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# Micropropagation of *Dioscorea alata* (cv. wonago I) from shoot tips and nodal explants

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| BAP | 6-Benzylamino purine |
| NaOCl | Sodium hypochlorite (local bleach) |
| EIAR | Ethiopian Institute of Agricultural Research |
| IBA | Indole -3-butyric acid |
| M.A.S.L | Meters above Sea level |
| MS medium | Murashige and Skoog |
| NAA | Naphthalene acetic acid |
| PGRs | Plant Growth Regulators |
| N.A.S | National Academy of Sciences |
| FAO | Food and Agricultural Organization |
| D.A.A.R.S | Department of Agricultural, Agricultural Research Service |
| KIN | kinetin |

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# Abstract

*Dioscorea alata L. (Yam) is a member of genus Dioscorea and monocotyledonous plant belonging to the Dioscoreaceae family which comprises about 600 species. D. alata is an important corp plant that produces tuberous roots used as a source of food and medicine. Inefficiency of traditional methods of propagation and lack of planting materials are the main constraints for implementing large-scale cultivation. The purpose of this study was, therefore, to develop a micropropagation protocol for D. alata L. from shoot tip and nodal explants. Explants were sterilized using different concentrations of NaOCl for different time exposure. MS culture media supplemented with different types and concentration of auxins and cytokinins were used for culture initiation, shoot multiplication and root induction. Sodium hypochlorite (NaOCl, 5.2% of active chlorine) at a concentration of 5 and 2 % at exposure time of 5 and 20 minutes gave 76.60±0.36%, of survived explants for nodal and 72.66±0.85% for shoot tip respectively*. The initiation media with a combination of 4x4x2x2 factorial; and multiplication of 3x3x2x2 factorial were used. From these, *BAP of 1.5 mg/l + NAA of 1.0 mg/l gave 93.2% for nodal and BAP of 1.0 mg/l + NAA of 1.0 mg/l gave 87.1% for shoot tip was found to be an optimum concentration for shoot induction. The combination of 2.0mg/l KIN, 1.0mg/l BAP and with 0.5mg/l NAA and 2.0mg/l KIN, 0.5mg/l BAP and with 0.5mg/l NAA was found to be the optimum concentration yielding 8.4± 0.21% and 9.5± 0.29% shoots per explants for shoot tip and nodal respectively for shoot multiplication. Half strength MS medium with 2.0 mg/l IBA gave the highest rooting percentage and with 2.0mg/l NAA gave optimum root number and length. Up on acclimatization and transplanting, 90% survival efficiency was observed on soil mix ratio of 2:2:1 decomposed coffee husk, sand and red soil respectively. There were no observable variations with respect to morphology and growth characteristics to the greenhouse raised parent plant. The results obtained in this study permit the development of mass propagation protocol that could enable large scale commercial production of this highly demanded Dioscorea cultivar and provide a possible system towards genetic improvement of the crop.*



Key words: *In vitro* propagation, MS medium, plant growth regulators.

# CHAPTER ONE

# 1. INTRODUCTION

## 1.1 Background

*Dioscorea alata* (water yam or greater yam), belongs to the Dioscoreaceae family which approximately 600 species. It is a family of seven genera; six genera are found in Madagascar, Central America, Indo-Malaysia, Micronesia and Europe and only one of which occurs in tropical and subtropical countries (Coursey, 1967; Ayensu, 1972). Greater yam (*D. alata L*.) is one of the important cultivated yams and an essential staple food crop for people in almost all tropical and subtropical countries (Han *et al*., 2000). *D. alata* can grow up to 2100 metres but is only important in areas below 1900 metres above sea level (NARI, 2006).

Yams are herbaceous, climbing, twining, perennial monocots (Coursey, 1976; Strausbaugh *et al*., 1977; Wagner *et al*., 1999). Most yams also produce aerial tubers or bulbils (Wagner *et al*., 1999). Both belowground tubers and bulbils are comprised of stem tissue (Martin, 1974; 1976). Among 600 species about 50 species are edible (Ayansu, 1972; Robertson and Lupien, 2008). Mie´ge and Demissew (1997) described eleven Dioscorea both wild and cultivated species are found and eight are economically most important species of yams cultivated as staple food for millions of peoples in tropical and subtropical countries (Coursey, 1967; Hahn, 1993; Muluneh, 2006; Edison *et al*., 2006 ; TariqulIslaml *et al*., 2008).

Yams are valuable source of carbohydrates, fibers and low level of fats which make them a good dietary nutrient; they are also processed into various staple intermediate and end product forms (Jaleel *et al*., 2007). Tubers have a dual agricultural function for nourishment as a source of food and also act as planting materials (Craufurd *et al*., 2006). It is mainly composed of complex carbohydrates and soluble dietary fiber. Together, they raise blood sugar levels rather very slowly than simple sugars and therefore, recommended as the low glycemic index healthy food (FAO, 2009). In addition, they are a good source of dietary fiber, potassium, which controls blood pressure, vitamin C, manganese and vitamin B6 (Wood, 1988; Hsu *et al*., 2002). Vitamin B6 is needed by the body to break down a substance called *homocysteine*, which can directly damage blood vessel walls (NAS, 1998; Morris *et al*., 2010).

Dioscorea species are a very important source of secondary metabolites used in pharmaceutical industry and medicine (Supriya *et al*., 2013**)**. The tubers of some species of *Dioscorea* are important sources of diosgenin, a chemical used for the commercial synthesis of sex hormones and corticosteroids, which are widely used for anti-inflammatory, androgenic and contraceptive drugs (Satour *et al*., 2007). Dioscorea species are one of the many traditional medicinal plants used for the treatment of infectious and non infectious health problems. Yam leaves and tubers are used to treat a variety of ailments. The leaves of air yam, Chinese yam, and water yam are used as a poultice for pimples and tumors and in bath water to soothe skin irritations and stings (Austin, 1999).

Yam (*D. alata*) is the most important family in terms of production and utilization; as a result, worldwide, Yam ranks at nine position among staple vegetable crops with about 58,754,533 tons produced globally from about 5,036,905 hectares with an average yield of 11,664.8 kg /Ha (FAO, 2012). Of which, most of the production, 56,500,776 tones with an average yield of 11,766.8 kg /Ha comes from African countries. According to recent statistics (FAO, 2012), large quantities of Yam are grown in West African countries, such as Nigeria (7,753,337 tones), Ghana (1,693,203tones), and Côte d'Ivoire (1,099,945 tones). Nigeria is the leading country from Africa and world. Ethiopia is an isolated center of yam cultivation in East Africa (Norman *et al.*, 1995), and the crop plays a vital role in local livelihood particularly in the densely populated areas of southern, southwestern, and western parts of the country across a range of agro-ecologies (Hildebrand *et al*., 2002; Tamiru, 2006). In Ethiopia 350,000 tones are produced annually from about 41,338 hectares with an average yield of 8,466.8 Kg/Ha (FAO, 2012).

The diversity under cultivation is further enhanced by the ongoing domestication of wild yam in various countries (Mignouna and Dansi, 2003; Scarcelli *et al*., 2006). Nevertheless, the extent of genetic diversity in many Dioscorea species and their relationships is yet to be investigated in detail. Attempts to characterize yam using morphological (Hamon and Touré, 1990b; Dansi *et al.*, 1999) and isozyme (Hamon and Touré, 1990a; Dansi *et al*., 2000b) markers did not give conclusive results due to their high degree of variability.

Chromosome counts are also variable in yams, ranging from 2n = 20 to 2n = 140 in the common food species (Hahn, 1995). About three varieties namely, Badi 2/81, Melko 56/7 and 3/81 are recognized in Ethiopia by Jimma Agricultural Research centre JARC/EIAR (EARO, 2004).

The conventional multiplication of *Dioscorea* species is by tuber seeds, a tuber fragment that grows and develops into a new tuber. The absence of viable seeds, the long period required for obtaining usable tubers and phytosanitary problems are some of the factors that limit the rapid conventional propagation and economic exploitation of *Dioscorea* species (Tschannen *et al*., 2005; Balogum *et al*., 2006). In traditional systems of yam cultivation (*Dioscorea* spp.), farmers often encounter shortages of yam planting material, especially following cyclones, droughts, and disease epidemics (Jill, 1989).

Yam anthracnose disease caused mostly by the fungus *Colletotrichum gloeosporioides* Jackson and Nwhoof (Nwakiti and Arene, 1978). Yam Mosaic Virus Disease caused by an aphid transmitted potyvirus (Mantell, 1980), and Water yam virus disease (*Dioscorea alata* virus) also the second commonly encountered fungal folial disease on yam (Amusa, 2000).

*In vitro* propagation of *Dioscorea* species has been increased and performed by using different types of explants such as immature leaves (Kohmura *et al*., 1995), zygotic embryos (Viana and Mantell, 1989), nodal cuttings (Alizadeh *et al*., 1998; Yongqin *et al*., 2003), bulbils (Asokam *et al*., 1983), roots (Twyford and Mantell, 1996), cells and protoplasts (Tor *et al*., 1998). However, few studies have been reported on the micropropagation and microtuberisation of *D. alata* (Mantell and Hugo, 1989; John *et al*., 1993; Jasik and Mantell, 2000; Borges *et al*., 2005). *In vitro* production of microtubers has been reported in a number of Dioscorea species (Jean and Cappadocia, 1991). *In vitro* propagation protocols developed in yam plant growth from nodal *and shoot* segments use semisolid culture media (Borges *et al*, 2004; Balogun, 2009).

*In vitro* propagation may help to overcome constraints related to the availability of high quality of planting material, disease free and drought resistant plants (Wheatley *et al*., 2005; Vaillant *et al*., 2005). Therefore, in the present investigation an attempt was made to see *Dioscorea species* (*D. alata) in vitro* propagations in order to increase the rapid production of disease free plant materials.

# 1. 2 OBJECTIVES

## 1. 2.1 General objective

To develop a micropropagation protocol that contributes for mass cultivation of Dioscorea *alata* L. (Yam) from shoot tip and nodal explants.

## 1.2.2 Specific Objectives:

- To determine the optimal NaOCl and time for surface sterilization of bleach (NaOCl) onYam shoot tip and nodal explants.
- To determine the optimal concentrations of BAP and NAA combinations on shoot initiation from shoot tip and nodal explants
- To determine the optimal combinations of KIN, BAP and NAA on shoot multiplication
- To test the effect of different auxin (IBA and NAA) concentrations on root induction
- To determine the percentage of survived plantlets under acclimatization.

# CHAPTER TWO

## 2. LITRATURE REVIEW

### 2.1. Botanical description of *D. alata*

*Dioscorea alata* (Greater Yam) are herbaceous, climbing, twining and perennial monocots (Coursey, 1967; Strausbaugh and Core, 1977; Wagner *et al*., 1999). Vines or the climbing stems reach up to 10m (30ft) without tendrils and use dead stems from the previous year's growth to climb into other vegetation (Morisawa; Wagner *et al*., 1999). Most yams also produce aerial tubers or bulbils (Wagner *et al*., 1999). Both belowground tubers and bulbils are comprised of stem tissue (Martin, 1974; 1976).

Yams are dioecious (Wagner *et al*., 1999) and produce very small flowers (Strausbaugh and Core, 1977; FNAA, 2009). Sexually produced fruits are 3- winged capsules (Strausbaugh and Core, 1977), but yam fruits are extremely rare and, if produced, are often sterile (Radford *et al*., 1968; Gleason and Cronquist, 1991; FNAA, 2009). Even when male and female water yams grew in close proximity, fruits were rare and seeds were typically aborted before reaching maturity (Martin, 1976). Leaves are large, elongate and heart shaped (Martin, 1976; Morisawa, 1999). Leaves long petioles, opposite (often with only 1 leaf persistent) blades to 20 cm (8 in) or more long, narrowly heart shaped, with basal lobes often angular. Leaf blades typically measure 2 to 6 inches (6-16 cm) long, 2 to 5 inches (4-13 cm) wide, and have entire margins. Bulbils may reach 4 inches (10 cm) long and 1.5 inches (4 cm) in diameter (Clewell, 1985; FNAA, 2009).

