PROTOCOL OPTIMIZATION FOR *IN VITRO* PROPAGATION OF SWEET POTATO (*Ipomoea batatas* L.) VARITIES USING SHOOT TIP CULTURE

MSc. THESIS

BELACHEW BEYENE BARI

SEPEMBER, 2016

JIMMA, ETHIOPIA

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BELACHEW BEYENE BARI

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I have incorporated the suggestions and modifications given during the internal defense and got the approval of my advisors. Hence, I hereby kindly request the Department to allow me to submit my thesis for external thesis defense.

Name and signature of student: Belachew Beyene _____

We the thesis advisors have verified that the student has incorporated the suggestions and modifications given during the internal thesis defense and the thesis is ready to be submitted hence, we recommended the thesis to be submitted for external defense.

Major Advisor: Gizachew Haile (PhD) Signature _____ Date _____

Co-advisor: Temesgen Matiwos (MSc, PhD Candidate) Signature _____ Date _____

Decision/Suggestion of Department Graduate Council (DGC)

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_____ _____ _____

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DEDICATION

To God Almighty and his blessed mother St. Mary.

STATEMENT OF THE AUTHOUR

By signing below, I declare that the work presented in the thesis manuscript entitled, "Protocol Optimization for *In Vitro* propagation of Sweet Potato *(Ipomoea batatas L.)* Varieties Using Shoot Tip Culture" for partial fulfilment of the requirements for the award of the degree of Master of Science in Plant Biotechnology at Jimma University is an authentic record of my own genuine work under the supervision of my major advisor Dr. Gizachew Haile and co-advisor Temesgen Matiwos.

Dully acknowledging all source materials I used in this thesis, I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic Degree, Diploma, or certificate. It can be deposited at the University Library to be made available to borrowers under rules of the library. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the Plant Science and Horticulture Department or the Dean of the School of Graduate Studies when in his/her judgment the proposed use of the material is in the interest of scholarship. In all other instances, however, permission must be obtained from the author.

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Signature: _____

Date of Submission: _____

Place: Jimma University

BIOGRAPHICAL SKETCH

The author, Belachew Beyene, was born from his father Ato Beyene Bari and his mother W/r Abebech W/Michael, on September 21, 1988 at Alemigena kebele, Gesha Woreda in Kefa zone of SNNPRs. He attended his elementary school at Deka Elementary School and secondary school education at Masha Senior Secondary School from 1997 to 2006. He joined Hosanna Teacher Training College in 2007 and graduated with diploma in natural science in July 2009. After his graduation he was employed by Gesha Woreda Education Office and he worked as a Biology teacher at Gesha Senior Secondary High School for one year. Then he joined Jimma University in 2010 and graduated with BSc degree in Biology in 2013. He joined the School of Graduate studies of Jimma University in September 2014 to pursue his MSc. study in Plant Biotechnology.

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LIST OF ABBREVIATIONS

BAP	Benzyl Amino Purine
CRD	Completely Randomized Design
IBA	Indol -3-Butyric Acid
LSD	Least Significance Difference
MS	Murashige and Skoog
PGRs	Plant growth Regulators
SPVD	Sweet Potato Virus Diseases

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ABSTRACT

*Conventional propagation methods of sweet potato (*Ipomoea batatas L.*) through stem cutting requires large amount of materials and space and an extended period to produce plants. There is also a high risk of disease transmission from generation to generation.* In vitro *propagation is the best alternative to overcome such limitations of conventional propagation. Thus, this study was conducted with the aim to optimizing a protocol for in vitro propagation of sweet potato varieties. For shoot initiation, shoot tip explants of Kulfo and Tulla varieties, the explants were cultured on MS basal medium that contained 30 g/l sucrose and 8 g/l agar supplemented with 0.0, 0.5, 1.0 and 2.0 mg/l BAP. For shoot multiplication, the initiated shoots were cultured on MS medium supplemented with 0.0, 0.5, 1.0, 1.5 2.0, 2.5 and 3.0 mg/l of BAP were used. For in vitro root inductions, micro-shoots were cultured on 1/2 MS media supplemented with 0.0, 0.1, 0.5, 0.75 and 1.0 mg/l IBA while for ex vitro rooting, in vitro multiplied micro- shoots were carefully excised and directly transferred to greenhouse for rooting as well as hardening simultaneously. Among BAP concentrations used for initiation, growth regulators free media resulted in 66.67% and 55.56% for Kulfo and Tulla respectively. Best shoot multiplication (5.33±0.00) shoots/explant with 7.82±0.02cm shoot length and 6.33 ±0.34 leaves/shoot was obtained on MS medium containing 1 mg/l BAP for Kulfo, while 2mg/l BAP resulted in a maximum of (6.78±0.19) shoots/explant with 9.70±0.00cm shoot length and 9.67±0.06 leaves/shoot for Tulla. The best* in vitro *rooted shoots was 100% with (8.27±0.05) cm root length for Kulfo and 88.89% with 8.24±0.05 cm root length for Tulla, on growth regulators free ½ MS medium. In* ex vitro *rooting experiment, best rooting response was 93.33% with 3.85±0.00 mean number of roots per shoot and 9.80±0.00 cm root length for Kulfo, whereas Tulla produced 86.70% rooted shoots with 2.52±0.00 number of roots and 8.40±0.00 cm root length. Among all plantlets planted in the glasshouse, 84% and 80% for* in vitro *rooted shoots, 93% and 86% for ex vitro shoots of Kulfo and Tulla varieties respectively were survived. It could be concluded that* in vitro *initiation of varieties Kulfo and Tulla, supplemented with BAP free MS media were the optimal concentrations. MS+1mg/l BAP was the optimum concentration for shoot multiplication of Kulfo, while Ms+2mg/l BAP was optimum for best multiplication of Tulla. For* in vitro *rooting, PGR free ½ MS medium were optimal for Kulfo and Tulla genotypes. Finally, further studies will be needed in* ex vitro *root induction rather than* in vitro *rooting for the sake of labour, time, better rooting system and cost reduction.*

Keywords: BAP, ex vitro rooting, IBA, MS medium, Micropropagation, Shoot tip culture

1. Introduction

Sweet potato (*Ipomoea batatas* (L.) Lam.) is a dicotyledonous plant which belongs to the family Convolvulaceae, genus *Ipomia*. Some of the species have fleshy roots, only sweet potato is edible. The cultivated *Ipomoea batatas* is a hexaploid of 2n = 6x = 90 with basic chromosome number x = 15 and is grown throughout the tropics with wide diversity (Jones, 1964).

It is the seventh most important food crop in the world following wheat (*Triticum aestivum*), rice (*Oryza sativa*), maize (*Zea mays*), potato (*Solanum tuberosum*), barely (*Hordeum vulgare*), and cassava (*Manihot esculenta*) (FAOSTAT, 2012). Globally it is grown in an area of about 8 million hectares with production of 104.45 million tons; average yield being 13 tons/ ha in 2014 (FAOSTAT, 2016 Africa's top producers are Uganda (1.7 million tons), Rwanda (980,000 t), Malawi (960,000 t) and Kenya (725,000 t) (Ewell, 2002). In Ethiopia sweet potato ranks the first in total production (42.84%) and the second in area coverage (25.43%) next to Irish potato among root and tuber crops cultivated. The total area under sweet potato cultivation in Ethiopia is 0.05 million ha with production of 0.39 million tons; average yield being 7.8 tons/ha in 2012 (CSA, 2012).

In some of the world's poorest nations, sweet potato is an important part of food security packages (Dagne *et al*., 2014). It is mainly cultivated for its expanded edible roots which contain high carbohydrate, minerals, vitamins, antioxidants and beta carotene to a large sector of the global population (Islam, 2006; USDA, 2007; Kapinga *et al*., 2011; Shonga *et al*., 2013). In some countries, the roots are processed to produce a wide variety of products: alcoholic and non alcoholic beverages, sweet desserts, snacks, a variety of convenience processed products, fast foods, multipurpose flour, starch and basic industrial raw material (Duvernaya *et al*., 2013). The crop has also high potential to reduce hunger, malnutrition and poverty since it gives better and early yield with less input (Lim *et al*., 2007).

Sweet potato is traditionally multiplied mainly by stem cuttings which are a slow process, and diseases may accumulate in the vine cuttings from generation to generation which could result in declining of root yield and loss of superior genotypes. In general, it requires large area, incurs high cost, consumes time and thus it is wasteful system.

Tissue culture technology can be used to produce high quality plants instead of the traditionally used cuttings and it is possible to produce large number of high quality propagules within short period of time unlike conventional techniques (Kwame *et al*., 2012). Moreover micropropagation of sweet potato offers significant advantages in the production of a very large number of clonal propagules within a short time, disease free plant material with the possibility of eliminating viral, bacterial and fungal infection and the production of high quality and uniform plantlets (Neja, 2009;Tekalign *et al*., 2012). Since the advent of *in vitro* techniques, a lot of interest has been generated in the recent year for the rapid multiplication of disease free clones of crop plants through shoot tip culture which are propagated vegetatively (Gong *et al*., 1998).

