EFFECT OF DIFFERENT COMBINATIONS OF PLANT GROWTH REGULATORS ON *IN VITRO* PROPAGATION OF YAM (*Dioscorea spp.)*

M.Sc. Thesis

BY

OBSSI DESSALEGN

March, 2013

Jimma University

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M.Sc. Thesis

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By

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This Thesis is dedicated to my family

STATEMENT OF AUTHOR

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BIOGRAPHICAL SKETCH

Obssi Dessalegn was born in May 1988 in Wombera, Metekel zone of Benishangul-Gumuz Regional State from his father Dessalegn Hora and his mother Gete Biru. He attended his elementary school at Wombera primary school from 1994 to 2000. He pursued his secondary school at Wombera senior secondary and preparatory school from 2000 to 2006. He joined Gondar University in December 2006 and graduated with BSc in Biotechnology in July, 2009. After graduation he was employed by Gondar University as assistant graduate from September 2009. He joined regular program of school of graduate studies of Jimma University College of Agriculture and Veterinary Medicine in September 2010 to pursue a study leading to M.Sc. degree in Plant Biotechnology.

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LIST OF ABBREVIATIONS AND ACRONYMS

2, 4-D = dichlorophenoxyacetic acid

2, 4, 5-T = trichlorophenoxyacetic acid

2-*i*p = isopentenyl-adenine

BAP = 6-Benzylaminopurine

CRD = Completely Randomized Design

FAO= Food and Agricultural Organization of the United Nations

GA_3 = Gibberellic Acid

IAA = Indole -3- acetic acid

IBA = Indole-3- butyric acid

JUCAVM = Jimma University College of Agriculture and Veterinary Medicine

Kin = Kinetin

MoA= Ministry of Agriculture

MoE= Ministry of Education

MS = Murashighe and Skoog

NAA = 1-naphthylacetic acid

P-CPA = *para*-chlorophenoxyacetic acid

REGWQ = Ryan-Einot-Gabriel-Welsch Multiple Range

TDZ = Thidiazuron

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EFFECT OF DIFFERENT COMBINATIONS OF PLANT GROWTH REGULATORS ON *IN VITRO* PROPAGATION OF YAM (*Dioscorea spp.)*

ABSTRACT

Yam is a multi-species tuber crop cultivated in Africa, Asia and parts of South America. It is an important crop in South and Southwestern parts of Ethiopia. Yam is propagated from seed tubers or sections of tuber and corms. Seed tubers are expensive, bulky to transport and the multiplication rate in the field is very low. Shortage of seed tubers for planting is one of the major constraints for yam production in Ethiopia. To overcome such problems and to increase production, in vitro *propagation has been implemented for many* Dioscorea species. *However, no information is available on work done on micropropagation of* Dioscorea spp. *in the country. The current study was, therefore, initiated to optimize type and concentration of plant growth regulators for shoot multiplication and rooting. Two experiments: shoot multiplication and rooting* in vitro *were carried out using a completely randomized design with five replications. For shoot multiplication, shoots initiated from nodal cuttings were treated with BAP in combination with NAA. For rooting, the micro-shoots were transferred to ½ MS media containing NAA and IBA. Data on number of shoots per explant, roots per shoot and related growth parameters were recorded and statistically analyzed. The results showed that MS media supplemented with 1.5mg/l BAP + 0.15 mgl^{-1} NAA gave an average of 6.40±0.28 shoots per explant with a mean shoot length of 2.0±0.11 cm. MS supplemented with BAP* (1.0 mg l^{-l}*) and NAA (0.15 mg l^{-l}) gave 5.40±0.28 with a mean shoot length of 1.84±0 .20 cm. There was no shoot growth on basal media without plant growth regulators, showing that cytokinin is needed to initiate multiple bud formation in the genotype. Plantlets grown on half strength MS media supplemented with 2.0 mg l^{-l} NAA and 0.5 mg l^{-l} IBA produced 5.7±0.27 roots per plantlet and an average root length of 4.63±0.23 cm. The second highest root number was recorded on 1/2 MS with NAA (2 mgl^{-1}) and IBA (0 mg l^{-1}), with an average of 4.8±0.27 roots per plantlet and an average root length of 3.52±0.20 cm. Thus MS media supplemented with 1.5mgl^{-1} BAP and 0.15 mg l^{-l} NAA can be recommended for shoot multiplication of this yam variety and 1/2 MS supplemented with2.0 mg l^{-l} NAA and 0.5 mgl^{-1} IBA for* in vitro *rooting. Besides the need of further optimization of this protocol, similar protocols for the other yam landraces are required.*

1. INTRODUCTION

Yam belongs to the genus *Dioscorea* in the family Dioscoreaceae. The family is believed to be among the earliest angiosperms and probably originated in Southeast Asia (Coursey, 1976). It is a multi-species tuber crop cultivated in Africa, Asia, parts of South America, as well as the Caribbean and the South Pacific Islands. It is a traditional crop that has long been cultivated in Southern, Western and Southwestern parts of Ethiopia as staple or co-staple with enset (*Ensete ventricosum*), cereals, and other root and tuber crops (Westphal, 1975; Edwards, 1991; Mie´ge and Demissew, 1997; Wilkin, 1998; Gemeda, 2000). About 600 species of yam have been recorded (Coursey, 1967; Govaerts *et al*., 2007), but the major cultivated ones are *D. alata, D. bulbifera, D. cayenensis, D. esculenta, D. opposita-japonica, D. nummularia, D. pentaphylla, D. rotundata* and *D. trifida* (Lebot, 2009). Yams have a basic chromosome number of x=10, but various levels of polyploidy exist even within the same species (Boussalem *et al*., 2006; Lebot, 2009).

The dominant region for yam production in the world is West Africa, where about 48 million tons (about 93% of the world's production) produced on 4 million hectares annually, and mainly in five countries Benin, Côte d'Ivoire, Ghana, Nigeria and Togo (Wilkin, 2001; Mulaama, 2004; FAO, 2009) of which Nigeria accounts for almost 67% (Hahn,1995). In Ethiopia, the total annual production of yam was estimated at about 277,000 metric tons from an area of about 68,000 ha, corresponding to a yield of about 4 tons per hectare (FAO, 2009). However, Gemeda (2000) reported that yam is more productive than the other tuber crops in the area, with an estimated yield of about 20 tons per hectare. Expanded production of yams in new areas or where it is produced in limited quantities in other parts of the tropics (Lebot, 2009; Asiedu and Sartie, 2010) including Ethiopia would contribute significantly to food security, improved health and increased income (Westphal, 1975; Mie´ge and Demissew, 1997).

As the crop is adapted to dry season planting (mainly at the onset of the dry season in October) early harvests in May fill a seasonal gap in food supply in Ethiopia (Tamiru *et al.*, 2005; Tamiru, 2006). The fact that it is preferred to the other root and tuber crops means yam is also an important cash crop, generating additional income for farm households (Tamiru, 2008). Nonetheless, yam is establishing itself as an important cash

crop in most localities. More importantly, it is the preferred food for honored guests and served during the main traditional celebration (Meskel) that coincides with the peak of harvesting, fetching high prices on markets (Tamiru *et al.,* 2005).

Dioscorea is a well-known edible and traditional medicinal plant. Traditional medicinal uses are a feature of *Dioscorea*, since the genus is rich in steroidal saponins (Kole, 2011). They are principally grown for food and have organoleptic qualities that make them the preferred carbohydrate food where they are grown. However, their storage organs (underground and/or aerial tubers) are also sources of proteins, fats, and vitamins for millions of people in Africa (Lebot, 2009). The mean protein content of yam is higher than the protein values of sweet potato, potato, cassava, taro and plantain (Hahn *et al*., 1987). When processed into flour, yams have a nutritional value comparable to cereals (Kole, 2011). The rhizomes of various species of *Dioscorea* have been used as an important ingredient for invigorating the spleen, kidney and stomach, promoting the body fluids and benefiting the lung, in addition to being used as a food crop (Wang *et al*., 2006). Certain species of yam are used for treating skin diseases and chitsinga and as sources of biologically active compounds in pharmaceutical industries (Kole, 2011).

Yam is propagated from seed tubers or sections of tubers and corms. Seed tubers are expensive, accounting sometimes for about as much as 50% of total variable cost (Manyong, 2000); they are bulky to transport and have extended dormancy period. The multiplication ratio in the field is very low (less than 1:10) compared, for instance, to some cereals (1:300) (Balogun, 2009). Traditionally farmers obtain seed tubers by selecting small tubers (e.g. 200–500 g) from each harvest (unfortunately these are often those produced by diseased plants by nematodes: root-knot (*Meloidogyne* spp), and yam nematode (Scutellonoma bradys) and Insects such as yam shoot beetle, often interact with fungi (*Botryodiplodia*, *Fusarium*) and bacteria (*Erwinia* spp.) to damage tubers in the field and in storage, yam tuber beetle (*Heteroligus meles*) and crickets.) (Aighewi *et al*., 2003a; Lebot, 2009). Diehl's (1982) survey report in Nigeria also showed shortage of planting material (owing to low reproductive rate) which may lead to future decline in yam production.

In Ethiopia there is shortage of seed tubers for planting, lack of formal seed supply system and specialization in the production of yam planting-materials (Tamiru *et al.,* 2005;

Tamiru *et al.,* 2008). Farmers mostly rely on their own planting-materials saved from the previous cropping season; some farmers partly meet their demand for seed tubers through purchases from local markets or exchanges with neighbors. This has led to a decrease in production (Tamiru *et al.,* 2008) due to insufficient quantity and poor quality of planting material.

In addition, farmers often encounter shortages of yam planting material, especially following droughts and disease epidemics. Productivity is hampered by pests and diseases and the limited availability and high cost of planting materials (Balogun *et al*., 2004). So, some farmers keep a reserve batch of seed yams (up to a third of the quantity planted) for replacement of seeds that do not germinate. Poor quality planting materials that germinate tend to carry disease and pest (viruses, fungi, nematodes and insects) from the storage barns to the field the next season resulting in low tuber yields, followed by poor shelf life (Ghosh *et al*., 1988; Asiedu and Sartie, 2010).

To overcome such problems and increase production, conventional methods such as partial sectioning, layering, vine rooting and minisett technique (Okoli *et al*., 1982) have been used to produce high amount of planting material. Minisett technique has significantly increased propagation rates, but it has been associated with less uniform and poor rate of sprouting when applied to white yam (Okoli *et al*. 1982; Wilson, 1989). The partial sectioning requires considerable manpower for the repeated examining and digging out of tubers to excise sprouted sections for field planting. In case of vine rooting technique, either tubers did not develop due to early senescence of rooted vines (Acha *et al*., 2004), or small tubers are produced when applied to *D. rotundata* (Okoli *et al*., 1982). The layering technique is unsuitable for farm use due to rigorous procedures involved (Acha *et al*., 2004) and it is genotype specific (Acha *et al*., 2004; Shiwachi *et al*., 2005b). So, other methods of rapid propagation such as micropropagation have been developed (Balogun *et al*., 2004) including production of microtubers from plantlets *in vitro* (Aighewi *et al*., 2003b; Feng *et al*., 2007).

