Micropropagation of *Colocasia esculenta* (cv. Bolosso I)

M.Sc. Thesis

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AARI-Areka Agricultural Research Institute

ANOVA-Analysis of Variance

BA-Benzyl Adenine

BAP-*6*-Benzylamino Purine

Ca.-calculated

CRD-Completely Randomized Design

Cv- Cultivar

DSMV-Dasheen Mosaic Viruses

EIAR-Ethiopian Institute of Agricultural Research

FAO-Food and Agriculture Organization

IAA-Indole-3-acetic acid

IBA- Indole-3-butyric acid

KIN- Kinetin

Mins- minutes

MoARD-Minister of Agricultural and Rural Development

MS- Murashige and Skoog Media

NAA- Naphthalene acetic acid

PGRs- Plant Growth Regulators

REGWQ- Ryan, Elinot, Gabriel, and Welsh model

SAS- Stastical Soft ware package for Science

SNNPS-South Nation Nationality and People Regional State

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ABSTRACT

Taro (*Colocasia esculenta* (L.) *is a perennial, aquatic and semi aquatic species, member of the family Araceae grown for its edible tuberous root. The application of conventional propagation techniques for Taro could not produce the high demand and quality planting materials due to its low productive capacity and diseases transmission. Therefore, this study was carried out to developed micropropagation protocol for Bolosso I typed cultivar of Taro using corm and sprout tip explants. MS media supplemented with different types and concentrations of plant regulators (BAP, kinetin, IAA, NAA and IBA) were used for initiation, shoot multiplication and root induction. A highly significant difference (P<0.0001) was observed between explant sources and among growth regulators for initiation, multiplication and root induction. Two percent NaOCl exposures for 15 and 20 minutes were found to be optimum for sterilization of sprout tip (83.33±0.85) and corm (66.63±0.51%) explants. Highest culture initiation was obtained on MS medium supplemented with 8 mg/l BAP (81.33±0.59), (76.67±0.48%) for corm and sprout explants, respectively. Maximum number of shoots (8.53±0.07/corm) and (5.8±0.39/sprout explant) was obtained on MS+8 mg/l BAP and 3mg/l IAA. The highest mean root number (6.9±0.09) and root length (11.25±0.5cm) per plantlet were recorded from MS media supplemented with 0.5mg/l IAA and 1.5mg/l IAA, respectively. Eighty percent survival efficiency was observed on the soil mix ratio of 1:2:2 (red: sand: coffee husk, respectively). This developed propagation protocol can be used to produce quality and true-to-type Taro planting material for large scale commercial production under natural condition.*

Key words: *Explant, MS media, Plant growth regulators, plantlet.*

CHAPTER ONE

1. INTRODUCTION

1.1 Background

Colocasia esculenta (L.) Schott (Taro, Elephant ear or Cocoyam) is a perennial, aquatic and semi-aquatic herbaceous species belongs to the family *Araceae*, originated in North Eastern India and Asia and then gradually spread worldwide (Hancock, 2004; Van Wyk, 2005). Taro is the second most important root staple crop interms of consumption (Singh *et al*., 2012) and is ranked fourth after sweet potato, Yam, and cassava interms of its production by weight with an estimated annual yield of over 229,088 tones worldwide (Bourke andVlassak, 2004).

Nutritionally, Taro crop is one of the most nutritious and easily digested crops where both the leaf and underground parts are important in the human diet (Tilahun, 2009; Hossain, 2012). Like many other root crops, Taro corms are high in moisture (63- 85 %), carbohydrate in the form of starch (47-74 %) protein (3-7%), potassium (higher than banana) and low in fat (Tilahun, 2009; Adane *et al*., 2013). The starch is 98.8 percent digestible, a quality attributed to its granule size, which is a tenth that of potato, making it ideal for people with digestive difficulties (Hanson and Imamuddin, 1983; John, 2007).

The propagation and ultimate production of Taro is adversely affected by biotic stresses such as viral diseases and pests (Singh *et al*., 2012). Of the various Taro diseases, Taro leaf blight (TLB) caused by the fungus-like Oomycete *Phytophthora colocasiae* Raciborski (*P. colocasiae*) is of prime importance because it can reduce corm yield by up to 50% (Singh *et al*., 2006; Singh *et al*., 2012) and leaf yield by 95% in susceptible varieties (Nelson *et al*., 2011). TLB can also deteriorate corm quality (Sar *et al.,* 1998). In addition to corm yield losses that occur as a consequence of the reduced leaf area (Jackson, 1999) in diseased plants, a corm rot caused by *P. colocasiae* may also occur (Brunt *et al.*, 2001).

Viruses are also one of the most important pathogens with some infections resulting in severe yield reductions and plant death for Taro crop. The main effect of virus infection in Taro is a reduction in corm size and quality, with yield losses of up to 20% being reported (Deo *et al*.,

2009). According to the same report, currently, there are five viruses reported which infect taro worldwide ; (i) Dasheen mosaic virus (DsMV) is a potyvirus with flexuous, rod shaped virions, which infects both the edible and ornamental aroids and is transmitted by aphids; (ii) Colocasia bobone disease virus (CBDV) is a cytorhabdovirus, which causes bobone disease; (iii) Taro bacilliform virus (TaBV) is a badnavirus, result in a range of mild symptoms including stunting, mosaic and down curling of the leaf blades. (iv)Taro vein chlorosis virus (TaVCV) is a nucleorhabdovirus, which causes distinctive veinal chlorosis symptoms. (v) Taro reovirus (TaRV) has been recently discovered. It has been detected in association with other viruses, yet no symptoms have been directly attributed to TaRV infection (Revill *et al.*, 2005).

There are no taro varieties known to be immune to DSMV (Ooka, 1994; Philemon, 1994) which is aphid-vectored virus that decreases corm yield by up to 60% (Savor, 2007) and leaf yield by 95% (Deo *et al*., 2009; Singh *et al*., 2012). Both fungal and bacterial diseases can eliminated from a given crop plants through the use of sanitary culture methods in the field, but viral diseases such as DSMV which are common for taro crop becomes a serious problems in Conventional Taro planting worldwide and must be eliminated *in-vitro* culture methods (Fukino *et al*., 2000).

In Ethiopia, a recent report indicated that the production of Taro has been declined significantly, in large part due to viral disease (Beyene, 2012). The common viral diseases recognized in Ethiopia are Dasheen mosaic virus (DsMV) which is a pot virus with flexuous, rod shaped virions, which infects both the edible and ornamental aroids and is transmitted by aphids and Taro bacilliform virus (TaBV) is a badnavirus, result in a range of mild symptoms including stunting, mosaic and down curling of the leaf blades (Revill *et al.*, 2005; Beyene, 2012). According to Beyene (2012) 36.9% of the Taro samples found to be positive to DsMV specific antibodies out of two hundred and ninety five symptomatic and non-symptomatic Taro leaf samples which were collected from major growing areas of south and southwest Ethiopia.

In addition, Taro's flower is normally dormant and seeds development is rare usually absent, as a result, farmers practice the conventional propagation method using stem cuttings (by cutting the top part of the corm or by planting cormels and dividing the edible corms) (Kifle, 2005; MoARD, 2009) leads to the accumulation of viral and bacterial diseases which reduce

productivity of the crop and may cause loss of genotypes (Nassar and Ortiz, 2007; Fujimoto, 2009). Besides, it is a labor intensive and lengthy process in terms of field preparation and planting of the parental materials (Kifle, 2005; Fujimoto, 2009).

The use of biotechnological approaches in particular tissue culture techniques is the best option in order to tackle all the problems mentioned above with regard to this crop. *In-vitro* culture provides a means of rapidly producing large quantities of healthy, identical propagates for large scale cultivation, eliminating diseases using meristem culture technology. Techniques such as meristem culture (Hu and Wang, 1983) and hot-water treatment of explants before *in vitro* culture (Langens-Gerrits *et al.* 1998) have been used to produce plants free from pathogen. It is also allowing for large scale production of crop plantlets within short period and limited space without seasonal dependency in contrast to conventional methods (Deo *et al*., 2009).