In addition, BAP had the greatest effect on shoot initiation; followed by the combination of BAP and NAA (Xiansong, 2010). According to Berihu (2013), maximum *in vitro* shoot intiation was obtained from MS media supplemented with 0.5mg/l BAP. Stfaan *et al.* (1994) and Yucesan *et al*. (2007) also reported the experiments using shoot tip as explant materials and involving combinations of two auxins and two cytokinins, the results showed that auxins, when used alone, had no effect on shoot induction since either NAA or IAA produced no shoots at all but both types of cytokinins, when used alone, were able to produce maximum number of shoots /explant and BAP was more effective. So far, *in vitro* shoot tip culture offers an opportunity for genetically uniform *in vitro* commercial propagation of sweet potato (Xiansong, 2010). There are some reports of shoot tip culture of sweet potato for mass propagations (Kuo *et al*., 1985; Kong *et al.*, 1998; Gong *et al.* 2005) but little information was available on *in vitro* propagation using shoot tip culture on Ethiopian cultivars purple-coloured sweet potato. Therefore, this study was initiated with the following objectives.

1.1. Objectives

General objective:-

- To optimize protocol for mass propagation of sweet potato genotypes under *in vitro* condition using shoot tip culture

Specific objectives:-

- ✓ To determine the optimum concentrations of BAP for shoot initiation.
- ✓ To determine the optimum concentration of BAP for shoot multiplication.
- ✓ To determine the optimum concentration of IBA for rooting.

2. LITERATURE REVIEW

2.1. Taxonomy and Description of sweet potato

Sweet potato and the wild species closely related to it are classified in the family Convolvulaceae, genus *Ipomoea*, Sub genus *Eriospermum* (formerly Batatas) (Austin and Huamán, 1996). It was botanically described in 1753 by Linnaeus as Convolvulus batatas, but Lamarck, (1791), reclassified the crop into the genus Ipomoea on the basis of the stigma shape and the surface of the pollen grains (Thottappilly and Loebenstein, 2009). Therefore, the botanical name of sweet potato was changed to *Ipomoea batatas* (L.) Lam. The different varieties of sweet potato are divided into two groups, dry flesh and moist flesh (Austin, 1987).

Approximately 900 different species of Convolvulaceae in 400 genera have been identified around the world. The closest relative of cultivated *Ipomoea batatus* is *Ipomoea trifida* which exists in 2x (diploid), 3x (triploid), 4x (tetraploid), or 6x (hexaploid) forms (Winter *et al*., 1992). This wild relative is believed to be one of the ancestors of the cultivated sweet potato. Sweet potato varieties exist in many colours of skin and flesh, ranging from white to deep purple, although white and yellow orange fleshed ones are the most common (Austin, 1987).

2.2. Economic importance of sweet potato

Roots and tubers, most notably cassava, sweet potato and potato are some of the most important primary crops worldwide. They play a critical role in the global food system, particularly in the developing countries, where they rank among the top ten food crops (Phillips *et al.,* 2004). Globally sweet potato is the seventh most important food crop in the world in terms of production (Lowenstein, 2009).

Sweet potato is mainly cultivated for its expanded edible roots which contain high carbohydrate and beta carotene. Hence, it prevents night blindness and deaths from weakened immunity due to vitamin A deficiency (Scott *et al*., 2000). Although it is cultivated mainly for the carbohydrate rich tubers, the foliage has the potential for use as vegetable and animal feed (Otoo *et al*., 2001). According to Duke (1983), the root of sweet potato is used as medicinal plant in folk remedy for asthma, burns, diarrhea, fever, nausea and tumor. The leaves of sweet potato are also source of nutrients including anti

oxidants, vitamin B, β carotene, boron, calcium, copper, cysteine, fiber, folic acid, iodine, iron, magnesium, manganese, niacin, phosphorous, protein, sulphur, tryptophan, tyrosine and Zinc (Gad and Kandil, 2008).

In some countries the roots are also processed to produce a wide variety of products: alcoholic and non alcoholic beverages, sweet desserts, snacks, variety of convenience processed products, fast foods, multipurpose flour, starch, animal feeds and basic industrial raw material (Woolfe, 1992). In Africa, particularly resource poor farmers, especially women grow sweet potato mostly for family consumption and for cash (Bashaasha *et al*., 1995).

2.4. Conventional propagation of sweet potato

Sweet potato is propagated asexually from vine cuttings or sexually from seed (Woolfe, 1992), but the latter is done only by breeding programs. Propagation of sweet potato is done by vegetative propagation using one of the following methods: sprouting of whole storage roots (sprouts are then used as planting materials), and use of stem or vine cuttings from plants used for production or from multiplication plots. In the latter method green vines of approximately 30cm length with at least three leaf nodes are planted into the soil (Obigbesan, 2009). However, the vine propagation method accumulates diseases during several cycles of field production. Furthermore, traditional method of cultivation using storage root and vine cuttings requires large quantity of planting materials, which is costly, time consuming and land demanding (Singh *et al*., 2006).

2.5. Tissue culture of sweet potato

Plant tissue cultures of sweet potato are done by placing one or more explants like node, shoot tip, leaves, petioles, meristem and embryos of plants into a pre-sterilized container of sterile nutrient medium. Sweet potato has long been considered a recalcitrant species for plant regeneration (Sihachakr *et al*., 1997). It is obstinate to regenerate producing adventitious plants from non meristematic tissue (Prakash, 1994). However, it is easy to micro propagate producing a higher number of plantlets within a short time compared to conventional mode of propagation. The technology has high fecundity, with production of thousands of sweet potato propagules in the same time it would take the conventional technique to produce tens or hundreds (ASARECA, 2008). Cavalcante *et al.* (1994) regenerated plants from lateral bud derived callus of sweet potato onto

regeneration medium supplemented with 2,4-D and BAP. Newell et *al.* (1995) also obtained callus and able to regenerate shoot onto medium containing NAA and BAP. Moreover, Zheng *et al.* (1996) reported that rapid and repetitive plant regeneration in only one genotype of 12 sweet potato i.e. PI318846-3 onto media devoid of hormones after using firstly an initiation medium supplemented with 2,4-D. Shoot regeneration from petiole-derived calli of sweet potato cv. Genki was also done using regeneration medium containing BAP only (Wang et al., 1999).

2.6. Principles of *in vitro* propagation

In general *in vitro* propagation is based on the principle of establishing and maintaining healthy micro shoots in culture for multiplication so that these produce rooted micro cuttings and plantlets (Liu and Bao, 2003). The basic principle of plant *in vitro* propagation of meristematic shoot tissues is to grow, manipulate and multiply identical plant cells, tissues and organs, which have been isolated from the mother plant sources. This approach involves techniques that are conducted in a controlled and aseptic environment; the cells, tissues and organs of a selected plant are isolated, surface sterilised and cultured in a growth promoting environment (Teng *et al*., 2002). There are generally five distinct steps applied in micropropagation (selection and preparation of mother plant, initiation of culture, multiplication, rooting and transfer to soil).

2.6.1. Composition of media for sweet potato culture

A nutrient medium is defined by its constituents of mineral salts, carbon source, vitamins, plant growth regulators and other organic supplements. An optimised culture media is essential for shoot growth, multiplication and root induction in tissue culture, as the media has to provide all nutrients and elements for *in vitro* growth of plants. Generally, sweet potato tissue culture media are made up of some or all of the following components: macronutrient, micronutrient, vitamins, sugar, and de ionized water, solidifying agents and growth regulators and other organic supplements (Smith, 2013). All these compounds full fill one or more functions in the *in vitro* growth of sweet potato plant (Murashige and Skoog, 1962).

Minerals (macro and micro salts): *In vitro* propagation of sweet potato plant requires combination of both macro and micronutrients for their growth. The macronutrients provide the following six major elements: Nitrogen, Phosphorus, Potassium, Calcium,

Magnesium and Sulphur in the form of salt in the media for satisfactory growth and morphogenesis (Fossard, 1976). The essential micro nutrients include the minor elements those are iron, Manganese, Zinc, Boron, Copper, Molybdenum, Cobalt, Iodine and others. The application quantity depends on the *in vitro* cultivation objectives; for example high formulations (full strength) media are better suited for shoot multiplication and elongation, whilst lower concentrations (1/2 or 1/4 strength) medium are generally used to promote root induction depending on plant species including sweet potatoes (Kim *et al*., 2003).The optimum concentrations of P, Mg, S and Ca range from 1-3 mM if other requirements for cell growth are provided (Smith, 2013). Copper and Cobalt are added to culture media at concentrations of 0.2 micromole iron and Molybdenum at 1 micromole for most sweet potato cultivars (Fossard, 1976).

Carbon and energy source: Sugars play an important role in micropropagation as an energy and carbon source as well as an osmotic agent. In Plant cell culture media, sucrose in the form of commercial sugar is the most preferred carbon source in tissue culture because it is cheap and readily available. Therefore, carbon source (e.g. sucrose, glucose, maltose or galactose) for *in vitro* plant metabolism has to be added to many culture media as the plantlets are not fully autotrophic and photosynthesis process is not adequate (Rahman *et al*., 2010). The most preferred energy source for micropropagation of sweet potato is sucrose at a concentration of 20-60g/l (Rai, 2007). Sucrose is cheap, easily available, readily assimilated and relatively stable and is therefore the most commonly source of energy *in vitro* propagated medium.

Plant growth regulators: In media constitute plant growth regulators or hormones are the critical in determining the developmental and growth pathway of the plant cells. There are five class of plant growth regulators; namely, Auxins, Cytokinins, Gibberellins, Abscisic acid and Ethylene. Of these plant growth regulators, the three most crucial classes used to control organ development and regeneration of explants are Auxins, cytokinins and gibberellins (Hartmann *et al*., 1990). The application and quantities of growth regulators depends on the *in vitro* culture objectives and plant species; e.g. callus formation, shoot regeneration and multiplication and elongation or rooting response. Cheong *et al.* (2009) reported that cytokinins (BA and kinetin), GA3 and auxin (NAA) in combination, were critical for maintaining viability and growth of meristem. The highest shoot initiation

frequency of 96% was obtained by combination of 0.1 mg/l BAP, 0.1 mg/l NAA, 0.1 mg/l KN and 0.1 mg/l GA3.

