kinetin are synthetically derived cytokinins. The effect of different levels of PGRs on *in vitro* propagation of sugarcane has been investigated. According to Behera and Sahoo, (2009), the highest percentage of callus induction was observed in Murashige and skoog (MS) medium supplemented with 2.5 mg/l, 2-4 D. The best response in terms of multiple shoot induction was observed on MS medium with BAP 2.0 mg/l + NAA 0.5 mg/l. When *in vitro* shootlets were inoculated on to the half-strength MS basal media supplemented with 3.0 mg/l NAA, rooting was more profuse with acclimatization response of 90%. Ather *et al. (*2009) proposed protocol for the micro propagation of sugarcane cultivar Thatta-10.

They initiated plantlets in which 100% calli were produced using 2, 4-D in the concentration of 3.0 mg/l with MS medium. BAP in the concentration of 1.0 mg/l was found best in terms of highest number of shoot regeneration. Maximum shoot elongation along with the highest number of root emergence was observed on the medium composed of MS with GA3 in the concentration of 3.0 mg/l. Optimum shoot length (8 mm) was obtained on MS medium containing 1.0 mg/l GA3, 0.5 mg/l Kin. Maximum number of roots (3.6) with maximum length (3.5 mm) was obtained at 1.0 mg/l IBA (Rashid *et al.*, 2009). Tarique *et al. (*2010) observed best results from the varieties Isd36 and Isd37 at 4.0 mg/l of 2, 4-D and the variety Isd16 at 3.0 mg/l of 2, 4-D for callus induction. 1.0 mg/l BAP + 0.5 mg/l NAA showed the best result for induction and multiplication of shoot. Best result of root formation was observed on MS medium supplemented with 5.0 mg/l of NAA. The variety Isd37 produced

the highest number of roots (13.47) at 5.0 mg/l of NAA. The plantlets were successfully transferred to soil with 85% survivability at normal temperature with 85% humidity. MS medium supplemented with auxin alone (3 to 4mg/l 2, 4-D) induces callus formation (3mg/l 2, 4-D alone produced embryogenic calli) and auxin-cytokine combination like 2, 4-D and IAA (both in 1mg/ l and 2mg/l concentration) with BAP (1mg/l) found very effective for somatic embryonic induction. While optimizing callus induction protocol, Ali *et al. (*2010) reported that application of 2.5 and 3mg/ l 2, 4-D produced genotype independent callus initiation as well as proliferation. Adding 3mg/l 2, 4-D and 1 mg/ l Kinetin produced maximum mass of calli.

However, most of the calli were friable and non-embryogenic. Adding 1.5mg/l of 2, 4-D is the best for shoot induction. Roots developed more conveniently on MS medium supplemented with NAA at 4-5 mg/ l along with 500 mg/l casein hydrolysate. In another study, the best callus induction was observed at 3.0mg/l, 2, 4-D with 10% coconut milk (CM). Best regeneration of shoot was achieved when they were cultured on MS medium supplemented with 1.0mg/l BAP and 0.5mg/l IBA. Among the different media tested with 3mg/l NAA and 5% sucrose supplemented media proved best production of roots (Gopitha *et al*., 2010).

2.9. Micro Propagation of Sugar Cane

The micro propagation technology has a vast potential to produce plants of superior quality, isolation of useful variants in well-adapted high yielding genotypes with better disease resistance and stress tolerance capacities (Brown and Thorpe, 1995). Certain type of callus cultures give rise to clones that have inheritable characteristics different from those of parent plants due to the possibility of occurrence of soma clonal variability (George, 1993), which leads to the development of commercially important improved varieties. Commercial production of plants through micro propagation techniques has several advantages over the traditional methods of propagation through seed, cutting, grafting and air-layering etc. It is rapid propagation processes that can lead to the production of plants virus free (Garcia-Gonzales *et al*., 2010). Micro propagation is currently the only realistic 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 (Feldmenn *et al*., 1994; Lee, 1987; Lorenzo *et al*., 2001; Krisnamurthi and Tlaskal, 1974). As a result of which plant regeneration through tissue culture technique would be a viable alternative for improving the quality and productivity in sugarcane. There are reports on tissue culture of sugarcane from different countries but the first attempts to regenerate plants through *in vitro* technique were made on sugarcane by (Naz, 2003; Heniz and Mee, 1969).

2.9.1. Shoot Proliferation and Multiplication

Shoot induction and multiplication is one of the most crucial stages of micro propagation method which is recognized by the formation, growth and proliferation of adventitious shoots from the primary explants. In the shoot proliferation stage, it consists of the establishment *in vitro* of suitable pieces of tissue, free from obvious contamination, whereas in the multiplication stage, each explant has expanded into a cluster of micro shoots (George *et al*., 2008). 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 brown tip, 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 (Pasqual *et al*., 2014). Frequency of shoot formation and further development were greatly influenced by the presence of auxins and cytokinin in the medium (Gopitha *et al*., 2010).

2.9.2. Root induction

Induction of roots in excised shoots and subsequent survival of plantlets in the soil are the most crucial steps for success of any micro propagation protocol. Root induction is the stage prior to acclimation 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). Adventitious and axillary shoots developed in cultures in the presence of a cytokinin generally lack roots. To obtain full

plants the shoots must be transferred to a rooting medium which is different from the shoot multiplication medium, particularly in its hormonal and salt composition. The number of shoot multiplication cycles after which the rooting exercise is to be started is governed by the number of plants to be produced through micro propagation and the available nursery facilities.

But this should not be open endlessly because the true to type production of *in vitro* plantlets decline as the number of shooting cycle increase tremendously. The role of auxins in root development is well established and has been reviewed by Scott (1972).Whilst root induction of any plant tissue is dependable on the proper amount and combination of rooting hormones (Doods and Roberts, 1985), the quality of plantlets in rooting stage is influenced by the type of growth hormone used and the concentration of salts in the medium. For *in vitro* rooting, nutrient salts in the medium have a dramatic effect both on rooting percentage and root number per micro cutting. Often, where shoot multiplication was induced on full-strength MS medium, the salt concentration was reduced to half. The promoting effect of diluted mineral salt solution on rooting is probably due to reduced nitrogen level.