Successful *in-vitro* multiplication have been reported from apical meristem and parenchymatous storage tissue (Chand *et al*., 1999; Li *et al*., 2002; Verma and Cho, 2010 and Hossain, 2012); auxiliary buds of developing suckers (Seetohul and Puchooa, 2005); shoot tip (Bhuiyan *et al*., 2011); shoot tips and petioles (20-30cm) (Hussain and Tyagi, 2006) and from meristem domes (Chien-Ying *et al*., 2008). In response, the present study aimed to develop an efficient micropropagation protocol for the selected Taro (*Colocasia esculenta* (L.) Schott) cv. Boloso I (ARC/064/96) using sprouted tip and corm base explants.

1.2. Objective of the study

1.2.1. General objective

To develop micropropagation protocol for *C. esculenta* cv. Bolosso I that contribute for mass propagation and in-situ conservation

1.2.2. Specific objectives

- To determine the effect of detergent (local bleach) for surface sterilization of Taro explants.
- To determine the effect of different BAP concentration alone and in combination with kinetin on culture initiation.
- To determine the effect of BAP alone and in combination with IAA on shoot multiplication.
- To test the effect of IAA, NAA, and IBA on *in-vitro* root induction of Taro plantlets.
- To determine the optimal acclimatization protocol

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Origin and distribution of Taro

Various lines of ethno-botanical evidence suggest that cultivation of Taro is thought to have originated in North Eastern India and Asia and then gradually spread worldwide (Hancock, 2004; Deo, *et al*., 2009). The putative area of origin of several other *Colocasia* species is Papua New Guinea which also represents a major centre of diversity of Taro (Edison *et al.*, 2006). As such, it is cultivated in more than 65 countries worldwide (USDA, 2001).

2.2. Taxonomy, morphology and Genetics of Taro

2.2.1. Botanical description and Taxonomy

Taro is sometimes known as Elephant ear or Cocoyam and scientifically known by *Colocasia esculenta* (L.) Schott. It is an emergent, perennial, aquatic and semi-aquatic herbaceous species belongs to the monocotyledonous family Araceae, sub-family Colocasioideae and genus *Colocasia* whose members are known as aroids (Van Wyk, 2005; Deo *et al*., 2009). *Araceae* includes about 100 genera and 1500 distributed species (Matthews, 2004; Van Wyk, 2005). Taro is one of the few edible and the most widely cultivated species grown for its starchy corms and it is a traditional staple food throughout the rural subtropical and tropical regions of the world (Van Wyk, 2005; Alex, 2013).

2.2.2. Morphology and Genetics

Taro is herbaceous plant, which grows to a height of 1-2 m. The plant consists of a central corm lying just below the soil surface, with leaves growing from the apical bud at the top of the corm and roots growing from the lower portion. Cormels, daughter corms and runners grow laterally (Deo *et al*., 2009). The leaf is peltate; the root system is fibrous and lies mainly in the top one meter of soil. The corm is a nutrient storage organ and shares the following characteristics with food storage organs in carrot, sweet potato and cassava: abundance of periderm, food storage in

large, thin-walled parenchymatous cells, poorly developed vascular bundles that are few in number, presence of latex cells and mucilage cells (Miyasaka, 1979 cited in Alex 2007).

Genetically, Using isozyme analysis Lebot and Aradhya (1991) reported the existence of two gene pools for cultivated Taro; one in Asia and the other in Pacific. Studies with Simple Sequence Repeats (SSR) markers (Noyer *et al*., 2003) and AFLP markers (Amplified Fragment Length Polymorphism) (Kreike *et al*., 2004) have confirmed the existence of these two distinct gene pools. Chromosome numbers reported for Taro from various regions include 2n = 22, 26, 28, 38 and 42. The most commonly reported chromosome numbers are: diploids 2n = 28 and triploids 3n = 42 (Lebot and Aradhya, 1991). Plants with 3n = 42 are referred to as alowane (male, large plant) and those of 2n = 28 are referred to as alokine (female, short plant) in Solomon Island (Deo *et al*., 2009).

2.3. Nutritional value of Taro

Taro is the second most important root staple crop after sweet potato in terms of consumption (Singh *et al*., 2012) and is ranked fourth root crop after sweet potato, yam and cassava in terms of its production by weight with an estimated annual production of over 229,088 tones worldwide (Bourke and Vlassak, 2004). Taro is one of the few crops where both the leaf and underground parts are important in the human diet and which contain high proportion of carbohydrate particularly amylopectin (Lee, 1999 cited in Alex, 2013; Hossain, 2012). Taro corm is an excellent source of carbohydrate, the majority being starch of which 17-28% is amylose, and the remainder is amylopectin (Oke, 1990). Taro is especially useful to people allergic to cereals and can be consumed by children who are sensitive to milk, and as such Taro flour is used in infant food formulae and canned baby foods (Ammar *et al*., 2009; Tilahun, 2009). The corm also is an excellent source of potassium (higher than banana), carbohydrate for energy, and fiber. When eaten regularly, taro corm provides a good source of calcium and iron (Mare, 2009; Tilahun, 2009). The Taro leaf, like most other higher plant leaves, is rich in protein. It contains about 7-23% protein on a dry weight basis. This is more than yam, cassava or sweet potato (FAO, 1999; John, 2007). It also an excellent sources of carotene, potassium, calcium, phosphorous, iron, riboflavin, thiamine, niacin, vitamin A, vitamin C and dietary fiber, which are important constituents of human diet (Hanson and Imamuddin, 1983; John, 2007).

2.4. Plant tissue culture

Tissue culture is alternatively called cell, tissue, organ culture through *in-vitro* condition (Rout *et al*., 2006). Tissue culture technique in plants is also called micropropagation is a practice used to propagate plants under sterile conditions or in a controlled environment, produce clones of the plants. In this processes, tissues or cells, either as suspensions or as is maintained under conditions conducive for their growth and multiplication. These conditions include proper temperature, proper gaseous and liquid environment and proper supply of nutrient (Akin-Idowu *et al*., 2009). Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, or (less commonly) roots often be used to generate a new plant on culture media given the required nutrients and plant hormones (George *et al*., 2008).

Micropropagation generally involves five distinct stages: preparation of mother stock, plants initiation of cultures, shoot multiplication, rooting of *in-vitro* grown shoots, and acclimatization. The first stage: establishment of mother stock plant depends on explants type or the physiological stage of the donor plant at the time of excision. Explants from actively growing shoots are generally used for mass scale multiplication. The second and third stage: shoot initiation and multiplication is crucial and it is achieved by using plant growth regulator cytokinin. The fourth stage: rooting elongated shoots, derived from the multiplication stage, are subsequently rooted either *ex-vitro* or *in-vitro* in auxins. In some cases, the highest root induction occurs from excised shoots in the liquid medium when compared with semi-solid medium. The fourth stage: acclimatization of *in-vitro* grown plants is an important step in micropropagation (Rout *et al*., 2006).

Shoot multiplication is increasing the number of shoots from a single shoot explant; it can be either by shoot culture on required type and concentration of growth regulators in a medium (Lizarraga *et al*., 1992). It is an essential component of micropropagation as it increases the number of shoots that will be rooted and acclimatized. Shoot multiplication can be enhanced with a relatively higher cytokinin (BAP) concentration to stop apical dominance of the shoot tip. Shoot induction and multiple shoot formation are known to be enhanced by BAP (El Far *et al*., 2009). It could be by node culture and shoot culture on different concentrations of cytokinins or

in combination with auxins which could be optimized for a respective variety. According to Kane (2005), optimum concentration of BAP for shoot multiplication can be made based on shoot multiplication rate, shoot length and frequency of genetic variation.