Micropropagation of yam offers the distinct advantage of large scale multiplication of high quality, clonally propagated planting materials (Ng, 1988; Asha and Nair, 2007). It provides many advantages over conventional methods including: (1) it enables mass propagation of specific species, (2) it helps to produce pathogen-free planting material, (3)

it enables clonal propagation of parental stock for hybrid seed production, and (4) it enables year-round nursery production (Hartmann *et al*., 2002).

In vitro propagation has been implemented for many *Dioscorea* species, such as *D. rotundata* (Balogun *et al*., 2006), *D. nipponica* Makino. (Chen *et al*., 2007), *Dioscorea esculenta* (Lour.) Burk, (Kharat *et al*., 2008), *D. hispida (*Behera *et al*., 2008*), Dioscorea alata* L.cv. Hatikhujia *(*Behera *et al*., 2010*),* wild yam (*Dioscorea wightii),* Mahesh *et al*, 2010, *Dioscorea fordii (*Yan *et al*., 2011) and they reported that the response of yam (*Dioscorea spp.*) towards *in vitr*o propagation depends on the presence and absence of auxin and cytokinin.

However, a protocol developed for one specific species of plant is not reproducible when applied to other genotypes even within the same species i.e. each genotype has its own requirements in all stages of *in vitro* propagation (Omar and Aouine, 2007). Likewise the response of yam *in vitro* is genotype specific (Balogun *et al*., 2004; Ahanhanzo *et al*., 2010). Despite its importance especially for food security, little has been done on the improvement of the crop in Ethiopia and no information on its propagation using *in vitro* techniques. The current study was, therefore, initiated with the following objectives:

- To optimize concentration of BAP in combination with NAA for shoot multiplication
- To determine the optimum concentration and combination of NAA and IBA for rooting.

2. LITERATURE REVIEW

2.1. Botany and Taxonomy of Yam (*Dioscorea spp.*)

The genus *Dioscorea* is genus of the family Dioscoreaceae and is the largest genus within this family of about 644 species (Govaerts *et al*., 2007). All *Dioscorea* species are *Dioecious* twining climbers producing dry capsules, although occasionally both male and female flowers can be found on the same plant. All species of economic importance are tuberous. The main food yam species belong to five different sections: Enantiophyllum (*D. alata*, *D. cayenensis*, *D. nummularia*, *D. opposita*, *D. rotundata* and *D. transversa*), Combilium (*D. esculenta*), Opsophyton (*D. bulbifera*), Macrogynodium (*D. trifida*) and Lasiophyton (*D. pentaphylla*) (Lebot, 2009).

The *Dioscorea spp*. produces a tuber as an annual underground storage organ, which shrivels away when regrowth commences and a new tuber can be formed simultaneously (Mulualem, 2008). Some species form perennial tubers, which become larger and more lignified as the plant ages. There is tremendous variation in size, form and number of tubers per plant within and between species. The shape of the tuber in species producing small tubers is generally regular and their skin usually thinner than species producing large tubers (Lebot, 2009).

The roots and the stems are renewed annually. Both type of species, annual or perennial-producing tubers; spend the dry part of the year in dormancy, which can vary from 1 to 6 months. The root system is very superficial. Several thick and long roots develop rapidly after sprouting and reach considerable distances, 3–4 m in radius around the plant, to ensure that the developing vine is anchored firmly. When plants are cultivated in mounds, this development is somewhat constrained. Some species, including the cultivated *D. esculenta*, produce roots near the surface that are armed with spines (Lebot, 2009). In some *Dioscorea spp.* the stems twine anticlockwise, are cylindrical and spineless and may climb up to 8 m. The leaves are large and simple, either opposite or alternate (Mulualem, 2008).

The structure of the yam tuber is highly variable, depending on the species. Both genetics and environment play significant roles in determining tuber shape and size. In case of *D. rotundata* tuber shapes are varied but the flesh of the white Guinea yam does not much vary in colour. The tuber skin is dark and smooth and nearly free of rootlets. Tuber of every imaginable shape has been produced (Coursey, 1967). Size of individual tuber may range from a few grams to over 50 kg, and tuber length of 2-3 m has been recorded. Most commercial yam tubers are more or less cylindrical in shape and covered by a thick layer of cork. Cracks are often present on the tuber surface. Some roots may be present on the tuber (Lebot, 2009).

2.2. Importance of Yam

Yams are source of carbohydrate, proteins, fats and vitamins for millions of people in West Africa (Kole, 2011). In countries where yams are generally cultivated, wild yams are used as food in times of shortage or famine (Coursey, 1967). These wild species, although consumed only under famine conditions, also makes enormous contribution to human welfare. Apart from food, *Dioscorea* species are also used in pharmaceutical industries as sources of biologically active compounds or their precursors. It regulates the female reproductive system, particularly during menstrual distress and menopause and is also used in treating infertility (Asiedu and Sartie, 2010). Certain wild species of yam are sold in the markets in Zimbabwe for treating skin diseases and chitsinga (physical disorder characterized by pain and swelling of the joints). It is an effective treatment for morning sickness when used with chaste berry and dandelion. It is also famed for its steroid-like saponins, which can be chemically converted to progesterone contraceptives and cortisone (Kole, 2011). The most frequently encountered medicinal use of yams in Madagascar is the treatment of burns, ulcers and other skin complaints with the bulbils of *Dioscorea spp.* (Kole, 2011).

Those wild species may serve as an important source of genetic variation in yam breeding work especially for resistance to pests and diseases. Further genetic improvement to reduce the bitter constituents in some of the species may render them more palatable and popular (Kole, 2011). A wide range of saponins and steroidal sapogenins (mostly diosgenin) have been extracted from various *Dioscorea spp*. with the aim of providing the pharmaceutical industry with compounds for oral contraceptives. Industrial production has

been undertaken in India and Mexico, with mixed results due to the increasing use of synthetic steroids (Coursey, 1967).

Chemical composition depends mainly on the species, the cultivar or wild form. Their protein, mineral and vitamin contents are higher (Hahn *et al*., 1987). The food value is composed of carbohydrates (starch, sugars, and fibers), proteins, minerals, vitamins and a negligible amount of lipids (Lebot, 2009). Nutritional qualities have been investigated for the major species, but there is less work on other *Dioscorea* species (Asiedu and Sartie, 2010).

2.3. Agronomy of yam

High yam yields depend on good planting material and husbandry and, in particular, timely weed control to permit establishment of a sufficient leaf area (Asiedu and Sartie, 2010). They also depend on adequate and near optimum temperatures (25–30°C). The most important constraints to production are the high labour requirements, the quality of the planting material and difficulties in mechanization (Lebot, 2009).

2.3.1. Soil preparation for yam cultivation

Yams, being light-loving and shade-sensitive plants, require sites which are well exposed to solar radiation (Shiwachi *et al*., 2005a). If planted in traditional agroforestry systems, they need to be established in the middle of the plot and to be staked in order to benefit from maximum sunlight (Asiedu and Sartie, 2010). Unlike cassava and sweet potato roots, which initially penetrate the soil and then expand, the yam tuber penetrates the soil while expanding. It is therefore important that the soil is light, well drained and friable. Land preparation is the most important input and necessitates almost half of the total 1800 man-hours/ha in West Africa (Hahn *et al*., 1987).

2.3.2. Nutrient requirement of yam

Degradation of soil fertility is the major constraint identified by growers in yam production in West Africa (Asiedu and Sartie, 2010). Although farmers perceive the decline in soil fertility as their most important difficulty in improving yield and profit, they

often lack suitable and practical solutions to correct the situation (Kang and Wilson, 1981).

Responses to fertilizers are erratic and usually much less significant than the effects of sett size or staking (Okoli *et al*., 1982). No responses to N fertilization or even depressive effects have been reported. Kang and Wilson (1981) reported no significant effect of NPK fertilizer on tuber yield at all three locations where their experiments were conducted and noticed some depression in the yield of plants grown on flats (Asiedu and Sartie, 2010). To complicate the situation, yams appear to depend on an effective mycorrhizal association to meet their P requirements. Apparently, yams respond well to N and K fertilizers, while their response to P is slight. This could be due to very efficient P uptake, possibly as a result of mycorrhizal contribution (Lebot, 2009).

2.4. Conventional Vegetative Propagation of Yam

Traditionally, yams are propagated by planting whole tubers or large pieces weighing 200 g or more, (Okoli *et al*., 1982; Balogun, 2009). Seed tuber can also used be as planting material but, seed tubers are expensive; accounting sometimes for about as much as 50% of total variable cost and the use of true seeds as propagules is restricted to research stations, mainly in crop improvement programs (Manyong, 2000). Many wild yams reproduce freely by seeds, but many cultivated varieties rarely or never produce viable, fertile seed (Winch, 2006). A sizable portion of otherwise consumable tubers are therefore reserved for planting yearly, and this leads to scarcity of planting materials. Multiplication ratio for seed yam production in the field is 1:10 compared to 1:300 in cereals. Planting materials alone constitute about 50% of production costs. Most farmers propagate yams by "milking" (Balogun, 2009). In this technique, tubers are harvested two thirds into the growing season without destroying the root system. This provides early yam for home consumption and market. There is regeneration of fresh small tubers from the corm at the base of the vine and these are used as planting materials for the following season (Lebot, 2009).

The major constraint of planting materials to yam production is being tackled by the development of more efficient propagation methods. These include partial sectioning technique, vein/rooted stem cutting and minisett technique (Okoli *et al*., 1982; Aighewi *et*

al., 2003a). Although the latter has significantly increased propagation rates, it has been associated with less uniform and poor rate of sprouting when applied to white yam (Okoli *et al*. 1982). Although multiplication rates are doubled using the partial sectioning technique, it requires considerable manpower for the repeated examining and digging out of tubers to excise sprouted sections for field planting. The rooted stem cuttings/vein cutting used for production of planting setts which not only accelerates propagation of selected clones but produces minitubers. These vine cuttings can be used to produce minitubers within 100–120 days, however, observed that some genotypes perform much better than others. In addition either tuber did not develop due to early senescence of rooted vines or small tubers are produced when applied to *D. rotundata* relative to other species (Acha *et al*., 2004; Shiwachi *et al*., 2005b).. Also, the layering technique is unsuitable for farm use due to rigorous procedures involved (Acha *et al*., 2004). And observed that some genotypes perform much better than others, i.e., it is genotype specific (Acha *et al*., 2004; Shiwachi *et al*., 2005b). To fill this gap rapid propagation, *in vitro* propagation developed (Balogun *et al*., 2004) including production of microtubers from plantlets *in vitro* (Aighewi *et al*., 2003b; Feng *et al*., 2007).