Auxins play an important role in many aspects of growth and differentiation of cells *in vitro* culture including cell enlargement, cell division, vascular differentiation, apical dominance, and root formation. The common auxins used *in vitro* culture media include: Indole-3- acetic acid (IAA), Indole -3- butyric acid (IBA), 2,4-dichloorophenoxy-acetic acid (2,4-D) and Naphthalene- acetic acid (NAA). The only natural occurring auxin found in plant cell or tissue is IAA and is sensitive to both heat and light.

Cytokinins play multiple roles in the control of plant development. In culture media, cytokinins proved to promote cell division, RNA synthesis induce shoot formation and axillary shoot proliferation and to retard root formation. Cytokinins are frequently reported to be difficult to dissolve and sometimes addition of few drops of 1N Hcl or 1N NaOH facilitate their dissolution. The most commonly used cytokinins in sweet potato regeneration are Zeatin, kinetin and BAP (Kreuze *et al*., 2008; Pido *et al*., 1995; Santa-Maria *et al*., 2009).

Gibberellins are involved in a wide range of developmental responses. They stimulate cell division and elongation (Harberd *et al*., 1998). In sweet potato regeneration through embryogenesis, gibberellins are usually applied to induce elongation of somatic embryos (Anwar *et al*., 2010). The timing of application of GA3 in tissue culture of sweet potato is very important and it is recommended that GA3 be applied only when somatic embryos are already mature or when shoots are already evident. When GA3 is added to *in vitro* culture media, it often diminishes or prevents the initiation of adventitious roots, shoots or somatic embryos. Thus, the prior treatment of callus or explants (George *et al*., 2008) with GA3, or the addition of GA3 to the medium together with auxins and cytokinins at concentrations which normally promote morphogenesis, is usually inhibitory (George *et al*., 2008).

Abscisic acid is added to *in vitro* culture media that is usually supplemented to inhibit or promote callus. It has also many roles in plants, such as the control of stomatal closure, regulation of water and ion uptake by roots, and of leaf abscission and senescence of plant. Hence, like other hormones, ABA has multifaceted effects *in vitro* (George *et al*., 2008). In tissue culture, ABA sometimes promotes morphogenesis or growth. More specifically,

ABA has been shown to control the expression of genes specific to embryo development and maturation.

Gelling agents (agar or Gelrite) in tissue culture for *in vitro* propagation traditionally solid media is favoured over liquid media with agar often being the preferred gelling agent. For any plant cells or tissues culture to be grown on the surface of the medium, it has to be gelled with agar. Agar has several advantages over other gelling agents; mixed with water, it easily melts in a temperature range of 60^0C to 100^0C and solidifies at approximately 45^0C and it forms a gel stable at feasible incubation temperatures. Agar gels do not react with media constituents and are not digested by plant enzymes. It is commonly used in media at concentrations ranging between 8 to 10 g/l. Another gelling agent used for commercial as well as research purposes is Gelrite. It is synthetic and used at 1.25-2.5 g/l produces clear gels which allows for accurate observation of root formation and are crucial aids for detecting contamination that may develop during the span of cultures in tissue culture (Williams and Taji, 1987).

2.6.2. Explants surface sterilization

Contamination in tissue culture can originate from two sources, both on the surface and in the tissues of explants or through faulty procedures in the laboratory. In meristem culture, most organisms would be eliminated due to its small size whereas in large explants (leaf, stem etc), most if not all microorganisms in the tissues may be carried over (Cassells, 2005). To avoid contamination, the explants have to be washed and cleaned up prior to surface sterilization by using liquid soap, commercial detergent, Kocide, Ridmol, Mancozium, Tween 20 or 80 etc with tap water. Although different sterilization agents such as $CaOCl_2$, H_2O_2, NaOCl, $HgCl_2$ and ethanol can be used for surface sterilization, ethanol, NaOCl and $HgCl_2$ are the most commonly used agents. Mercuric chloride is a very strong sterilant yet Gopal *et al.* (1998) disinfected the single nodal cuttings of different cultivars with a mixture of 0.1% Mercuric chloride and 0.1% Sodium lauryl sulfate for 5 minutes.

The shoot tips obtained from green house grown plants should be surface disinfected for 3 minutes by soaking in a calcium hypochlorite (10% commercial bleach) solution with a small amount of detergent (e.g. Tween- 20 or Tween-80). According to Chandra (1993), ethanol is a mild surface sterilant and 70% is recommended for initial use. Sodium

hypochlorite has turned out to be a better sterilant than calcium hypochlorite due to bleaching effects of the later and hence has been extensively used for sweet potato sterilization. Amongst the two sterilants i.e. NaOCl and $HgCl_2$, NaOCl was found better for controlling the infection and it had not any adverse effect on explants even in long duration. Gopal *et al.* (1998) have reported the use of $HgCl_2$ for 5 minutes, it being a strong sterilant was used by them in combination with Sodium Lauryl Sulphate. Villafranca *et al*. (1998) surface sterilized the sprouts with 1% sodium hypochlorite contain 3-4 drops of Tween-20 solutions for 10 minutes.

2.6.3. *In vitro* intiation and multiplication of sweet potato

Effect of different concentration of cytokinins with auxin studied on establishment stage for shoot explants cultured *in vitro* had attributed to the mode of action of BAP as cytokinins at 1.0 mg/l on promotion both cell division and growth of axillary shoots in plant tissue culture of sweet potato (Newell *et al*.,1995). Shoot regeneration from bud derived calli of sweet potato was also done using regeneration medium containing BAP only (Wang *et al*., 1999). Furthermore, Oggema *et al.* (2007) regenerated shoots from leaf callus initiated onto medium containing 2; 4-D and BAP at 0.5mg/l or BAP alone medium contain 1 mg/l.

Once the aseptic cultures are established, next step is to develop methods for continuous production of efficient multiplication. Plant growth hormones are, perhaps, one of the prominent factors that influence the rate of multiplication efficiency. For instance, application of cytokinins eliminates apical dominance; there by stimulating the growth of lateral buds (George *et al*., 2008). The most commonly used cytokinins for shoot proliferation in sweet potato genotypes are Benzylaminopurine (BAP) at 2mg/l, Isopentenyl-adinine (2-ip) 0.5 mg/l, furfurylaminopurine (Kinetin) 0.5 mg/l, Thidiazuron (TDZ) 1 mg/l and Zeatin 1.5 mg/l supplemented on MS medium (Goussard, 1981).

The optimal cytokinins or growth regulators required for specific morphogenic response arises with the sweet potato genotype under investigation. This is because; the endogenous level of growth regulators vary with the genotype of the species and largely influence the requirement of the exogenous hormones in the plant system (Fatima *et al*., 2009). Explants with multiple shoots proliferated on MS medium containing; BAP had showed good performance of sweet potato (Sharma, 2007). He also revealed the

effectiveness of the combination treatments namely 2 mg/l BAP + 0.01mg/l, NAA + and 0.1mg/l GA3 + 0.05 mg/l Kn for shoot elongation. Multiple shoots obtained from lateral bud explants on BAP containing media did not elongate when transferred to medium containing GA3 0.1mg/l + Kinetin 0.05mg/l. However, when transferred to 1mg/l BAP and 0.1mg/l GA3 maximal shoot elongation was occurred (Venkataiah *et al*., 2006).

2.6.4. Rooting and Acclimatization of sweet potato

In vitro propagation of plants, adventitious root induction and formation are fundamental for successful production of viable plantlets. Root induction of any *in vitro* culture is dependent on the proper amount of rooting growth hormones. The most commonly used auxins for root induction includes indole-3-butyric-acid (IBA), Indole-3-acetic-acid (IAA) and α-naphthalene-acetic-acid (NAA), with IBA being the most effective for root induction and most commonly used for sweet potato genotypes. Different concentrations of auxins or combinations have been applied depending on rooting treatments (Deklerk *et al*., 1997). For *in vitro* root induction the micro cuttings or shoots are maintained in a culture media with low mineral concentrations (½ MS or less) and low auxins concentrations to stimulate adventitious root induction (Kim *et al*., 2003). The best rooting induction and elongation sweet potato occurred on medium containing 0.05mg/l NAA and 0.1mg/l IBA (Hartmann *et al*., 1990).

In vitro propagation has mainly five steps: culture establishment or initiation, multiplication, rooting of micro shoots and acclimatization. However, *in vitro* rooting process is expensive and can even double the final price of *in vitro* propagated plants. Therefore, the simultaneous *ex vitro* rooting and acclimatization have been trialed as there is a reduction in a cost by avoiding the *in vitro* rooting, reduction in labor and the time of establishment from laboratory to soil. It also gives the plants better developed root system (McClelland *et al*., 1990; Kim, *et al*., 1998; Martin, 2003; Thomas and Schiefelbein, 2005; Tileye *et al*, 2007; Xu *et al*, 2008). Ranaweera *et al*. (2013) reported 71% cost reduction using *ex vitro* rooting of tea (*Camellia sinensis* L.) compared to *in vitro* rooting phase. Under the same circumstances, root formation and acclimatization occur simultaneously in a controlled environment such as a greenhouse, conditions similar to those used for acclimatization of *in vitro* rooted plants (Hazarika, 2003).