Flowers are small, occasionally, male and female arising from leaf axils on separate plants (i.e., it's a dioecious species), male flowers in panicles to 30 cm (1 ft) long, female flowers in smaller spikes. Fruit a 3-parted capsule; seeds winged (Coursey, 1967). Staminate flowers occur in a zigzag pattern along a rachis up to 10 inches (25 cm) long; pistillate flowers occur in 4- to 20- flowered inflorescences that may reach 14 inches (35 cm) long (FNAA, 2009).

## 2.2 Origin of Dioscorea species

Dioscorea species (yam) was thought to originate in southern Asia, but recent genetic studies have identified Melanesia as its centre of origin, and this region remains the centre of diversity (Lebot, 1999).

Its existence in northern Australia is believed to be due to human introduction before the continent was separated from New Guinea by sea level rise 10,000 years ago. It is believed to have been among several Asian crops introduced to Madagascar by Austronesians some 2,000 years ago, and from there spread into mainland East Africa (Lebot, 2009). It is not clear whether *D. alata* was established in West Africa before European contact, but it has since come to rival African species both there and in the Caribbean.

## 2.3 Domestication of Dioscorea species

Domestication has been a traditional farmers practice in West Africa (Scarcelli *et al.,* 2005). Guinea yams (*Dioscorea cayenensis-rotundata* complex; *D. rotundata* Poir. and *D. cayenensis* Lam.) have been described as resulting from a process of domestication of wild yams of the section *Enantiophyllum* by African farmers (Mignouna and Dansi, 2003). Guinea yams were domesticated about 7000 years ago and over the years farmers have selected genotypes that best suit their needs, and thus have generated a large number of traditional cultivars.

White yam (*Dioscorea rotundata*) originated in Africa and is the most widely grown and preferred yam species. Yellow yam (*Dioscorea cayenensis* Lam.) is also native to West Africa and very similar to the white yam in appearance.

Water yam is believed to be a true cultigen that might have been domesticated in Indo-China from *Dioscorea hamiltoni* and *D. persimilis (*Agbaje *et al., 2005*). Genetic improvement programs at IITA (Nigeria) and at the Central Tuber Crops Research Institute (CTCRI, India) have been developing high yielding *D. alata* and *D. rotundata* varieties with pest and disease resistance to meet farmers' requirements.

There is tremendous genetic variability in yam. Contrary to the situation in other crops where the deployment of improved varieties has led to loss of diversity and a narrowing of the genetic base, some kind of domestication of semi-wild yam species is still ongoing in West African countries, which continually augments the germplasm diversity (Mignouna and Dansi, 2003).

## 2.4 Distribution of *D. alata*

*Dioscorea* is a pantropical genus and different species have been independently distributed on each continent (Coursey, 1967). *D. alata* (greater yam or water yam) is the most widespread species. The economically most important yam species include *D. alata*, *D. rotundata* and *D. cayenensis* widely distributed in the tropics and sub-tropics although a few species of minor economic importance are found in the warmer regions of the temperate zone (Coursey, 1967). *D. alata* L. (Water yam or greater yam) is the species most widely spread throughout the world and in Africa is second only to white yam in popularity and thought to have been introduced clonally in Africa ( Lebot *et al.*, 1998; Mignouna and Dansi, 2003). According to Lebot *et al.*, (2005), it is the most widely distributed species in the humid and semi-humid tropics. *D. alata* is less highly regarded than the indigenous *D. rotundata* in West Africa (Brunnschweiler *et al*., 2004). It is an important food in Africa, the Caribbean, and especially Melanesia where it has considerable social and cultural importance (Lebot *et al.*, 2005). Ethiopia is considered as only country for yam distribution and cultivation from east Africa (Norman *et al*., 1995). In Ethiopia, Yam cultivated mostly in western part of the country and the utilization is different from most West African countries, where the tuber is consumed boiled, mashed, fried, roasted, and baked. Tubers are also processed into flour, flakes, chips and dry roasted slices (Tamiru *et al.*, 2008).

## 2.5 Economic Importance of Dioscorea species

Several Dioscorea species are widely cultivated and the major species used as food worldwide are *D. alata L.*, *D. esculenta* (Lour) Burkill, *D. cayenensis* Lam, *and D. rotundata* Poir. At least 20 others are used as foods in times of famine, and a similar number have medicinal and other uses (Demissew and Miege, 2006). Cultivated species are the sources of food in some countries (Coursey, 1976).

*Dioscorea alata* L. is an important tuber crop and is a staple food for millions of peoples in tropical and subtropical countries (Edison *et al*., 2006). Root and tuber crops are the most important food crops after cereals. Yam is considered to be the most nutritious of the tropical root crops (Wanasundera and Ravindran, 1994).

It contains approximately four times as much protein as cassava, and is the only major root crop that exceeds rice in protein content in proportion to digestible energy (Bradbury and Holloway, 1988).

The amino acid composition of yam protein is suboptimal in sulfur-containing amino acids (cysteine and methionine), but the overall rating for essential amino acids is high and superior to sweet potato (Splittstoesser *et al*., 1973; Bhandari *et al*., 2003). Yam is also a good source of vitamins A and C, and of fibre and minerals. Its relatively low calcium content is related to low concentrations of calcium oxalate, an antinutritional factor (Bradbury and Holloway, 1988). It is also low in the antinutrients phytate (Wanasundera and Ravindran, 1994) and trypsin inhibitor (Bradbury and Holloway, 1988). Yams are one of the most highly regarded food products in tropical countries of West Africa and are closely integrated into social, economic, cultural and religious aspects of communities (Okigbo and Ogbonnaya, 2006).

The ritual ceremony and superstition often surrounding yam and its utilization in West Africa is a strong indication of the antiquity of use of this crop (Norman *et al.*, 1995). Aside their high values as a food source, some species of yam have been used medicinally to treat diseases like diabetes mellitus, coronary disorders and in preventing high hypercholesterolemia (Undie and Akubue, 1986). Yam starch takes longer time to break down compared with other starchy tubers like potato and sweet potato, which makes it a safer source of carbohydrate for diabetics.

It is a good source of manganese, a vital micronutrient. A number of *Dioscorea* wild species are sources of compounds used in the synthesis of sex hormones and corticosteroids (Coursey, 1967). Some yam species such as *D. piscatorum* have toxic properties that allow them to be used in the production of insecticides. An insecticide from *D. piscatorum* is used in controlling insect pests of rice in Malaysia. Extracts from *D. deltoidea* is used in the production of anti-lice shampoo in India (Coursey, 1967).

## 2.6 Propagation of *D. alata*

### 2.6.1 Vegetative propagation

Yam tuber grows from a corm-like structure located at the base of the vine. Occasionally this corm remains attached to the tuber after harvest and sprouts will develop from it. When the

corm separates from the tuber sprouting occurs from the tuber near to the point at which the corm was attached (Huber, 1998).

The crop is propagated using tuber pieces and mini-tubers which are affected by a range of pathogens. In Nigeria, the number one producer in the world, the farmers dedicate 35% of their harvest to renew their yam's fields (Ng, 1988). To overcome the lack of yam's seeds, techniques of multiplication based on the mini-fragmentation of tubers were evaluated. Those new methods for seed yam production have been developed by international institute of tropical agriculture (IITA) (1993) and the Nigerian researchers. However, the interesting clones stemming from such method are little spread because of the important viral contaminations and the slowness of regeneration. Instead of the traditional methods of obtaining yams seeds, other methods were focused. Technologies for microtubers production in yam crop show a great potential as alternatives for propagation (Fotso *et al*., 2013). Microtubers may be used for planting material production, plant breeding and germplasm conservation programs (Mbanaso *et al*., 2007; Balogun, 2009), germplasm exchange since they are more tolerant to light and temperature variations and less prone to damage than shoots (John *et al*., 1993).

Microtubers have a potential to be integrated into seed yam programs (Balogun *et al*., 2006; Chen *et al*., 2007). Microtubers are particularly convenient for handling, storage and distribution. Unlike micro propagated plantlets, they do not need a time consuming hardening period in a greenhouse and may be adapted easily to large scale mechanised planting in the field (Coleman *et al*., 2001; Pruski *et al*., 2003).

### 2.6.2 Conventional propagation and its constraints

Yam propagation by seeds using conventional methods is slow and not adequate for rapid multiplication.

Seed yam should be produced by planting sets cut from a larger tuber which will provide whole seed yam of a suitable size as planting material; a relatively high level of care has to be applied because cut surfaces increase the likelihood of tuber rot and desiccation. Minisetts of 25 g have proved successful (Kalu *et al*., 1989).

Tuber yield is drastically reduced by viral and nematode infections; through infected tubers it is transmitted to the next generation (Ng, 1992) and it also deteriorates the quality of the tuber (Mitchell and Ahimed, 1999). *In vitro* propagation may help to overcome constraints related with availability of high quality of planting material (Vaillant *et al*., 2005). For their nutritional value yams are used as staple food. But many reasons restrict production of tuber such as lack of agronomic constraints, phytosanitary problems and lack of healthy planting materials. Tissue culture technique provides a way to increase the rapid production of disease free plant material.

Anthracnose disease of yam has had a considerable impact on yam production world-wide (Nwakiti and Arene, 1978). This is caused mostly by the fungus *Colletotrichum gloeosporioides* (Nwakiti and Arene, 1978). On susceptible yam cultivars, symptoms appeared at first as small dark brown or black lesion on the leaves, petioles and stems.

Yam Mosaic Virus disease is caused by an aphid-transmitted potyvirus that infects several species of Dioscorea, particularly *D. alata*, *D. cayenensis*, *D. rotundata* and *D. trifida*. The symptoms observed in each host can be vein banding, curling, mottling, green-spotting, flecking etc (Mantell, 1980; IITA, 1993). Water yam virus disease (*Dioscorea alata* virus) is more commonly found on *D. alata*. Symptoms included chlorosis vein banding, flecking and leaf puckering (IITA, 1993). Concentric leaf spot disease: Concentric leaf spot disease of yam has been reported to be the second commonly encountered fungal folial disease on yam in south western Nigeria (Amusa, 2000). The causal agent has been identified as *Sclerotium rolfsii* (Amusa, 2000).

## 2.7 Plant Tissue Culture

Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions (Thorpe, 2007) often to produce the clones of plants.

A single explant can be multiplied into several thousand plants in relatively short time period and space under controlled conditions, irrespective of the season and weather on a year round basis (Akin-Idowu, 2009).

The technology had been successfully used for large scale commercial production and supply of pathogen free plants of varied plant genotypes and species (Tsay, 2002), germplasm conservation of rare and endangered species, production of secondary metabolites, as well as rapid and large-scale multiplication of elite genotypes (Baskaran and Jayabalan, 2005). It also serves as an important tool for genetic manipulation, protoplast fusion, embryo rescue and other modern procedures that enhance plant improvement and other basic researches (Chaudhari, 2004; Van, 2009).

### 2.7.1 Micropropagation

Micropropagation is the application of tissue culture technology for propagation of any economically important plant species. Micropropagation starts with the selection of plant tissues (explant) from a healthy, vigorous mother plant (Murashige, 1974). Any part of the plant (leaf, apical meristem, bud and root) can be used as explants. It offers an alternative to vegetative propagation and is mainly aimed at enhancing the rate of multiplication. Micropropagation can be done through i) shoot bud proliferation, ii) adventitious shoot production, iii) meristem culture and nodal culture, iv) *In vitro* tuberization and v) somatic embryogenesis (George *et al*., 2008). *In vitro* regeneration or micropropagation is the practices of rapidly multiplying stock plant material to produce large numbers of progeny plantlets. The integrated approaches of plant culture systems will provide the basis for the future development of novel, safe, effective, and high quality products for consumers (Abera, 2009).