2.5. *In* V*itro* Propagation

In vitro propagation is the rapid methods developed to address limitations of the traditional practices in plant propagation. It has been used in clonally propagated plants for different purposes such as alleviating the problem of planting material availability. The technique of mass production of planting material *in vitro* is called micropropagation. It is an *in vitro* technique of true-to-type mass production of planting material. Such a true-to-type *in vitro* mass production of planting material requires a well optimized efficient protocol which could depend on the cultivars or clones (Hartmann *et al*., 2002).

Multiplication of yam by *in vitro* growth of nodal segments is a practical way for rapid clonal multiplication and some tissue culture companies like Vitrobio Valenda SL in Spain are involved in commercial propagation of yam through those protocols developed and there is also mass propagation in some African and Arabian countries but, in tropical countries, only a few agricultural research stations can afford to do it (Omar and Aouine, 2007). *In vitro* techniques are also used for the rapid propagation of virus-free clonal material (Mantell *et al*., 1978).

2.5.1. Nutrient composition for plant tissue culture

One of the most important factors governing the growth and morphogenesis of plant tissues in culture is the composition of the culture medium. In fact, the basic nutrient requirements of cultured plant cells are very similar to those of whole plants. Plant tissue and cell culture media are generally made up of the following components: macronutrients, micronutrients, vitamins, amino acids or other nitrogen supplements, sugar(s), other undefined organic supplements, solidifying agents or support systems and growth regulators. White (1953), Murashige and Skoog (1962), Gamborg *et al*. (1968), Schenk and Hilderbrandt (1972), Nitsch and Nitsch (1969) and woody plant medium (Lloyd & McCown's, 1981) are some of the media formulation used in tissue culture. Murashige and Skoog's MS medium, Schenk and Hildebrand's, SH medium and Gamborg's B-5 media are high in macronutrients, while the other media formulations contain considerably less of the macronutrients.

2.5.1.1. Macro- and micro-nutrients

The macronutrients provide the six major elements-nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S)-required for plant cell or tissue growth (Bhojwani and Razdan, 1996). The optimum concentration of each nutrient for achieving maximum growth rates varies considerably among species (George *et al.*, 2008). The essential micronutrients for plant cell and tissue growth include iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo) (George *et al*., 2008). In Murashige and Skoog medium, an ethylene diaminetetraacetic acid (EDTA)-iron chelate is used to bypass the problem of precipitation (Khanna, 2003).

2.5.1.2. Carbon source and vitamins

The carbon and energy requirement of tissue under *in vitro* condition should also be provided by including the sources in the media (George *et al*., 2008). 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 but fructose being

somewhat less effective (Razdan, 2003). Sucrose concentrations of culture media normally range between two and three percent (Khanna, 2003).

Normally, plants synthesize the vitamins required for their growth and development (Razdan, 2003). Vitamins are required by plants as catalysts in various metabolic processes (George *et al.*, 2008). When plant cells and tissues are grown *in vitro*, some vitamins may become limiting factors for cell growth (Bhojwani and Razdan, 1996). The vitamins most frequently used in cell and tissue culture media include thiamine (B_1), nicotinic acid, pyridoxine (B_6) and myo-inositol (Khanna, 2003).

2.5.1.3. Gelling agents

Agar is the most commonly used gelling agent for preparing semisolid plant tissue culture media. Agar has several advantages over other gelling agents (George *et al.*, 2008). First, when agar is mixed with water, it forms a gel that melts at approximately 60°-100° C and solidifies at approximately 45°C; thus, agar gels are stable at all feasible incubation temperatures (Razdan, 2003). Additionally, agar gels do not react with media constituents and are not digested by plant enzymes (Bhojwani and Razdan, 1996). The agar concentrations commonly used in plant tissue culture media range between 0.5 and 1.0%; these concentrations give a firm gel at the pH's typical of plant tissue culture media (Khanna, 2003). Another gelling agent commonly used for commercial as well as research purposes is Gelrite. This product is synthetic and should be used at 1.25-2.5 g/liter, resulting in a clear gel which aids in detecting contamination.

2.5.1.4. Plant growth regulators

Plant growth regulators, also known as plant hormones, are substances naturally produced by plants that control normal plant functions, such as root growth, fruit set and drop, growth and other development processes. In addition to the nutrients, it is generally necessary to add one or more growth hormones, such as auxins, cytokinins and gibberellins to mediate many changes in the physiological state of plant tissues and regulate the growth and development of the cells (Khanna, 2003) to support growth of tissues and organs (Bhojwani and Razdan, 1996). In tissue cultures, by manipulating the

types and levels of plant hormones it is possible to directly regulate the pattern of growth under *in vitro* condition. Even though it is hard to make reliable generalizations about the response of plant hormones, the following broad generalizations were made by Khanna (2003) about of growth regulators: (1) Auxins usually promote root initiation and callus growth but inhibit root growth and lateral growth, (2) cytokinins promote shoot proliferation and cell division but inhibit root initiation and (3) gibberellins promote elongation and may overcome dormancy.

Auxins and cytokinins are the two most commonly used hormones in tissue culture. In tissue culture, auxins have been used for cell division and root differentiation. The auxins commonly used in tissue culture are indole acetic acid (IAA), indole-3- butyric acid (IBA), naphthalene acetic acid (NAA),dichlorophenoxyacetic acid (2,4-D), *para*-chlorophenoxyacetic acid(p-CPA) and trichlorophenoxyacetic acid (2,4, 5-T). IBA and IAA are widely used for rooting and, in interaction with a cytokinin, for shoot proliferation (Bhojwani and Razdan, 1996). Cytokinins are concerned with cell division, modification of apical dominance, shoot differentiation, etc. These compounds are also used for shoot proliferation by the release of axillary buds from apical dominance. More commonly used cytokinins are benzylamino purine (BAP), isopentenyl-adenine (2-*i*p), kinetin, thidiazuron (TDZ) and zeatin. Cytokinins are generally dissolved in dilute HCl while Auxins are usually dissolved in either ethanol or dilute NaOH (Bhojwani and Razdan, 1996).

2.5.2. Nutrient requirements for different stages in plant tissue culture

Murashige (1974) subdivided the sequential stages of micropropagation into three (Stage 1, 2, and 3). Since then Stage 0 and 4 were added (George and Sherrington, 1984). Such stages are as follows: Stage 0: preparation of the mother plant and is an initial step of micropropagation in which the stock plant used to culture initiation are grown for at least 3 month under carefully monitored greenhouse condition. Stock plants are grown at relatively low humidity and watered either with irrigation tubes of by capillary sand beds or mats. This stock plant preconditioning stage also includes measures to be adopted for reduction of surface and systematic microbial contaminants (Razdan, 2003).

Stage I: establishment of the aseptic culture (initiation of the aseptic culture). In this stage an explants (i.e. shoot tip, lateral bud, leaf segment, etc.) is surface sterilized and cultured on nutrient medium. The objective of this stage is to obtain a clean or contamination free cultures that can be used in the following stage, regardless of the amount of growth attained (Razdan, 2003). The nutritional requirements are usually very simple and the cultures are incubated either under light or dark conditions according to the method of propagation (Razdan, 2003; Omar and Aouine, 2007).

Stage II: Multiplication stage: This is the most important stage in any propagation program since it determines the number of produced plants. In this stage, the number of propagules is multiplied by repeated sub- and reculture until the desired (or planned) number of plants is attained. The chemical formulation and physical status of nutrient medium as well as the incubation conditions are of prime significance (Omar and Aouine, 2007).

Stage III: Rooting stage (regeneration of whole plant): Shoots produced from the previous stage are separated and individually rooted in a relatively high auxin containing media. In this stage, a good root system is initiated and complete plants are achieved.

Stage IV: hardening for subsequent field planting (acclimatization stage). This stage may be included with the pervious stage. Plants developed through tissue culture are heterotrophic, lack cuticle on their epidermis, as well as having non-functional stomata. Such plants cannot survive the outside unfavorable conditions. Thus, they need hardening and acclimatization, where they receive a special treatment before they can be transferred to the soil in order to stimulate photosynthesis, cuticle development and their stomata's starts functioning. Probably this is the most important stage in the whole process. Thus, it should be conducted under proper conditions regarding soil, light, temperature and irrigation (Razdan, 2003).

The nutrient requirements for these different stages in tissue culture differ from species to species even different genotypes of the same species do have different requirements for all stages i.e. each specific genotype of the same species has its own requirement in all previous stages (Razdan, 2003; Omar and Aouine, 2007).

2.5.2.1. Mother plant preparation

Various workers established mother plants of yam in greenhouses and used as a source of explants. Omar and Aouine (2007) established mother plants from healthy seed yams (weighing 200 ± 5 g) which had broken dormancy and were planted singly in 40 cm black polybags filled with sterilized top soil and kept in a greenhouse. Light watering with tap water was carried out twice daily until sprouts were produced. Young healthy sprouts were removed from mother plants after two months of growth, deleafed, and cut into smaller pieces (Mbanaso *et al*., 2007).

The tubers of various yam genotypes were put in a greenhouse for sprouting to obtain the mothers plants and the explants were obtained from the established experimental site after 35 to 60 days after germination (Behera *et al*., 2008; Behera *et al*., 2009; Behera *et al*., 2010).

Vines/top shoot cuttings having 5-8 nodes, excised from greenhouse grown, 4-month-old healthy plants of about 2 m height, raised from the tubers under uniform manorial conditions, served as source of explants (Adeniyi *et al*., 2008). Vines/shoot top cuttings, single nodal segments (1-2 cm) were excised and used as explants for in vitro experiments (Asha and Nair, 2007).

2.5.2.2. Initiation of aseptic culture

Out of the various treatments tried, the treatment with $HgCl_2$ (0.1%) for 4 minutes followed by washing thrice with sterile water and then dipping in ethanol (70%) for 1 minute exhibited maximum establishment of aseptic as well as proliferating cultures, i.e., 78.67 and 76.00%, respectively(Kharat *et al*., 2008; Ahanhanzo *et al*., 2010).

Mwirigi *et al*. (2010) reported that sequential sterilization that involved the use of bleach concentration at levels of 40% for 30 minutes followed by a concentration of 20% for 20 minutes gave the best results with 85% of the explants surviving. They have reported that

there was no significant difference between the concentrations 0.5 mgl^{-1} and 1.0 mgl^{-1} BAP. The two concentrations gave the best results for shoot initiation.