The amount of sugar in the rooting medium kept equal with that of multiplication media. This may be due to the fact that sucrose play major role in differentiation of good vascular tissues and accelerates lignification in addition to the nutritional role. 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 gets metabolized (Woodward and Bartel, 2005).

Therefore, reports of different authors for various sugarcane species show that successful root induction and root number depends upon the source of the explants, optimum concentration of growth regulators, and selection of appropriate size of shoot propagule. Usually there is

sufficient residual cytokinin in the shoots, thus little or no cytokinin is required for root induction (Singh *et al*., 2013). Even though, the rooting percentage was varying, depend on the species and propagule size various optimum auxin concentrations' were obtained for successful in vitro rooting of different sugarcane species. In most of the cases roots are induced on *in vitro* regenerated shoots on auxin enriched half strength MS medium (Singh *et al*., 2012).

2.9.3. Acclimatization of *In Vitro* Propagates to *In Vivo* Condition

The success of any in vitro regeneration protocol largely depends on the survival and growth performance of micro propagated plantlets which in turn depends on the proper acclimatization (hardening) procedures. The process of gradual adaptation of tissue culture raised plants to greenhouse or field conditions is called acclimatization or hardening off. In vitro raised plants usually lack well developed structures of roots like root hair, well developed vascular system. These roots frequently die or collapse after the plantlets are removed from cultures and new, functional lateral and adventitious roots are formed during acclimatization. Once plantlets are generated by tissue culture, they have to be transferred to the greenhouse or field (Cardoza, 2008). During *in vitro* culture, plantlets grow under very special conditions in relatively higher air humidity, limited inflow of CO2 and outflow of gaseous, higher saccharides as carbon and energy sources.

These special conditions during *in vitro* culture result in the formation of plantlets of abnormal morphology, anatomy and physiology (Pospisilova *et al*., 1999). The plants multiplied *in vitro* are exposed to a unique set of growth conditions (high levels of inorganic and organic nutrients and growth regulators, sucrose as carbon source, high humidity, and low light and poor gaseous exchange) which may support rapid growth and multiplication. Therefore, gradual acclimatization is necessary for these plants to enhance the survival transition from culture vials to the greenhouse and then to field condition. After *ex vitro* transfer, these plantlets might easily be impaired by sudden changes in environmental conditions (Lesar *et al*., 2012). Therefore, after *ex vitro* transplantation plants usually need some period of acclimatization with gradual lowering in air humidity (Bolar *et al*., 1998).

2.10. Problems Associated With *In Vitro* Propagation of Sugarcane

Despite several advantages of applying micro propagation in sugarcane such as quick spread of newly released varieties (Jalaja, *et al*., 2008), rejuvenation of old deteriorated varieties; easy transportation of seed material; high cane productivity and sugar yield etc., this technique is not gaining popularity up to the desired extent due to the various constraints. Contamination of cultures by microbes is a severe problem that not only reduces the frequency of shoot culture initiation from the source explants but also the total number of shoots produced at various cycles due to loss of cultures (Lal *et al*., 2014). Further, young meristematic tissues of sugarcane contain high levels of phenolic substances which are enormously released in the medium during culture initiation.

The phenolic in oxidized form generally cause mortality of the explants probably by inducing tissue oxidation, retard tissue growth or even promote abnormal pattern of cell development (Ishaq and Ehirim, 2011). Vitrification also termed as vitricence, glassiness or hyper hydration has been identified as a physiological disorder of shoot cultures (Constantine, 1986). Phenolic compounds and oxidative enzymes can be generated during normal plant metabolic processes, and the concentration will be increased in response to abiotic stresses. Moreover, the inevitably mechanical damage during explants preparation is thought to be a key factor attributed to browning during callus induction. Accumulation and oxidation of phenolic compounds thought to be the main reason for inhibition of growth and a decrease in the regeneration ability of plant cells.

2.11. The Role of Activated Charcoal in Sugarcane Propagation

Previous studies on sugarcane shoot tip tissue culture browning mainly focused on the harmful effects and controlling strategies. However, there is little report on factors and cellular structure changes related to browning in sugarcane shoot tip culture. The use of activated charcoal can make a major difference in the success or failure of a given tissue culture attempt (Pan and van Staden, 1998). Activated charcoal (AC) is composed of carbon arranged in a quasigraphitic form in small particle size. It is a porous and tasteless material and is distinguished from elementary carbon by removal of all non-carbon impurities and the

oxidation of carbon surface (Budavari, 1996). Activated charcoal is often used in plant tissue culture to improve cell growth and development (Pan and van Staden, 1998).

The addition of AC to both liquid and semi-solid media is a recognized practice and its influence in growth and development may be attributed mainly to the adsorption of inhibitory substances in the culture medium. The difficulty in using AC in medium is that in addition to adsorbing unwanted substances, it may adsorb needed hormones (Fridborg *et al*., 1978). The most crucial impact of adding AC to the culture media is a drastic dip in concentration of PGRs and other organic supplements. This is due to the adsorption of these chemicals by AC (Ebert and Taylor, 1990). The addition of AC to the culture medium significantly reduced the discoloration of embryo explants and the culture media.