Therefore, different varieties of *Dioscorea species* susceptible to different environmental factors can be overcome by in *vitro* regeneration and micropropagation (Jova *et al*., 2012). *In vitro* propagation of *Dioscorea* species has been increased and performed by using different types of explants such as immature leaves (Kohmura *et al*., 1995), zygotic embryos (Viana and Mantell, 1989), nodal cuttings (Alizadeh *et al*., 1998; Yongqin *et al*., 2003), bulbils (Asokam *et al*., 1983), roots (Twyford and Mantell, 1996), cells and protoplasts (Tor *et al*., 1998).

However, few studies have been reported on the micropropagation of *D. alata* (Mantell and Hugo, 1989; John *et al*., 1993; Jasik and Mantell, 2000; Borges *et al*., 2005). *In vitro* production of microtubers has been reported in a number of Dioscorea species (Jean and Cappadocia, 1991). *In vitro* propagation protocols have been developed in yam plant from nodal *and shoot* segments useing semisolid culture media (Borges *et al.*, 2004; Balogun, 2009).

## 2.7.2 Explants

The explant types which are extensively used in commercial micropropagation are shoot-tip, meristem-tip, nodal or axillary bud culture. This consists of a piece of stem with axillary bud culture with or without a portion of shoot. In some plants, leaf discs, intercalary meristems from nodes, small pieces of stems, immature zygotic embryos and nucleus have also been used as explants to initiate cultures. The explants source should be free of disease causing pathogens, vigor and conformity of the variety, and elimination of somaclonal variants are critical for maintaining plant quality (Abraham, 2009; Feyissa, 2010). Explants can be a portion of the shoot, leaves, or some cells from a plant, or can be any part of the tissue from an animal. In brief, the tissue is harvested in an aseptic manner, often minced, and pieces placed in a cell culture dish containing growth media.

Over time, progenitor cells migrate out of the tissue onto the surface of the dish. These primary cells can then be further expanded and transferred into fresh dishes (Orth *et al*., 2000). Nodal and mature tip shoot explants were used for rapid propagation of yam (Chaturvedi *et al*., 1982). *In vitro* production of microtubers has been attracted for plant propagation in yam because of its easy handling especially in international germplasm exchange.

The use of nodal segments as initial explants for the *in vitro* propagation of different Dioscorea species has been reported as in *D. floribunda* (Lakshmisita *et al*., 1976; Sinha and Chaturvedi, 1979), *D. bulbifera* (Forsyth and van Staden, 1982), *D. composita* (Datta *et al*., 1982), *D. oppositifolia* and *D. pentaphylla* (Poornima and Ravishankar, 2007) and in *D. alata* and *D. rotundata* (Mantell *et al*., 1978; Nair and Chandrababu, 1996).

## 2.7.3. Basal media and plant growth regulators

Plant tissue culture media should generally contain some or all of the following components: macronutrients, micronutrients, vitamins, amino acids or nitrogen supplements, carbon sources, undefined organic supplements, growth regulators and solidifying agents. According to the International Association for Plant Physiology, the elements in concentrations greater than 0.5 mM/l are defined as macroelements and those required in concentrations less than 0.5 mM/l as microelements (de Fossard, 1976).

It should be considered that the optimum concentration of each nutrient for achieving maximum growth rates varies among species. In order to assess the potential of the cultures to produce leaves and micro plants, the basal MS must be supplemented with different concentrations of growth regulators and sucrose (Tariqul Islam1 *et al*., 2008). The type and the concentration of hormones used depend mainly on the species of the plant, the tissue or organ cultured and the objective of the experiment (Ting, 1982).

Plant growth regulators are important in plant tissue culture since they play vital roles in stem elongation, tropism, and apical dominance. Moreover, proportion of auxins to cytokinins determines the type and extent of organogenesis in plant cell cultures (Skoog and Miller, 1957). The high concentration of auxins generally favors root formation, whereas the high concentration of cytokinins promotes shoot regeneration. A balance of both auxin and cytokinin leads to the development of mass of undifferentiated cells known as callus (Ting, 1982).

*D. alata L.*(Yam) clone were cultivated in a culture medium containing inorganic salts and vitamins as proposed by Murashige and Skoog (1962) (MS). Cistein (20 g/l), sucrose (30 g/l) and agar at a final concentration of 8 g /l were added to culture medium. Nodal segments showing axillary buds were obtained from *in vitro* plants in the third subculture. The culture were under a temperature of 25 ± 2°C and artificial illumination by means of white fluorescent lamps that provided an intensity of 60 µ mol/ $m^2.s^1$. The photoperiod corresponded to 16 h of light and 8 h of darkness (jova *et al*., 2011).

## 2.8 Stages of micropropagation

### 2.8.1 Stage 0: Preparation of donor plant

Any plant tissue can be introduced *in vitro*. To enhance the probability of success, the mother plant should be *ex vitro* cultivated under optimal conditions to minimize contamination in the *in vitro* culture (Cassells, 2005). It is possible to seed *in vitro* culturing that, seed development, dormancy and germination are controlled by specific endogenous growth promoting and inhibiting compounds (Hartman *et al*., 1997), and there is a correlation of hormone concentration with specific developmental stages, effects of applied hormones and the relationship of hormones to metabolic activities ( Pedroza-manrique *et al*., 2005).

### 2.8.2 Stage I: Initiation stage

In this stage an explant is surface sterilized and transferred into nutrient medium. Generally, the combined application of bactericide and fungicide products is suggested. The selection of products depends on the type of explants to be introduced.

The surface sterilization of explants in chemical solutions is an important step to remove contaminants with minimal damage to plant cells (Husain and Anis, 2009). Many factors are known to influence the proliferation response *in vitro.* These may include the presence or absence of growth regulators either singly or in combination, type of explants, and genotype (Staba, 1982; Ondo *et al*., 2007).

Jova *et al*., 2005, investigated the effect of temporary immersion system on formation of micro tuber in *D. alata.* They reported best shoot proliferation was observed in MS medium + 2mg/l kinetin + 1.0 mg/l BAP + 0.5 mg/l NAA + 100 mg/l ascorbic acid where 90% explants showed proliferation. The individual effects of sucrose, plant growth regulators and basal salt medium formulations on microtuber induction and development were investigated (Alizadeh *et al.,* 1998) and reported BA at 1.25 mg/l and 2.5 mg/l strong inhibitory effects on microtuber induction while promotive effect was shown by NAA and IBA at 5.0 mg/l.

Primary callus was induced by (Shu *et al*., 2005) culturing stems, leaves, petioles on MS medium supplemented with 0.5-2.0mg/l BA + 0-2.0mg/l NAA and best callus formation was observed in medium with 0.5mg/l BA + 2.0mg/l 2,4-D from stem explants.

Cytokinin and Auxin are known to promote callus formation in tissue culture (Skoog and Armstrong, 1970). The addition of optimum amount of cytokinin however, might reduce shoot induction rate by inhibiting the availability of the required endogenous amount of auxin for shoot initiation, and rather callus formation is promoted (Akisoshi *et al.,* 1983*).*

### 2.8.3 Stage II: Multiplication stage

The aim of this phase is to increase the number of propagules through subcultures until the desired (or planned) number of plants is attained (Saini and Jaiwal, 2002). Kadota and Niimi (2004), reported that liquid medium was superior to solid medium in terms of shoot proliferation, 6.9 number of node produced in liquid medium where 2.1 node produced in solid medium in *D. japonica.*

6-Benzylaminopurine at 0.44 mg/l produced highest number of nodes (Ondo *et al*., 2007) In another way, some studies have reported that addition of 2.0 mg/l KIN to culture media reduces multiplication rate (node number) of certain *D. cayensis* clones (Ondo *et al*., 2010).

According to Adesoye *et al*., (2012) shoot tip and node explants were cultured on MS supplemented with three concentrations of BAP and kinetin. Kinetin induced single shoots in both explants type whereas BAP induced multiple shoots. The number of shoots and the percentage shoot induction increased with the BAP concentration (Abraham, 2009). Kinetin was only slightly effective in shoot proliferation and 2iP was ineffective. The use of 2iP has increased shoot proliferation in some species but has resulted in no increase or poor growth in other species ( Demeke and Hughes, 1990).

*D. wightii* was propagated using nodal segment as explants, BA and kinetin was used for the multiplication of nodal segment. Callus initiation was observed in MS medium supplemented with 0.15-1.75mg/l BA, 0.75-5.0mg/l kinetin, 0.15-0.30 mg/l 2iP and shoot formation was observed in all growth regulators tested in BA, Kinetin and 2iP (Mahesh *et al*., 2010).

Poornima and Ravishankar (2007) used nodal segments to propagate *D. Oppositifolia* and *D pentaphylla* and reported multiple shoots produced on MS medium with 8.8 mg/l BAP and 0.3% activated charcoal. *In vitro* regeneration and multiplication of *D. alata* was studied by Borges *et al*., (2004) that high rates (100%) of explants regeneration was observed in MS medium with 1.5% manitol, 1mg/l BAP and 2g/l activated charcoal. An improved method of *in vitro* propagation of *D. bulbifera* from nodal segment on MS medium reported that the increasing concentration of kinetin increases shoot formation per node (Forsyth and Staden, 1982).

### 2.8.4 Stage III: Rooting stage

In tissue cultures auxins have been used for cell division and root differentiation. The auxins commonly used in root induction are: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA).

Of these, IBA and IAA are widely used for rooting and, in interaction with a cytokinin, for shoot proliferation (Abera *et al*., 2010). Ovono *et al.*, (2009) reported that presence of kinetin (2mg/L) reduce the shoot length, root length, node numbers but root length was increase when

sucrose concentration increased 3%-5% in case of *D. cayenensis -D. rotundata* complex. Root initiation from *in vitro* regenerated shoots obtained from nodal explants studies revealed that half of MS medium supplemented with IAA and 2% of sucrose; regeneration of roots from 100% inoculated shoot explants was observed (Morwal, 2000).

Demeke *et al.* (1992) reported that the auxin such as IAA, IBA and NAA, enhanced root production of *in vitro* proliferated shoot tips. A high rooting percentage was obtained in most of the treatments with the exception of the control and IAA at 0.57 gm/l however, callus was evident in most of the treatments. Indole -3- Butyric acid at 0.49 gm/l showed a high rooting percentage with minimal callus. In most of the treatments there was a direct association between high rooting capacity and high callus production. At low auxin levels, thin and long roots were observed. Increasing the auxin concentration resulted in the production of greater amounts of callus and thick and short roots. Omer *et al*., (1995) reported a similar observation. NAA and IBA to induce rooting from *in vitro* raised shootlets of *D.hispida*, and they observe highest rooting on half strength MS basal medium + 2mg/l NAA+ 2g/l Ac and 2mg/L IBA + 2g/l Ac in half strength MS basal medium induce second highest rooting (Behera *et al.*,2008).

For rooting *in vitro* micro shootlets of *D.oppositifolia* inoculated on half MS medium supplemented with 2mg/l NAA and profuse rooting was observed on this medium (Behera *et al*., 2009). 2mg/l NAA in combination with 0.2 and 0.5 mg/l BA produced root in *D. esculenta* (Belarmini and Rosario, 1991). Poornima and Ravishankar (2005) reported that efficient rooting was observed on MS medium +0.5mg/l NAA after 30 days. Sucrose concentration when raised 3% to 8% an increase in root number was observed and when sucrose concentration increased 3% to 5% root length also increased (Ondo *et al*., 2007).