Enlargement and subsequent break of axillary buds of nodal explants cultured on MS media supplemented with 8.8 μM BAP and 0.3% activated charcoal was observed (Poornima and Ravishankar Rai, 2007).

MS medium supplemented with BAP (1and 2 mgl^{-1}) was best medium for shoot induction. After 4–7 days in culture, the lateral buds displayed visible growth (Chen *et al.,* 2007). Behera *et al*. (2008) reported that bud break was noticed within 8-10 days after culture MS media supplemented with (1, 1.5, 2 mgl^{-1} BAP).

2.5.2.3. Multiplication of propagules *in vitro*

The nodal segments of 15mm length produced higher percentage of shoot (77.33%) (Kharat *et al*., 2008; Ahanhanzo *et al*., 2010).

Sucrose levels greater than 20 g l^{-1} in culture media appeared to be a prerequisite for optimal *in vitro* plantlet growth of *D. composita* micro plants (Alizadeh *et al*., 1998).

Nodal explants of tender stem cuttings of *D. nipponica* Makino were planted on basal media supplemented with hormone combinations at different concentrations. After 4–7 days in culture, the lateral buds displayed visible growth, and most of them grew into 35–50 mm long shoots within 4 weeks. Multiple buds growing on a suitable initiation medium developed into plantlets, with part of the plantlet producing microtubers. Media containing only BAP hormonal supplements, the rate of shoot induction and shoot height increased with increasing hormone concentration up to 2.0 mgl^{-1} and then dropped at 4.0 mgl^{-1} as the BAP became super optimal. And media combination of 2.0 mg/l BAP and 1.0 mg/l NAA shows highest shoot induction frequency (Chen *et al.,* 2007).

Ahanhanzo *et al*.(2010) reported that BAP (0.5 mg l^{-1}) induced a significant increase ($p < 0.05$) in leaf number of varieties *Kounondakou* and *Gnon-boya* and a significant increase ($p < 0.01$) in the height of *in vitro* plants for three varieties. They have reported that there was significant interaction between genotype and cytokinin type. The results recorded

during the second week of culture showed that BAP improved better axillaries buds sprouting for all yam varieties studied and facilitated the appearance and the development of the stems and leaves of some variety (Ahanhanzo *et al*., 2010).

Growth inhibitory effect of kinetin on shoot numbers of *D. oppositifolia* and *D. pentaphylla* was observed. But the promotive effects of kinetin (46.4μM) on plantlet growth for *D. bulbifera*, which increased the number of shoots per plantlet. The results obtained indicated that no break in leaf growth was observed on the control medium (without cytokinin) but media with BAP presented a good plants aerial part development (Belarmino and Gonzales, 2008).

The results of Yan *et al*.(2011) showed significantly higher shoot length, frequency of proliferation of *Dioscorea fordii* in MS basal medium supplemented with 1.0 mg l^{-1} BAP, 0.1 mg l^{-1} NAA, 30 g l^{-1} sucrose and 1.5 g l^{-1} AC in liquid culture) where 5.5 shoots per explants were recorded compared to the other treatments for the independent variables NAA/BA at 1:10 (0.1 mg l^{-1} NAA and 1.0 mg l^{-1} BA), 1:1 (1.0 mg l^{-1} NAA and 1.0 mg l^{-1} BA) and 10:1 (1.0 mg l^{-1} NAA and 0.1 mg l^{-1} BA).

The highest shoot induction of 75% was obtained in the medium containing 0.10 μM NAA + 0.20 μM BAP and there was significant NAA x BAP interaction, indicating that the effectiveness of each of the phytohormone in inducing shoots and plantlets was influenced by the presence or absence of the other (Adeniyi *et al*., 2008).

Ramierez-Magon *et al*. (2001) has been reported that when *Spathiphyllum floribundam* was cultured on media supplemented with BAP in combination with IAA, there was increase in shoot multiplication from 1.8 shoots per cultured explant to average of 11.6 shoots per explant.

Explants grown in media with 0.5 mgl^{-1} BAP and 0.01mgl^{-1} NAA which is the only combination test, showed the highest rate of multiplication and survival as compared with explants in media with other growth regulators (Thankappan and Patell, 2011). A notable growth pattern was observed 4 to 5 weeks post-inoculation. High multiplication rate of 21 segments that can be cultured were obtained in 10 to 12 weeks of inoculation from nodal

segments. Multiple shoots were noticed and up to 9 shoots were observed from single node without callus formation (Thankappan and Patell, 2011).

Of the combination tested by Behera *et al*.(2008), MS +BAP (2.0 mgl^{-1}) + NAA (0.5 mgl^{-1}) with 100mgl^{-1}ascorbic acid, elicited optimal response in which an average of 6 ± 0.18 shoot lets with a mean shoot length of 5 ± 0.29 cm per explants was recorded on *Dioscorea hispida*. The second best shoot multiplication 4.5 ± 0.12 was obtained in the medium MS + Kinetin (1.5$mg1^{-1}$) + NAA (0.5 mg^{-l}) + 100 mg^{-l} ascorbic acid with a mean shoot length of 4 ± 0.29 cm.

Behera *et al*. (2009) reported when *Dioscorea oppositifolia* cultured on MS+ Kinetin (2.0 mgl^{-l}) +BAP (1.0 mgl^{-1}) + NAA (0.5 mgl^{-1}) with 100mgl^{-1}ascorbic acid an average of 10.5 ± 0.51 shootlets with a mean shoot length of 5.4 ± 0.24 cm per explants were obtained. Followed by Kinetin (1.5mgl^{-1}) + BAP (1.0 mg l^{-l}) + NAA (0.5 mg^{-l}) + 100 mg^{-l} ascorbic acid where 5.5 ± 0.43 shootlets were obtained with a mean shoot length of 4.2 ± 0.21 cm.

Behera and his coworker reported higher multiplication of 9.5 ± 0.61 shootlets of *Dioscorea alata* with a mean shoot length of 6.7 ± 0.44 cm per explants on MS supplemented with Kinetin (2.0 mg^{-1}) + BAP (1.0 mg^{-1}) + NAA (0.5 mgl^{-1}). And 6.5 ± 0.42 shoots on MS + Kinetin (2.0 mgl^{-1}) + BAP (0.5 mgl^{-1}) + NAA (0.25 mgl^{-1}) with a mean shoot length of 5.8 ± 0.63 cm (Behera *et al*., 2010).

The frequency and rate of bud break and multiplication depends on the cytokinin type and its concentration either alone or in combination with an auxin (Poornima and Ravishankar, 2007), with the combination of 1.5mgl^{-1} BAP and 0.02mgl^{-1} IAA giving the best results.

Mwirigi *et al*. (2010) reported that when 0.5 mgl^{-1} BAP alone was used a mean number of 1.1 shoots was obtained. Addition of 0.02 mg l^{-1}NAA on 0.5 mgl^{-1} BAP gave the best shoot formation with an average of 2.1 shoots followed by 1.0 mgl^{-1} BAP + 0.04 mgl^{-1} NAA with an average of 1.6. In general, the effectiveness of each phytohormone in inducing plantlet regeneration was influenced by the presence or absence of the other but the best treatment was found to be a combination of 0.5 mgl^{-1} BAP +0.02 mgl^{-1} IAA and hence this level is recommended for further work (Mwirigi *et al*., 2010), but Kharat *et al*.

(2008) report that Shoot regeneration was highest (81.33%) in medium with BAP (0.3 mg l^{-1}) and NAA (0.25 mg l^{-1}).

2.5.2.4. Rooting of propagules

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyam, 1970). The plantlets propagated with axillary segments could easily be rooted and transplanted and were found suitable for the ex vitro rooting to produce mini tubers. The number of roots and leaves and the height of each young sprout were determined after 5 weeks in culture (Ahanhanzo *et al*., 2010).

Behera *et al*. (2008) obtain highest rooting in 1/2 MS + 2.0 mgl^{-1} NAA where about 90% cultures responded with an average number of 5.2 ± 0.28 roots per plantlet and an average root length 3.5 ± 0.12 cm on *D. hispida*. And 75% response was recorded in MS medium supplemented with 1.5 mgl^{-1} of NAA + 2.0 gl^{-1} activated charcoal.

There were reports that rooting was better in the culture which had combination of 1/2 MS+2.0 mgl^{-1} NAA where about 90% cultures responded with an average number of 6.5 ± 0.30 roots per plantlet and an average root length 4.5 ± 0.16 cm was recorded, followed by 1.5mgl^{-1} of NAA (Behera *et al*., 2009).

Rooting of *Dioscorea alata* L.cv. Hatikhujia was highest on 1/2 MS + 2.0 mgl^{-1} NAA where about 92% cultures responded with an average number of 5.5 ± 0.48 roots per plantlet and an average root length 5.2 ± 0.26 cm. The second highest response (77%) was recorded at 2.0 mgl^{-1} of IBA (Behera *et al*., 2010). Emergence of root primordia was observed from the shoot base from 6 to 8 days after inoculation followed by a rapid root growth.

NAA was found more effective than IBA in induction of rooting as days required for rooting was only 6-8 as against 10 to15 in the case of IBA (Behera *et al*., 2008; Behera *et al*., 2009). NAA at higher concentration (1.5 and 2.0 mgl^{-1}) and IBA (2.0 mgl^{-1}) was the best for induced rooting (Behera *et al*., 2010). *In vitro* rooting was efficiently obtained

within a period of 30 days on MS medium with 2.67 μM NAA and MS basal medium (Poornima and Ravishankar, 2007).

MS medium with 0.5 mgl^{-1} IBA and 0.01mgl^{-1} NAA, induce profuse root resulting in 12.5 to 14.5 cm in root length in 8 to 10 weeks and there was no rooting on media free of auxin (basal media) (Thankappan and Patell, 2011).

Compared with the plantlet without microtubers, the plantlets with microtubers produced roots more easily with the highest rate being 100% (with 0.5 mgl^{-1} IBA). Maximum rooting of the plantlets without microtubers (94.67%) was on a medium with 1.0 mgl^{-1} IBA and 0.5 mgl^{-1} NAA. Compared with the control, low concentrations of IBA could increase the rooting frequency in regenerated plantlets without microtubers, but 2.0 mgl^{-1} IBA was optimal only for plantlets without microtubers, and thus decreased the overall rooting frequency (Chen *et al.,* 2007).

2.5.2.5. Acclimatization

Rooted plantlets grown *in vitro* were washed thoroughly to remove the adhering gel, transplanted to sterile poly pots (small plastic cups) containing pre-soaked vermiculite and maintained inside growth chamber set at temperature 28^0C and 70-80% relative humidity. After three weeks they were transplanted to earthen pots containing mixture of soil + sand + manure in 1:1:1 ratio and kept under shade house for a period of three weeks for acclimatization. The potted plants were irrigated with Hoagland's solution every 3days for period of 3 weeks. Survival rate of the plantlets were recorded after 3 weeks (Behera *et al*., 2009).