3. MATERIALS AND METHODS

3.1. Description of the Study Area

This study was carried out in Plant tissue culture laboratory of Tigray Biotechnology Center (TBC) in Mekelle. Mekelle is the capital city and commercial center of the Tigray National Regional State in the northern Ethiopia. The town is located at $39^0$33'E longitude and $13^0$32'N latitude, situated in the extension of the central highlands of Ethiopia. The altitude of Mekelle is between 1965 m and 2220m above sea level. The town is bounded by mountain ranges in the east and north. Climatologically, the area is classified as "Woina Dega" (temperate) with an effective temperature between 14^{0C} and 20^{0C} (Ethiopian Mapping Agency, EMA, 1981), which for most of the time is comfortable. It has a moisture index (P/ET) ranging in between 0.25 and 0.5, which indicates moderately dry area. The altitude varies from 2220 m at eastern side to 1965 m in the northwestern side of the town (lower reach of Illala River) (Gebremedhin Berhane, 2002).

3.2. Plant Materials and Explant Preparation

The sugarcane (variety SP-70) grown at Wonji Corporation was used for this experiment. After the explant separated from the mother plant it was disinfected or sterilized by spraying chemicals while it was in the field. The explant was then sterilized in autoclave for about 50^{oc} for 2 hours to remove any contaminants like bacteria and fungi. After that it was planted in coco peat for about 2-4 weeks to sprout buds for initiation and the upper part of the explant was removed then, apical meristem buds were excised to1- 2cm length by 0.25cm diameter from the explant. The excised buds of the explant was then washed thoroughly with tap water and soap solution to remove all the traces of dust particles and it was further washed in sterile water for 10 minutes and immersed in70 % alcohol for one minute and 30 seconds followed by another treatment in 0.1% (w/v) mercuric chloride (HgCl2) for another 5 minutes. Finally, the bud cuttings were washed thoroughly 3 to 5 times with sterile distilled water before inoculation in to sterilized media pre-packed in culture tubes. All the above operations were performed under aseptic conditions in laminar airflow cabinet.

3.3. Culture Medium Preparation

Full strength Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) was used as a culture medium. Culture medium was prepared by combining 30g/l sucrose and 5-6 g/l agar as a gelling agent to the MS basal medium. The pH of the medium was adjusted to 5.8 using 1 N KOH and 1 N HCl before being gelled and autoclaved at 121^{oC}, 15 psi for 20 minutes. While molten 40 ml of the medium, it was dispensed into glass culture jar for culturing and stored under aseptic condition until use for culturing.

3.4. Culture Initiation

After sterilization, the explant was unfurled (open out) to remove the part which is damaged by the sterilant till the size of the explant is approximately pencil size (2cm).There were seven treatment, and in each treatment eight bottles were prepared, one explant was inoculated per bottle containing MS basal medium supplemented with BAP (0, 0.25. 0.5, 1.0, 2.0 mg/l), in combination with, IBA (0, 0.5, mg/l). MS medium without plant growth regulator was used as control. Cultures were incubated in a dark for three weeks. All treatments were done in a completely randomized design in three replications.

3.5. Shoot Induction and Regeneration

For shoot induction and multiplication, ten treatments with eight bottles containing one explant per bottle was transferred to MS basal medium containing 30g/l sucrose as a carbon source and 5-6 g/l agar as a gelling agent supplemented with 0, 0.5, 1, 2, 3 mg/l of BAP combined with 0, 0.5, 1mg/l IBA. All the cultures were incubated in a growth room with a 16h: 8h photoperiod (2000-3000 Luxes) and the temperature was maintained at 25 ± 2^{oC} with 70-80% relative humidity in the culture room. MS medium without PGR was considered as control. All treatments were done in a completely random design in three replications.

3.6. Rooting

For rooting, a total of six treatment having eight bottles containing one explant per each bottles was transferred to rooting media. Elongated micro shoots having three to four leaves

and measuring about 5-6 cm in length were excised from culture tube and aseptically transferred to half-strength (1/2 MS) medium having 30 g/l sucrose, and 5-6 g/l agar supplemented with (0,0.5,1,2,3,4, mg/l) of NAA. The control was without NAA. The culture tube was capped carefully, labeled properly and maintained in a growth chamber for four weeks at a temperature of 25 ± 2^0C and 16:8h hour photoperiod provided by white florescent tube to observe rooting parameters. The rooting experiment was done in completely randomized design in three replications.

3.7. Acclimatization

During acclimatization a total of 120 plantlets was transferred to green house from the six treatments that were in the rooting culture media and each treatment were contain 24 plantlets after sub culturing. After 30 days of culture on rooting media, the rooted plantlets were taken out of the culture bottles using forceps with extreme care to avoid any mechanical damage to the plantlets and root system. They were thoroughly washed under running tap water to remove any traces of agar that prevent the absorption of nutrients from the acclimatization culture substrates by roots. The plantlets were carefully planted in plastic pots filled with autoclaved garden soil, farmyard manure and sand (2:1:1). Holes were made by inserting a sterile stick in the middle of the potting mix and the roots was gently inserted in the hole. The pots were covered with transparent plastic bags (in order to keep humidity) at the first two weeks with random holes for air circulation. This provides for increasing the survival rate of micro propagated plants.

The plantlet were thoroughly watered and kept in poly house under humidity of 80-90 in the first week, 70-80 for the second week, 60-70 for the third week and 50-60 % for the fourth 4 weeks. The temperature was also adjusted at 25 ± 2^0c, 26 ± 2^0c, 27 ± 2^0c and 27 ± 2^0c for the first, second, third and fourth week respectively. Plastic cover was removed partially after a week and completely after two weeks. Finally, after four weeks percent of plantlets successfully hardened were calculated by the number of survived shoot lets and died shoot lets from the total transferred plantlets.

3.8. Data Collected

The following parameters were recorded from the experiments:

Percent of initiated explants: The percentage of initiated explants was calculated and recorded in each replication from each treatment.

Shoot response: The response of each shoot explant was calculated by subtracting responsive explants in the shooting media from the total transferred explants from initiation culture media.

Shoot number: The number of micro shoots in each treatment from each replication was counted and recorded.

Shoot length: length of each initiated/proliferated shoot was measured in centimeters from base to the tip using ruler (cm) after four weeks of culturing.