Shoot clonal multiplication is followed by rooting which is an essential step in making the shoot completely photo autotrophic and independent of the *in-vitro* nutrient supply (Geleta, 2009). The rooting step can be attained within the minimum time which can be within a week by either transferring the shoots into a medium free of growth regulators or different concentrations of auxins which are optimized for a particular variety. Before planting in the outdoor garden, *in-vitro* shoots are gradually subjected to hardening (acclimatization) for the maximum survival in the natural environment (George *et al*., 2008; Geleta, 2009).

2.5. Applications of plant tissue culture

Plant tissue culture has been successfully used for large scale commercial production and supply of true-to-type, pathogen-free plants of varied plant genotypes and species (Debergh and Maene, 1981), and to conserve germplasm of rare and endangered species, production of secondary metabolites, as well as rapid and large-scale multiplication of elite genotypes (Fay, 1992; Baskaran and Jayabalan, 2005). Techniques such as meristem culture (Hu and Wang, 1983; Funkino *et al*., 2000) and hot-water treatment of explants before *in-vitro* culture (Hol and Vander Linde, 1992; Langens-Gerrits *et al*., 1998) have been used to produce plants free from pathogens. Plantlets induced from meristem culture are genetically stable due to the highly organized apical meristem. Genetic stability of regenerated plants is offering another advantage of clonal propagation for rapid multiplication of identical (uniform) shoots *in-vitro* (Kane, 2005). Tissue culture is also useful for rapid propagation of cultures. This result in large number of plantlets propagated *in-vitro* without infection by pathogens and it is much faster than conventional methods (Lindsay and Jones, 1996).

Plant cell culture is a flexible system that is easily manipulated to increase product yields (Roberts and Shuler, 1997). Cultures of plant cells are not limited by environmental, ecological or climatic conditions (Zhong *et al*., 1995). The capability to cultivate plant callus and organs in liquid media has also made an important contribution to modern plant biotechnology with

respect to the production of commercially valuable compounds such as secondary metabolites (Su and Lee, 2007). Callus obtained from the transgenic plants can be grown in simple, chemically defined liquid media to establish transgenic cell suspension cultures for recombinant protein production (Su, 2006).

In addition, Propagation of some commercial plants which are difficult to reproduce conventionally by seed or vegetative propagules is realized by *in-vitro* tissue culture techniques (Ziv, 1998). Moreover, tissue culture is an essential component of genetic transformation. It is, therefore, a valuable tool of biotechnology that enables fast *in-vitro* propagation of root crops and others (Geleta, 2009).

In Ethiopia, tissue culture is recently introduced technology where researches were done in plants such as *Phytolacca dodecandra* (Demeke *et al*., 1992); *Ensete ventricosum* (Negash *et al.*,2000); *Aframomium corrorima* (Tefera and Wannakrairo, 2004; Eyob, 2009); *Manihot esculenta* (Beyene *et al*., 2010; Berhanu, 2011); *Taverniera abyssinica (*Abera *et al*.,2010); *Ipomoea batatas* (Feyissa and Dugassa, 2010); *Zingiber officinale* (Ayenew *et al*., 2012); *Eragrostis tef* (Mekibeb *et al*., 2012); *Vanilla planifolia (*Mengesha *et al*., 2012); *Solanum tuberosum* (Fufa and Diro, 2013); *Ananas comosuss* (Mengesha *et al*., 2013); *Coccinia abyssinica* (Bekele *et al*., 2013; Yambo and Feyissa, 2013) and *Artemisia annua* (Hailu *et al*., 2013).

2.6. Plant tissue culture media and plant growth regulators

Plant material can only grow *in-vitro* when provided with specialized media (Abraham, 2009). The main components of most plant tissue culture media are mineral salts, sugar as carbon source and water. Other components may include organic supplements, growth regulators, a gelling agent (Gamborg *et al*., 1968; Gamborg and Phillips, 1995). Although, the amounts of the various ingredients in the medium vary for different stages of culture and plant species, the basic MS (Murashige and Skoog, 1962) and LS (Linsmaier and Skoog, 1965) are the most widely used media.

During the past decades, many types of media have been developed for *in-vitro* plant culture (Torres, 1989). Media compositions have been formulated for the specific plants and tissues

(Nitsch and Nitsch, 1969). Some tissues respond much better on solid media while others on liquid media. In general, the choice of medium is dictated by the purpose and the plant species or variety to be cultured (Ahloowalia and Savangikar, 2002).

Another important factor in determining growth of *in-vitro* tissue is a group of organic substance to which plant growth regulators (PGRs) belong. Plant growth regulators, particularly auxins and cytokinins, regulate the regeneration of shoots and induction of roots *in-vitro*. The type and concentration of these nutrient components and PGRs needed in either stock solution or medium are based on genetic makeup of the plant for the regulation of the growth and development of the plant (Feyissa *et al*., 2005; Abera *et al*., 2010).

2.7. Gelling agents

The growth of cultures and production of shoots or roots is strongly influenced by the physical consistency of the culture medium. Gelling agents are usually added to the culture medium to increase its viscosity as a result of which plant tissues and organs remain above the surface of the nutrient medium (Ahloowalia and Savangikar, 2002). Many gelling agents are used for plant culture media, for example, agar, 'Agarose', and 'Gellan gum', and are marketed under trade names such as 'Phytagel, Gelrite' (Sigma Co., Merck and Co. Inc, Kelco division), and 'Gel-Gro' (ICA Biochemicals). Agar is the most commonly used gelling agent for preparation of solid and semi-solid media. It contributes to the matrix potential, the humidity and affects the availability of water and dissolved substances in the culture containers (Debergh, 1983). Various brands and grades of agar are available commercially, which differ in the amounts of impurities, and gelling capacity. Agar brands vary widely in price, performance and composition. It is the actual use and experience, which ultimately determines the choice of agar brand in a specific system and for a plant species. It is usually unnecessary to use high purity agar for large-scale micropropagation; cheaper brands of agar have been successfully used for industrial scale micropropagation (Debergh, 1983).

2.8. Explants selection and sterilization techniques

Explant is defined as plant parts which excised from the stock for the purpose of plant tissue culture (micropropagation) (Feyissa *et al*., 2005). All parts of a plant have been used successfully

as a source of explants. In practice, the explant is removed surgically, surface sterilized and placed on a nutrient medium to initiate the mother culture, that is multiplied repeatedly by subculture (George *et al*., 2008).

The explants which are commonly used in commercial micropropagation are shoot-tip, meristem-tip, nodal or auxiliary bud culture. In some plants, leaf discs, intercalary meristem 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. Since the meristem culture is usually health, vigorous, and physiologically active part, is preferable as the source of explants (George *et al*., 2008; Abraham, 2009).

In case of Taro, successful *in-vitro* multiplication have been reported from apical meristem and parenchymatous storage tissue (Chand *et al*., 1999; Li *et al*., 2002; Verma and Cho, 2010 and Hossain, 2012); Auxiliary buds of developing suckers (Seetohul and Puchooa, 2005); shoot tip (Bhuiyan *et al*., 2011); shoot tips and petioles (20-30cm) (Hussain and Tyagi, 2006) and from meristem domes (Chien-Ying *et al*., 2008).

Another very important technique in plant tissue culture is sterilization and use of sterilant chemicals. Plant tissue culture media, which are rich in sucrose and other organic nutrients, this condition, supports the growth of many microorganisms like bacteria and fungi these microbes generally grow much faster than the cultured tissue and finally kill it (Abera, 2009; Bekele *et al*., 2013). There are several possible sources of contamination of the medium: i) culture vessels, ii) the medium itself, iii) the explants, iv) the instrument used to handle the explants (Abera, 2009; Abraham, 2009). Except the explants, the rest materials can be controlled by autoclaving at 121^0C and 15Pa pressure for 15-20 min. Explants carries a wide range of microbial contaminants, to avoid this source of infection the tissue must be thoroughly surface sterilized before planting it on the nutrient medium. To disinfect plant tissues, various sterilizing agents such as sodium hypochlorite (NaOCl, 0.025% -0.25%), calcium hypochlorite (CaOCl, 9-10%), hydrogen peroxide (H_2O_2) solution (3%-10%) and others like bromine water (1%-2%), silver nitrate ($AgNO_3$, 1%) and mercuric chloride (HgCl, 0.1%-1%) have been used (Feyissa *et al*.,

2005). Diluted household bleach can also be used for this purpose, which normally contains 5.25% NaOCl (Abraham, 2009; Bekele *et al*., 2013).