About 90% of the rooted plantlets established in the greenhouse within 2-3 weeks of transfer. The plants grew well and attained a 6-8 cm height within 4 weeks of transfer. The acclimatized plants were established in the field condition and grew normally without morphological variation (Behera *et al*., 2010).

The acclimatization procedure described by Behera *et al*. (2008) resulted in a high survival rate of *D. hispida* plantlets. There was 90% survival rate of the plant lets that were rooted

in NAA supplements medium where the plantlet exhibited healthy growth. After three weeks in vermiculite medium the plants were transferred to field.

The acclimated plantlets with 5–8 roots showed obvious growth one week after being removed from culture flasks and transferred to sterilized soil in the greenhouse. One month after transferring, the survival rate of the plantlets was greater than 91%, and the young plants grew vigorously in the greenhouse. Each surviving plant increased in height and number of leaves and could be transplanted into the field after 4 weeks (Chen *et al.,* 2007).

3. MATERIALS AND METHODS

These experiments were carried out in the tissue culture laboratory of Jimma University College of Agriculture and Veterinary Medicine (JUCAVM), during 2011-2012. The materials used and the methods followed are listed as follows.

3.1. Planting Materials

Yam variety Aw-004/00 (*Dioscorea spp.*) was used as a source of explant. The variety is believed to be widely distributed in Southern and Southwestern part of Ethiopia and it is the only released variety of yam in the country. This variety was released by Hawassa Agricultural Research Center in 2010. It is characterized by high yield (25 t/ha on farmers' field) and it is preferred by farmers (MoA, 2010). The variety was obtained from Hawassa Agricultural Research Center.

3.2. Mother Plant and Explants Preparation

The seedlings of Aw-004/00variety were established and grown in the greenhouse and they were grown until they form 15 or more nodal segments per plantlet. The growing nodal segments were cut from the seedling and prepared by removing extra leaf sheaths and then taken to laboratory for surface sterilization.

Explants (5-10 cm nodal segments) were washed under running tap water and then treated with 0.3% a fungicide (Kocide) solution for 20-30 minutes. Then, the explants were treated with mercuric chloride (0.1 % $HgCl_2$ for 5 minutes) solution followed by 70% ethanol for 1 min. The explants were then thoroughly rinsed three times with sterile distilled water and the explants were reduced to one node size (about 1-2 cm) and placed on MS media containing 1 mg/l BAP under aseptic condition in laminar flow cabinet, which has been used as initiation media for yam (Chen *et al.*, 2007; Mwirigi *et al*., 2010). The explants started initiation within 4-8 days after culturing.

3.3. Media Preparation and Culturing

Murashige and Skoog (MS) (1962) basic media was used for all experiments (Appendix 3). The MS media contain 30g/l sucrose as a carbon source and 0.8% agar as a gelling agent. The stock solutions were mixed in a required proportion along with growth regulators and sucrose. The volume was made up by adding double distilled water. The pH of the medium was adjusted to 5.8 by using either 0.1 N HCl or NaOH before autoclaving at 121^0C for 15 minutes. The volume was finally adjusted and required amount of agar was added into the medium. The agar in the medium was completely melted by gentle heating in a microwave oven and 30-40 ml of medium was poured into pre sterilized glass culture jar with a capacity of 120 ml and plugged with aluminum foil. The media were autoclaved at 121^0C (15 minutes at 15 psi) and then allowed to cool to a room temperature and stored in transfer rooms until used. Surface sterilized explants were cultured in culture jar containing 30-40 ml media supplemented with $1mgl^{-1}$ BAP and one explant per jar was used for shoot initiation. The cut ends of explants were kept in such a way that maximum contact with the medium can be created. Then the initiated explants were transferred to glass jars with a capacity of 350 ml (560 x 355 mm) containing 50-60 ml media for shoot multiplication. Similarly ½ MS media were prepared and 30-40 ml media were poured into pre sterilized glass culture jars with a capacity of 120 ml for rooting.

3.4. Culture Conditions

All the aseptic activities such as surface disinfection of explants, preparation and inoculation of explants and subsequent sub-culturing were carried out in the laminar air flow cabinet. The working table of laminar air flow cabinet and spirit lamp were sterilized by swabbing with 70% ethanol. All the required materials like media, spirit lamp, lighter and glass ware were kept under the clean laminar air flow hood. The cultures were maintained at 16 hour photoperiod, temperature of 25 ± 2^0C, humidity of 60-70% and light intensity of 2000 lux ($\approx$30 µmol m^{-2} s^{-1}) provided by florescent light which is used by Behera *et al*.(2008).

3.5. Treatments and Experimental Design

All the experiments were laid down in a completely randomized design (CRD**)** with five replications (five jars each with three explants , a total of 15 explants for multiplication and each with two shoots, a total of 10 shoots per treatment for rooting were used).

3.5.1. Experiment 1: Effects of BAP and NAA combination on shoot multiplication

In this experiment, MS medium containing 3% sucrose, 0.8 % agar, and different levels of BAP and NAA was used to test the effects of different plant growth regulators on shoot multiplication. Four concentrations of BAP (0.5, 1, 1.5 and 2 mg l^{-1}) and NAA (0, 0.15, 0.25, and 0.5 mgl^{-1}which is similarly NAA concentration used by Kharat *et al*. (2008)) in 4x4 factorial combinations were used. Basal medium (without plant growth regulators) was included as a control.

3.5.2. Experiment 2: Effect of NAA and IBA combinations on *in vitro* rooting

Under this experiment, the effect of auxin types and concentrations on rooting of *in vitro* shoots were studied. Accordingly, ½ MS medium supplemented with five concentrations of IBA (0, 0.5, 1, 1.5, 2 mgl^{-1}) and five concentrations of NAA (0, 0.5, 1, 1.5, 2 mgl^{-1}) in 5x5 factorial combinations were tested.

3.5.3. Acclimatization

Plantlets with three to four leaves were transplanted to plastic pots filled with sterilized nursery medium in equal ratio of manure, forest soil and sand (1:1:1) after agar is completely removed from the roots by washing under tap water(Appendix Figure 3**)**. The plants were sprayed three times with water every morning, midday and evening for a period of three weeks and then covered with polyethylene plastic sheet in the greenhouse. After four weeks, percent of acclimatized seedlings or plantlets were recorded.

3.6. Data Collected

Data were collected on growth parameters of *in vitro* generated plantlets after 40-45 days of culturing on multiplication media and four weeks after culturing on rooting media. Number of nodes, number of shoots, and number of leaves were counted from five jars each containing three explants, a total of 15 explants (Appendix Figure 2). Number of roots and root length were counted from five jars each with 2 shoot, from a total of 10 shoots per treatment. The average number of nodes/explant, average number of shoots/explant, mean shoot length, average number of leaf/explant, average number of roots/shoot and mean of root length were computed from the collected data.

3.7. Data Analysis

The collected data were analyzed using SAS (version 9.2*)* software (SAS Institute Inc. 2008). The data were subjected to analysis of variance (ANOVA) and mean separation was done using procedure of REGWQ for significant means at a probability of 5%.

4. RESULTS AND DISCUSSION

This study was conducted to investigate the effect of plant growth regulators on *in vitro* propagation of yam via nodal culture. The results obtained are presented and discussed as follows:

4.1. Effects of BAP and NAA Combination on Shoot Multiplication

Analysis of variance showed that BAP and NAA combinations were highly significantly different ($p< 0.0001$) for shoot multiplication (Appendix 1). The response of nodal explants cultured on different shoot multiplication media is presented in Table.1. The results showed that culture medium devoid of plant growth regulators (control) failed to stimulate any shoot multiplication in the cultured explants within a period of five weeks. MS medium supplemented with growth regulators (BAP in combination with NAA) produced better results in terms of shoots /explant, average shoot length and average number of nodes per explant.

Among the combination tested, 1.5 mgl^{-1} BAP + 0.15 mgl^{-1} NAA resulted in the highest response in which an average of 6.40±0.28 shoots per explant with a mean shoot length of 2.0±0.11 cm was obtained (Table 1; Fig 1A). The second best shoot multiplication (5.40±0.28 shoots per explants and mean shoot length of 1.84±0.20 cm) was obtained on MS medium supplemented with BAP (1.0 mgl^{-1}) + NAA (0.15 mgl^{-l}) (Fig.1B). MS medium supplemented with BAP (1.5 mgl^{-l}) + NAA (0.25 mgl^{-l}) resulted in 4.33±0.34 shoots/explant with a shoot length of 4.0±0.12 where number of shoots per explant is significantly lower than that of the first two combinations of plant growth regulators whereas the shoot length is significantly improved (Table .1, Fig.2A).

Table 1: Shoot multiplication in nodal explants of *Dioscorea spp*. cultured on MS medium supplemented with various concentrations of BAP and NAA.

Treatments (mg^{-l})		Mean No of shoots/explant ±S.D.	Mean shoot length (cm) ±S.D.	Mean No of nodes/explant ±S.D.	Mean No of leaf/explant ±S.D.
BAP	NAA				
0	0	0.00^{h}±0.0	0.00^{i}±0.00	0.00^{h}±0.00	0.00^{h}±0.00
0.5	0	2.60^{gf}±0.28	1.07^{fg}±0.08	5.2^{ef}±0.56	$1.16(14.2)^{cd}$±0.13
0.5	0.15	4.53^{c}±0.38	1.42^{e}±0.16	6^{d}±0.53	$1.16(13.87)^{cd}$±0.11
0.5	0.25	2.20^{g}±0.30	1.61^{de}±0.12	6.20^{d}±0.56	$1.26(17.13)^{abc}$±0.01
0.5	0.5	0.00^{h}±0.0	0.00^{i}±0.00	0.00^{h}±0.00	$(0.00)^{h}$±0.00
1	0	3.60^{d}±0.30	1.45^{e}±0.19	6.00^{d}±0.53	$1.18(7.87)^{bcd}$±0.03
1	0.15	5.40^{b}±0.28	1.84^{cd}±0.20	7.00^{c}±0.23	$1.2(10.2)^{abcd}$±0.08
1	0.25	2.67^{efg}±0.24	2.6^{b}±0.15	7.53^{cb}±0.30	$1.3(11.6)^{ab}$±0.03
1	0.5	3.47^{d}±0.3	1.06^{fg}±0.18	3.40^{f}±0.28	$0.91(7.67)^{g}$±0.06
1.5	0	4.33^{c}±0.24	1.75^{cd}±0.12	7.07^{c}±0.43	$1.2(14.94)^{abcd}$±0.02
1.5	0.15	6.40^{a}±0.28	2.0^{c}±0.11	7.8^{b}±0.18	$1.26(17.27)^{abc}$±0.03
1.5	0.25	4.33^{c}±0.34	4.0^{a}±0.12	9.6^{a}±0.28	$1.32\ (19.67)^{a}$±0.01
1.5	0.5	3.20^{de}±0.30	1.37^{e}±0.13	4.6^{f}±0.28	$0.91\ (7.13)^{g}$±0.05
2	0	3.27^{d}±0.28	0.77^{h}±0.13	3.8^{g}±0.38	$0.95\ (14)^{fg}$±0.03
2	0.15	4.13^{c}±0.38	1.104^{f}±0.21	5.87^{de}±0.38	$1.05(14.93)^{ef}$±0.05
2	0.25	3.53^{d}±0.38	1.64^{de}±0.12	6^{d}±0.53	$1.1\ (18.67)^{de}$±0.05
2	0.5	3.07^{def}±0.36	0.8^{gh}±0.22	3.67^{g}±0.24	$0.94\ (7.13)^{g}$±0.05
CV%		8.3	9.7	6.9	5.4