Root response: The responsive explants were calculated by subtracting the number of responsive explants in rooting media from the total transferred explants from shooting media.

Root length: The length of responsive roots was measured using cm after four weeks.

Root number: Number of roots was counted from individual treatments in each replication after four week of culturing.

Survival rate of plantlets: The rate of survival of plantlets was evaluated after transferring to green house from each treatment in each replication and changed in to percentage.

3.9. Data Analysis

Data was subjected to analysis of variance (ANOVA) using statistical package SAS. Difference between treatments was considered significant at $p<0.05$.

4. RESULTS AND DISCUSSION

4.1. Initiation/Culture Establishment

Shoot tip of sugar cane variety Sp-70 were excised aseptically and cultured on MS nutrient medium supplemented with different concentrations and combinations of cytokinins and auxins for culture establishment. The percent establishment varied between different concentration of cytokinine and auxin hormones. Explant viability (responsiveness) were the most relevant criteria for determining the best combination and concentration of phyto hormones. As indicated in(appendix.3), the number of viable explants recorded in each replication at individual treatment was varied from treatment to treatment and highest rate of viable explants with mean percentage of (83.33$\pm$7.22%) was observed on MS media supplemented with 0.5mg/l BAP (Table-1). Explants inoculated in MS medium with no growth hormone shows the lowest mean percentage of responsiveness (16.67$\pm$7.22%) (Table.1). All viable explants were able to proliferate shoots in both control and cytokinin supplemented MS medium. However the initiation percentage and the mean responsiveness of the plantlets were found to be different in different combinations and concentrations of cytokinin as indicated in (Table.1.).

Even though the explants inoculated in MS medium without growth hormones shows a response to some extent this may be due to its endogenous hormone since plants have naturally produced growth hormones but the plantlets which give some response were not morphologically good and they were inactive in time of development. The plantlets generated from the media without growth hormone were not deep green in their appearance and their multiplication rate was low when compared with plantlets grown in medium supplemented with cytokinin. In addition to this, the plantlets observed in this hormone free media plantlets were not vigorous enough to multiply in the next stage of the micro propagation process. This indicates the application of plant growth hormones was necessary to induce the rate of proliferation of sugarcane in tissue culture. The result of this study agrees with the study of Anon (1985) who indicated that normal shoot initiation and development was obtained in two cultivars of sugarcane CO-62171 and CO-7201 with BAP at 0.5 mg/l only. But according to Dhumale *et al*. (1994) the highest rate of shoot initiation was recorded with BAP at 3 mg/l

and NAA at 1 mg/l. Biradar *et al.* (2008) also reported that auxiliary bud is the most suitable explant for initiation with MS medium containing BAP at 2 mg/L concentration. As it is observed from the table for initiation of culture or culture establishment the highest response of explants was recorded when the explant is treated with cytokines only as indicated in treatment two (T2) (Table.1) and when the concentration of cytokinin is higher than the concentration of auxins in the MS media this is also shown in treatment four (T4) (Table.1.).In such cases higher cytokines levels have been used, whereas, normal development of the shoot from the bud meristem might require very low levels of the growth regulators, as in case of (Sreenivasan and Jalaja, 1983).

The rate of multiplication is high in adventitious shoots and somatic embryogenesis but the occurrence of soma clonal variation among the plantlets is probably due to incipient callus is a drawback that's why the present study were not used the same concentration of cytokinin and auxin during shoot initiation. In order to get true to type plantlets, the present study used axillary buds through meristem shoot tip culture. The reason might be that shoot tip is much safer and fast growing portion of the plant and the result of this study were effective for optimizing culture initiation and producing high viable explants during this experiment.

Table 1: Effect of Auxin and Cytokine (mean + SD) in Establishment of Culture

Treatment code	Hormone combination		Mean of responsive explant	Mean percentage of responsive explant
	BAP	IBA		
T0	0	0	1.33 ± 0.58^{e}	16.67 ± 7.22^{e}
T1	0.25	0	$2.33\pm0.58a^{cd}$	29.17 ± 7.22^{cd}
T2	0.5	0	6.67 ± 0.58^{a}	83.33 ± 7.22^{a}
T3	1	0	3.00 ± 000^{c}	37.5 ± 000^{c}
T4	1	0.5	5.33 ± 0.58^{b}	66.67 ± 7.22^{b}
T5	2	0	1.67 ± 0.58^{de}	20.83 ± 7.22^{de}
T6	2	0.5	3.33 ± 0.58^{f}	41.67 ± 7.22^{fc}

Means followed by the same letter with in a column are not significantly different at 5% significance level.

4.2. Shoot Multiplication

The result of the different combinations and concentrations of BAP (0, 0.5, 1, 2, 3 mg/l) and IBA (0.5, 1 mg/l) on the shoot multiplication is presented in Table 2.Various concentrations of cytokine (BAP) and auxins, (IBA) were used in different concentration and combinations for shoot regeneration. As it observed from results presented in (Appendix table. 4), the number of viable explant recorded, the number of shoots counted and the measured shoot length was different in each treatment in each replications. During this investigation shoot formation was highly influenced by concentrations and type of the growth regulators used in the experiment. Among different concentrations and combinations for shoot multiplication, best performance was observed when explants were cultured on MS media supplemented with 1mg/l BAP and 0.5mg/l IBA (Table-2).At this combination the mean percentage of explant that produced shoots was 91.67%. In the MS medium supplemented with 1mg/l BAP and 0.5mg/l IBA the highest mean responsiveness of the explants was 7.33±0.57(Table.2)

In relation to this the responsiveness of the explants obtained in this study, it was also observed that deep green appearance of the plantlets, elongated stem, established basement of the plantlets as well as low level of phenolic compounds. Generally better shoot regeneration was obtained in MS medium with lower auxin concentration and this result is in agreement with that of Kharinarain *et al*. (1996); who obtained maximum shooting by reducing auxin concentration in MS medium. Alam *et al.* (2003) also obtained good regeneration on MS medium containing low auxin and high cytokinins. In fact, cytokinins are capable of overcoming apical dominance and release lateral buds from dormancy thereby enhance shoot multiplication (George *et al*., 2008).