Rooting frequency was higher in the solid medium but number of roots produced by each shoots was greater in liquid medium and the roots produced in gellan gum medium was longest (Kadota and Niimi, 2004). In hormone free medium within 10 days all the shoots produced root, when medium was supplemented with 4.9mg/l IBA induced fastest rooting with higher number of roots per plant was observed (Chen, 2003).

Rooting was observed in MS medium with 2.67 mg/l IBA and developed tuber on MS medium with 8.8mg/l BAP. Plantlets of *D. Oppositifolia* where nodal segment was used as explants and cultured on MS medium supplemented with BAP and NAA (Behera *et al*., 2009). Plantlets

regenerated on solid MS medium with 0.2mg /l BAP and half MS medium with 0.5 mg/l NAA favoured root formation in regenerated shoots in *D. Zingiberensis* (Heping *et al*., 2008).

### 2.8.5 Stage IV: Acclimatization Stage

Under greenhouse condition, plantlets are acclimatized or hardened before being transferred to the field to develop adequate root systems and leaf structure to withstand field environment. This is achieved through a gradual decrease in relative humidity and increase in light. The hardening chamber needs high illumination (4,000-10,000 lux), high humidity (90-100%) through misting systems as well as cooling and heating systems (Feyissa *et al*., 2005; Abraham, 2009). They were watered twice a week for 2 weeks, then once a week until plant maturity. Harvesting was performed 6 weeks after transfer to soil. The size and the number of mini tubers per plant were recorded (Bazabakana *et al*., 1999).

Micropropagated plants of *D. japonica* were transferred to pots containing 1:1vermiculite and soil (v/v) mixture under green house condition about 80% of the plants survived (Kadota and Niimi, 2003). Micropropagated plants were transferred to the pots containing mixture of soil + sand + manure in 1:1:1 ratio and 90% plants survived (Behera, 2009). Rooted plantlets were transferred to the pots containing sand, compost and mould mixture (1:1:2), after 8 month acclimatized plants produced tuber (Bazabakana, 1999). Rooted plantlets were transferred to soil rite (equal proportion of decomposed coir and peat moss) for acclimatization (Jova, 2005).

# CHAPTER THREE

## 3. MATERIALS AND METHODS

### 3.1. Plant materials

The plant materials of *D. alata* were collected from Awassa Agricultural Research Center and the tubers were grown under greenhouse condition at average temperature of $25^0$c $\pm$ $2^0$c. The tubers were planted on sterilized soil that had a mixture of coffee husk, sand and red soil at a ratio of 2:2:1 respectively (Figure 1.) Very young, healthy and vigorous plants were used as a source of explants. Whole experiment was conducted at Jimma University, Natural Science College in the laboratory of plant tissue culture.


Figure 1: Mother plants in green house condition at average temperature of $25^0$c $\pm 2^0$c and light intensity of 4,000-10,000 lux.

## 3.2 Growth regulators stock preparation

All the plant growth regulators were prepared by weighing 100 mg powder and dissolved in 3-5 drops of 1N NaOH and 1N HCl for auxins and cytokinins, respectively. Then, the final volume was adjusted to 100 ml stock by adding sterilized distilled water. All the stock solutions were stored in refrigerator at +4$^{o}$C temperature to be used for a maximum of one month period.

## 3.3 Culture medium preparation

Sterilized distilled water was used for the preparation of culture medium. MS powder (*Murashige and Skoog medium*, 4405.19g/l conc.) was weighed for one litter, sucrose and growth regulators were added to the double distilled water kept in a vessel. The final volume was adjusted in a graduated cylinder/beaker by adding double distilled water. The pH of the solution was adjusted to 5.7-5.8 using 0.1 N HCl or 1N KOH. For solidification of the medium, agar powder (Tissue culture grade; agar-agar type) 0.8% (w/v) was added to the luke warm solution and then, melted for proper dissolving. Then the medium was poured in glass vessels borosilicate test tubes. After plugging, the test tubes were covered with aluminum foil and autoclaved.

## 3.4 Tissue Culture Experiments

### 3.4.1. Plant material collection and sterilization

Shoot tip and nodal segment explants (1.5-2.0cm) were obtained from healthy and vigorously growing tuber sprout. The excised explants were washed three to five times with tap water. Kept in fungicide (0.3% Mancozeb) solution for 25 minutes and transferred to the laminar flow hood cabinet and surface sterilized in 70% (v/v) Ethanol for 30 seconds, then the treated explants were rinsed repeatedly four times with sterilized distilled water and surface sterilized further using NaOCl (5% ) disinfectant solutions for the specified time, as per the treatment combinations. The sterilized shoot and nodal explants were further cut down to 10 to 15 mm size by removing all the dead and chlorine affected tissue prior to culture.

### 3.4.2 Culture initiation

Shoot tip and nodal segment explants was inoculated in magenta jar or large test tubes containing full MS (Murashige and Skoog, 1962) media supplemented with BAP (0.5, 1.0, 1.5 and 2.0, mg/l) and NAA (1, 1.5, 2.0, and 2.5 gm/l). The combination of each concentration with 3% sucrose (30 gm/l), and Agar agar (8 gm/l) one explants per jar was cultured with 5 replicates per treatment. The cultures were maintained at $25 \pm 2^0$C with a 16 hour photoperiod at a light intensity of 2500-3000 lux from cool white florescent 60 watt bulbs. There were 4x4X2x2 factorial combinations in Completely Randomized Design (CRD) where hormone free media was used as control.

### 3.4.3. Shoot Multiplication

All healthy initiated shoots was transferred to shoot multiplication MS fresh media supplemented with a treatment combination of three different concentrations of KIN (1, 2.0 and 3.0 mg/l) and three different concentrations of BAP (0.5, 1.0, and 1.5mg/L) alone and or in combination with 0.5 mg/l NAA at each concentration were used and four replicates with best proliferated shoots each was cultured in each medium. Therefore, the experiment was 3x3x2x2 factorial combinations in CRD and treatment with free PGRs.

### 3.4.4. Rooting

The regenerated shoots was cultured on half-strength MS media supplemented with NAA (0.5, 1, 1.5, 2.0 and 2.5 mg/l) and IBA (0.5, 1, 1.5, 2.0 and 2.5 mg/l) for root induction. Well developed micro shoots with minimum length of 1.10 cm obtained from experiment three were transferred to experiment four for rooting. For rooting experiment, agar solidified (0.8% agar-agar) half strength MS basal medium added with 3% sucrose was supplemented with different concentrations of IBA and NAA.

### 3.4.5. Acclimatization

Rooted plantlets were removed from the medium. The roots were carefully washed with running tap water and warm water. The plantlets were then planted into pots (4.5 cm x 3.5 cm) filled with a mixture of coffee husk, sandy and red soil in 2:2:1 proportions respectively and acclimatized for 6 weeks under natural diffuse sunlight and 70% humidity in a glasshouse.

They were then treated in light polyethylene tunnel covered by 70 % shade net above it and without shade net. The system was designed to give high humidity (80 - 90 %) to prevent desiccation for ten days, prior to their transfer to a shade house. In the tunnel, the water was sprayed daily without creating water logging.

## 3.5 Experimental design and Treatments

All experiments were laid in a Completely Randomized Design (CRD) with factorial treatment combinations, having three replications per treatment and five explants per jar under each replicate. All the experiments were repeated two times to ensure reproducibility of the results and the average of these two were considered for analysis. Prior to laying the multiplication and rooting experiments, sufficient explants were made to multiply till the desired numbers of explants are gained. At all times, explants were cultured on a PGR-free medium prior to their use for an experiment; so as to avoid any sort of carryover effects from the previous culture medium they were retained. Controls were set for each experiment with zero concentration of the analyte considered.

## 3.6 Data Recording and Data Analysis

After sterilization experiment, the number and percentage of explants affected by contamination and tissue death was recorded during the first two weeks of culture for shoot tip and nodal explants independently. For the second experiment, the number and/or percentage of explants forming shoot buds was recorded after four weeks for shoot tip and nodal explants independently. Number of shoots proliferated from each shoot bud on multiplication media was counted at three weeks interval during sub culturing. The number of roots (including the main roots and their branches), shoot length and the length of the roots was recorded after three weeks of culture for experiment four.

Average of the data collected from the two repetitions for each experiment were independently subjected to statistical analysis using the SAS statistical software (version 9.2) and ANOVA was constructed, followed by mean separation using appropriate procedures (REGWQ).

# CHAPTER FOUR

## 4. RESULTS AND DISCUSSION

Results obtained from experiments on micropropagation of *D. alata (Yam)* shoot tip and nodal explants namely; surface sterilization, shoot bud induction, shoot multiplication, and rooting of micro shoots and acclimatization of plantlets are presented and discussed in the following sections.

### 4.1 Effects of NaOCl concentrations and exposure time on sterilization of *D. alata* explants

The effect of different concentrations of sodium hypochlorite solutions and different time durations of explants exposure to the sterilants were evaluated for determining the most effective treatment combination on sterilization of shoot tip and nodal explants. The analysis of variance showed that the concentration of sodium hypochlorite solution, time duration of explants exposure to the sterilants and interaction of concentrations to time duration had highly significant effect ($p < 0.0001$) on both of contamination and tissue death of shoot tips and nodal explants. The highly significant difference was also revealed between the two types of explants (treatment: *explants:- = $p < 0.0001$) indicating that the level of contamination and tissue death was influenced by explants type and the mean average value for contamination and tissue death of shoot tips exceeded that of node explants.

The highest rate of contaminant free culture (76.60±0.36% and 72.66±0.85%) was obtained from treatment combinations of 3 and 2 % concentration of NaOCl and 5 and 20 minute exposure duration, respectively for shoot tip explants. For nodal explants 3 % concentration and 20 minute exposure duration were found to be the most effective treatment combination with mean average result of 72.00±0.20% contaminant free cultures (Table 1). Increasing the sterilant concentration from 1.0 to 3.0% active chlorine maintaining 20 minute exposure duration constant had reduced the rate of contamination from 88.00 ± 1.00% to zero and from 90.00 ± 0.50% to zero for shoot tips and nodal explants, respectively.

This effect was mainly attributed to the high level of tissue death i.e., 77.40 ± 0.45% for shoot tips and 70.00 ± 1.00% for nodal explants caused by the maximum concentration of the sterilant solution.

Exposure duration of explants to the sterilant chemical also had significantly affected the effectiveness of the chemical in that it increased in time from 10 to 20 minute at 3 % constant concentration had decreased the percentage of clean lively culture from 36.50 ± 0.50 to 22.60 ± 0.60 and from 50.00 ± 0.30 to 30.00 ± 0.50 for shoot tip and nodal culture, respectively increasing the rate of tissue death (Table 1). The rate of tissue death severely affected shoot tip explants than nodal explants as shoot tip tissues are relatively young and more susceptible to the chemical action.