(a-i means having the same letters in a column were not significantly different at $P < 0.05$ level. S.D.-Standard deviation of mean, means in the brackets were the actual data while the one outside of the brackets were transformed data using log transformation)

The dependency of cultured nodal explants of *Dioscorea spp.* on the presence or absence

of cytokinin and auxin for shoot multiplication has already been established (Adeniyi *et al*., 2008; Mwirigi *et al*., 2010). This has also been recently reported in the case of micropropagation of other yams like *D. composite* (Alizadeh *et al*., 1998), *D. oppositifolia* (Behera et al., 2009), *Dioscorea alata* (Behera *et al*., 2010). In this experiment, on MS media supplemented with 1.5 mgl^{-1} BAP alone, 4.33 shoots per explant was obtained. When 0.15 mgl^{-1} NAA was added in the presence of 1.5mgl^{-1} BAP, a significant improvement in the number of shoots per explant (6.4,) was recorded showing the existence of interaction effect between BAP and NAA. This result confirms the reports of Chen *et al*. (2007) and Adeniyi *et al*. (2008) where media supplemented with BAP and NAA combination induced best shoot multiplication, indicating that the effectiveness of each of the phytohormone in inducing shoots multiplication was influenced by the presence or absence of the other.

It has been also reported that when Kenyan yam was cultured on media with BAP supplement alone, a limited proliferation of explants with an average of 1.1 shoots per cultured explant was observed while addition of 0.02 mgl^{-1} NAA produced an average of 2.1 shoots per explant (Mwirigi *et al*., 2010).

Figure 1: Shoot multiplication. Shoot multiplied on BAP 1.5mg/l &NAA 0.15**(**A). Medium supplemented with 1 mg/l BAP & 0.15 mg/l NAA (B).

In this study only single cytokinin was found to be used for shoot multiplication but some authors suggested that the combination of two cytokinins with auxin were needed for producing higher number of multiple shoots. Behera and his coworkers in 2009 obtain an average of 10.5 ± 0.51 shoots on MS+ Kinetin (2.0 mg^{-l}) +BAP (1.0 mgl^{-1}) + NAA (0.5 mgl^{-1}) with ascorbic acid 100mg^{-l} followed by MS + Kinetin (1.5mgl^{-1}) + BAP (1.0 mg^{-l})

+ NAA (0.5 mg^{-l}) + 100 mg^{-l} ascorbic acid where 5.5 ± 0.43 shootlets were obtained. In 2010 the same authors reported 6.5 ± 0.42 shootlets on Kinetin (1.5mgl^{-1}) + BAP (1.0 mg^{-l}) + NAA (0.5 mg^{-l}) + 100 mg l^{-l} ascorbic acid with different yam species which is completely different from present study in terms of methodology but similar outcome with best result of the present study.

In other reports a combination of single cytokinin with auxin was needed for better production of multiple shoots. Behera *et al*. (2008) reported that BAP (2.0 mgl^{-1}) + NAA (0.5 mgl^{-1}) with 100mgl^{-1}ascorbic acid, producing an average of 6 ± 0.18 shoot lets per explants. They also reported that the second best shoot multiplication (4.5 ± 0.12) on a medium supplemented with BAP (1.5$mg1^{-1}$) + NAA (0.5 mg^{-l}) + 100 mg^{-l} ascorbic. Similarly Thankappan and Patell (2011) reported highest rate of multiplication on media with 0.5 mgl^{-1} BAP and 0.01mgl^{-1} NAA which was the only hormone combination they have used. Yan *et al*. (2011) obtained 5.5 shoots per explant on 1.0 mg l^{-1} BAP + 0.1 mg l^{-1} NAA + 30 g l^{-1} sucrose in liquid culture which is in agreement with the second best result of this study.

4.2. Effect of NAA and IBA Combination on Rooting

Analysis of variance showed that there was highly significant difference between treatments ($p< 0.0001$) for rooting (Appendix 2). There was also highly significant interaction between NAA and IBA. The rooting responses of shoots on different media, including mean number of roots/shoot and mean root length over a period of four weeks were significantly different (Table-2). There was no rooting in case of shoots planted on auxin free (basal) medium (Fig. 3, C; Table-2). Similarly, at lower level of NAA (0.5 mgl^{-l}) there was hardly any rooting in the cultured shoots during the four weeks of observation period. However higher concentration of NAA (1.5 and 2.0 mgl^{-l}) and IBA at some concentration tested responded well (Table 2.).

Rooting was best on cultures which had combinations of 1/2 MS+2.0 mgl^{-l} NAA + 0.5 mgl^{-l} IBA where an average number of 5.7±0.27 roots per plantlet and an average root length of 4.63±0.23 cm was recorded (Table. 2, Fig. 3 A. Appendix Figure 2 A). The second highest rooting was recorded on 1/2 MS + NAA (2 mgl^{-1}) + IBA (0 mgl^{-1}) with an

average of 4.8±0.27 roots per plantlets and an average root length of 3.52±0.20 (Fig.3 B). As shown in Table.2 NAA*IBA was more effective than their separate effects on rooting.

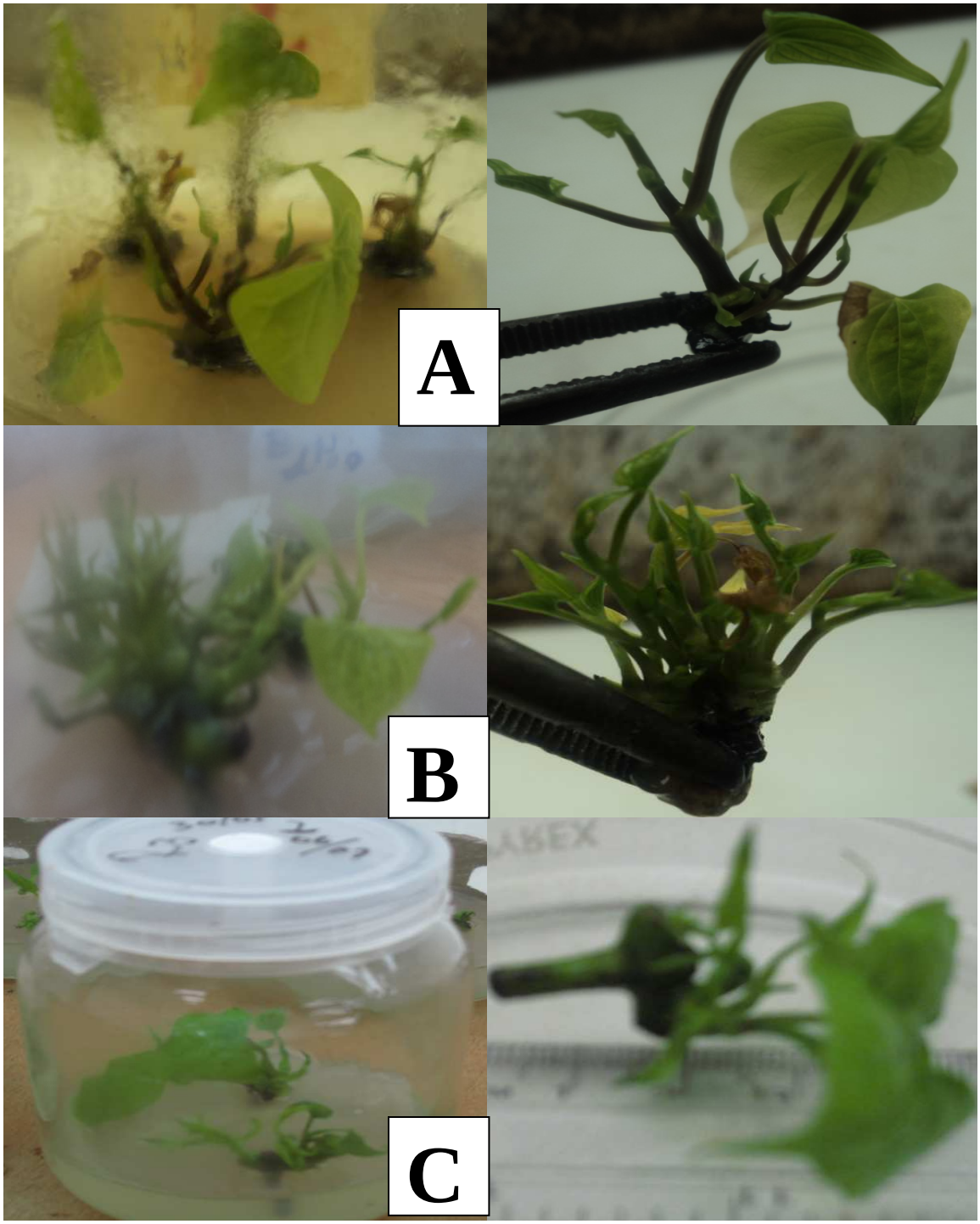

Figure 2: Shoot multiplication when it is inside and out of the jar. MS medium + 1.5 mg/l BAP + 0.25 mg/l NAA (A). MS + 1.5 mg/l BAP + 0.15 mg/l NAA (B). MS + 1 mg/l BAP + 0.15 mg/l NAA (C).