The positive effect of this result indicates the significance of adding the two growth regulators in combination rather than alone in shoot multiplication medium. Many authors also reported that, different combination and concentration of cytokinin and auxin hormones were used in MS medium for multiple shoot regeneration from shoot tip culture of various sugar cane varieties. For example, Islam *et al*. (1982) reported the positive effects of BAP+IBA combination on shoot formation in sugarcane and Yutaka *et al.* (1998) reported that combination of phytohormones often determine the course of morphogenesis, i.e., shoot

organogenesis and embryogenesis. Shoot regeneration potential of shoot tip culture directly influenced by the concentration and type of cytokinin and auxin used. High Cytokinin to auxin ratio favors shoot regeneration in *in vitro* culture. In fact, cytokinins are capable of overcoming apical dominance and release lateral buds from dormancy thereby enhance shoot multiplication (George *et al*., 2008). The result found from this study opposes by the findings of Ali *et al*. (2008) who achieved best shoot multiplication for sugarcane genotype BL-4 on MS medium amended with 0.50 mg/l BAP + 0.25 mg/l kinetin. But some authors like Tarique *et al*. (2010) found the same result with this study in MS media supplemented with 1.0 mg/l BAP + 0.5 mg/l IBA and these concentration were best regarding regeneration of shoot from the explant. Khan *et al*. (2009) also observed maximum shoot multiplication on MS medium augmented with 1 mg/l BAP + 0.1 Kinetin and 1 mg/l BAP + 0.5 Kinetin in sugarcane genotype CPF-237 and HSF-240, respectively.

Apart from the explant responsiveness the number of shoots generated from the inoculated explants was also affected by the concentrations and the growth regulators applied in the MS medium during shoot multiplication stage. The result of this finding indicates the highest number of shoots produced from the inoculated explants was found in the MS medium supplemented with 1mg/l BAP and 0.5mg/l IBA with mean shoot number of 5.33±0.57 (Table-2). The result of this study is not in line with the results of Behara *et al*. (2009) who reported 12.4 ± 1.90 and10.5 ± 1.31 average numbers of usable shoots in the MS medium supplemented with BAP at 2.0mg/l and IBA at 1.0mg/l. But the result of this finding is supported by different previous authors such as Varakagoda *et al*. (2007), MS media supplemented with 1.0 mg/l BAP and 0.5 mg/l IBA were best regarding regeneration of shoot number and multiplication.

In this study it was also observed that length of shoot was highly influenced by concentration and type of growth hormones used during the experiment and the highest shoot length was obtained in the MS media supplemented with 1mg/l BAP and 0.5mg/l IBA. In this combination the maximum recorded mean shoot length was 5.47±1.31cm (Table-2). From the result of the present study it is important to note that if hormonal combinations and concentrations supplemented in the MS medium with 1mg/l BAP and 0.5mg/l IBA produces maximum number of shoots, this combination also possible to produce moderate or even

sometimes optimum shoot length. This idea is supported by many authors such as (Behara *et al.*, 2009; Tarique, (2010). The result of the current study disagrees with the reports of Behara and Sahoo (2009) who recorded 6.2 ± 0.37 cm and 4.0 ± 0.61cm for the two top performing hormone combinations, BAP (2.0 mg/l) + IBA (0.5mg/l) and BAP (2.0mg/l) + IBA (1.0mg/l), respectively used for shoot multiplication of sugarcane. The reports of Tarique *et al*. (2010) was similar to the result of the current study i.e. 4.7cm shoot length was recorded on MS media supplemented with 1.0 mg/l BAP and 0.5 mg/l IBA during the multiplication of shoots.

Table 2: Effect of Different Concentration and Combination of Cytokines and Auxin in Shoot Regeneration

Treatment Code	Hormone combination		Mean of Responsive explant	Mean Percent of responsive explant	Mean of shoot number	Mean of shoot length
	BAP	IBA				
T0	0	0	1.67 ± 0.57^{f}	20.83 ± 7.22^{f}	1.00 ± 00^{e}	2.20 ± 00^{d}
T1	0.5	0	6.67 ± 0.57^{ab}	83.33 ± 7.22^{ab}	4.67 ± 0.57^{a}	4.8 ± 1.00^{ab}
T2	1	0	5.33 ± 0.57^{c}	66.67 ± 7.22^{c}	3.00 ± 00^{bc}	3.5 ± 1^{bcd}
T3	1	0.5	7.33 ± 0.57^{a}	91.67 ± 7.22^{a}	5.33 ± 0.57^{a}	5.47 ± 1.31^{a}
T4	2	0	5.67 ± 0.57^{c}	70.83 ± 7.22^{c}	2.33 ± 0.57^{cd}	3.7 ± 1^{bc}
T5	2	0.5	6.67 ± 0.57^{ab}	83.33 ± 7.22^{ab}	4.67 ± 0.57^{a}	4.8 ± 1.1^{ab}
T6	2	1	6.00 ± 0.57^{bc}	$75.00\pm00b^{c}$	3.67 ± 0.57^{b}	3.8 ± 0.96^{bc}
T7	3	0	2.67 ± 0.57^{e}	33.33 ± 7.22^{e}	1.67 ± 0.57^{de}	2.5 ± 0.3^{cd}
T8	3	0.5	3.67 ± 0.57^{d}	45.83 ± 7.22^{d}	2.33 ± 0.57^{cd}	3.2 ± 0.1^{cd}
T9	3	1	4.33 ± 0.57^{d}	54.17 ± 7.22^{d}	2.67 ± 0.57^{c}	3.3 ± 0.2^{cd}

Means followed by the same letter with in a column are not significantly different at 5% significancelevel

4.3. Root Induction

The regenerated shoots were used for root induction in the root forming media. The rooting response of *in vitro* regenerated shoots was tested on half strength MS basal medium supplemented with different concentrations of NAA. Different concentrations of NAA were used for root regeneration and the number of responsive explants, root numbers as well as the measured root length was showed variability between each treatment which was done in the three replications (Appendix table.5). In Half-strength MS medium supplemented with 1mg/L NAA showed best rooting response. Mean percent of responsive explants was (95.83%) and the maximum mean responsiveness was 7.67±0.58 (Table 3). Control media without growth hormone showed no response for rooting in the same way as indicated by (Rashid *et al.*, (2009).