Likewise, reported study on sterilization of Taro explants with sodium hypochlorite showed an effective result in the concentration of NaOCl (2%) without harming the cellular tissue and with highest survival rates as well as the percentage of green buds that developed shoots after five weeks. Seetohul and Puchooa (2005) revealed that NaOCl (2.5%) showed better result than NaOCl (2.0 %) and eliminate all the surface contaminants, but, it was toxic to cellular tissue of taro (Seetohul and Puchooa, 2005).

Sodium hypochlorite is one of the common disinfectants used in plant tissue culture to promote surface sterilization and improve the establishment of aseptic cultures (Seetohul and Puchooa, 2005). This is, because its mechanism of action causes biosynthetic alterations in cellular metabolism and phospholipids destruction, formation of chloramines that interfere in cellular metabolism, oxidative action with irreversible enzymatic inactivation in bacteria and lipid and fatty acid degradation (Estrela *et al*., 2002).

2.9. Propagation of root crop plants

Root and tuber crops are used as staple food in most countries in the world but their contribution to the energy supply of the population varies within a large range depending on the country (0 to 56% with a world mean of 5). Many species and varieties are consumed but three species (namely; cassava, Irish potato and sweet potato) provide 93% of the root and tuber crops used for direct human consumption in the world (UNIFEM, 2002).

The main advantages of propagating root crops as a staple food compared with cereals are that they are cheaper source of energy, can be cultivated easily and provide more dietary energy per hectare at a lower cost (principally because of reduced labor inputs).They generally require a comparatively low level of husbandry (UNIFEM, 2002).

The relative importance of individual root crops varies both by region and country. For example yams are a major food crop in West Africa, the Caribbean, the south Pacific Islands, South-East Asia, India and some parts of Brazil. Cassava is particularly important in South America, West

East, Central and South Africa and Oceania. Taro plays an important cultural role in the diet of the people of the pacific Islands, West Africa, Oceania and the West Indies and West Africa (UNIFEM, 2002).

2.9.1. Conventional propagation of Taro

Taro is mainly vegetative propagated (Strauss *et al*., 1979), but may also reproduce sexually (Ivancic, 1992). Due to vegetative/clonal propagation, there is almost no genetic variation within the cultivars although somatic mutations may occur thus increasing their vulnerability to pest and diseases or changes in climatic conditions (Ivancic, 1992). Sexual hybridization of Taro is well documented and techniques for pollinating and growing seedlings have been established (Tyagi *et al*., 2005). Sexual hybridization is one way to generate new cultivars with improved qualities (Strauss *et al*., 1979). Even though sexual hybridization of Taro is promising, it is a labor intensive and lengthy process in terms of field preparation, planting of parents, induction of flowering, pollination, development and maturation of fruit heads and seed harvesting (Tyagi *et al*., 2005; Deo *et al*., 2009).

2.9.2. Micropropagation of Taro

Micropropagation is the application of tissue culture technology for propagation of any economically important plant species. The primary advantage of micropropagation is the rapid production of high quality, disease-free and uniform planting material (Abraham, 2009). 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 (Feyissa *et al*., 2005; Abraham, 2009). Reported studies in regard to micropropagation of Taro in each consecutive stage are presented in the following sections.

2.9.2.1. Culture initiation

Both BAP and kinetin are the two groups of cytokinins which are very important for culture initiation. The relative value of BAP for shoot proliferation has been well documented for various species (Abraham, 2009; Omer *et al.*, 1995 cited in Bekele *et al*, 2013) while Kinetin

was only slightly effective in shoot proliferation as compare to BAP. In Taro, as reported by Seetohul and Puchooa (2005) the initiation media test with IAA, showed more healthy and vigorous growth with highest average number of Taro leaves and roots. The study also showed that MS media supplemented with IAA at concentrations above 15 mg L^{-1} did not support growth of Taro explants as compared to basal MS media. The other report by Chand *et al.* (1999) indicated that explants of Taro cultured on modified MS medium plus 2.6 M (0.6 mg /L TDZ) grew more vigorously than on media including BA. Chien-Ying *et al.* (2008) also reported that BA at concentration of 8 mg/l has been optimum for *in-vitro* initiation Taro corm explants.

2.9.2.2. Shoot multiplication

The relative value of BAP for shoot multiplication and elongation has been well documented for various species (Abraham, 2009; Bekele *et al*., 2013). For the case of Taro, different studies undertaken on various cultivars. The study by Chien-Ying *et al.* (2008) indicated that the highest shoot formation frequency (30.5-99.5%) with highest number of shoots record could be possible from full MS media containing BA and IAA. Hossain (2012) showed that a combination of NAA and BAP under an incubation photoperiod of 16 hr coupled with temperature of 24°C ± 2°C produced successful shoot regeneration and most suitable for *in-vitro* culture of Taro (*C. esculenta*). The study also by Hussain and Tyagi (2006) indicated maximum rate of shoot number resulted from MS media containing 3% sugar + 0.5 mg/l BAP + 0.1mg/l NAA and 0.8% agar. As clearly observed these reported studies higher concentration of cytokinins particularly BAP with low concentration of auxins is more effective and produced better results on Taro multiplication.

2.9. 2.3. Root induction

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, sometimes, interaction with cytokinins used for root proliferation (Feyissa *et al.*, 2005; Abera *et al*., 2010).

Demeke *et al.* (1992) reported that the auxins such as IAA, IBA and NAA, enhanced root production of *in-vitro* proliferated shoot tips culture, 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. IBA at lower concentration (0.49 gm/l) showed a high rooting percentage with minimal callus.

In Particular, for Taro (*C. esculenta*) root initiation percent as well as number of roots per culture increased with the increase of IAA levels up to 0.5 mg/l IAA and then declined (Bhuiyan, 2011). The other study on plantlet development through somatic embryogenesis and organogenesis in plant cell cultures of *C. esculenta* by Verma and Cho (2010) stated 100% root induction from MS medium augmented with 2μM IAA in a week and 10±5 roots per shoot with 8-15cm length after four weeks of planting.

In general, most authors reported that *in-vitro* propagation protocol for different Taro genotype, however, no one deal or reported for the selected Ethiopian Taro variety (cv. Bolosso I).

2.9.2. 4. Acclimatization

It is a final step in plant tissue culture at which plantlets are transferred to the external environments. Plantlets resulted from tissue culture are usually acclimatized or hardened in green house and other similar environment before transfer to the field (George *et al*., 2008). In green houses, plantlets are acclimatized or hardened before being transferred to the field to develop adequate root systems and leaf structure to with stand 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%) as well as cooling and heating systems (Feyissa *et al*., 2005; George *et al*., 2008; Abraham, 2009). The report of Verma and Cho (2010) showed that 100% survival rate of Taro plantlets and vigorous growth was observed after fully acclimatized plants (15-20cm length) transferred to the field after four weeks of planting.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Plant material

Selected healthy corms of Taro (*C. esculenta* cv. Boloso I) were collected from root crops research division, Areka Agricultural Research Institute (AARI) located in Areka town, Bolosso Sore district of Wolaita zone, South Nations Nationalities and Peoples' Regional State (SNNPRS). The corms were then planted and grown on sterilized soil that had a mixture of red soil, coffee husk and sand at a ratio of 1:2:2, respectively under green house conditions of Natural Science College, Jimma University. The established mother stockplant was daily watered with tap water and sprayed with 0.3 % Mancozeb at eight days interval to control fungal infection. Very young, healthy and vigorous sprouted and basal parts of matured Taro (4-5 month old) were used as a source of explants (Figure 1).