**Table1: Interaction effect of Sodium hypochlorite concentrations and its time of exposure on sterilization of Shoot tip and Nodal explants of *D. alata.***

| Con. (%) | Time(min) | % of Contamination | | % of Clean culture | | % of Tissue Death | |
|---|---|---|---|---|---|---|---|
| | | Shoot tip (Mean±Std) | Nodal (Mean±Std) | Shoot tip (Mean±Std) | Nodal (Mean±Std) | Shoot tip (Mean±Std) | Nodal (Mean±Std) |
| 0 | 0 | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ | 0.00±0.00$^{k}$ | 0.00±0.00$^{o}$ | 0.00±0.00$^{k}$ | 0.00±0.00$^{k}$ |
| 1 | 5 | 88.00±1.00$^{b}$ | 90.00±0.50$^{b}$ | 12.00±0.50$^{j}$ | 10.00±0.20$^{n}$ | 0.00±0.00$^{k}$ | 0.00±0.00$^{k}$ |
| 1 | 10 | 84.60±0.28$^{c}$ | 87.00±0.50$^{c}$ | 13.13±0.80$^{j}$ | 11.50±0.50$^{m}$ | 0.66±0.04$^{j}$ | 1.50±0.10$^{j}$ |
| 1 | 15 | 81.66±0.28$^{d}$ | 84.00±0.50$^{d}$ | 15.90±0.10$^{i}$ | 13.00±0.34$^{m}$ | 0.83±0.07$^{j}$ | 3.00±0.20$^{i}$ |
| 1 | 20 | 78.40±0.52$^{e}$ | 81.00±0.50$^{e}$ | 18.00±0.50$^{h}$ | 15.00±0.20$^{l}$ | 1.20±0.15$^{i}$ | 4.00±0.20$^{i}$ |
| 1.5 | 5 | 73.33±0.06$^{f}$ | 78.00±0.50$^{f}$ | 21.70±0.88$^{g}$ | 16.50±0.50$^{k}$ | 1.66±0.15$^{i}$ | 5.50±0.50$^{h}$ |
| 1.5 | 10 | 71.00±1.32$^{g}$ | 74.60±0.40$^{g}$ | 22.50±1.00$^{f}$ | 18.00±0.20$^{j}$ | 2.16±0.15$^{h}$ | 7.40±0.40$^{g}$ |
| 1.5 | 15 | 68.00±0.50$^{h}$ | 72.00±0.50$^{h}$ | 24.00±0.50$^{f}$ | 19.50±0.50$^{i}$ | 2.66±0.15$^{h}$ | 8.50±0.50$^{g}$ |
| 1.5 | 20 | 65.00±0.50$^{i}$ | 69.00±0.20$^{i}$ | 25.50±0.70$^{e}$ | 21.00±0.50$^{i}$ | 3.16±0.15$^{g}$ | 10.00±1.00$^{f}$ |
| 2 | 5 | 60.50±1.00$^{j}$ | 65.83±0.72$^{j}$ | 27.50±0.43$^{d}$ | 23.00±1.00$^{h}$ | 4.00±0.20$^{f}$ | 11.00±0.50$^{f}$ |
| 2 | 10 | 58.00±0.50$^{k}$ | 62.96±0.30$^{k}$ | 28.50±0.50$^{d}$ | 27.50±0.50$^{g}$ | 4.50±0.10$^{e}$ | 12.50±0.50$^{e}$ |
| 2 | 15 | 48.00±0.50$^{l}$ | 59.66±1.25$^{l}$ | 35.33±1.60$^{c}$ | 46.00±1.00$^{d}$ | 5.33±0.15$^{d}$ | 14.00±0.50$^{d}$ |
| 2 | 20 | 12.00±0.50$^{m}$ | 57.00±0.50$^{m}$ | 72.66±0.85$^{b}$ | 72.00±0.20$^{a}$ | 5.00±0.26$^{d}$ | 12.00±0.50$^{e}$ |
| 3 | 5 | 9.50±0.50$^{n}$ | 17.00±0.20$^{n}$ | 76.60±0.36$^{a}$ | 70.00±1.00$^{b}$ | 4.63±0.15$^{e}$ | 11.00±0.50$^{f}$ |
| 3 | 10 | 8.50±0.50$^{n}$ | 11.00±0.50$^{o}$ | 36.50±0.50$^{c}$ | 50.00±0.30$^{c}$ | 55.00±0.50$^{c}$ | 43.00±0.50$^{c}$ |
| 3 | 15 | 6.50±0.50$^{o}$ | 7.66±0.20$^{p}$ | 25.20±0.40$^{e}$ | 35.00±1.00$^{e}$ | 68.30±1.12$^{b}$ | 57.00±0.50$^{b}$ |
| 3 | 20 | 0.00±0.00$^{p}$ | 0.00±0.00$^{q}$ | 22.60±0.60$^{f}$ | 30.00±0.50$^{f}$ | 77.40±0.45$^{a}$ | 70.00±1.00$^{a}$ |
| CV | | 1.15 | 0.85 | 2.49 | 2.08 | 2.47 | 3.23 |

*Means with the same letters in a column are not significantly different from each other by Ryan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ) at α= 5 % .*

Generally, occurrence of high contamination rate of culture at a relatively lower concentration and shorter exposure time treatment combinations was possibly due to the insufficiency of sterilant concentration and exposure duration to remove or kill the contaminant agents.

High concentration of NaOCl and long exposure duration of explants in the sterilant solution resulted better removal of microbes due to the powerful oxidant property of active chlorine that disintegrates the lipids in the cell wall of bacteria and fungi.

The effect of the sterilant chemical could also alter or denature the shape and function of microbial enzymes (George *et al*., 2008). However, the increase in sterilant concentration and exposure time above certain optimum limit cause loss of explants because of the oxidant chemical ingredient killing the plant tissue as well. Hence, the optimum treatment combination (concentration and time) for effective sterilization of explants should be determined based on the two aspects of the observations, i.e. a relatively minimum level of contamination as well as tissue death that gives the maximum percentage of clean lively culture as indicated in Table 1.

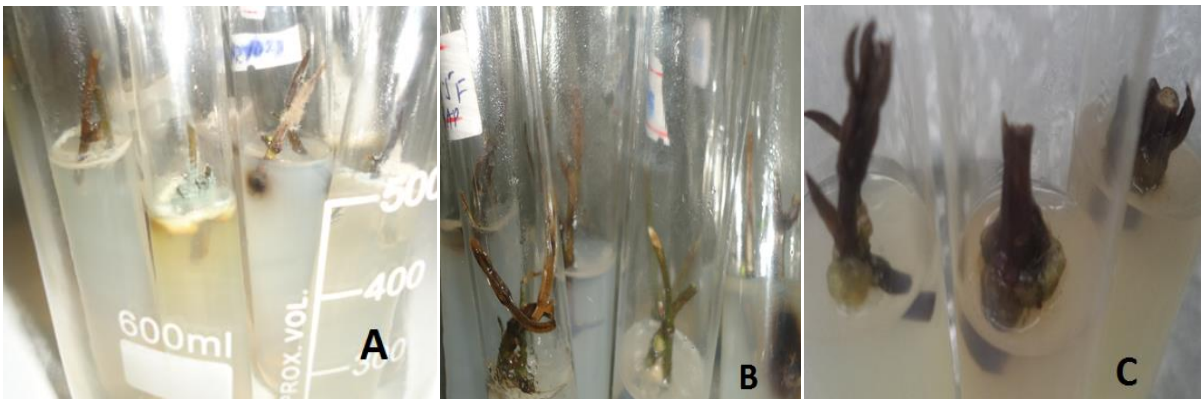


**Figure 2:** Response of cultures to different concentrations of NaOCl, (A) 1% sodium hypochlorite concentration and 5 minute exposure time (B) 3% NaOCl and 10 minute exposure time and above results for tissue death (C) 3% NaOCl and 5 minute exposure time

According to Tariqul Islaml *et al*., (2008), effective chlorine concentration with 3% and Tween 20 (2 - 3 drops) for 15 min minimizes the level of contamination from nodal explants. This is in agreement with present result of 3 % active chlorine in NaOCl solution for 5 minute. Similarly, the nodal *D. alata* segments disinfected with 0.1% mercuric chloride (HgCl2) for 5 minute as effective treatment to remove microbes from shoot tip and nodal explants on initiation of aseptic culture *in vitro* (Supriya, 2013). The use of mercuric chloride, however, is highly discouraged as such chemicals have been known to have a serious harmful residual

effect to both human and the environment. Thus, the trend is to try for the substitute with a relatively safe or less harmful chemical such as NaOCl solution as employed in this study.

## 4.2 Effect of different concentrations and combinations of BAP and NAA on shoot initiation from nodal and shoot tip explants of *D. alata*

Aseptic shoot tips and nodal cultures were transferred on MS media fortified with different concentrations of BAP in combination with NAA for 3 weeks to determine optimum medium for shoot induction of *D. alata.* The interaction between BAP and type of explants had very highly significant effect ($P < 0.0001$) on the shoot induction rate. Interaction effect of explants type with BAP and NAA on rate of shoot induction was found to be highly significant (BAP*NAA* Explant).

The response of shoot tip and nodal explants to a given concentration of BAP was not the same; the nodal explants gave greater response than shoot tip explants (Table 2). The nodal explants gave greater response than shoot tip explants (Table 2). Although it requires further investigation to be carried out, this might be related to the variation in endogenous level of auxin in shoot tip- and nodal sections of the given genotype (Hopkins and Huner, 2004).

The highest rate of shoot induction (93.20±1.07%) was obtained on MS medium supplemented with 1.5 mg/l of BAP and 1.0 mg/l of NAA froms nodal explants while shoot tip (87.25±0.53%) on MS containing 1.0 mg/l of BAP and 1.0mg/l of NAA (Table 2). MS supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA for shoot tip and 1.5 mg/l BAP with 1.0 NAA for nodal initiation were found to be the optimum media for *in vitro* shoot initiation of *D. alata* (Table 2).

From all treatments the minimum rate of shoot induction was observed on MS containing 0.5 mg/l of BAP and 2.5 mg/l of NAA (8.12±0.39% and 7.90±0.14%) for shoot tip- and nodal explants. Shoot bud development capacity of both shoot tip- and nodal cultures increased with the increase in concentration of NAA from zero to 2 mg/l and reduced with further addition of NAA Table 2).

**Table 2 Effects of BAP and NAA on *in vitro* shoot induction rate of *D. alata* from shoot tip and nodal explants culture on MS.**

| CONC. OF PGRS | | Explants | |
|---|---|---|---|
| BAP(mg/l) | NAA(mg/l) | Shoot tip | Nodal |
| 0 | 0 | 6.55±0.99$^{q}$ | 43.67±0.63$^{h}$ |
| 0.5 | 1 | 25.95±0.85$^{n}$ | 41.25±0.90$^{i}$ |
| 0.5 | 1.5 | 33.65±0.47$^{k}$ | 54.75±1.07$^{e}$ |
| 0.5 | 2 | 43.67±0.63$^{h}$ | 62.75±0.82$^{d}$ |
| 0.5 | 2.5 | 8.12±0.39$^{p}$ | 7.90±0.14$^{p}$ |
| 1 | 1 | 87.12±0.62$^{a}$ | 64.00±0.81$^{c}$ |
| 1 | 1.5 | 82.25±0.53$^{b}$ | 72.57±0.84$^{b}$ |
| 1 | 2 | 27.40±0.43$^{m}$ | 50.70±0.52$^{f}$ |
| 1 | 2.5 | 20.60±0.48$^{o}$ | 36.82±0.23$^{j}$ |
| 1.5 | 1 | 77.25±0.53$^{c}$ | 93.20±1.07$^{a}$ |
| 1.5 | 1.5 | 30.60±0.48$^{l}$ | 30.62±0.68$^{l}$ |
| 1.5 | 2 | 55.02±0.68$^{g}$ | 33.65±0.47$^{k}$ |
| 1.5 | 2.5 | 37.12±0.62$^{j}$ | 27.15±0.75$^{m}$ |
| 2 | 1 | 67.32±0.53$^{e}$ | 43.07±0.83$^{i}$ |
| 2 | 1.5 | 64.25±0.52$^{f}$ | 20.57±0.46$^{o}$ |
| 2 | 2 | 73.47±0.49$^{d}$ | 25.95±0.85$^{n}$ |
| 2 | 2.5 | 41.10± 0.84$^{i}$ | 45.92±0.09$^{g}$ |
| **CV** | | 1.98 | 1.78 |

Means within a column followed by the same letters are not statically significant at $p< 0.01$ by Ryan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ).

The addition of optimum amount of auxin, however, together with relatively less amount of cytokinin might reduce shoot induction rate by inhibiting the availability of the required endogenous amount of auxin for shoot initiation as cytokinins do not act alone unless combined with auxins (George *et al*., 2008).