This finding was in agreement with the reports of Lal and Singh, (1999) who found that 1.0 mg/l of NAA as best for rooting response in sugarcane. Cooke, (2002) also showed low auxin concentration as most suitable one for rooting in sugarcane. Rooting response was lower with increasing auxin concentration. This might be related to the fact that higher concentrations of NAA is inhibitory both to root induction and elongation in sugarcane (Biradar *et al*., 2009). This inhibition of root initiation and elongation at higher concentration of auxins may be due to deposition of ethylene. Because auxins of all types stimulate plant cell to produce ethylene, especially when high amount of synthetic auxins are used. Ethylene is also known for retarding root elongation (Weiler, 1984; Pau and Chi, 1993).

Contrary to this point, however, some authors found positive rooting response with high concentrations of auxins. For example, (Khan *et al*., 2009; Gopitha *et al*., 2010; Behera and Sahoo, 2009) found that better rooting response when used 3mg/l of NAA. Anbalagan *et al*. (2000) and Nadgauda, (2002) reported high concentration i.e. 5.0 mg/l of NAA or combination of two auxins NAA and IBA for rooting in sugarcane was the best. In addition to the effect of auxin concentration for the induction percentage of roots and root responsiveness the number of roots generated from the shoot explants depends on the auxin concentration used in the half MS media. As it is observed from the result of the study, the maximum mean number of roots was obtained from half MS medium augmented with 1mg/l of NAA. With

regard to mean number of roots per shoot, half strength MS media and 1 mg/l NAA registered its superiority over the other treatments by exhibiting 7.33±0.57 root per shoots. Bekesha *et al*. (2002) observed 85% rooting response with an average number of roots per shoots of 15±0.5 on half MS supplemented with 5mg/l NAA. But this finding is supported by Khan *et al*. (2006) who reported the highest root induction with best root growth at ½ MS medium containing 1 mg/l of NAA and 60 g/l sucrose. Based on the result obtained from this study the numbers of roots generated from the shoots of the explant were depended on auxin concentration as in case of root response and root percentage.

As we can see in (Table 3), hormone levels that produced maximum number of roots in the above results (Table3) produced maximum root length. The effect of different combination and concentrations of auxin hormone was observed in different treatment on the shoot explant. Half strength of MS media contained 1mg/l NAA produced best root length and this combination resulted in the maximum mean root length of 8.4±0.2 (Table 3). Alam *et al*. (2003) also reported best rooting response was recorded at 2.5mg/l IBA having 1.1 cm root length. Whereas some findings were agree with the result of this current study. For example Lal and Singh (1999) found that 1.0 mg/l of NAA resulted of best root response.

Table 3: Effect of Different Concentrations and Combinations of NAA in Root Induction

Treatment Code	Hormone concentration	Mean of responsive explant	Mean percentage of responsive explant	Mean of root number	Mean of root length
	NAA(mg/l)				
T0	0	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{e}
T1	0.5	6.0 ± 1.0^{bc}	75.0 ± 12.50^{bc}	5.0 ± 1.0^{b}	6.7 ± 0.8^{bc}
T2	1	7.67 ± 0.58^{a}	95.83 ± 7.22^{a}	7.33 ± 0.57^{a}	8.4 ± 0.2^{a}
T3	2	6.67 ± 0.58^{ab}	83.33 ± 7.22^{ab}	6.33 ± 1.16^{ab}	7.1 ± 0.8^{b}
T4	3	5.67 ± 0.58^{bc}	70.83 ± 7.22^{bc}	3.33 ± 0.57^{c}	5.6 ± 0.8^{cd}
T5	4	$5.0+00^{c}$	62.50 ± 0.0^{c}	3.0 ± 1.0^{c}	4.5 ± 0.8^{d}

Means followed by the same letter with in a column are not significantly different at 5% significance level.

4.4. Acclimatization

In vitro developed plantlets have shown morphological and physiological abnormalities due to the *in vitro* culture conditions (Pospisilova *et al*., 1999) and direct transfer of *in vitro* plantlets to *ex vitro* environment may result in rapid wilt and death (Lesar *et al*., 2012). Therefore, acclimatization is essential for the survival and successful establishment of plantlets (Deb and Imchen, 2010). For this study sugar cane variety SP-70 plantlets were used. The rooted plantlets were taken carefully out of the culture jar and washed with running tap water gently to remove traces of culture medium particularly sucrose.

Since the plantlets were from high sucrose concentration medium and the chance of fungal attack could potentially be minimized through washing and they were successfully acclimatized in the mixture of autoclaved soil, manure and sand (2:1:1). Result showed that the survival rate was 98% (Appendix table.6) after four weeks of acclimatization by maintaining high humidity (80-90%) at 25 ± 2^{0c} in the initial one to two weeks and in 50-60 relative humidity at 27 ± 2^{0c} in the three to four weeks. Ali *et al*.(2008); found 96% survival rate for sugarcane plantlets acclimatized on substrate mixture composed of farmyard manure and soil in 2:8 ratio. Ather *et al.* (2009) also reported 85% sugarcane platelet survival when acclimatized on mixture of soil + sand + compost in 1:1:1 ratio.