Acclimatization of *in vitro* propagated plants to the *ex vitro* environment is a critical step for successful propagation. It is ultimately depending on their ability to withstand the conditions transferring from *in vitro* to *ex vitro* because the *in vitro* environments are highly conducive than *ex vitro* environment. Rooted and un rooted micro shoots are removed from the culture vessel, agar is washed away completely to remove a potential source of contamination and the micro plants are transplanted in to a standard pasteurized rooting or soil mix in small pot. Acclimatization of the micro-shoots was performed as described previously (Pal *et al*., 2007; Alam *et al*., 2010; Venkataiah *et al*., 2006) and kept for one month's in green house or glasshouse condition.

2.7. Shoot organogenesis in sweet potato

Organogenesis is the production of organs, either directly from explants or from a callus culture without the production of somatic embryos. Organogenesis relies on the inherent plasticity of plant tissues, and is regulated by altering the components of the medium (Slater *et al*., 2008). Direct shoot organogenesis in sweet potato explants has been demonstrated in many studies (Gong *et al*., 2005; Gosukonda *et al*., 1995a; Santa-Maria *et al*., 2009). Regeneration efficiencies ranged from 2% - 93% depending on the cultivar. Dessai *et al*. (1995) used a modified protocol with only 2, 4-D in the first medium. They achieved 10% to 83% regeneration efficiency with 15 out of the 25 tested cultivars. The optimal type of auxins and cytokinins used in each stage of the protocol may vary among cultivars.

2.8. Factors that affect *in vitro* culture of sweet potato

2.8.1. Plant genotype

Genotype has been shown to be a major limiting factor in establishment of plant regeneration in sweet potato. Many cultivars give low or no embryogenic responses at all during *in vitro* culture (Desamero *et al*., 1994). Triqui *et al*. (2007) found that three out of six sweet potato cultivars were completely recalcitrant to regeneration even after experimenting with them on medium supplemented with three different types of auxins. In addition different laboratories report different results for the same variety indicating that regeneration of sweet potato is difficult to reproduce (Moran *et al*., 1998). Inconsistencies in regeneration responses within the same cultivar may be due to a variation in the

developmental and physiological stage of *in vitro* plants, affecting the cultural behaviour of explants (Jones *et al*., 2007; Triqui *et al*., 2007).

2.8.2. Type of plant organ

Apart from the overall genotype of the plant, the plant organ used as explants also has an effect on regeneration efficiency (Dodds *et al*., 1992). Although plant growth regulators may help to induce regeneration, cell in some parts of the plant appear to be partially predetermined to a particular morphogenetic pathway so that it takes only a slight change in environment to induce the tissues of some organs to form an adventitious meristem or somatic embryo instead of progressing to become a differentiated cell within the mother intact plant. In sweet potato, somatic embryogenesis can be initiated from anther derived callus (Tsay and Tseng,1979), leaf, storage root discs (Newell *et al*., 1995), shoot tip, stem internodes (Song *et al*., 2004), root explants (Liu and Cantliffe,1984) and lateral buds (Jarret *et al*.,1984). When using different types of sweet potato explants, various groups obtained different results.

2.8.3. Age and size of explants

Apart from genotype and type of plant organ, the age and size of the explants has also been implicated in influencing regeneration efficiency of sweet potato. Triqui *et al*.(2006) found that embryogenic response in sweet potato highly depended on the size of lateral buds incubated. Only buds with a size of 0.5 to 1 mm were suitable for embryogenic induction. They found that buds greater than 1 mm in length had a tendency to form non embryogenic green callus, while those under 0.5 mm failed to grow or develop in any manner. The age of explants is also an important factor. Dessai *et al*. (1995) observed that sweet potato leaves from the fourth position downward (older) generally responded poorly performance. Differences in regenerative abilities of leaves of varying ages may be the result of differences in internal auxins, cytokinins and/or Abscisic acid (ABA) levels. Also the high metabolic activity in young developing plants may contribute to organogenesis *in vitro* culture (Ritchie and Hodges, 1993).

2.8.4. Physical environment

Optimization of the micro environment is a key step *in vitro* propagation and ensures the production of good quality plantlets that have high chances of surviving the *exvitro* conditions in greenhouse and ultimately the natural environment. Light and temperature

cycles, necessary for optimum growth of cultured plantlets, are programmable and controllable according to the individual plant species' requirements (Johnson, 1996).

Plant development and growth is influenced by wavelength, intensity and the duration of light and this can be controlled under *in vitro* propagation according to plant species and the *in vitro* culture objectives. Light is also an important factor for phototropism, morphogenesis and photosynthesis (Read and Preece, 2003). It has been observed that light can enhance shoot growth and root formation in some species, whilst other species preferred darkness for root induction (Kumar, 2003). Plants have a system of sensory photoreceptors that monitor changes in the ambient light environment (Gyula *et al*., 2003). Light energy for photosynthesis is generally measured by photosynthesis photon flux density (PPFD) on the growing plantlet (Ibaraki and Nozaki, 2005). In plant *in vitro* culture systems, low light intensities (15-65 $\mu molm^{-2s-1}$) are normally used, as exposure to high PFD can result in photo inhibition and photo oxidative damage to the fragile photosynthetic apparatus (Kodym and Zapata, 1998). Plants respond differently to the spectral wavelength of light and the relationships become even more complex due to interactions with other factors such as temperature, photoperiod and light intensity (Mack, 2009).

A maximum shoot regeneration and number of shoots per culture were obtained at 16h photoperiod of 4000 lux light intensity at room temperature for different type of sweet potato cultivars. Ali *et al*. (2008) showed the fluorescent light having 2500-lux light intensity and $26^0C \pm 1^0C$ incubation temperature with 16/8 hour light/dark period was optimal. Benisheikh *et al*. (2012) reported that a 16/8hours light/dark photoperiod at 27 ± 2^0C temperature with 2000-3000 Lux light intensity was optimal for cultures placing at 25-30 cm fluorescent light.

Plant development in micropropagation also depends on the optimum temperature range for the physiological process of respiration, but these optimum temperatures vary according to species and genotypes. The biophysical and biochemical process of photosynthesis is also temperature dependent and is a major determinant of the rate of growth of plants (Gomes *et al*., 2006). In nature, the perception of ambient temperature allows for the maintenance of plant homeostasis, thereby buffering against potential disruptive effects on cellular stability (Franklin, 2009). However, in culture a constant temperature regime is normally maintained and this may have effects on the development

of *in vitro* plants. Culture temperatures between 20°C and 27°C are most commonly applied to *in vitro* culture of sweet potato (Read and Preece, 2003).

3. MATERIALS AND METHODS

3.1. Plant Materials

The study was conducted using two orange fleshed sweet potato genotypes, Kulfo (Lo 323) and Tulla (CIP 420027). The genotypes were selected based on their agronomic performance and they are newly introduced and being widely distributed to farmers, in various parts of Ethiopia including Hawassa as a candidate to alleviate vitamin-A deficiency in humans' nutrition. The average productivity of Tulla at Hawassa Research Centre was 28.5ton/ha, whereas Kulfo gives 27 ton/ha. The genotypes were released by South Agricultural Research Institute (SARI) in 2005.

The materials were obtained from Hawassa Agricultural Research Centre, Southern Nations Nationalities and Peoples Regional State (SNNPRs), Ethiopia. Vine cuttings of about 25 cm long were planted and grown in greenhouse at the College of Agriculture and Veterinary Medicine, Jimma University. The mother plants were watered twice per day and allowed to grow for one month after which actively growing shoot tips were collected and prepared as source of explants.

3.2. Media Preparation

3.2.1. Stock solution and plant growth regulators preparation

MS media (Murashige and Skoog, 1962) supplemented with various plant growth regulators were used. Stock solutions of the macro salts, micro salts, vitamins, iron source and plant growth regulators (1mg: 1ml) were prepared and stored at $+4^{o}C$ in refrigerator for immediate use. Plant growth regulators, IBA was dissolved using a drop of ethanol and cytokinins (BAP) by NaOH before making up the final volume with distilled water. The dissolved solution was poured into labeled volumetric flask to be fully dissolved and finally stored in refrigerator for later use.

3.2.2. Culture Medium Preparation

The culture medium was prepared from their respective stock solutions, contained with the appropriate amount of 30 g/l sucrose and plant growth regulators (BAP and IBA) were added to the medium as required at various concentrations. The mixture was stirred using magnetic stirrer and the volume was adjusted using ddH_2O. Then, pH was adjusted in all cases to 5.8 using 1M NaOH and 1M Hcl. Finally, 8.0 g/l agar was added and heated to

melt throughout the experiment. Before autoclaving, the media were dispensed into sterilized culture jars. The media were steam sterilized using autoclave machine at a temperature of 121^{o}C with a pressure of 0.15 Kpa for 15 minutes and transferred to the culture room and stored until for later use.

3.2.3. Sterilization and Initiation of Cultures

Healthy vine shoot tip of Kulfo and Tulla sweet potato genotypes were collected as an explant. The explants were then washed with soap solution with distilled water until the foam rejected. Then the explants were taken into sterilized laminar airflow cabinet, and dipped in 70% ethanol for 1min in a sterilized jar and washed using sterile distilled water three times for 5 min. They were then sterilized with 1% (v/v) commercial bleach (Berekina) solution containing 3–4 drops of Tween-20 for 15 minutes and rinsed 4 times with sterile double distilled water each for 5min with gentle shacking to remove the chemical residue under aseptic laminar air condition. The damaged parts were excised off using a sterile scalpel and about 1–1.5cm long explants were introduced into the nutrient media. The cultures were maintained at room temperature with 16/8h light and dark photoperiod respectively and used cool white fluorescent lamps in the growth room.