Table 2: Influence of different combination of NAA and IBA on rooting of *in vitro* generated shoot lets of *Dioscorea* spp. (Aw- 004/00)

Plant growth regulators (mg-l)		Mean numbers of root /shoot ±S.D.	Mean root length (cm) ±S.D
NAA	IBA		
0	0	0.00^{l} ±0.00	0.00^{o}±0.00
0	0.5	$0.45(1.8)^{hij}$ ±0.04	0.84^{n}±0.09
0	1	$0.45(1.8)^{hij}$ ±0.04	0.92^{nm}±0.04
0	1.5	$0.57(2.7)^{efg}$ ±0.0.03	1.09^{klm} ±0.03
0	2	$0.5(2.2)^{ghi}$ ±0.04	1.18^{kl} ±0.03
0.5	0	$0.23(0.7)^{k}$ ±0.07	0.75^{n} ±0.03
0.5	0.5	$0.43(1.7)^{ij}$ ±0.04	1.05^{lm}±0.09
0.5	1	$0.52(2.3)^{fgh}$ ±0.04	1.28^{jk} ±0.05
0.5	1.5	$0.53(2.4)^{fg}$ ±0.03	1.42^{ij} ±0.04
0.5	2	$0.43(1.7)^{ij}$ ±0.04	1.29^{jk} ±0.02
1	0	$0.38(1.4)^{j}$±0.04	1.64^{gh} ±0.16
1	0.5	$0.5(2.2)^{ghi}$ ±0.04	1.74^{fg} ±0.16
1	1	$0.56(2.6)^{efg}$ ±0.03	1.52^{hi} ±0.05
1	1.5	$0.58(2.8)^{efg}$ ±0.03	1.60^{ghi} ±0.10
1	2	$0.5(2.2)^{ghi}$±0.04	1.91^{ef} ±0.06
1.5	0	$0.68(3.8)^{cd}$±0.03	1.98^{e} ±0.06
1.5	0.5	$0.72(4.3)^{bc}$ ±0.02	2.90^{c} ±0.11
1.5	1	$0.63(3.3)^{de}$ ±0.0.03	2.40^{d} ±0.06
1.5	1.5	$0.59(2.9)^{ef}$ ±0.03	2.12^{e} ±0.07
1.5	2	$0.57(2.7)^{efg}$ ±0.03	1.74^{fg} ±0.12
2	0	$0.76(4.8)^{ab}$ ±0.02	3.52^{b} ±0.20
2	0.5	$0.82(5.7)^{a}$ ±0.0.02	4.63^{a} ±0.23
2	1	$0.72(4.3)^{bc}$ ±0.02	2.90^{c} ±0.16
2	1.5	$0.52(2.3)^{fgh}$ ±0.04	1.96^{e} ±0.08
2	2	$0.28(0.9)^{k}$ ±0.06	1.47^{hij} ±0.06
	CV%	6.97	5.8

(a-o, means having the same letter in a column were not significantly different ($p < 0.05$). S.D.-Standard deviation of mean, means in the brackets is actual values while the one outside are transformed values using log transformation).

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyam, 1970). In the present study best rooting was observed on half strength MS with NAA (2.0 mgl^{-l}) and IBA (0.5 mgl^{-l}). In

this study the combinations of NAA and IBA were used but some authors suggested that auxin NAA or IBA alone is needed to profuse rooting on *Dioscorea hispida* (Behera *et al*., 2008), *Dioscorea oppositifolia l.* (Behera *et al*., 2009), *Dioscorea alata* (Behera *et al*., 2010) and reported that NAA is more effective than IBA.

On the other hand some authors reported that combination of auxin NAA and IBA was needed for better production of roots in *Dioscorea prazeri* (Thankappan and Patell, 2011), *Dioscorea nipponica* (Chen *et al.,* 2007) who obtained best rooting on a medium containing 1.0 mgl^{-1} IBA and 0.5 mgl^{-1} NAA and 0.5 mgl^{-1} IBA and 0.01mgl^{-1} NAA respectively. In addition Behera *et al*. (2008) reported 5.2 ± 0.28 roots per plantlet and an average root length of 3.5 ± 0.12 cm for *D. hispida.* Behera *et al*. (2009) reported 6.5 ± 0.30 roots per plantlet and an average root length 4.5 ± 0.16 cm. Similarly Behera *et al*. (2010) obtained 5.5 ± 0.48 roots per plantlet and an average root length 5.2 ± 0.26 cm on ½ MS + 2 mg/l NAA without IBA, which is the second best in the present study.

4.3. Acclimatization

About 86% of the rooted plantlets exhibited establishment in the greenhouse within 3-4 weeks after being transferred to the sterilized soil medium (1:1:1 sand, forest soil and dried and decomposed manure, Appendix Figure.4). This result is almost similar with that of Behera *et al*. (2008), Behera *et al*. (2009) and Behera *et al*. (2010) who reported about 90% of the rooted plantlets established in the greenhouse within 2-3 weeks of transfer on similar medium. Chen *et al.* (2007) also reported 91%, survival rate of the plantlets one month after acclimatization and the young plants grew vigorously in the greenhouse.

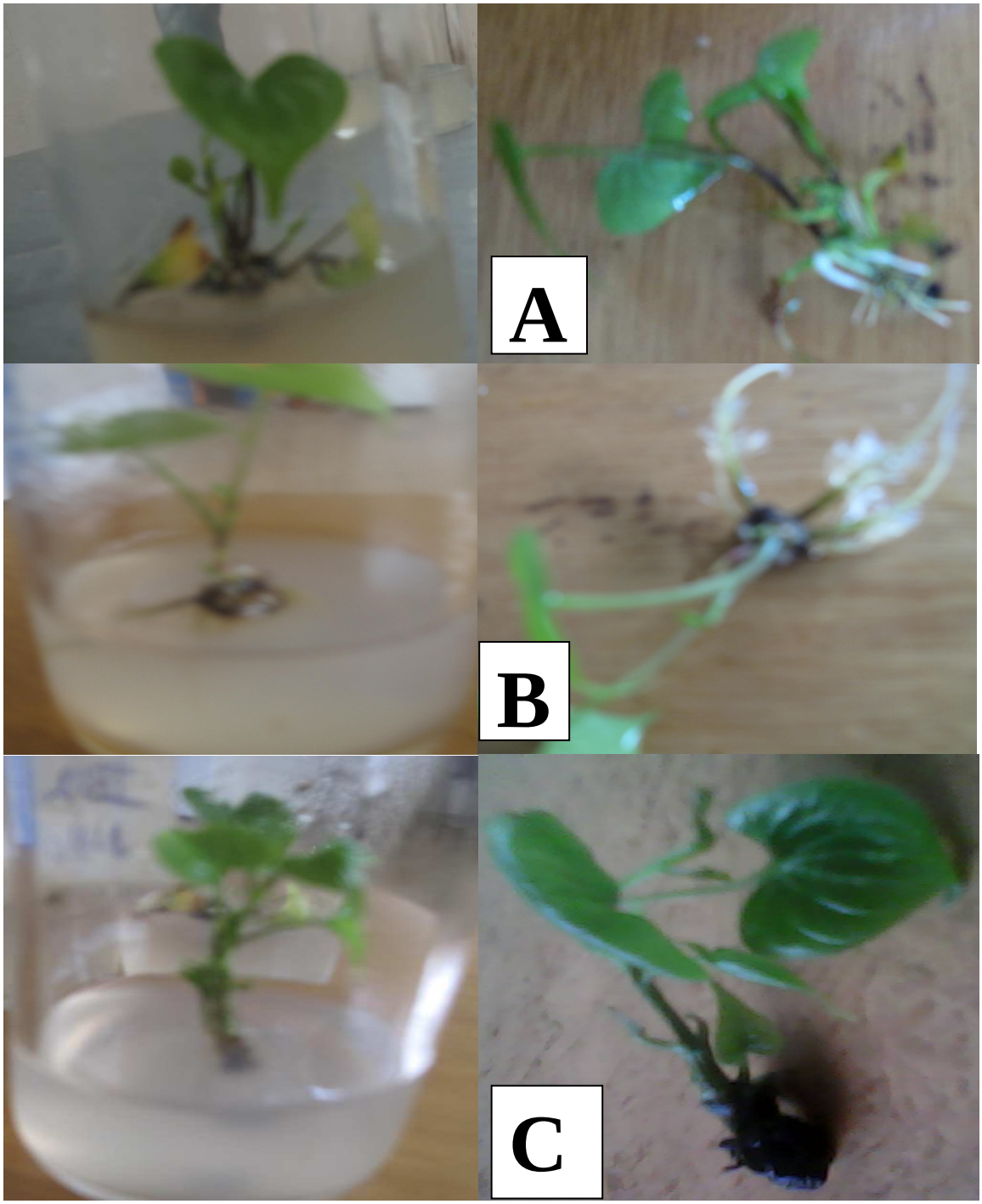

Figure 3: Rooting of *in vitro* shoots rooted plantlets on 1/2 MS medium supplemented with 2mg/l NAA + 0.5 mg/l IBA(A). Half MS + 2 mg/l NAA + 0 mg/l IBA (B). Control, medium free of auxin (i.e. NAA &IBA at zero level) (C)**.**

5. SUMMARY AND CONCLUSION

Yam (*Dioscorea species*) is a traditional crop that has long been cultivated in southern, western and southwestern part of Ethiopia as staple or co-staple with cereals and other root and tuber crop. It would contribute significantly to food security, improved health and increased income and fill a seasonal gap in food supply in Ethiopia as it is adapted to dry season planting.

Shortage of planting material is a major constraint for yam production, owing to low reproductive rate which may lead to future decline in yam production. In addition to this yam tubers are bulky to transport and have extended dormancy period which are some of the problems in yam propagation. To address this limitation *in vitro* propagations were developed for many *Dioscorea species*, but the protocol developed for one genotype is not reproducible when applied to other genotype of the same species and there is no any *in vitro* propagation protocol for yam in Ethiopia. Therefore, the present study was carried out with the objectives of optimizing the best combination of BAP and NAA for shoot multiplication and NAA and IBA for *in vitro* rooting of specific variety of yam. The study was done at the tissue culture laboratory of JUCAVM.

Variety, Aw- 004/00 was used as a planting material which is high yielding (25 t/ha) and the only released variety of yam in Ethiopia. The planting materials were obtained from Hawassa Agricultural Research Center and established in green house. After the mother plant was established in the green house the explants were prepared from this by removing the extra leaf sheath and taken to lab for sterilization. The disinfection of the explant was carried out by first washing the explants with tap water to reduce the microbial load. Then three consecutive sterilizations were carried out in the hood, 20 min in 0.3% kocide, 5 min in 0.1 % mercuric chloride followed by 70% ethanol for 1min and finally rinsed three times in deionized and autoclaved water.

Two separate experiments (shoot multiplication and *in vitro* rooting) which were laid out in a factorial arrangement using CRD with five replications and five jars each with three explants, a total of 15 explants per treatment were conducted for shoot multiplication. Five jars each with two shoots, a total of 10 shoots per treatment was used for rooting. Nodal

buds from greenhouse raised mature yam were used as explant source. Full strength solid MS medium containing different concentrations and combinations of BAP (0.5, 1, 1.5, and 2 mg l^{-1}) and NAA (0, 0.15, 0.25 and 0.5 mgl^{-1} for shoot multiplication and NAA (0.0, 0.5, 1.0, 1.5 and 2mg l^{-1}) and IBA at similar concentration for rooting were used. Data based on number of shoot and root, length of shoot and root were collected.