As it was observed during the study, plantlets were well acclimatized by increasing humidity from 80-90% in the first 10 -15 days by maintaining the temperature at 25 ± 2^{0c} and by reducing relative humidity to 50-60%, and increasing temperature to about 27 ± 2^{0c}. This is because the plantlets need high humidity during the first stage of their development and they do not strong enough to expose to high temperature whereas after a while they become hard and should be treated with low humidity and temperature must be raised since they are ready to field condition.

5. SUMMARY, CONCULUSION AND RECOMONDATION

5. 1. Summary and Conclusion

Lack of steady supply of adequate quantity quality and disease free sugarcane planting materials is one of the most challenging issues for attaining the intended production plans of the Ethiopian Sugar Industry using the conventional method of propagation. *In Vitro* mass propagation of sugarcane through shoot tip or apical meristem explant ensures rapid multiplication of true to type and disease free planting materials of sugarcane within a short period of time and Plant growth regulators play an essential role in determining the development pathway of plant cells and tissues in culture medium.

The present study was carried out to standardize protocol of micro-propagation of sugarcane sp-70 for large scale production of sugarcane planting material. Standardization and optimization of culture establishment shoot multiplication; root induction and acclimatization of plantlets were the major objectives of the study. It was observed in the present study that an efficient protocol is needed for any new variety or clone to get rapid shoot initiation, shoot multiplication, root induction and acclimatization. Initiation of explant was found to be maximum in BAP (0.5 mg/lit) in MS medium with mean responsiveness (6.67 ± 0.58) and mean percentage of responsiveness (83.33 ± 7.22). For shoot proliferation and multiplication MS medium supplemented with 1mg/l BAP and 0.5 mg/l IBA, was found to be the best compared to other media prepared and the mean responsiveness, mean percentage of responsiveness mean of shoot number and mean of shoot length were (7.33 ± 0.57), (91.67 ± 7.22), (5.33 ± 0.57) and (5.47 ± 1.31) respectively.

For root induction ½ MS medium supplemented with 1mg/l NAA was appropriate hormone concentration and mean responsiveness, mean percentage of responsiveness, mean of root number and mean of root length were (7.67 ± 0.58), (95.83 ± 7.22), (7.33 ± 0.57) and (8.4 ± 0.2) respectively. Maximum survival percentage (98%) was found after the plantlets treated in the green house for about 30 days by treating the plantlets at relative humidity of 80-90% four about half weeks at temperature of 25 ± 2^0c and by maintaining humidity at 50-60% with27 ± 2^0c in the third and fourth weeks. During the experimental activities of this study

different problems were occurred such as contamination of culture media, plantlet contamination and browning of the explants in the growth room. But these problems were solved by further sterilization of culture medium and the materials used as well as repeated sub culturing of planets during the experimental studies. From the present study it can be concluded that MS media supplemented with 0.5 mg/lit BAP is best for shoot initiation while 1mg/l BAP and 0.5mg/l IBA for multiplication and for root induction ½ strength MS media supplemented with 1 mg/lit NAA is best. For commercial production of sugarcane ½ strength MS is found to be more economically viable. It is also possible to conclude that for shoot initiation and shoot multiplication best result is obtained by lowering auxin concentration and increasing cytokinin concentration and for rooting good result is found by lowering cytokinin concentration and by increasing auxin concentration or auxin only. From the study conclusion can be drown for acclimatization i.e. ultimate hardening well rooted plantlets grown on plastic pots contain soil, manure and sand treated with balanced temperature and relative humidity for about 30 days assured a survival rate of 98% in the greenhouse.

Thus, the optimized protocol can be used to develop healthy and multiple shoots profuse root system for the sugarcane variety sp-70 and can play a key role in the stage of rapid supply of quality planting material of the sugarcane varieties and minimizes the current challenge of the sugar Industry.

5.2. Recommendation

- From this study it is possible to recommend that, for micro-propagation of sugarcane variety sp-70, 0.5 mg/l BAP can be used for best shoot initiation.
- For shoot induction and multiplication combination of 1 mg/l BAP and 0.5mg/l IBA can be used and 1mg/L of NAA are suitable for rooting of plantlets.
- Hardening of plantlets in plastic pots contains soil, manure and sand in 2:1:1 at optimum temperature and relative humidity was good enough method to produce high quality plantlets in the green house.
- To get true type, fast growing and disease free plantlets, apical meristem shoot tip obtained from the explant was effective rather than the other part of the plant.

- This study can serve as baseline for germplasm conservation and further studies on this sugarcane species.
- Further studies on identifying the dry weight of the micro shoots of this variety of sugarcane during multiplication should be carried out.
- Further studies on the performance of the tested cytokinins and auxins in combination and individually should be carried out for better result on shoot initiation and multiplication.
- For better rooting results, the combination effect of the studied auxins and with other hormone (auxins and/or cytokinins) should be investigated.
- Since the objective of this study was to optimize protocol for culture establishment or shoot initiation, shoot multiplication, rooting and determining the survival rate of plantlets in green house,
- Other, factors causing low success in micro-propagation such as contamination, tissue browning because of phenolic oxidation in the study should be dealt with in the future.

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7. APPENDICES

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

Constituents	Amount(gm/L)
Macronutrients	
NH_4NO_3	1.65
KNO_3	1.9
$CaCl_2.2H_2O$	0.44
$MgSO_4.7H_2O$	0.37
KH_2PO_4	0.17
Micronutrients	
H_3BO3 6.2	0.0062
$NaEDTA.2H_2O$	0.0372
$MnSO_4.4H_2O$	0.0223
$FeSO_4.7H_2O$	0.0278
$ZnSO_4.7H_2O$	0.0086
KI	0.00083
$Na_2MoO_4.2H_2O$	0.00025
$CoCl_2.6H_2O$	0.000025
$CuSO_4.7H_2O$	0.000025
Vitamins	
My inositol	0.1
Glycine	0.002
Niacin	0.005
Pyridoxine	0.005
Thiamine	0.0001
Other organic supplements	
Sucrose	30
Malt extract	0.1
Activated charcoal	0.5 and 5
Agar	6

Appendix Table Cont.……

Glutamine	0.1
Proline	0.05
Cysteine	0.05
Biotin	0.001

Appendix Table 2.Amounts and Concentration of Plant Growth Regulator Hormones

Plant growth regulator	Concertation(mg/ml)	Amount(mg/l)
BAP	(10.75)	0,0.25,0.5,1,2,3 and 4
NAA	(10.5)	0,0.5,1, 2,3 and 4
IBA	(1.5)	0, 0.5, 1.