3.3. Experiment 1. Effect of Different Concentration of BAP on Shoot Initiation

The sterilized explants were cultured on basal MS medium supplemented with various concentrations of BAP (0.5, 1.0, 1.5, and 2.0 mg\l). Murashige and Skoog medium without PGRs were used as control for shoot initiation from shoot tip explants. The experiment was laid down in completely randomized design in factorial arrangement (2X5 treatments, 2 genotype and 5 levels of BAP) with nine regenerated shoots per treatment. After 3 weeks, data on shoot intiation and shoot length were recorded.

3.4. Experiment 2. Effect of Different Concentrations of BAP on Shoot multiplication

For shoot multiplication experiment the initiated shoots were taken after 3 weeks of first culture and then cultured on hormone free MS basal medium for two weeks to avoid carry over effects. Medium supplemented with different concentrations of BAP was used for shoot multiplication. In this experiment, BAP (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg\l) were used. The experiment was replicated three times arranged in completely randomized design (2x7 two genotypes and seven levels of PGRs) with 15 regenerated shoots per treatment. The cultures were placed in white florescent light room adjusted at 16/8 h

light/dark at room temperature. Data on shoot number, shoot length and leaf number were recorded after 5 weeks of culture.

3.5. Experiment 3. Comparison of *in vitro* and *ex vitro* on root formation

For *in vitro* rooting experiment, multiplied shoots were cultured on hormone free MS medium to avoiding carry over effect. Then shoots of 1 cm or more long were transferred to ½ MS medium containing, 0.0, 0.1, 0.5, 0.75 and 1.0 mg/l IBA. Completely randomized design (CRD) with factorial arrangements and three replications of 15 shoots per treatment was used for *in vitro* rooting. Another one treatment was conducted to test *ex vitro* root induction from multiplied shoots in order to compare with *in vitro* rooting experiment and replicated three times. Then about 2 cm of 150 in *vitro* multiplied micro-shoots for both cultivars were carefully excised and directly transferred to greenhouse for rooting as well as hardening simultaneously. After one month, mean number of roots, mean length and percentage of rooted plantlets in each treatment were recorded for both *in vitro* and *ex vitro* treatments.

3.6. Acclimatization

Plantlets with well developed root and leaf systems were washed with tap water to remove adhering media and sucrose attached on the roots of plantlets. Twenty five plantlets from each genotype were transferred to plastic pots in green house containing hardening medium composed of soil, compost and sand (1:1:2) ratio,- respectively. The plants were placed in pots covered with transparent plastic bags (in order to keep humidity) and irrigated using sprayer every day. Plastic cover were removed partially after a week and completely removed after two weeks. Finally after 30 days, the survival rates of the plantlets were evaluated by counting the number of successfully acclimatized plants.

3.7. Data Analysis

SAS software (SAS, 2008 9.2 version) was used for data analysis and for significantly different treatments, mean separation was done with Least Significance Difference (LSD) at or below the probability level of 0.01 (at 1% significance level).

4. RESULTS AND DISCUSSION

4.1. Effects of BAP on Shoot Initiation

Analysis of variance revealed that the interaction effects of genotype and BAP were highly significant ($p<0.01$) for percentage of shoot initiation and shoot length (Appendix 1). Similarly, the analysis of variance indicated that the effect of BAP concentrations were highly significantly different ($p<0.01$) both for percentage of shoot initiation and shoot length The two sweet potato genotypes did not show any significant difference in the percentage of shoot initiation *in vitro* plantlets on MS medium.

The genotype Kulfo gave the higher (77.78±0.23) percentages of explants showing shoot initiation with average shoot length of 4.40±0.11cm on MS media supplemented with 0.5 mg/l BAP (Table1, Fig 2). An average percentage of shoot initiation (66.67±0.00) was observed on growth regulators free medium; whereas, Tulla produced maximum shoot initiation percentage (88.89±0.23) with 4.47±0.15 cm shoot length on MS medium supplemented with 1.0 mg/l BAP. The minimum percent of shoot initiation (33.33±0.00) was observed on 2mg/l BAP for both genotypes. A significant percentage of shoot initiation (55.56±0.39) was observed on free medium (Table1, Fig1). No significant difference was observed in both genotypes between hormones free, 0.5 and 1.0mg/l of BAP on percent of shoot induction. Therefore, hormone free medium could be taken as the best option economically as it reduces the cost of the growth regulator.

In current study, it was found that increase in the concentration of BAP (0.5-2mg/l) increased the percentage of explants showing shoot induction from 77.78±0.23 and 66.67±0.00 to 88.89±0.23 for Kulfo and Tulla genotypes, respectively. However, it started declining as the concentrations kept increasing to 2mg/l. Shoot initiation of both Kulfo and Tulla genotypes were significantly low at the maximum concentration of BAP. This is due to high concentration of PGRs that leads to metabolic inhibition. Moreover, lower shoot induction was for Kulfo than Tulla genotypes under both cytokinins used suggesting variation due to genotypic difference on the same PGRs and an endogenous cytokinins concentration difference which affects the frequency of shoot organogenesis. Gosukonda

et al. (1995) found that different sweet potato varieties respond differently to *in vitro* shoot induction media.

The differences in shoot length (cm) for treatments were highly significant. The maximum and the minimum shoot lengths for Kulfo on BAP 0.5mg/l and 2.0mg/l concentrations were (4.40±0.11 and 2.03±0.07), respectively, whereas the maximum shoot lengths (4.47±0.15 cm) and the minimum shoot length (2.13±0.05 cm) were resulted for Tulla genotype at BAP 1.0mg/l and growth regulator free levels, respectively. As the BAP concentrations increased, the shoot lengths were also increased till it reached to the optimum (0.5mg/l for Kulfo and 1.0 mg/l BAP for Tulla) and starts to decline as the BAP concentration kept increasing. The lengths of shoots of the developing plantlets were influenced by the concentration of BAP in the culture medium. Lower concentration of BAP led to increased length while an increased concentration decreased the length of the plantlets. The reduction in the shoot length with the increased in BAP concentration in the culture medium may be as a result of inhibitory effect of BAP at higher concentration on shoot elongation. According to George *et al*. (2008) higher concentration of BAP inhibits shoot elongation.

The current result is in conformity with the finding of Sowal *et al*. (2002) who reported the effectiveness of low concentration of BAP to result in rapid shoot initiation due to the activation of tRNA cytokinins resulting in rapid proliferation of shoot primordial. In similar study, Khalafalla *et al.* (2007) also reported that BAP at the concentration of 5mg/l gives low number of shoot regeneration of sweet potato explants and they concluded that shoot initiation and shoot lengths decreases with BAP concentrations increase.

Percent shoot regeneration of 66.67 and 55.56 were obtained in growth regulators free media for both Kulfo and Tulla genotypes. The present study also agrees with the study of Tasew (2011), who reported the highest shoots regenerated on growth regulators free medium from leaf calli of Beletech and Awassa-83 varieties. This result is also in agreement with the work of Otani *et al*. (1996) who reported that synthetic cytokinins inhibit shoot elongation at higher concentrations and who pointed out that endogenous concentration of PGRs could affect growth. Possible justification for the concentration of cytokinin increased beyond the optimal need of the plant; they inhibit the release of endogenous cytokinins and assimilation of the given nutrients by inhibiting the activities of enzymes. They observed that the higher frequency of shoot regeneration was obtained

on the hormone free medium and the percentage of shoot regeneration was reduced with an increase in BAP concentration.

The present study is in contrary with the findings of Otani and Shimada (1988) who showed that shoot regeneration from leaf calli of *Ipomoea trichocarpa* was induced only on the medium supplemented with more than 2 mg/l BAP, and the one with 10 mg/l was the most effective. The present results from Tulla's showed much higher shoot induction percentage than the work of Xiansong (2010), who obtained the percentage of plant regeneration of shoots of the genotype of purple-fleshed sweet potato (cv. "Zishu No.10") is a low rate (20.83%) at BAP 1.0 mg/l. Sato *et al*. (1999) also obtained that the petiole calli of Genki sweet potato variety that had been cultured on the regeneration medium supplemented with 3.0 mg/l BAP gave a higher regeneration frequency than those cultured on the hormone free medium. The difference could be due to differences in genotypes, may be related to the endogenous levels of hormone and type of explant used in various explants.

A maximum shoot length of 4.40 and 4.47 cm was obtained for both Kulfo and Tulla genotypes, respectively. The current findings were in line with Berihu (2014), who obtained an average shoot length of 3.96 and 3.86 cm from Kulfo and Tulla genotypes using bud culture. Garcia *et al*. (2005) also reported the effectiveness of low concentration of cytokinin for shoot proliferation from shoot tip explants in *I. batatas* L.cv.