The combination of 0.5mg/l BAP and 2.5mg/l of NAA gave no or very low response of shoot bud induction rather than callus formation while 1.0mg/l BAP with 1.0mg/l NAA produced the highest shoot induction (Table 2). This recalls the knowledge that, it is the ratio of auxin to cytokinin, not the absolute level of cytokinin that initiates shoot bud growth (Hartmann *et al*., 2009).

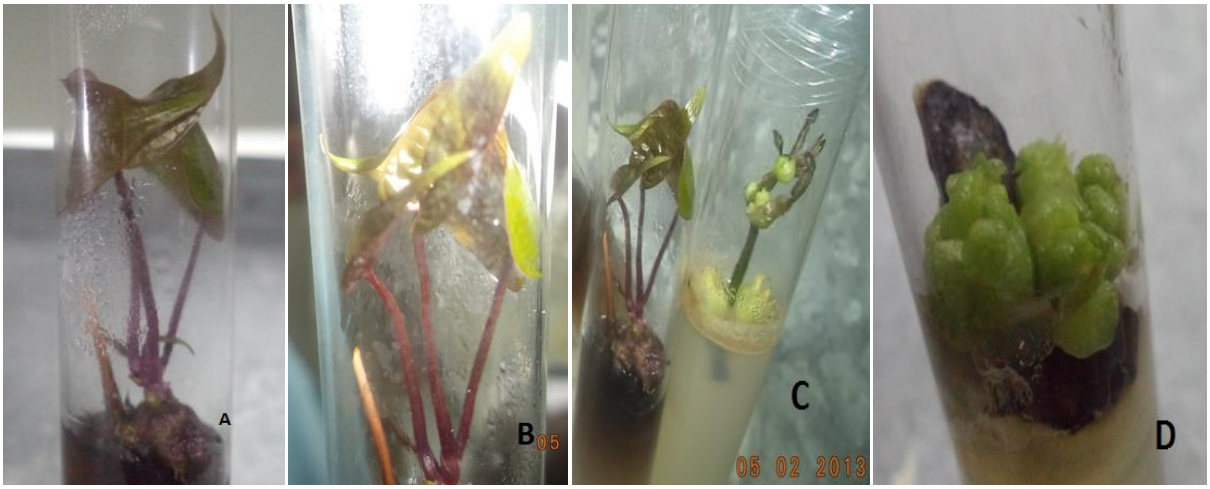


**Figure 3**: Effect of BAP and NAA on Shoot induction, (A and B) Shoot induction on 1.5 mg/L of BAP and 1.0 mg/L NAA (c) shoot induced on 1.0mg/ L BAP and 1.0 mg/L NAA *(*D) callus formation at the base of explant

The present result of shoot induction rate is in agreement with the report of Fotso *et al*., (2013) who considered 0.5 mg/l BAP with 1.0 mg/l NAA as the best supplemented medium for *D. alata* for nodal cuttings derived shoot regeneration.

## 4.3 Effect of different concentrations and combinations of KIN, BAP and NAA on shoot multiplication of *D. alata*

The ANOVA revealed that the concentration of KIN and BAP both alone and in combination with NAA had a highly significant effect ($p < 0.0001$) on shoot multiplication rate. Shoot buds raised from both shoot tip- and nodal explants responded almost similarly on shoot multiplication indicating the non - significant effect of explants at this stage. The proliferation rate had shown a progressive increase from the first sub - culture to the second by an average of 16.6%.

In this study, the best result (number) of shoot proliferation from nodal (9.50±0.29) was obtained on MS containing 2.0 mg/l of KIN, 0.5 mg/l of BAP and 0.5 mg/l of NAA (Table 3). Nevertheless, the micro shoots from both shoot tip and nodal in this medium were less vigorous and bushy as compared to MS prepared with 2.0 mg/l of KIN +1.5mg/l of BAP + 0.5mg/l of NAA (Figure 1) resulting in the production of 7.00±0.29 shoots (Table 3).

**Table 3 Effect of different concentrations and combinations of KIN, BAP and NAA treatments on shoot multiplication of Yam *(D. alata)***

| Level of PGRs (mg/l) | | | Shoot tip | | Nodal | |
|---|---|---|---|---|---|---|
| | | | Shoot number | Shoot length | Shoot number | Shoot length |
| KIN | BAP | NAA | Mean ±Std Dev | Mean ± Std Dev | Mean ± Std Dev | Mean ± Std Dev |
| 1 | 0.5 | 0 | 2.00±0.18$^{l}$ | 1.60±0.18$^{fg}$ | 2.0±0.18$^{ij}$ | 1.30±0.14$^{ij}$ |
| 1 | 1 | 0 | 2.50±0.08$^{k}$ | 2.00±0.18$^{f}$ | 1.50±0.08$^{kl}$ | 2.05±0.12$^{h}$ |
| 1 | 1.5 | 0 | 2.70±0.14$^{kji}$ | 2.70±0.14$^{e}$ | 2.70±0.14$^{gh}$ | 3.40±0.18$^{fe}$ |
| 2 | 0.5 | 0 | 3.00±0.08$^{hji}$ | 3.00±0.08$^{de}$ | 7.00±0.29$^{b}$ | 5.30±0.18$^{b}$ |
| 2 | 1 | 0 | 3.20±0.08$^{gh}$ | 5.00±0.14$^{a}$ | 3.20±0.08$^{f}$ | 3.80±0.18$^{e}$ |
| 2 | 1.5 | 0 | 3.50±0.35$^{g}$ | 3.30±0.08$^{cd}$ | 3.50±0.35$^{f}$ | 2.50±0.18$^{g}$ |
| 3 | 0.5 | 0 | 4.30±0.25$^{ef}$ | 1.60±0.14$^{fg}$ | 1.30±0.18$^{l}$ | 1.60±0.14$^{ij}$ |
| 3 | 1 | 0 | 3.00±0.18$^{hji}$ | 3.00±0.18$^{de}$ | 3.00±0.18$^{gf}$ | 3.00±0.18$^{f}$ |
| 3 | 1.5 | 0 | 3.15±0.26$^{hgi}$ | 3.15±0.26$^{cd}$ | 3.15±0.26$^{gf}$ | 1.20±0.18$^{j}$ |
| 1 | 0.5 | 0.5 | 3.50±0.11$^{g}$ | 3.50±0.11$^{c}$ | 3.50±0.11$^{f}$ | 3.00±0.11$^{f}$ |
| 1 | 1 | 0.5 | 4.50±0.21$^{e}$ | 4.50±0.21$^{b}$ | 4.50±0.21$^{d}$ | 4.30±0.21$^{d}$ |
| 1 | 1.5 | 0.5 | 5.00±0.29$^{d}$ | 5.10±0.43$^{a}$ | 5.00±0.29$^{c}$ | 4.75±0.42$^{c}$ |
| 2 | 0.5 | 0.5 | 6.40±0.18$^{c}$ | 4.57±0.09$^{ab}$ | 9.50±0.29$^{a}$ | 6.20±0.18$^{a}$ |
| 2 | 1 | 0.5 | 8.40±0.21$^{a}$ | 5.30±0.29$^{a}$ | 6.80±0.18$^{b}$ | 4.50±0.21$^{dc}$ |
| 2 | 1.5 | 0.5 | 7.00±0.29$^{b}$ | 3.50±0.18$^{c}$ | 2.40±0.18$^{ih}$ | 3.00±0.25$^{f}$ |
| 3 | 0.5 | 0.5 | 4.00±0.29$^{f}$ | 1.30±0.18$^{g}$ | 4.00±0.29$^{e}$ | 1.30±0.08$^{ij}$ |
| 3 | 1 | 0.5 | 2.60±0.08$^{kj}$ | 2.60±0.08$^{e}$ | 1.80±0.18$^{kj}$ | 2.50±0.18$^{g}$ |
| 3 | 1.5 | 0.5 | 2.05±0.20$^{l}$ | 1.80±0.18$^{f}$ | 1.50±0.08$^{kl}$ | 1.70±0.29$^{ih}$ |
| CV | | | 5.48 | 6.33 | 6.12 | 6.83 |

Percentage increases/decreases in shoot number, shoot height, root number and root height per explants is given by plus or minus (±). Means within a column followed by the same letters are not statically significant at $p< 0.01$ by Ryan –Einot - Gabriel - Welsch Multiple Range Test (REGWQ).

In MS containing 2.0 mg/l of KIN, 1.0 mg/l of BAP and 0.5mg/l of NAA 8.40±0.21 shoots from shoot tip with a good morphological appearance were observed (Figure 4). Shoot buds on MS prepared with 1.0 mg/l of KIN, 0.5 mg/l of BAP and 0.5mg/l of NAA have changed into callus while as the concentration of KIN or BAP increases at the same concentration of NAA had given better and good shoot proliferation of adventitious origin (Figure 4).

An increase in number of shoots per shoot bud culture with increased concentration of KIN and BAP from 0.5 up to 3 mg/l of KIN and 0.5 to 1.5mg/l of BAP respectively might be due to the effect of both KIN and BAP in releasing primordia of lateral buds from dormancy or breaking apical dominance by inhibiting the level of endogenous auxins. Cultures on a higher level of above 2.0 mg/l of KIN and 1.0 mg/l of BAP had developed into bushy and ill defined shoot buds and they did not respond when subcultured on the same medium.

The present results are in agreement with those of Behera *et al*., (2010) and Borges *et al*., (2009 ) who reported best proliferation rate of *D. alata* on MS + 2 mg/l KIN + 1.0 mg/l BAP + 0.5 mg/l NAA.

Similar results reported by Jova *et al*., (2005), that investigated the effect of temporary immersion system on formation of micro tuber in *D. alata.* They reported best shoot proliferation was observed in MS medium + 2mg/l kinetin + 1.0 mg/l BAP + 0.5 mg/l NAA + 100 mg/l ascorbic acid where 90% explants showed shoot multiplication.

On the other hand, Behera *et al*., (2008) reported that of the combinations tested BAP (2.0mg/l) + NAA (0.5 mg/l ) with ascorbic acid 100 mg/l, elicited optimal response in which an average of 6.0± 0.18 shoot lets with a mean shoot length of 5.0± 029 cm per explants were recorded on *Dioscorea hispida Dennst* species. In the present study among all the combinations and concentrations, the longest shoots 6.20±0.18 and 5.30±0.29 were observed on the medium containing 2.0 mg/l of KIN+ 1.0 mg/l of BAP + 0.5 mg/l of NAA and 2.0mg/l of KIN + 0.5mg/l of BAP + 0.5 mg/l of NAA for both nodal and shoot tip explants respectively.