Solvent: 95% ethanol or NaOH

Appendix Table 3.Data Obtained From Result of Initiation

s/n	Treatment code	Hormone combination		Explants initiated In each replication			Responsive explant per treatment			Responsive explant in %	Non responsive explant		
		BAP	IBA	R1	R2	R3	R1	R2	R3		R1	R2	R3
1	T0	0	0	8	8	8	1	2	1	16.66	7	6	7
2	T1	0.25	0	8	8	8	2	2	3	29.16	6	6	5
3	T2	0.5	0	8	8	8	7	6	7	83.33	1	2	1
4	T3	1	0	8	8	8	3	3	3	37.5	5	5	5
5	T4	1	0.5	8	8	8	5	5	6	66.66	3	3	2
6	T5	2	0	8	8	8	1	2	2	20.83	7	6	6
7	T6	2	0.5	8	8	8	3	4	3	41.66	5	4	5

NB. 1 explant per bottle and 8 bottles per treatment for each replication

Appendix Table 4. Data Obtained From Result of Shoot Multiplication

s/n	Treatment code	Hormone combination		No. of bottle taken/sample size			Responsive Or shoot formed explants			Responsive explant in %	Shoot number per replication			Shoot length per replication in cm		
		BAP	IBA	R 1	R 2	R3	R 1	R 2	R3		R1	R 2	R 3	R1	R2	R3
1	T_0	0	0	8	8	8	2	2	1	20.83	1	1	1	2.2	2.2	2.2
2	T1	0.5	0	8	8	8	7	6	7	83.33	5	4	5	5.8	3.8	4.8
3	T2	1	0	8	8	8	5	5	6	66.67	3	3	3	3.5	2.5	4.5
4	T3	1	0.5	8	8	8	7	7	8	91.67	5	6	5	5.4	4.2	6.8
5	T4	2	0	8	8	8	5	6	6	70.83	3	2	2	3.7	2.7	4.7
6	T5	2	0.5	8	8	8	6	7	7	83.33	5	4	5	4.8	3.7	5.9
7	T6	2	1	8	8	8	6	6	6	75.00	4	3	4	2.8	3.9	4.7
8	T7	3	0	8	8	8	2	3	3	33.33	1	2	2	2.8	2.2	2.5
9	T8	3	0.5	8	8	8	3	4	4	45.83	2	2	3	3.1	3.3	3.2
10	T9	3	1	8	8	8	4	4	5	54.17	2	3	3	3.1	3.5	3.3

NB. 8 bottles per treatment with one explant per bottle and it was done in three replications for each treatment.

Appendix Table Cont.……

Appendix Table 5. Data Obtained From Result of Rooting

s/n	Treatment code	NAA	sample size/bottle no per treatment	Responsive explant per replication			Responsive explant in %	Root number per micro shoot			Root length/ cm/		
				R1	R2	R3		R1	R2	R3	R1	R2	R3
1	T_0	0	24	0	0	0	.0000	0	0	0	0	0	0
2	T1	0.5	24	5	6	7	75.0000	5	4	6	5.9	6.7	7.5
3	T2	1	24	7	8	8	95.8333	7	7	8	8.4	8.2	8.6
4	T3	2	24	6	7	7	83.3333	5	7	7	7.1	6.3	7.9
5	T4	3	24	6	5	6	70.8333	3	3	4	5.6	4.8	6.4
6	T5	4	24	5	5	5	62.5000	3	2	4	4.5	3.7	5.3

NB. 8 Bottle per treatment per replication and one plantlet per bottle. NB. All steps done in 3 replications

Appendix Table 6.Data Recorded For Acclimatization

Treatment code	No of planted explants per treatment	No survived explant per treatment
T0	0	0
T1	18	17
T2	23	23
T3	20	20
T4	17	17
T5	15	14
Total planted explants	93	91
Survived explant in %	91/93*100 = 97.84 = 98%	

Appendix of Table cont…….

Appendix Table 7. Analysis of Variance for Initiation

Variables	DF	SS	Mean SQ	F-value	P-value
Explant responsive	5	68.94	13.79	49.64	<0.001
Responsive in %	5	10772.6	2154.5	49.64	<0.001

DF= Degree of freedom, SS= Sum Square, Mean SQ= Mean Square

Appendix Table 8.Analysis of Variance for Shoot Multiplication

Variables	DF	SS	Mean SQ	F-value	P-value
Explant responsive	9	94	10.44	34.8	<0.001
Responsive in %	9	14687.5	1631.94	34.8	<0.001
Shoot Number	9	54.13	6.02	22.56	<0.001
Shoot Length	9	29.05	3.23	4.64	<0.001

DF= Degree of freedom, SS= Sum Square, Mean SQ= Mean Square

Appendix Table. 9. Analysis of Variance for Root Induction

Variables	DF	SS	Mean SQ	F-value	P-value
Explant responsive	5	108.5	21.7	65.1	<0.001
Responsive in %	5	16953	3390.6	65.1	<0.001
Root Number	5	104.5	20.9	31.35	<0.001
Root Length	5	130.76	26.15	60.35	<0.001

DF= Degree of freedom, SS= Sum Square, Mean SQ= Mean Square

Appendix Figure 1: Shoot Initiation (A) and Shoot Multiplication (B) stage

Appendix Figure 2: Rooting Stage (C) and Acclimatization (D).