Figure 1 Mother stockplant grown under green house condition

3. 2 Explants collection and sterilization

After growing a healthy and vigorous plant materials, the apical shoot of mature plants between 4-6 months old (ca. 1.5-2.0cm) and newly sprouted auxiliary shoots (1.5-2.0 cm) segments were used as source of explants (Figure 1). The excised explants material were thoroughly washed with tap water and outer leaves are removed until inner cleaner section appeared. Explants were then surfaced-wiped with 70% ethanol for a maximum of one minute. Outer leaves were separated from the dome in a circular fashion using a sterile surgical blade under a laminar flow hood cabinet. They were then washed in four different levels of sodium hypochlorite solution (NaOCl), namely 1.0%, 1.5%, 2% and 2.5% with exposure duration of 5, 10, 15 and 20 minutes. To maximize the efficacy of the sterilant chemical, some drops of Tween-60 (as a wetting agent) was added into all the sterilant solutions prior to treatment. Then, the treated explants were rinsed repeatedly five times with sterilized distilled water under aseptic condition. Finally, the explants which lack any sign due to the effects of the sterilant were then transferred to culture jars and cultured in a plant growth regulator free conditioning medium of full strength MS medium (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose, and solidified with 8 g/l agar-agar. Thus, this experiment was laid in a 4x4x2 factorial combination in CRD. The four factors evaluated in this experiment were four concentrations (1, 1.5, 2 and 2.5% active chlorine) of sterilant solution, four durations (5, 10, 15 and 20 minute), and two types of explants (corm and sprout tip).

3. 3 Stock solution preparation

Different growth regulators stock solution such as 6-benzyl amino purine (BAP), α-naphthalene acetic acid (NAA), indol-3-butyric acid (IBA) and indol-3-acetic acid (IAA) were prepared by weighing and dissolving the powder in double distilled water at the ratio of 1mg PGRs/1ml double distilled water. For dissolution process, 3-4 drops of 1N NaOH, 1N HCl and 96% ethanol were used based on the requirement of the growth regulators (NaOH/96% ethanol for auxins, HCl for cytokinins. The volume was adjusted by adding sterilized distilled water. Finally, all the growth regulators stock solutions were stored in a refrigerator at a temperature of 0 to +4^{o}C.

Culture medium was prepared by taking the proper amount of MS powder (Murashige and Skoog medium, 4405.19 gl^{-1} conc.) solidified with 8 gm/l agar and then 3% sucrose (w/v) as carbon source was added at 5.7 pH. The pH of the medium was adjusted by using some drops of 0.1N HCl or 0.1N NaOH, gelled with 0.8% (w/v) agar. The media were then sterilized by autoclaving at a temperature of 121 ^{0}C with a pressure of 15Pa for 15 minutes. Then, the culture media were incubated in the dark at 25 ± 2^{o}C to minimize the effects of light.

3.4 Tissue culture experiment

3.4.1. Culture initiation

Sterilized corm base and auxiliary bud segment (sprouted tip) explants were inoculated in 8-12cm culturing jar and test tube respectively in full MS (Murashige and Skoog, 1962) media supplemented with BAP (4, 6, 8, and 10 mg/l), kinetin (1, 2 and 3 mg/l), sucrose (30 g l^{-1}) and 0.8 % agar (Sigma, St Louis MO, USA). One explant per jar was cultured for each treatment and six jars considered as one. Then, each treatment was replicate four times. The cultures were incubated in the light condition for three weeks. Then, all explants were transferred to fresh media in order to increase the initiation capability of the culture and incubated for another three weeks under light conditions. Data on the percentage of initiated shoots per explant were recorded after 3 weeks of culture. So, there were 4x3x2 factorial combinations in CRD including free growth regulator (0) as a control.

3.4.2 Shoot multiplication

Shoot buds derived from those explants that had responded well to the initiation media were transferred singly onto 8-12 cm culture jars. For this experiment, the shoot multiplication media used were composed of MS basal medium supplemented with 30g/l sucrose and different concentrations of BAP (0, 2, 4, 6, 8, 10, and12 mg/l) each alone and interacting at each concentration with (1, 2 and 3 mg/l) IAA. The experiment was thus being arranged in a 6x3x2x2 factorial in CRD. After 3-4 weeks of culture average numbers of shoots per explants were recorded. Then, from cultures each proliferated shoot (sprouts) were divided and subcultured separately on to a fresh medium of similar composition for further growth. Then, after 3 weeks an average heights (cm) of the micro shoots were recorded.

3. 4. 3 Rooting

For root induction experiment, three auxins type (IAA, NAA and IBA) were tested. Well developed microshoots with minimum length of 2.0 cm were transferred on fresh media of rooting media. For this experiment, agar solidified (0.8% agar-agar) half strength MS basal medium with 3% sucrose was supplemented with different concentrations of IBA, IAA and NAA. Therefore, the experiment was laid with treatment of three concentrations for each of auxins (0, 0.5, 1 and 1.5 mg/l). Thus, the treatment combination for this experiment was 3 x 3 x 3 factorial combination in CRD, where 3- concentration of NAA, IAA and IBA

3.4.4 Acclimatization

After completing the root induction experiment, all the plantlets which were successfully induces root were removed from the rooting media for acclimatization. For the case, the roots were carefully washed with running tap water to remove the gelling agent raised from the *in-vitro* media and the plantlets were then planted into pots (45 cm x 35 cm) filled with sand, decomposed coffee husk and red soil mixes. Five plantlets were grown in soil mix ratio of 2:2:1; four plantlets were grown in soil mix ratio of 1:2:2 and 1:1:1. Four survived plantlets were obtained from soil mix ratio of 2: 2: 1, sand, coffee husk and red soil, respectively. Three survived plantlets were obtained from each other soil mix ratio. Finally, the survived plantlets percentages from each soil ratio were recorded using the formula of: Survived plantlets = survived plantlets divided by total grown plantlets and multiplied by 100.

3.5. Experimental design and data analysis

The experimental design were set to be 4x4x2, 4x3x2, 6x3x2 and 3x3x3 factorials for sterilization, shoot initiation, shoot multiplication, and rooting, respectively. Where, treatment was one factor and explants were another factor. The experiments were laid in Completely Randomized Deign (CRD) with factorial treatment combinations. Data collected from each experiment were subjected to statistical analyses using the SAS statistical software (version 9.2) and ANOVA were constructed, followed by mean separation using appropriate procedures of Ryan, Elinot, Gabriel, and Welsh model (REGWQ).

CHAPTER FOUR

4. Result and Discussion

4.1 Effects of NaOCl concentrations and exposure time on sterilization of *C. esculenta* explants

The analysis of variance showed that the concentration of active chlorine in sodium hypochlorite solution, time duration of explants exposure to the sterilant and interaction of concentrations to time duration had very highly significant effect ($P < 0.0001$) on survived with contamination free culture of both explants (Appendix I A and B). Very highly significant difference had also been revealed between the two types of explants (treatment * explants = $P < 0.0001$) which revealed that the level of tissue survived with contamination was influenced by the concentration of NaOCl and duration of exposure time and the mean average value for survived tissue with contamination free of sprout tip exceeded the corm base explants.

The highest rate of alive clean survived culture 83.33±0.85 % and 76.67±0.92% was obtained from treatment concentration of 2% active chlorine in NaOCl solution with fifteen and ten minute exposure duration respectively for sprouts tip culture. For corm culture, 2% active chlorine and 20 minute exposure duration was found to be the most effective treatment combination with average result of 66.63±0.51% clean culture followed by the same concentration with time duration of 15 minutes with mean average value of 60.00±1.00% (Table 1).