Genotypes	BAP Conc.(mg/l)	Percentage of Shoot Initiation(Mean±SD)	Shoot length (cm) (Mean±SD)
Kulfo	0	66.67^{ab}±0.00	3.00^{d}±0.10
	0.5	77.78^{a}±0.23	4.40^{a}±0.11
	1	55.56^{ab}±0.39	3.41^{c}±0.07
	1.5	55.56^{ab}±0.39	2.18^{e}±0.07
	2	33.33^{b}±0.00	2.03^{e}±0.07
Tulla	0	55.56^{ab}±0.39	2.13^{e}±0.05
	0.5	66.67^{ab}±0.00	4.13^{b}±0.12
	1	88.89^{a}±0.23	4.47^{a}±0.15
	1.5	33.33^{b}±0.00	4.10^{b}±0.10
	2	33.33^{b}±0.00	3.50^{c}±0.05
	CV	5.74	2.82

Table 1: Effect of BAP on Shoot Initiation of Kulfo and Tulla Varieties

Note: BAP= Benzyl Amino Purine. Means with the same letter in the same column are not significantly different at 0.01 probability level, CV= Coefficient of Variation.

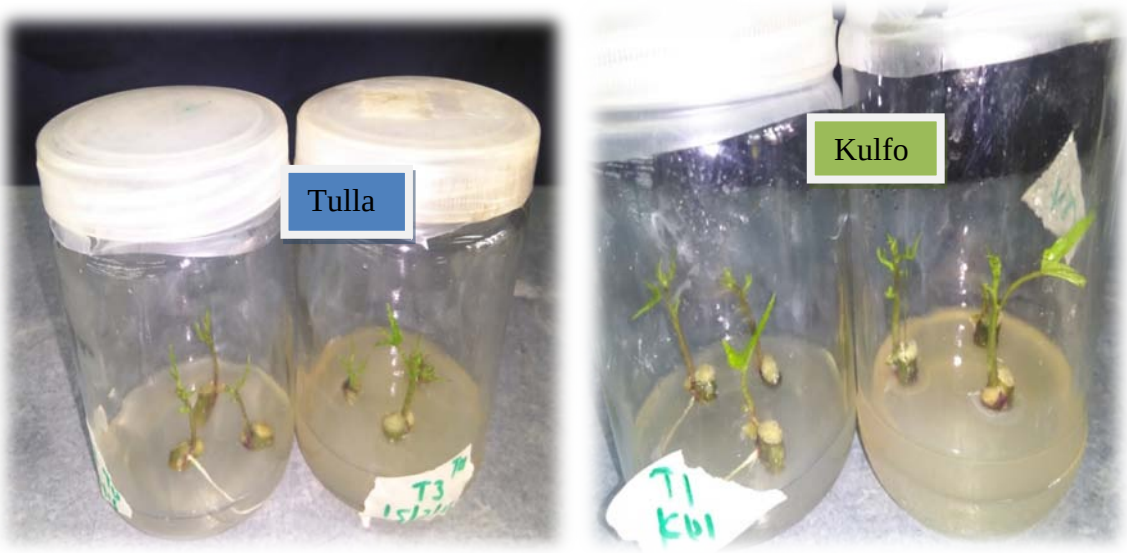

Figure2. *In vitro* shoot intiation of Tulla and Kulfo on MS+1.0 mg/l BAP on hormone free MS medium

4.2. Effects of BAP on shoot multiplication of two genotypes

Analysis of variance revealed that the interaction effects of genotype and BAP were highly significant ($p<0.01$) for number of shoots/explant, shoot length and number of leaves/shoot (Appendix 2). The genotypes were also highly significant different for number of shoots per explant, shoot length and number of leaves per shoot.

The effect of BAP on shoot multiplication showed that the Kulfo variety performed best with 5.33 ±0.34 shoots/explant and 7.82 ±0.02 cm average shoot length and 6.33 ±0.34 leaves/ shoot on MS medium supplemented with 1.0mg/l BAP (Table 2; Fig.3). Tulla produced 6.78±0.19 shoots/explant with 9.70 ±0.00 cm shoot length and 9.67 ±0.06 leaves/shoot on MS media fortified by 2mg/l BAP (Table2; Fig.2). The minimum (1.00 ±0.00) shoots with 2.11±0.12cm shoot length and 2.78 ±0.19 leaves was formed for Kulfo genotypes, whereas Tulla produced 1.33±0.33 shoots/explant with 2.94±0.02cm shoot length and 3.11±0.19 leaves/ shoot on MS media supplemented with 3.0mg/l BAP.

In this finding of Kulfo, it was showed that as the concentration of BAP increased from 0.5-3mg/l, number of shoots/explant, shoot length/explant and leaf number also increased from 3.67±0.33 to 5.33±0.34, 6.51±0.04 to7.82±0.02, and 3.00 ±0.33 to 8.22±0.19,

respectively and then started declining as the concentrations kept increasing to 3mg/l. In addition, Tulla showed continuously increased proliferation of shoot number, shoot length and number of leaves/explant from 3.00±0.33 to 6.78±0.19, 5.82±0.01 to 9.70 ±0.00, 3.56 ±0.20 to 9.67±0.34, respectively and also declined when BAP increased to 3.0 mg/l. The result revealed that shoot number, shoot length and leaves numbers /explant were decreased when the concentration of BAP increased, shoot formation of two genotypes was significantly lower at the highest used concentration of BAP; the effect of BAP at 0.5, 1.0 and 1.5 mg/l was significantly higher than its effect at 2.0, 2.5 and 3.0mg/l of both varieties showing similar trend to BAP, i.e., higher number of shoot per explant at 0.5, 1.0 and 1.5mg/l and lower number of shoot per explant at 2.0, 2.5 and 3.0mg /l (Table 2).

Moreover, shoot number obtained was lower for Kulfo than Tulla varieties, suggesting variation due to genotypic difference or varietal responses to the same PGRs, and also an indication that exogenous growth regulator supplement inhibited elongation and multiple shoot formation which affects the frequency of shoot organogenesis. This requires that novel or modified *in vitro* regeneration procedures must be developed for each genotype because of the significant variations in response to hormone treatments. Augmenting the culture medium at BAP 1.0 mg/l and 2mg/l showed highest value 5.33±0.00 and 6.78±0.19 number of shoots/explant and, 7.82±0.02 and 9.70±0.00 shoot length for Kulfo and Tulla varieties, respectively.

BAP is an essential exogenous PGR in sweet potato shoot tip culture reported by Tang and Wang (1994). However, BAP at higher concentrations not only reduced the number of shoots but also resulted in stunted growth of the shoots. As the BAP increased (>2.5 mg/l) the shoots showed bushy and distorted growth. This agrees with the previous findings on different genotypes of sweet potato by Geleta and Tileye (2011) and Neja (2009).

The current result is in line with Addisu (2013) who obtained the highest mean number of shoots on MS medium containing 1.0 mg/l BAP and 2.0 mg/l BAP for Awassa-83, Beletech, Adu and Barkumie sweet potato genotypes. According to Onuoch and Onwubiku (2007), BAP has physiological behavior that exhibit inhibitory effects on shoot elongation and multiplication at higher concentrations. Tassew (2012) also reported that as compared to BAP alone and the medium supplemented with combination of the two growth regulators (BAP and GA3), has resulted in less number of shoots per node and BAP is most effective on Beletech and Awassa-83 varieties. As the concentration of GA3

increased while maintaining constant BAP, degeneration of shoot to calli was observed

Genotypes	BAP Conc. (mg/l)	Shoot number (MEAN±SD)	Shoot length (MEAN±SD)	Leaf number (MEAN±SD)

instead of shoot during shoot multiplication.

A maximum shoot multiplication of 6.78±0.19 shoots/explant and 9.70±0.00 cm shoot length was obtained for Tulla genotype at BAP level of 2.0 mg/l. The present result of Tulla is different from the results of Tasew (2012) who obtained only degenerated shoots to calli and 2.50±0.50, 2.70±0.21, 3.30±0.26 and 3.10±0.23 cm shoot lengths were obtained at 2 mg/l BAP both from petiole and leaf calli of Beletech and Awassa-83 varieties, respectively. The difference could be due to differences in genotypes response to PGRs and type of explant used.

Table 2: Effect of BAP on Shoot Multiplication of Kulfo and Tulla Genotypes

	0	$2.00^{ef} \pm 0.00$	$5.10^{g} \pm 0.10$	$3.22^{gh} \pm 0.19$
	0.5	$3.67^{c} \pm 0.33$	$6.51^{e} \pm 0.04$	$3.00^{gh} \pm 0.33$
Kulfo	1	$5.33^{b} \pm 0.00$	$7.82^{b} \pm 0.02$	$6.33^{d} \pm 0.34$
	1.5	$2.67^{de} \pm 0.33$	$6.44^{e} \pm 0.03$	$5.11^{e} \pm 0.01$
	2	$4.33^{c} \pm 0.00$	$7.20^{d} \pm 0.10$	$8.22^{b} \pm 0.19$
	2.5	$2.33^{de} \pm 0.34$	$5.20^{g} \pm 0.10$	$5.44^{e} \pm 0.20$
	3	$1.00^{g} \pm 0.00$	$2.11^{j} \pm 0.12$	$2.78^{h} \pm 0.19$
	0	$1.33^{fg} \pm 0.34$	$4.31^{h} \pm 0.01$	$4.22^{f} \pm 0.19$
Tulla	0.5	$3.00^{d} \pm 0.08$	$5.82^{f} \pm 0.01$	$3.56^{g} \pm 0.20$
	1	$4.33^{c} \pm 0.34$	$7.48^{c} \pm 0.02$	$9.22^{a} \pm 0.19$
	1.5	$5.33^{b} \pm 0.05$	$9.68^{a} \pm 0.02$	$5.67^{e} \pm 0.33$
	2	$6.78^{a} \pm 0.19$	$9.70^{a} \pm 0.00$	$9.67^{a} \pm 0.06$
	2.5	$5.11^{b} \pm 0.20$	$5.85^{f} \pm 0.01$	$7.33^{c} \pm 0.34$
	3	$1.30^{fg} \pm 0.23$	$2.94^{i} \pm 0.02$	$3.11^{gh} \pm 0.19$
	CV	8.11	0.93	4.73

Note: BAP=Benzyl Amino Purine. Means with the same letter in the same column are not significantly different at 0.01 probability level.