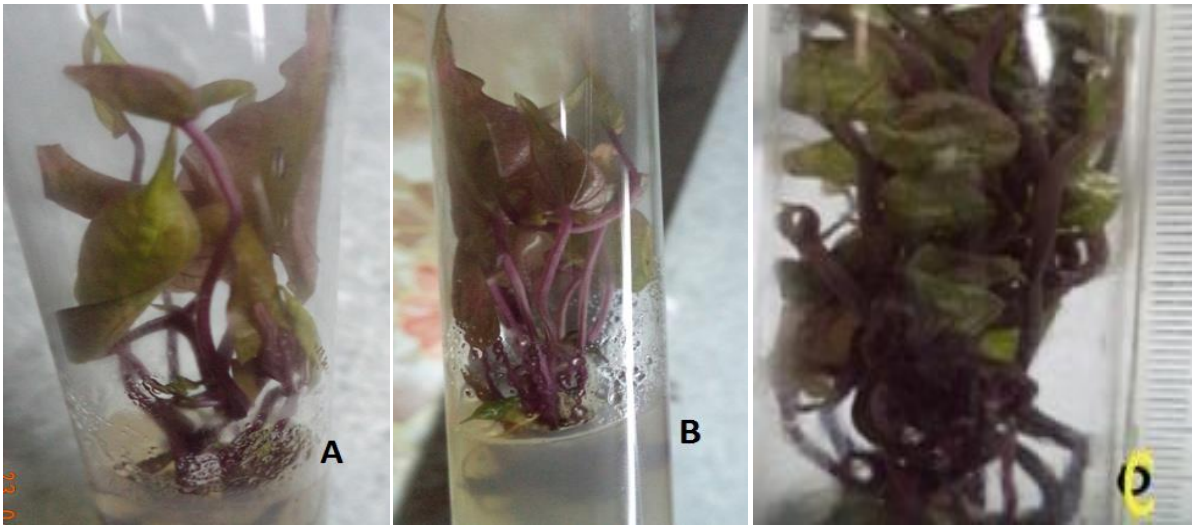


**Figure 4**: *In vitro* shoot multiplication of *D. alata*, (A and B ) Shoots multiplied on 2.0 mg of KIN 1.0 mg/L of BAP and 0.5 mg/L NAA; (C) Shoots multiplied on 2.0 mg/L of BAP and 0.5 mg/L BAP with longest shoot

## 4.4 Effect of different concentrations of IBA and NAA for in vitro root initiation of *D. alata*

Higher rooting percentage 90.70±0.99% on half - strength MS medium at 2.0 mg/L of IBA followed by 86.75±0.64% at 2.0mg/L of NAA with minimum callusing (Table 4). Among the given concentrations the above root inducing auxin hormones higher concentration resulted in less rooting percentages with greater diameter of callusing. Naphthalene acetic acid (NAA) at a concentration of 0.5 mg/l resulted in less percentage of rooting 48.62±0.85% that was less than the root induced from the same amount of IBA. Regenerated shoots with average height of 2-5cm cultures were sub cultured on a medium prepared for rooting purpose on half-strength MS medium of the above different auxins at different concentrations.

The longest shoots 5.00±0.18cm were obtained from a medium that contained 2.0 mg/L of NAA followed by and 4.30±0.08cm from 1.0 mg/l of IBA. Smallest shoot height 1.40±0.08cm were obtained from 1.0 mg/l of NAA. The highest mean number 8.30±0.21 of roots were obtained from 2.0 mg/l of NAA followed by 10.61±0.21 on 2.0 mg/l IBA. Highest concentrations of auxins resulted in less number of root. Relatively, less number of roots 1.20±0.18 was obtained from IBA at 2.5 mg/l (Table 4).

**Table 4: Effect of various concentrations of IBA and NAA on rooting of proliferated shoots of *D. alata* cultured on half - strength MS medium.**

| Conc. of PGRs (mg/l) | | Rooting Percent (Mean ± SD) | Shoot height (cm) (Mean ± SD) | Root number (Mean ± SD) | Root length(cm) (Mean ± SD) |
|---|---|---|---|---|---|
| NAA | IBA | | | | |
| 0 | 0 | $0.00\pm0.00^{l}$ | $1.20\pm0.08^{h}$ | $0.00\pm0.00^{i}$ | $0.00\pm0.00^{g}$ |
| 0 | 0.5 | $52.80\pm1.04^{g}$ | $1.50\pm0.08^{g}$ | $2.20\pm0.18^{g}$ | $2.00\pm0.18^{e}$ |
| 0 | 1 | $67.00\pm0.91^{e}$ | $4.30\pm0.08^{b}$ | $4.30\pm0.29^{e}$ | $1.20\pm0.16^{f}$ |
| 0 | 1.5 | $76.20\pm0.83^{c}$ | $2.00\pm0.21^{f}$ | $6.20\pm0.24^{c}$ | $2.40\pm0.18^{d}$ |
| 0 | 2 | $90.70\pm0.99^{a}$ | $3.40\pm0.11^{c}$ | $10.61\pm0.21^{b}$ | $4.00\pm0.24^{b}$ |
| 0 | 2.5 | $73.40\pm0.87^{d}$ | $2.50\pm0.08^{e}$ | $1.20\pm0.18^{h}$ | $1.10\pm0.21^{f}$ |
| 0.5 | 0 | $48.62\pm0.85^{j}$ | $3.00\pm0.18^{d}$ | $1.50\pm0.18^{h}$ | $1.25\pm0.05^{f}$ |
| 1 | 0 | $52.40\pm0.71^{h}$ | $1.40\pm0.08^{hg}$ | $3.50\pm0.18^{f}$ | $3.00\pm0.18^{c}$ |
| 1.5 | 0 | $63.10\pm0.73^{f}$ | $2.50\pm0.18^{e}$ | $4.05\pm0.23^{e}$ | $5.00\pm0.18^{a}$ |
| 2 | 0 | $86.75\pm0.64^{b}$ | $5.00\pm0.18^{a}$ | $8.30\pm0.21^{a}$ | $2.00\pm0.18^{e}$ |
| 2.5 | 0 | $47.70\pm0.72^{k}$ | $3.50\pm0.18^{c}$ | $5.00\pm0.21^{d}$ | $4.00\pm0.18^{b}$ |
| CV | | 1.34 | 5.13 | 5.25 | 7.41 |

Percentage increases/decrease in shoot height, root number and root height per explants is given by plus or minus (±). Means within a column followed by the same letters are not statically significant at **α=** 5 % by Ryan - Einot – Gabrie Welsch Multiple Range Test (REGWQ).

In this study, half - strength MS medium supplemented with NAA (0.5 1.0, 1.5, 2.0 and 2.5) and IBA (0.5 1.0, 1.5, 2.0 and 2.5) were evaluated and relatively 2.0 mg/L for both IBA and NAA were given good rooting percentage and the second highest percentage (76.2%) was at 1.5mg/l of IBA.

These results are in agreement with those reported by Behera *et al.*, (2010) concerning the better performance of half strength MS for *in vitro* rooting of *D. alata* in terms of rooting with the addition of 2.0 mg/l of either IBA or NAA . Also said, at lower level of NAA (0.5 mg/l) treatments, there was hardly any rooting in the cultured shoots during the 4 weeks of observation. However NAA at higher concentration (1.5 and 2.0 mg/l) and IBA (2.0 mg/l) was the best for inducing root.

Behera *et al.,* (2008) also noted the same result on different species of Dioscorea (shootlets of *D.hispida*) on 2mg/l of NAA and 2mg/l of IBA in half strength MS basal medium induce (80%) second highest rooting. For rooting Behera *et al*., (2009) *in vitro* micro shootlets of *D. oppositifolia* inoculated on half MS medium supplemented with 2mg/l of NAA and profuse rooting was observed on this medium.


Figure 5: Root induced on different auxins (A and B) rooting on half - strength medium with 2. 0 mg/L IBA (C and (D) rooting on half strength medium with 2. 0 mg/l NAA

## 4.5 Acclimatization *in vitro* derived plantlet

Production of plantlets with profuse rooting in *Invitro* is essential for successful establishment of regenerated plants in soil (Ohyma, 1970). Later, plants were transferred to earthen pots containing mixture of different types of soil and organic compounds for explants development.

The establishment of *in vitro* plantlets under different environmental conditions was greatly affected in terms of survival percentage of plantlets. In the present study, employing the acclimatization procedure indicated in the methodology, the plantlets were transferred to pots containing 1:1:1: coffee husk, sand and red soil respectively showed 75% survival efficiency.

Micro propagated plants were transferred to the pots containing mixture of coffee husk + sand + red soil in 2:1:1 ratio and 82.5% plants survived and the one transferred to the pots containing mixture of coffee husk + sand + red soil in 2:2:1 ratio about 90% plants survived. The plantlets transferred under net house conditions resulted in the best establishment, whereas no plantlets could be established under direct field conditions.


**Figure 6:** Acclimatization on 2:2:1 coffee husk, sand soil and red soil mix (A) plantlets ready to transfer on sterile soil mix and (B and C) transplanted and established plantlets in pot/ polytene tube ( after two weeks).

Two node vine cuttings have been used for seed tuber production in *D. alata, D. rotundata and D. esculenta* under pot culture conditions (Raviet *et al*., 2010). In the present study best rooting were observed on half strength MS with IBA (2.0 mg-l) than NAA (2.0 mg-l) which is, different from the result of Chen *et al*., (2003) on *D. zingiberenesis* that best rooting were observed at IBA than NAA. Therefore the *in vitro* micropropagation technique described here may be highly useful for raising quality planting material of *D. alata* (cv. Hatikhujia) for commercial and off season cultivation which will help in reducing the quantity of where tubers reserved for use as seed tuber.

# CHAPTER FIVE

# 5. CONCLUSION AND RECOMMENDATIONS

## 5.1 CONCLUSION

From different concentration of sterilant used for sterilizing yam nodal and shoot tip explants two and three percent sodium hypochlorite with five and 20 minute exposures of time was optimum for nodal and shoot tip explants of *D. alata*.

The combined effects of different concentrations of BAP, as a source of cytokinin, together with NAA were evaluated for enhancing *D.alata* shoot induction. The maximum percentage of shoot induction (93.20±1.07%) and (87.312±0.62%) was observed on an MS medium supplemented with 1.5.mg/l of BAP and 1.0mg/l of NAA for nodal and 1.0 mg/l of BAP and 1.0 mg/l of NAA for shoot tip explants respectively. Nodal explants gave better shoot induction response than shoot tip. BAP combined with greater number of NAA (auxin) resulted in very low shoot induction.

For mass propagation of *D. alata*, multiplication protocol development is indispensible. MS basal medium supplemented with 2.0 mg/l of BAP and 0.5 mg/l of NAA resulted in 9.50±0.29 shoot number from nodal with best and vigor morphological appearance. MS containing 2.0 mg/l of BAP and 1.0 mg/l with 0.5 mg/l of NAA also produced 8.40±0.21% shoot number with good morphological appearance from shoot tip. Best rooting percentage was achieved on half strength MS basal media containing 2.0 mg/l of IBA which resulted mean values of 90.70 ± 0.99% with 10.61±0.61 root number, followed by half strength MS basal media containing 2.0 mg/l of NAA which resulted 86.75±0.64 with 8.30 ±0.21 root number, 5.00±0.18cm shoot height and 2.00 ± 0.18cm root length. Root induction and unnecessary callus formation occurs almost at all stages, shoot induction, multiplication and rooting stages without using any auxin hormone.

This indicates that naturally the plant has high concentration of endogenous auxin. Thus, it was difficult to manage the ethylene biosynthesis that occurs due to high concentration of auxins that results in growth retardation, so it needs further study in near future (Akisoshi *et al.*, 1983).

Those plantlets well performed *in vitro* showed 90% survival efficiency after hardening and acclimatization on soil mixture by ratio of 2:2:1 decomposed coffee husk, sand and red soil respectively. Therefore, this protocol could be useful for large-scale production of highly demanded *D. alata* cultivar and provide a possible system towards genetic improvement and high disease free yield of the crop using nodal as well as shoot tip explants sources.

## 5.2 RECOMMENDATIONS

- Low cost and locally available sterilant for surface sterilization such as berekina should be tried.
- By applying the protocol developed to address the current development needs with regard to expansion of *D. alata* plantations in the country, thereby responding to the great demands of the producers.
- Micropropagation is important for production of virus free plantlets so; in the future studies on virus indexing should also be given attention and incorporated.
- Field performance of tissue culture raised Yam plantlets can be tried during the season of *D. alata* tubers are in dormant condition.
- Improved hardening procedure can be tried to address maximum field establishment in Yam plantlets raised through *in-vitro* technique.
- Using the developed protocol, Yam plantlets should be multiplied and distributed to farmers for large scale production.

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# APPENDICES

## I. Sterilization experiment SAS result

Summary of analysis of variance for the effect of concentration of Sodium hypochlorite (NaOCl 5%) and duration of explants exposure to different concentration of NaOCl on contamination percent of explants after a week.