The sterilized explants were cultured on MS media supplemented with 1mg/l BAP for shoot initiation and the initiated explants were transferred to multiplication medium containing different concentration of BAP & NAA. Then data was collected after five weeks of culturing on multiplication media on the bases of shoot number as a major parameter. From MS Medium supplemented with 1.5mg/l BAP + 0.15 NAA 6.40±0.28 shoot lets with a mean shoot length of 2.0±0.11 cm per explant was recorded and 5.40±0.28 shoots with a mean shoot length of 1.84±0.20 cm from 1mg/l BAP + 0.15 NAA. There was no any multiplication on basal medium.

Shoots that have been formed on multiplication medium and having two or more nodes were transferred into rooting medium for development of plantlets. Data collected after four week of transfer on rooting media. On half strength MS medium supplemented with 2mg/l NAA + 0.5 IBA 5.7±0.27 roots per plantlet and an average root length 4.63±0.23 cm was recorded followed by 2mg/l NAA + 0mg/l IBA that is almost similar to 1.5mg/l NAA + 0.5mg/l IBA where 4.8±0.27 roots per plantlets and an average root length of 3.52±0.20 were recorded. There is no any rooting on basal medium (medium free of plant growth regulators). In addition NAA was better than IBA for *in vitro* rooting of this variety. The rooted plantlets were transferred to sterilized soil for further hardening with a 1:1:1 mixture of sand, forest soil and dried and decomposed manure for further hardening. After four weeks of acclimatization 86% survival was recorded.

Generally A-w 004/00 gave 6.4 shoots per explant within 4-5 week of culturing. Thus, the optimized protocol is useful for *in vitro* propagation of this specific variety of yam planting material. MS media supplemented with 1.5mg/l BAP + 0.15 NAA for shoot multiplication and ½ MS supplemented with 2mg/l NAA + 0.5 IBA for rooting were the best growth regulator combinations for micropropagation of this specific variety of yam and these media combination can be recommended for further use.

As this experiment was done for the first time for this specific variety of yam and it was only to optimize growth regulators, so, additional studies are required to further improve the protocol optimized .i.e.:

- Optimization of other components of media other than growth regulators required.
- Determining the subculture stage at which maximum shoot multiplication will be obtained.
- Studies on genetic stability of the *in vitro* regenerated plantlets.
- Virus indexing, the possibilities of genetic transformation and cryopreservation.

Besides this *in vitro* propagation protocol for the other existing yam landraces are required to obtain clean planting material and conservation of yam landraces.

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7. APPENDICES

Appendix 1: Mean squares for shoot growth parameters as affected by combination of BAP and NAA

Source of variation	DF	Number of shoots/explant	Number of nodes/explant	Number of leaves/shoot	Average shoot length
BAP	3	17.14***	33.85***	0.32605706***	7.1***
NAA	3	25.64***	75.65***	1.24430662***	9.8***
BAP* NAA	9	2.76***	4.58***	0.29455963***	0.89***
CV (%)		8.3	6.9	5.4	9.7

***= highly significant at P< 0.0001, P = probability value, MS= mean square, DF= degree of freedom, CV = Coefficient of variation, BAP= 6-benzylaminopurine, NAA= α-naphthalene acetic acid

Appendix 2: Mean squares for root growth parameters as affected by combination of IBA and NAA

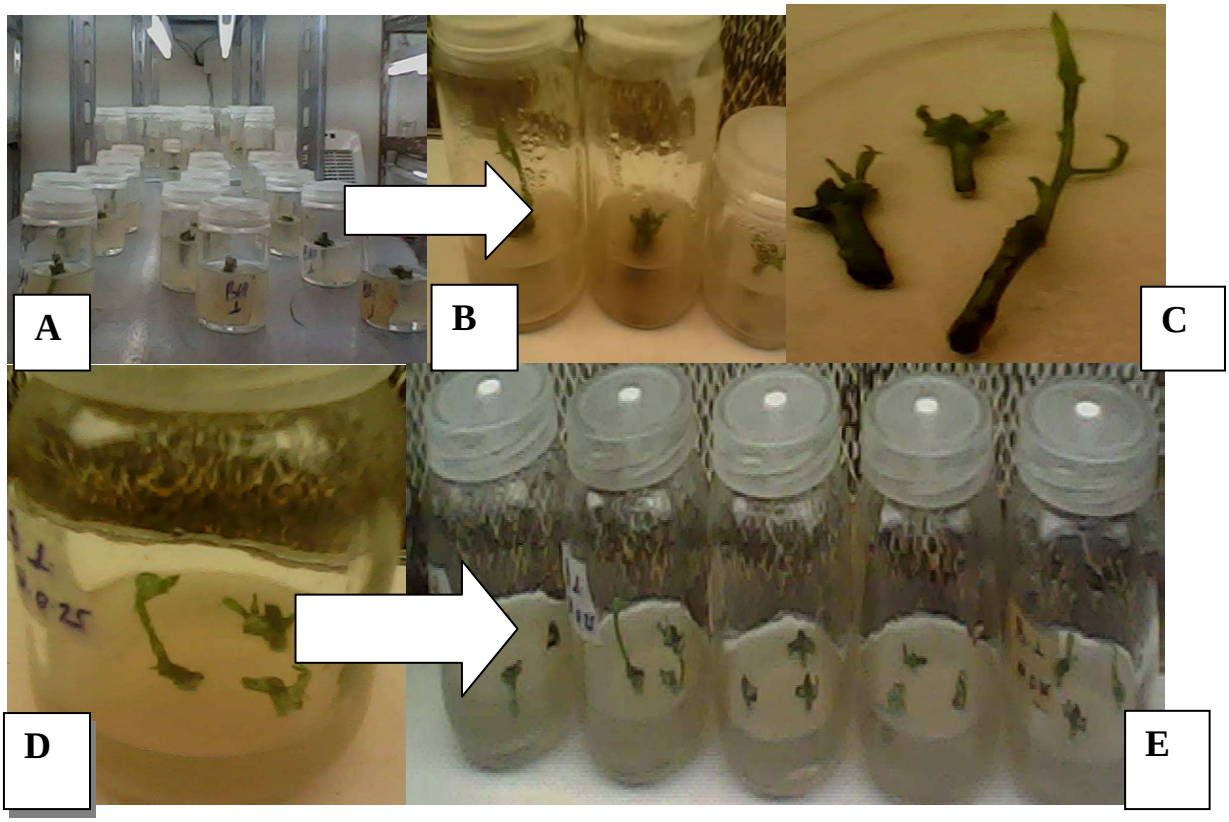
Source of variation	DF	Number of root / shoot	Average root length
NAA	4	0.31***	17.38***
IBA	4	0.16***	2.09***
NAA*IBA	16	0.12***	2.09***
CV (%)		6.97	5.8

***= highly significant at P< 0.0001, P = probability value, MS= mean square, DF= degree of freedom, CV = Coefficient of variation, IBA= indole-3-butyric acid, NAA= α-naphthalene acetic acid

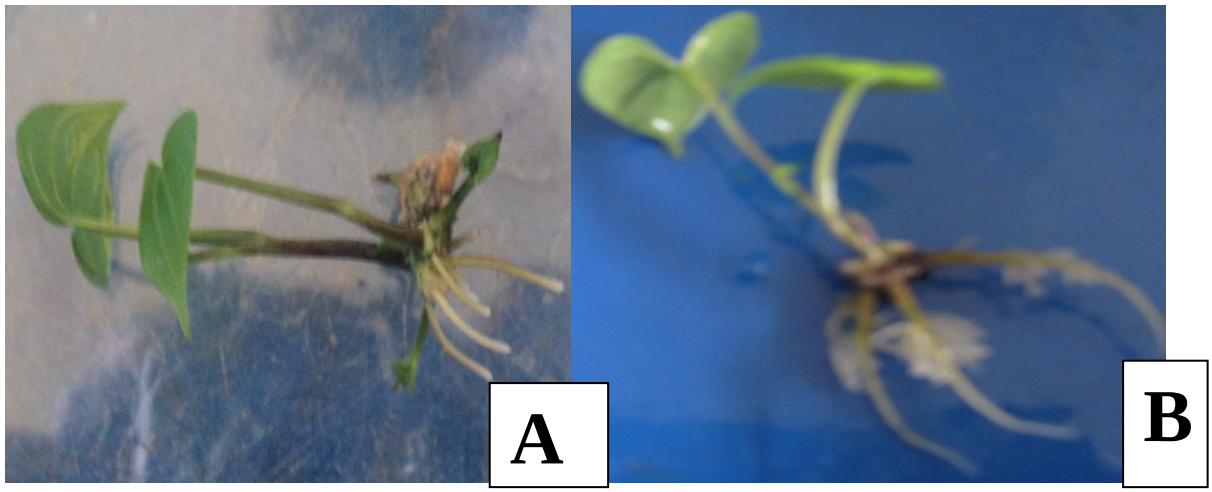
Appendix 3: Components of modified Murashighe and Skoog medium (1962)

Constituents	Concentration (mg/l)
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.H_2O$	440
KH_2PO_4	170
$MgSO_4.7H_2O$	370
H_3BO_3	6.2
$NaEDTA.2H_2O$	37.3
$FeSO_4.7H_2O$	22.3
$MnSO_4.4H_2O$	27.8
$ZnSO_4.7H_2O$	8.6
KI	0.83
$NaMoO_2.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Nicotinic acid	0.5
Pyridoxine	0.5
Thiamine	0.1
Myo-inositol	100
Sucrose	30,000
Agar	8,000

Source: Khanna, 2003



Appendix Figure 1: Procedures of initiation to multiplication. Culture initiation on BAP 1mgl^{-1} (A). Number of initiated explant used for multiplication per jar (B&C). Initiated shoot on multiplication media (D). Initiated culture cultured on multiplication media composed of similar combination of BAP & NAA (five jars per treatment) (E).



Appendix Figure 2: Rooting when outside the jar. Rooted plantlets on NAA 2mgl^{-1} + 0.5 IBA (A). Rooted plantlets on NAA 2mgl^{-1} (B).

Appendix Figure 3: Soil media preparation for acclimatization. Sand (A). Dried and decomposed manure (B)**.** Forest soil (C)**.** Sterilized 1:1:1 combination of sand: manure: soil (E&F). Plantlets covered by plastics soon after their transfer on to the soil (G).

Appendix Figure 4. Plants after four weeks of acclimatization.