Figure 3: *In vitro* shoot multiplication of Tulla on MS+2.0 mg/l BAP and after 5 weeks of culture

Figure 4: *In vitro* shoot multiplication of Kulfo on MS+1.0 mg/l BAP after 5 weeks of culture

4.3. Comparison of *in vitro* and *ex vitro* rooting of *in vitro* generated micro-shoots

The analysis of variance indicated that the interaction effect of genotype and IBA application were highly significant ($p< 0.01$) for number of roots per shoot and root length of the two sweet potato Varieties (Appendix 3). The genotypes showed very highly significant difference ($p< 0.01$) in number of roots and average root length. The effect of IBA and *ex vitro* rooting also showed highly significant differences on rooting percentage**.**

Kulfo gave the highest (100±0.00) *in vitro* rooting on 1/2 MS media with PGRs free and 0.5mg/l IBA, and the minimum *in vitro* rooted shoots (33.33±0.01) were recorded on 1/2 MS medium containing 1mg/l IBA, while 93.33±0.00 root formation was obtained from *ex vitro* rooting, whereas Tulla produced a maximum of *in vitro* roots 88.89±0.00 when supplemented with ½ MS free media and at 0.1mg/l IBA concentration (Table 3). From the rooting experiment, *ex vitro* could be taken as the best option that replaces *in vitro* rooting formation economically as it reduces the cost of the growth regulator. This implies the less difficulty of rooting in sweet potato even without and/or with less concentration of auxin. It is due to the ability of sweet potato to propagate through vegetative means and although it is possible that there were high endogenous auxin concentration in the explanted organ (Benmahioul *et al.*, 2012).

The impact of IBA on percent root formation of both Varieties showed similar trend to IBA, i.e., higher root formation on PGRs free medium, 0.1 and 0.5 mg/l and lower root formation at 0.75 and 1mg/l IBA (Table 3). By increasing the concentration of IBA from 0.1mg/l to 1mg/l, percentage of rooted shoots decreased continuously from 100% to 33.33% in Kulfo, and discontinuously decreased from 88.89% to 44.44% in Tulla. This result indicates that each genotype requires different concentrations based on the amount of their endogenous auxin concentration.

For mean number of roots and length of roots (cm), the effect of growth regulator had exerted significant effect. Kulfo gave the highest (7.44±0.38) number of roots/shoot with (6.22±0.11) cm average root length on 0.5mg/l IBA and a minimum of 3.33±0.33 and 4.09±0.11cm number of roots and root lengths, respectively, on ½ MS medium supplemented with 1.0mg/l IBA (Table 3). On the similar media constituents, 6.00±0.00 mean number of roots/shoot with 6.85±0.02cm average root length was observed for Tulla. As the concentration of IBA increased, number of root and the length of roots were

significantly reduced for both genotypes. This indicates that rooting was highly influenced by the concentrations of IBA used. Hence, appropriate amounts of auxin in the rooting medium are crucial for root induction. This agrees with the work of Geleta and Tileye (2011) on Awassa-83, Guntute and Awassa local varieties.

The current results are supported by the findings of Berihu (2013) who reported 93 % up to 100% *in vitro* rooting on PGRs free medium for Kulfo and Tula genotypes using lateral bud culture. The present findings also agrees with the results obtained by Addisu (2013) that resulted in highest mean root lengths exhibited by Adu (7.29), Barkumie (7.48), Beletech (7.12) and Awassa-83 (7.05) cm in PGR free medium.

The highest (7.44 and 6.00) number of roots/shoot were obtained at 0.5 and 0.1mg/l IBA for Kulfo and Tulla genotypes, respectively. These results are also in conformity to that of Tasew (2012) obtained the highest (6.80 and 6.97) mean number of roots on hormone free MS medium for Awassa 83 and Beletech varieties, respectively. The present result for Tulla disagrees with Addisu (2013) work who obtained the lowest mean number of roots per explant for Barkumie (3.76) and Awassa-83 (4.60) in the medium supplemented with 0.1 mg/l IBA level. This is due to that each genotype responded differently due to their endogenous auxin amount (Neja (2009).

Roots developed through *ex vitro* rooting were significantly longer than those developed *in vitro*. The highest (9.80±0.00 and 8.40±0.00) cm root length were recorded at *ex vitro* rooting formation and (8.27±0.05 and 8.24±0.05) cm root length were observed *in vitro* rooting of both Kulfo and Tulla genotypes, respectively. This indicated that *ex vitro* rooting gave the best results in rooting response and root lengths. Furthermore, the rooting quality is better in *ex vitro* rooted plantlets which thought to enhance the chance of survival of plantlets in the greenhouse conditions. It is a cost effective technique and could save labour, time and energy and better rooting system in plant propagation system and is used to simplify the procedure because no *in vitro* rooting step under sterile conditions is required, owing to the fact that rooting and acclimatization take place simultaneously, previously the experiment was conducted by Martin (2003; Benmahioul *et al. (*2012); Ponnusamy and Van Staden (2013); Ranaweera *et al*. (2013); Mahipal *et al. (*2015).

The roots developed from *ex vitro* rooting system were non fragile like naturally developed root system. However, *in vitro* developed roots have been found to be thick,

fragile and easily breakable during handling. This makes the *ex vitro* rooting method more suitable compared with the *in vitro* development of roots. There are evidences that roots growing in agar medium show structural abnormalities (Kataoka, 1994) and often lack root hairs which affect their establishment infield soil under commercial -scale cultivation (Debergh and Maene, 1981). Similar problem has also been observed in case of *in vitro* developed micro- shoots of blue honeysuckle (Karhu, 1997).

In absence of IBA treatment, 93.33% and 86.7% *ex vitro* rooted plants were obtained for Kulfo and Tulla genotypes, respectively, which could be acclimatized simultaneously. Similar studies showed that *ex vitro* root induction was successfully done by many researchers in *Passiflora edulis* (Mahipal *et al.,* 2015); *Malus zumi* (Jin *et al.,* 2008); *Hagenia abyssinica* (Tileye *et al*., 2007) and so forth. These findings are similar to that of Mahipal *et al.* (2015) who reported 100% rooting response for *ex vitro* experiments and Mangal *et al.* (2015) experiment who reported 70 % of shoots for *Jatropha curcas* rooted *ex vitro.* Micro shoots of both sweet potato varieties rooted better under *ex vitro* condition than *in vitro.*

Table 3: Effect of IBA on Rooting of Sweet potato Varieties (Kulfo and Tulla)

Genotypes	IBA (mg/l)	Rooting % (Mean ±SD)	No.of roots (Mean±SD)	Root length (Mean±SD)
Kulfo	0.0	100^{a}±0.00	1.67^{i}±0.00	8.27^{b}±0.05
	0.1	88.89^{ab}±0.00	4.22^{d}±0.19	7.19^{c}±0.03
	0.5	100^{a}±0.23	7.44^{a}±0.38	6.22^{e}±0.11
	0.75	66.67^{abc}±0.00	5.22^{c}±0.19	4.99^{f}±0.01
	1	33.33^{c}±0.01	3.33^{ef}±0.33	4.09^{g}±0.11
	Ex	93.33^{a}±0.00	3.85^{de}±0.00	9.80^{a}±0.00
Tulla	0	88.89^{ab}±0.24	2.11^{hi}±0.19	8.24^{b}±0.05
	0.1	88.89^{ab}±0.23	6.00^{b}±0.00	6.85^{d}±0.02
	0.5	77.78^{ab}±0.23	5.11^{c}±0.19	5.08^{f}±0.07
	0.75	66.67^{abc}±0.00	4.11^{d}±0.19	3.90^{h}±0.00
	1	44.44^{bc}±0.39	3.11^{fg}±0.19	3.41^{i}±0.01
	Ex	86.70^{ab}±0.00	2.52^{gh}±0.00	8.40^{b}±0.00
	CV	4.05	5.14	0.87

Note: Ex= *Ex vitro* rooting, IBA=Indol-3-Butyric acid. Means with the same letter in the same column are not significantly different at 0.01 probability level, CV= Coefficient of Variation.