Table 1. Interaction effect of sodium hypochlorite concentrations and its time of exposure on sterilization of corm base and sprout tip of *C. esculenta*.

Conc. (%)	Time (min.)	Clean alive culture (%)		Contamination (%)		Tissue death (%)	
		corm	sprout tip	corm	sprout tip	corm	sprout tip
		Mean ±S. D.	Mean ±S. D.	Mean ±S. D.	Mean ± S. D.	Mean ±S. D.	Mean ±S. D.
0	0	0.00^{m}±0.00	0.00^{m}±0.00	100^{a}±0.00	100^{a}±0.00	0.00^{l}±0.00	0.00^{j}±0.00
1	5	13.33^{l}±0.75	20.00^{l}± 0.20	86.66^{b}±0.76	80.00^{b}± 0.26	0.00^{l}±0.00	0.00^{j}±0.00
1	10	18.15^{k}±0.10	26.66^{k} ±0.76	81.15^{c}±0.40	73.33^{c}±0.45	0.00^{l}±0.00	0.00^{j}±0.00
1	15	26.66^{j}±0.81	33.33^{j} ±0.98	73.33^{d}±0.65	66.66^{d}±1.15	0.00^{l}±0.00	0.00^{j}±0.00
1	20	33.33^{i}±0.61	40.00^{i}±0.10	66.86^{e}±0.23	60.00^{e}±0.26	0.00^{l}±0.00	0.00^{j}±0.00
1.5	5	35.33^{h}±0.61	43.33^{h} ±0.58	64.00^{f}±0.91	56.66^{f}±0.49	6.66^{k}±0.20	0.00^{j}±0.00
1.5	10	40.00^{g}±0.80	46.66^{g} ±0.61	46.66^{g}±0.41	53.33^{g}±0.40	10.00^{j}±0.18	0.00^{j}±0.00
1.5	15	46.34^{f}±0.57	53.33^{f} ±0.61	40.00^{h}±0.43	43.33^{h}±0.61	13.66^{i}±0.28	3.33^{i}±0.15
1.5	20	50.00^{e} ±0.17	60.26^{e} ±1.51	33.33^{i}±0.35	33.33^{i}±0.35	16.00^{h}±0.50	6.66^{h}±0.30
2	5	53.33^{d} ±0.40	66.66^{d} ±0.92	26.66^{j}±0.57	23.26^{j}±0.64	20.00^{g}±1.00	10.00^{g}±0.36
2	10	55.00^{c} ±1.20	76.67^{b} ±0.92	20.00^{k}±0.40	10.00^{k}±0.87	25.00^{f}±1.14	13.33^{f}±0.45
2	15	60.00^{b} ±1.00	83.33^{a} ±0.85	13.33^{l}±0.40	3.33^{l}± 0.66	26.66^{e}±0.28	13.33^{f}±0.45
2	20	66.63^{a} ±0.51	73.33^{c} ±0.51	6.66^{m}±0.57	3.33^{l}±0.66	26.66^{e}±0.28	23.33^{e}±0.40
2.5	5	53.33^{d} ±0.40	60.00^{e} ±0.32	6.66^{m}±0.57	0.00^{m}± 0.00	40.00^{d}±0.50	40.00^{d}±0.57
2.5	10	50.10^{e} ±0.17	52.00^{f} ±0.26	3.33^{n}±0.57	0.00^{m}± 0.00	46.66^{c}±1.52	48.00^{c}±1.65
2.5	15	46.66^{f}±0.57	43.67^{h}±0.35	0.00^{o}±0.00	0.00^{m}±0.00	53.34^{b}±0.75	56.33^{b}±0.75
2.5	20	40.00^{g} ±1.80	33.34^{j}±0.15	0.00^{o}±0.00	0.00^{m}±0.00	60.00^{a}±1.00	66.67^{a}±1.33
CV		1.81	1.41	1.27	1.46	3.13	3.96

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

As indicated in Table 1 above, 1.5% NaOCl and 2 % NaOCl with time exposure of 15-20 minutes, in all the cases, reduce the presence of contaminants in the *in-vitro* culture media with higher percentage of clean alive culture. In the other case, NaOCl with higher concentration (2.5%) with long exposure duration (15 and 20 minutes) did eliminate all the surface contaminants. However, it was toxic to cellular tissue of Taro (Figure 2 d) because of its mechanism of action causes biosynthetic alterations in cellular metabolism and phospholipids destruction in cell wall of bacteria and fungi (Seetohul and Puchooa, 2005). On other hand, NaOCl (1.5 % and 2%) did not eliminate all the contaminants as some bacteria and fungi were observed in the culture media but, the survival rates as well as the percentage of green buds that developed shoots after two to three weeks of culture from both explants were highest (Figure 2 b and c). In lower concentration of NaOCl (1%), with lower exposure time (five and ten minutes), highest percentage of contamination were observed while shoot death is highly low in both explants. This is due to insufficiency of sterilant concentrations and exposure time to kill surface contaminants mainly fungi and bacteria those are strongly associated to the culture explants.

The results of this study is in line with the study done by Seetohul and Puchooa (2005) who reported 2% active chlorine (NaOCl) with 15 exposure time resulted highest percentage of contamination free *in-vitro* culture for axillary bud sucker explants of Taro after washed for one hour with running water and treated with 15 minutes in a solution of benlate at 0.06% on a shaker. The present study while developed optimal sterilant concentration with time duration of (2% NaOCl/ with 15 and 20 minutes) for sprout tips and corm base explants after treated with 70% v/v ethanol alcohol for a minute prior to NaOCl treatment.

Hossain (2012) also similarly reported using meristem and parenchymatous storage tissues of Taro explants after treated with 70 % ethanol for three minutes followed by soaking in 1.5% chlorine water with few drops of Tween 20 for ten minutes of exposure as effective sterilization combination. The same author, in 2009 also showed 1.5 % NaOCl with time duration of 10 minutes in the presence of 70% ethanol alcohol as best sterilization combination for *C. esculenta* var. Latiraj.

In other study, such as Tyagi and Hussain (2006) showed that 0.05% $HgCl_2$ with Tween 20 for ten minutes as best and effective treatment to remove microbes from the plant surface as compared to sodium hypochlorite solution. Study on Coccame species, *Xanthosoma sagittifolium* (L) Schott by Bari and Paul (2007) also supported sterilization using mercuric chloride. However, mercury chloride is a very toxic chemicals for human, unsafe and unfriend with the environment and hence difficult to discard after sterilization process. Thus, it is better to substitute and use with relatively safe and easily available sterilant chemical such as sodium hypochlorite/bleach/ solution to disinfect cultured explants as used in this study (Bari and Paul, 2007).

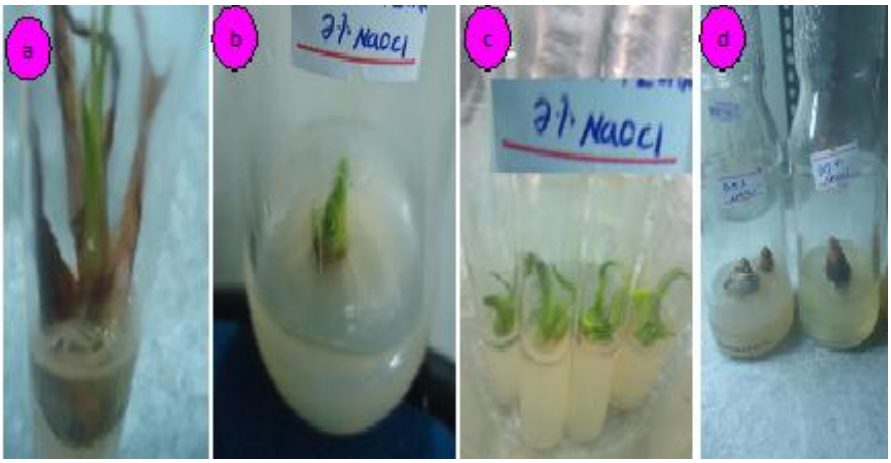

Figure 2. Response of cultures to different concentrations of NaOCl: (A) 1% sodium hypochlorite concentration and 5 minute exposure time results contamination (B and C) 2% sodium hypochlorite concentration and 15 minute exposure time results contamination free culture (D) 2.5 % sodium hypochlorite concentration and 20 minute exposure time results for tissue death of Taro explants.