**Appendix I: Summary of analysis of variance for the interaction effect of NaOCl with Exposure time on percentage of contamination of *D. alata* shoots tip and nodal explants**

**Appendix I a: Summary of ANOVA table for the interaction effect of NaOCl with Exposure time on percentage of contamination from shoot tip explant of *D. alata***

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Na | 3 | 41100.85 | 13700.28 | 35685.1 | <.0001** |
| Dur | 3 | 2575.84 | 858.61 | 2236.43 | <.0001** |
| Na*Dur | 9 | 2377.44 | 264.16 | 688.06 | <.0001** |

R - Square = 0.99CV % = 1.15, TIVCONT mean =53.70, ** = highly significant at α = 5 %, DF = degree of freedom, TIVCONT= Tissue with contamination

*= shows the interaction effects of Sodium Hypochlorite (NaOCl) with exposure time.

**Appendix I b: Summary of ANOVA table for the interaction effect of NaOCl with Exposure time on percentage of contamination from nodal explants of *D. alata***

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Na | 3 | 40943.21 | 13647.73 | 51749.8 | <.0001** |
| Dur | 3 | 776.84 | 258.94 | 981.88 | <.0001** |
| Na*Dur | 9 | 75.57 | 8.39 | 31.84 | <.0001** |

R - square = 0.99CV % = 0.85, TIVCONT mean =59.80, ** = highly significant at α = 5 %, DF = degree of freedom, TIVCONT= Tissue with contamination

*= shows the interaction effects of Sodium Hypochlorite (NaOCl) with exposure time.

**Appendix I c: Summary of ANOVA table for the interaction effect of NaOCl with Exposure time on percentage of clean culture from shoot tip explant of *D. alata***

| Source | DF | Type III SS | Mean Square | F Value | Pr > F** |
|---|---|---|---|---|---|
| Na | 3 | 6012.02 | 2004.00 | 4084.91 | <.0001** |
| Dur | 3 | 1069.16 | 356.38 | 726.45 | <.0001** |
| Na*Dur | 9 | 8762.81 | 973.64 | 1984.65 | <.0001** |

R - Square = 0.99 CV % = 2.49, CONFR mean =28.09, ** = highly significant at a = 5 %, %, DF = degree of freedom, CONFR = contamination free

*= shows the interaction effects of Sodium Hypochlorite (NaOCl) with exposure time.

**Appendix I d: Summary of ANOVA table for the interaction effect of NaOCl with Exposure time on percentage of clean culture from nodal explants of *D. alata***

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Na | 3 | 10234.12 | 3411.37 | 9879.62 | <.0001** |
| Dur | 3 | 351.37 | 117.12 | 339.20 | <.0001** |
| Na*Dur | 9 | 7035.75 | 781.75 | 2264.01 | <.0001** |

R - Square = 0.99 CV % = 2.08, CONFR mean =28.11, ** = highly significant at a = 5 %, %, DF = degree of freedom, CONFR = contamination free

*= shows the interaction effects of Sodium Hypochlorite (NaOCl) with exposure time.

**Appendix I e: Summary of ANOVA table for the interaction effect of NaOCl with Exposure time on percentage of tissue death from shoot tip explant of *D. alata***

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Na | 4 | 22182.07 | 5545.51 | 46659.4 | <.0001** |
| Dur | 4 | 3319.50 | 829.87 | 6982.50 | <.0001** |
| Na*Dur | 8 | 6172.77 | 771.59 | 6492.13 | <.0001** |

R - square = 0.99CV % = 2.47, TD mean =13.91, ** = highly significant at α = 5 %, DF = degree of freedom, TD = tissue death

*= shows the interaction effects of Sodium Hypochlorite (NaOCl) with exposure time.

**Appendix I f: Summary of ANOVA table for the interaction effect of NaOCl with Exposure time on percentage of tissue death from nodal explants of *D. alata***

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Na | 3 | 13492.81 | 4497.60 | 16991.0 | <.0001** |
| Dur | 3 | 1985.11 | 661.70 | 2499.77 | <.0001** |
| Na*Dur | 9 | 3875.07 | 430.56 | 1626.57 | <.0001** |

R - square = 0.99CV % = 3.23, TD mean =15.90, ** = highly significant at α = 5 %, DF = degree of freedom, TD = tissue death

*= shows the interaction effects of Sodium Hypochlorite (NaOCl) with exposure time.

## II. Shoot initiation experiment SAS result

**Appendix II: Effect of different levels of BAP and NAA on shoot induction of shoot tip and nodal explants *D. alata* cultured on MS medium after four weeks.**

**Appendix II a: Summary of ANOVA table for the interaction effect of BAP with Kinetin on percentage of shoot initiation from shoot tip explant of *D. alata* (Yam)**

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| BAP | 3 | 10147.40 | 3382.46 | 5221.52 | <.0001** |
| NAA | 3 | 11926.02 | 3975.34 | 6136.73 | <.0001** |
| BAP*NAA | 9 | 13293.97 | 1477.10 | 2280.21 | <.0001** |

R - square = 0.99 CV % = 1.98, mean of shoot initiation =45.95, ** = highly significant at α = 5 %, DF = degree of freedom, PERSHINN= % shoot initiation, explant = shoot tip explants

* = shows the interaction of the treatments, *** = highly significant at α = 5 %,

**Appendix II b: Summary of ANOVA table for the interaction effect of BAP with Kinetin on percentage of shoot initiation from nodal explant of *D. alata* (Yam)**

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| BAP | 3 | 4139.56 | 1379.85 | 1955.88 | <.0001** |
| NAA | 3 | 8011.06 | 2670.35 | 3785.10 | <.0001** |
| BAP*NAA | 9 | 15953.31 | 1772.59 | 2512.57 | <.0001** |

R - square = 0.99 CV % = 1.78, mean of shoot initiation =43.85, ** = highly significant at α = 5 %, DF = degree of freedom, PERSHINN= % shoot initiation, explant = nodal explants

* = shows the interaction of the treatments, *** = highly significant at α = 5 %,

## III. Shoot multiplication experiment SAS result

**Appendix III: shoot multiplication**

**Appendix III a: Summary of ANOVA table for the interaction effect of KIN, BAP and NAA on percentage of shoot number from shoot tip explants of *D. alata* (Yam)**

| Source | D F | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| KIN | 2 | 62.81 | 31.40 | 721.85 | <.000 1** |
| BAP | 2 | 0.69 | 0.34 | 7.97 | <.0009** |
| NAA | 1 | 57.60 | 57.60 | 1323.92 | <.0001** |
| KIN*BAP*NAA | 12 | 90.67 | 7.55 | 173.66 | <.0001** |

R - square = 0.98 CV % = 5.48, mean of shoot number =3.80, ** = highly significant at α = 5 %, DF = degree of freedom, PERSHN= % of shoot number

* = shows the interaction of the treatments, *** = highly significant at α = 5 %,

**Appendix III b: Summary of ANOVA table for the interaction effect of KIN, BAP and NAA on percentage of shoot number from nodal explants of *D. alata* (Yam)**

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| KIN | 2 | 112.34 | 56.17 | 1199.22 | <.0001*** |
| BAP | 2 | 29.03 | 14.51 | 309.92 | <.0001*** |
| NAA | 1 | 9.82 | 9.82 | 209.79 | <.0001*** |
| KIN*BAP*NAA | 12 | 178.02 | 14.83 | 316.71 | <.0001*** |

R - square = 0.99CV % = 6.12mean of shoot number =3.53, *** = highly significant at α = 5 %, DF = degree of freedom, PERSHN= % of shoot number

* = shows the interaction of the treatments, *** = highly significant at α = 5 %,

**Appendix III c: Summary of ANOVA table for the interaction effect of KIN, BAP and NAA on percentage of shoot length from shoot tip explants of *D. alata* (Yam)**

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| KIN | 2 | 39.85 | 19.92 | 526.43 | <.0001*** |
| BAP | 2 | 14.35 | 7.17 | 189.67 | <.0001*** |
| NAA | 1 | 9.17 | 9.17 | 242.36 | <.0001*** |
| KIN*BAP*NAA | 12 | 41.81 | 3.48 | 92.07 | <.0001*** |

R - square = 0.98CV % = 6.33mean of shoot length =3.06, *** = highly significant at α = 5 %, DF = degree of freedom, PERSHL= % of shoot length

* = shows the interaction of the treatments, *** = highly significant at α = 5 %,

**Appendix III d: Summary of ANOVA table for the interaction effect of KIN, BAP and NAA on percentage of shoot length from nodal explants of *D. alata* (Yam)**

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| KIN | 2 | 65.44 | 32.72 | 790.32 | <.0001*** |
| BAP | 2 | 4.37 | 2.18 | 52.83 | <.0001*** |
| NAA | 1 | 11.20 | 11.20 | 270.56 | <.0001*** |
| KIN*BAP*NAA | 12 | 68.16 | 5.68 | 137.19 | <.0001*** |

R - square = 0.98CV % = 6.83mean of shoot length =2.97, *** = highly significant at α = 5 %, DF = degree of freedom, PERSHL= % of shoot length

* = shows the interaction of the treatments, *** = highly significant at α = 5 %,

## IV. Root induction experiment SAS result

**Appendix IV: ANOVA table for the interaction effect of IBA and NAA on percentage of rooting, root number, root length and shoot height of *D. alata* (Yam)**

**Appendix IV a: Summery of ANOVA table for the interaction effect of IBA and NAA on percentage of rooting of *D. alata* (Yam)**

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| NAA | 5 | 16139.09 | 3227.81 | 4992.06 | <.0001*** |
| IBA | 5 | 20341.31 | 4068.26 | 6291.86 | <.0001*** |
| NAA*IBA | 0 | 18341.31 | 5063.26 | 8196.86 | <.0001*** |

R - Square = 0.99 CV % = 1.34, mean of rooting percentage =59.87, *** = highly significant at α = 5 %, DF = degree of freedom, PERR= percentage of rooting

* = shows the interaction of the treatments, *** = highly significant at α =5%,

**Appendix VI b: ANOVA table for the interaction effect of IBA and NAA on root number of *D. alata***

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| NAA | 5 | 166.15 | 33.23 | 766.87 | <.0001*** |
| IBA | 5 | 166.37 | 33.27 | 767.88 | <.0001*** |
| NAA*IBA | 0 | 166.84 | 33.27 | 767.88 | <.0001*** |

R - Square = 0.99CV % = 5.25, mean of rooting percentage =3.95, *** = highly significant at α = 5 %, DF = degree of freedom, PERRN= percentage of root number

* = shows the interaction of the treatments, *** = highly significant at α =5%,

**Appendix VI c: ANOVA table for the interaction effect of IBA and NAA on root height of *D. alata***

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| NAA | 5 | 39.89 | 7.97 | 398.93 | <.0001*** |
| IBA | 5 | 27.95 | 5.59 | 279.53 | <.0001*** |
| NAA*IBA | 0 | 21.29` | 3.59 | 138.53. | <.0001*** |

R - Square = 0.98CV % = 5.13, mean of rooting percentage =2.75, *** = highly significant at α = 5 %, DF = degree of freedom, PERRH= percentage of root height

* = shows the interaction of the treatments, *** = highly significant at α =5%,

**Appendix VI d: ANOVA table for the interaction effect of IBA and NAA on root length of *D. alata***

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| NAA | 5 | 67.20 | 13.44 | 439.18 | <.0001*** |
| IBA | 5 | 37.31 | 7.46 | 243.83 | <.0001*** |
| NAA*IBA | 0 | 32.41 | 3.35 | 136.83 | <.0001*** |

R - Square = 0.98CV % = 7.41, mean of rooting percentage =2.35, *** = highly significant at α = 5 %, DF = degree of freedom, PERRL= percentage of root length

* = shows the interaction of the treatments, *** = highly significant at α =5%,