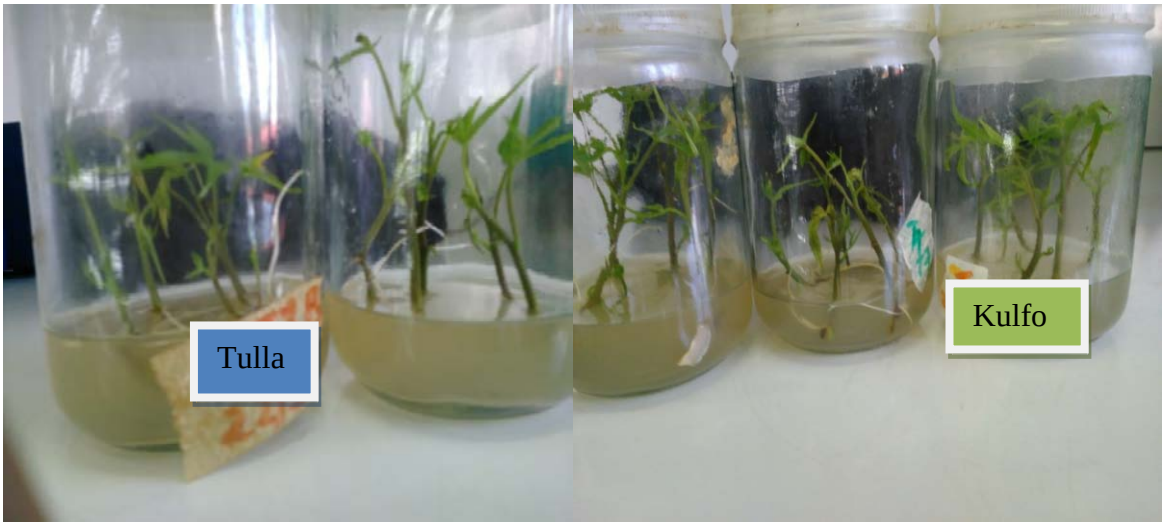

Figure 5: *In vitro* rooting of Tulla and Kulfo Varieties on PGRs free ½ MS medium

Figure 6: *Ex vitro* rooted and acclimatized plantlets of A) Tulla and B) Kulfo genotypes

4.4. Acclimatization of Plantlets

The *in vitro* as well as *ex vitro* rooted plantlets were hardened in the green house. After one month of acclimatization, 84% and 80% of plantlets were survived and successfully established from *in vitro* experiments of Kulfo and Tulla Varieties, respectively. In contrast to *in vitro* rooting, 93% and 86% of the *ex vitro* rooted plantlets were successfully survived and acclimatized (Fig 6). The difference in survival rate of the two Varieties might be due to differences in adaptation to the new environment. Better survival of *ex vitro* rooted plantlets than *in vitro* rooted plantlets may be attributed to higher root length comparable to that of *in vitro* root formation (Kumar *et al*., 2014). Baskaran and Van Staden (2013) also reported that *ex vitro* rooting in micropropagation technique could overcome the problems during acclimatization prior to transplanting in the field conditions.

The current result is in harmony with the finding of Berihu (2014) who reported 81.25% and 70.59 % successfully survived plantlets for Kulfo and Tulla Varieties, respectively, using the mixture of moist red soil, sand soil, and compost in the ratio of 1:2:1. Tasew (2013) also obtained 80% - 90% of plantlets transferred to sterilized soil were acclimatized after one month. However, this result is in contrary with the finding of Kwame *et al*. (2012) work reported that the highest survival of 66.7% and 67% plantlets for Kemb-36 and Tainurey sweet potato genotypes, respectively, using the combination of red soil and rice husks as mixture media.

Figure 7: Acclimatizated plantlets of Tulla and Kulfo genotypes in the green house.

A= Plantlets covered with plastic bags (1st week of acclimatization)

B and C= Acclimatizated plantlets of two genotypes after one month in greenhouse

5. SUMMARY AND CONCLUSION

Sweet potato (*Ipomoea batatas (L.)* Lam.) is economically important tuberous roots crop that belongs to the family Convolvulaceae. It is mainly cultivated for its expanded edible roots which contain high carbohydrate, minerals, vitamins, antioxidants and beta carotene to a large sector of the global population. Globally it is grown in an area of about 8 million hectares with production of 104.45 million tons; average yield being 13 tons/ ha in 2014, and the total area under sweet potato in Ethiopia is 0.06 million ha with production of 2.7 million tons; average yield being 45 tons/ ha in 2015.

Conventional propagation methods of sweet potato through stem cutting require large amount of materials and space for propagation and an extended period to produce plants. These limitations prevent an efficient and rapid production of sweet potato to meet the current market demand for sweet potato. In addition, there is also a high risk of disease transmission and high cost of labourer.

Thus, Plant tissue culture technology provide a novel way to produce high quality plants instead of the traditionally used cuttings and it is possible to produce large number of high quality propagules within short period of time. Therefore, the present study was initiated to optimize a suitable protocol for *in vitro* propagation of sweet potato through shoot tip culture.

The protocol involves three subsequent experiments; viz. shoot initiation, shoot multiplication and root induction were carried out. All experiments were arranged in a completely randomized design. Data was collected for number of shoots formed; shoot length (cm) and leaf number per shoot after 5 weeks of culture. For *in vitro* root induction experiment, half MS media fortified with IBA were used. For *ex vitro* experiment, the *in vitro* multiplied micro-shoots were carefully excised and directly transferred to greenhouse for rooting as well as hardening simultaneously. After 30 days, mean length, mean number of roots and percentage of rooted plantlets for each treatment were recorded for both *in vitro* and *ex vitro* treatments.

Among BAP concentrations used for shoot initiation, growth regulators free, 0.5mg/l and 1.0mg/l medium were found to be optimum concentration for Kulfo and Tulla genotypes, respectively. No significant difference was observed between growth regulators free, 0.5 and 1mg/l BAP for shoot initiation of both two varieties. Thus, hormone free medium

could be as the best option for shoot intiation of these genotypes economically as it reduces the cost of the growth regulators.The highest frequencies of initiated shoots per explant (77.78% and 88.89 %) and shoot length (4.40 and 4.47) cm were the best performances for Kulfo and Tulla varieties, respectively.

The effects of BAP concentrations on shoot multiplications were also highly significant. Kulfo gave a maximum of 5.33 ±0.00 shoots/ explant with 7.82±0.02 cm shoot length and 6.33±0.34 leaves/shoot for BAP concentration of 1.0mg/l. However, Tulla produced a maximum of 6.78±0.19 shoots/explant with 9.70 ±0.00cm shoot length and 9.67±0.06 leaf/shoot at 2mg/l BAP concentration. This multiplication difference might be contributed from either genotypic difference or endogenous hormone concentration difference between genotypes.

For *in vitro* rooting, MS medium with growth regulators free, 0.1mg/l and 0.5mg/l IBA concentrations, respectively, resulted in the highest value for percentages of shoots rooted for both two genotypes. IBA at 0.5mg/l and 0.1mg/l could be taken as the best performance (7.44±0.38 and 6.00±0.00) for number of roots/shoot of Kulfo and Tulla genotypes, respectively. For root length hormone free medium showed the best performance for *in vitro* rooting of these genotypes. Although, statically no different was observed between *in vitro* and *ex vitro* root inductions of both genotypes, but higher root length (cm) response were recorded at *ex vitro* rooting than *in vitro* rooting. About 84% and 80% of the *in vitro* rooted plantlets of Kulfo and Tulla genotypes, respectively, were acclimatized successfully, whereas, 93% and 86% of the *ex vitro* rooted plantlets of Kulfo and Tulla genotypes, respectively were survived and acclimatized successfully.

Future line of work/ Recommendation;

- Further studies will be required to improve the optimized protocol using other type plant growth regulators with the aim of increasing multiplication efficiency.
- Further studies will be use *ex vitro* root instead of *in vitro* induction for the sake of labour, time and energy, better rooting system and cost reduction.
- Agronomic performance of sweet potato derived from tissue culture regenerated plantlets should be tested.

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7. APPENDICES

Appendix Table1: ANOVA for the effect of 6-BAP on shoot intiation of two Varieties

Source	DF	% of intiation	Shoot length
		MS	MS
Var	1	0.03	3.26**
BAP	4	0.64**	3.27**
Var*BAP	4	0.19**	2.08**
CV		5.74	2.82

Note = **, highly significant at 0.01 level of probabilities. Var = Variety, MS = Mean square, DF = Degree of freedom, CV = Coefficient of Variation.

Appendix Table 2. ANOVA for the effect of 6-BAP on shoot multiplication of two Varieties

Source	DF	Shoot number	Shoot length	Leaves number
		MS	MS	MS
Var	1	7.44**	6.26**	16.09**
BAP	6	15.12**	26.80**	32.47**
Var*BAP	6	4.46**	3.74**	1.24**
CV		8.11	0.93	4.73

Note = **, highly significant at 0.01 level of probabilities. Var = Variety, MS = Mean square, DF = Degree of freedom, CV = Coefficient of variation.

Appendix Table 1. ANOVA for the effect of IBA on rooting of two genotypes

Source	DF	% of rooted shoot	root number	root length
		MS	MS	MS
IBA	5	0.74**	15.08**	27.02**
Var	2	0.01	1.93**	5.48**
Var *IBA	5	0.71**	3.17**	0.40**
CV		4.05	5.14	0.87

Note = **, highly significant at 0.01 level of probabilities. Var = Variety, MS = Mean square.

Appendix Table 4: Full MS basal medium stock solution composition

Constituents	Concentration (g/l)
NH_4NO_3	16.50
KNO_3	19.00
CaCl2.2H2O	4.40
$MgSO_4.7H_20$	3.70
KH_2PO_4	1.70
$ZnSO_4.4H_2O$	0.86
H_3BO_3	0.62
$MnSO_4.4H_2O$	2.23
$CuSO4.5H_2O$	0.03
KI	0.86
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	4.40
Na-EDTA	3.73
$FeSO_4.7H_2O$	2.23
Nicotinic acid	0.50
Thiamin (B1)	0.50
Pyridoxine (B6)	0.50
Glycine	2.00
Sucrose	30.00
Agar	8.00
Myo-inositol	0.10