4.2 Effect of different concentration and combination of BAP and Kinetin on shoot initiation from corm and sprout tip explants of *C. esculenta*

The analysis of variance obtained from the data (Appendix II) showed that the interaction with BAP and type of explant had very highly significant effect ($P < 0.0001$) on shoot induction rate. Interaction effect of explants type with BAP on rate of shoot induction was found to be highly significant (BAP*explants) (Appendix II A and B). The response of sprout tip and corm base explants in a given combination of BAP was not the same; corm explants gave higher response than sprout tip explants (Table 2).

Maximum shoot induction percentages were achieved on MS medium supplemented with 8 mg/l BAP alone with mean initiation percentage of 81.33±0.59 and 76.67±0.48 for corm explant and sprouts culture, respectively (Table 2). The culture medium devoid of phytohormones (control) failed to response to the bud break of sprout tip explants; mean no any sign of initiation was observed even after two months of culture period while the other explant, the corm were showed slight sign of proliferation after the same period of culture. It is a good indication for the importance plant growth regulators in *in-vitro* plant growth.

Of all basal media containing BAP and Kinetin as a combination, the high shoot induction percent were observed on MS media supplemented with 8 mg/l BAP and 2 mg/l Kinetin with mean present of initiation 65.00±0.55 and 55.00 ± 0.69 from corm and sprout tip explants, respectively. From all given combination, the minimum rate of shoot induction was recorded on MS medium containing 10 mg/l BAP and 3 mg/l Kinetin with an average present of 28.33 ± 0.84 % and 25.00± 0.47% for corm and sprout tip explants, respectively.

Table 2. The effects of different concentrations of BAP and Kinetin alone and in combination on MS medium for percentage of shoot induction of *C. esculenta* sprout tip and corm explants

Conc. of PGRS		Explant	
		corm	sprout tip
BAP (mg/l)	Kin (mg/l)	Mean±STD	Mean±STD
0	0	4.17^{s}±0.11	0.00^{t}±0.00
0	1	20.83^{r}±0.83	16.67^{s}±0.63
0	2	21.66^{r} ±0.41	18.33^{r}±0.83
0	3	23.33^{q}±0.48	20.00^{q}±1.58
4	0	66.67^{c}±0.44	63.33^{c}±0.46
4	1	43.33^{l}±2.31	31.67^{n}±0.48
4	2	45.00^{k}±0.57	38.33^{k}±0.84
4	3	41.66^{m} ±1.22	35.00^{m}±0.63
6	0	78.33^{b}±0.64	71.67^{b}±0.67
6	1	55.00^{i}±0.82	45.00^{i}±0.62
6	2	56.67^{h} ±3.15	46.67^{h}±0.81
6	3	53.33^{j}±0.23	43.33^{j}±0.80
8	0	81.33^{a}±0.59	76.67^{a}±0.48
8	1	63.33^{e}±2.51	50.00^{f}±0.82
8	2	65.00^{d}±0.55	55.00^{d}±0.69
8	3	61.00f±0.81	48.33^{g}±0.43
10	0	58.33^{g}±0.82	51.67^{e}±0.88
10	1	40.00^{n}±1.11	36.66^{l}±0.31
10	2	33.33^{o}±0.66	30.00^{o}±0.55
10	3	28.33^{p}±0.84	25.00^{p}±0.47
CV		1.24	2.38

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*

As indicated in the Table 2 above, in all cases, shoot bud proliferation increases with an increase level of BAP concentration alone from 0 to 8 mg/l for both explants. However, further addition of BAP concentration alone and in combination showed lower culture initiation percentage with poor bud breaking for both corm and sprout tip explants. Therefore, MS basal media containing 8.0 mg/l BAP alone were found to be optimum media for *in-vitro* shoot initiation of *C. esculenta* for both corm and sprout tip explants (Table 3). This indicated that BAP alone is more effective than kinetin alone and in combinations with kinetin for *in-vitro* initiation stage of Taro.

The result of this study is in accordance with the study of Chien-Ying *et al*. (2008) on MS media containing 8 mg/l of BA for shoot proliferation of Dasheen (*C. esculenta*) whereas slightly in contrast to the study of Alex (2007) who reported 10 mg/l of BAP as effective media for *in-vitro* initiation of Taro while in this study MS media containing above 8mg/l BAP showed low or poor sign of shoot induction rate for the selected genotype of Taro as used in this study. This deviation might be as a result of variation in genotype of the stock plant used. According to George *et al*. (2008), the effect of phytohormones used during *in-vitro* multiplication in plant tissue culture has been varied with plant genotype. Alex (2007) also similarly showed that response in shoot proliferation varied within the three Taro varieties namely: Dasheen, wild and eddoe, Dasheen typed variety showed better result in terms of initiation and shoot bud number comparatively with other stated varieties. Beyene (2009) showed difference in shoot induction percent for two varieties of cassava on the same media composition. According to him, the maximum average shoot induction percent (84.0 %) from 'Kello' and (82.76%) from 'Qulle', variety were obtained on media supplemented with 2mg/l BAP, 1mg/l GA3 and 0.01mg/l NAA. This indicated that shoot initiation performance varied with the particular genotype of the same species.

On the other hand, the lower percentage of shoot induction with increasing level of BAP above optimal concentration in accordance with the work of other root crops such as anchote (Bekele *et al*., 2013; Yambo and Feyissa, 2013), cassava (Beyene *et al*., 2010; Onuoch and Onwubiku, 2007) and sweet potato (Dugassa and Feyissa, 2010). Those reports stated that using BAP above a certain optimum concentration decreased the shoot production either by inhibition of shoot initiation or by encouraging callusing in the *in-vitro* culture plant. Here, such an effect was observed, when higher concentration of BAP (above 8 mg/l) alone was used.

Figure 3. *In-vitro* shoots induction of Taro on MS+8 BAP mg/l from corm explants (A -C) and sprout tip (D-F).

4.3. Effect of different concentration and combination of BAP and IAA on shoot multiplication of *C. esculenta*

The analysis of variances revealed that the concentration of BAP alone and in combination with IAA had very highly significant effect ($P < 0.0001$) on shoot multiplication rate (Appendix III).

In this study, the culture media devoid of phytohormones (control) showed minimal shoot multiplication rate with mean number of shoots per explant 1.93±0.3 and 1.63±0.06 from corm and sprout tip culture, respectively. On the other hand, media containing growth regulators showed better result in terms of average shoot and average shoot length/explants. Media supplemented different concentration of BAP alone exhibited different rate of multiplication.

Of all combination tested, the highest multiplication results were found on MS media contained 8 mg/l BAP and 3mg/l IAA, with mean number of shoots 8.53± 0.07 and 5.80±0.39 from corm and sprout tip explant, respectively (Table 3). With regard to length of shoots, longest shoots (6.7 ±0.10 cm) were also observed on the same media (Table 4 and Figure 5 A). The second best multiplication media was achieved from MS medium supplemented with 8 mg/l BAP and 2 mg/l IAA with a mean shoot number (7.67±0.14) with average length of (5.87±0.14 cm) from corm explant. For sprout tip explant, an average shoot (5.13±0.38) and mean length (5.27±0.03cm) was recorded on the same media (Table 3 and Figure 5).

Table 3. Effect of different concentrations and combinations of *BAP and **IAA treatments on shoot multiplication of *C. esculenta*.

Levels of PGR		Corm		Sprout tip	
BAP (mg/l)	IAA (mg/l)	Shoot number Mean ± Std Dev	Shoot length Mean ± Std Dev	Shoot number Mean ± Std Dev	Shoot length Mean ± Std Dev
0	0	1.93^{m}±0.30	1.57^{o}±0.21	1.63^{l}±0.06	1.30^{k}±0.3
0	1	2.87^{l}±0.21	2.00^{no}±0.20	1.83^{kl}±0.15	1.45^{k}±0.09
0	2	2.93^{l}±0.12	2.34^{nm}±0.07	2.00^{kjl}±0.00	2.24^{ji}±0.0.24
0	3	3.00^{l}±0.00	2.67^{lmk}±0.15	2.20^{kjl}±0.10	2.30^{ji}±0.17
2	0	3.53^{k}±0.15	2.80^{jlmk}±0.20	2.27^{jhi}±0.08	2.44^{ji}±0.80
2	1	3.67^{jk}±0.15	3.00^{jlik}±0.22	2.33^{jhi}±0.28	2.50^{ghi}±0.30
2	2	3.43^{k}±.12	3.37^{jih}±0.20	2.33^{kjhi}±0.29	2.87^{fihg}±0.30
2	3	3.73^{jk}±0.12	3.4^{ih}±0.21	2.40^{jhi}±0.00	3.20^{feg}±0.40
4	0	3.83^{jk}±0.15	3.54^{gih}±0.12	2.43^{jhi}±0.15	3.43^{feg}±0.15
4	1	3.87^{jk}±0.15	3.83^{gfh}±0.15	2.43^{jhi}±0.21	3.83^{de}±0.16
4	2	3.87^{jk}±0.21	3.5^{gih}±0.0.46	2.53^{ghi}±0.15	3.5^{fe}±0.46
4	3	4.0^{jk}±0.16	3.8^{gfh}±0.17	2.6^{ghi}±0.09h	3.83^{de}±0.15
6	0	4.15^{ji}±0.11	3.6^{gih}±0.20	2.67^{bhi}±0.07	3.52^{fe}±0.07
6	1	4.67^{hg}±0.29	3.8^{gfh}±0.17	2.77^{gh}±0.05	3.47^{feg}±0.16
6	2	5.67^{f}±0.15	4.63^{dce}±0.15	3.90^{e}±0.01	4.6^{c}±0.10
6	3	6.00^{ed}±0.00	5.00^{c}±0.24	4.43^{d}±0.12	4.63^{c}±0.15
8	0	6.13^{e}±0.06	4.20^{fe}±0.19	4.53^{cd}±0.15	4.2^{dc}±0.53
8	1	7.27^{cb}±0.02	4.80^{dc}±0.20	4.93^{cb}±0.10	4.37^{dc}±0.06
8	2	7.67^{b}±0.14	5.87^{b}±0.14	5.13^{b}±0.38	5.27^{b}±0.03
8	3	8.53^{a}±0.07	6.7^{a}±0.10	5.80^{a}±0.39	5.56^{a}±0.21
10	0	3.83^{jk}±0.20	3.2^{jik}±0.17	2.20^{kjl}±00	3.13^{fhg}±0.32
10	1	4.93^{g}±0.06	4.37^{dfe}±0.21	3.37^{f}±04	3.4^{feg}±0.06
10	2	6.67^{d}±0.21	3.82^{gfh}±0.14	4.00^{e}±00	3.47^{feg}±0.15
10	3	7.07^{c}±0.29	4.07^{gf}±0.31	4.54^{cd}±0.31	2.9^{fihg}±0.10
12	0	2.73^{l}±0.25	2.8^{jlmk}±0.2	2.16^{jhi}±0.15	2.3^{4ji}±0.21
12	1	3.53^{k}±0.08	3.13^{jlik}±0.17	$2.5g^{jhi}$±0.10	2.8^{jhi}±0.26
12	2	3.87^{jk}±0.15	3.0^{jlik}±0.27	2.57^{ghi}±0.03	2.5^{jhi}±0.10
12	3	4.47^{hi}±0.11	2.6^{lm}±0.27	2.97^{gf}±0.09	2.2^{j}±0.11
CV		3.64	5.57	5.59	6.96

(*BAP = 6-Benzylaminopurine; **IBA = Indole-3-acetic acid). Data are given as means ± SD; *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)*

As indicated in Table 3 above, with increase the concentration of BAP alone from zero to 8 mg/l, an increase in number of shoots per shoot bud culture was observed. This might be due to the effect of cytokinin (BAP) in releasing lateral bud shoots through breaking apical dormancy by inhibiting the effect of high level of edophytic auxins (Hailu *et al*., 2013). The highest shoot multiplication result in terms of shoot number and length per explant from MS media supplemented with 8 mg/l BAP and 3 mg/l IAA agrees with the report of Chien-Ying *et al*. (2008) using 8 mg/l of BA and 3mg/l IAA. This is however, slightly in contrast to earlier reports in *C. esculenta* by Alex (2007) as reported that better proliferation occurred on medium containing 10 mg/l of BAP with mean number of 5.9 shoots per explants. This difference might be as a result of the genotype plant used. On the other hand, further increases in BAP concentration to 10 mg/l and 12 mg/l, shoots become few in number, very short and starts to develop abnormal morphological appearance of stem and leaves. This might be resulted from the use of higher level of cytokinins (BAP) above the optimum level. The physiological effects using higher concentration of BAP on plants put by Barrie (1984) who reported that synthetic cytokinins are inhibitory to shoot growth at high concentration (above optimum). The inhibition of *in-vitro* growth with increasing BAP concentration above certain optimal concentration is in accordance with the micropropagation works on other tuber crops such as anchote (Bekele *et al*., 2013; Yambo and Feyissa, 2013), cassava (Beyene *et al*., 2010; Onuoch and Onwubiku, 2007) and sweet potato (Dugassa and Feyissa, 2010).

From combination media tested, medium containing 8 mg/l of BAP with 2 -3 mg/l IAA elicited optimal response in terms of average shoot numbers and length as compared to media containing only BAP, of which, MS media containing 8 mg/l BAP and 3mg/l IAA showed a relatively highest response in terms of quality shoots, average shoots and length, in which, shoots with very good morphological appearance (reasonable shoot height, stem thickness and leaf structure in comparison with the other combinations) were obtained. A comparatively lower response in shoot number, average height and bad-looking shoots were observed when BAP at higher concentration combined with higher auxins concentration (Figure 4 G and H). This might be related with the interacting effect of the two growth regulators at this level of concentration or due to the use of supra-optimal concentration of BAP. Here combination of a relatively lower auxin with a relatively higher cytokinin promote the shoot number and shoot height on this stage.

The average number of shoots and length obtained from the present study were in agreement with the work of Chien-Ying *et al.* (2008) who recorded an average of 5.9 shoots per explants using MS + 8 mg/l of BA + 3 mg/l IAA while in this work, using BAP instead of BA in combination with 3 IAA mg/l an average number of shoots 8.53 with an average length of shoot 6.7 cm was recorded. It is also near to the reports in *C. esculenta* (cv. local Jhankhri) by Kambaska and Sahoo (2008), who recorded an average shoot of 6.5±0.4 and mean plantlet length of 5.2 ± 0.33 cm from MS media supplemented with 3 mg/l BAP and 1mg/l IAA. A medium with free growth regulators and a medium with high concentration of BAP alone and in combination with IAA resulted in low multiplication rate (Table 4). Length of shoots that were obtained from sprout tip explant was slightly shorter than from corm base explants.

Adding IAA in multiplication media improved response in shoot numbers and length for Coccame species (*C. esculenta and X. sagittifolium*) Seetohul and Puchooa (2005); Bari and Paul (2007); Kambaska and Sahoo (2008); Virendra and Cho (2010) and Chien-Ying *et.al.* (2008).

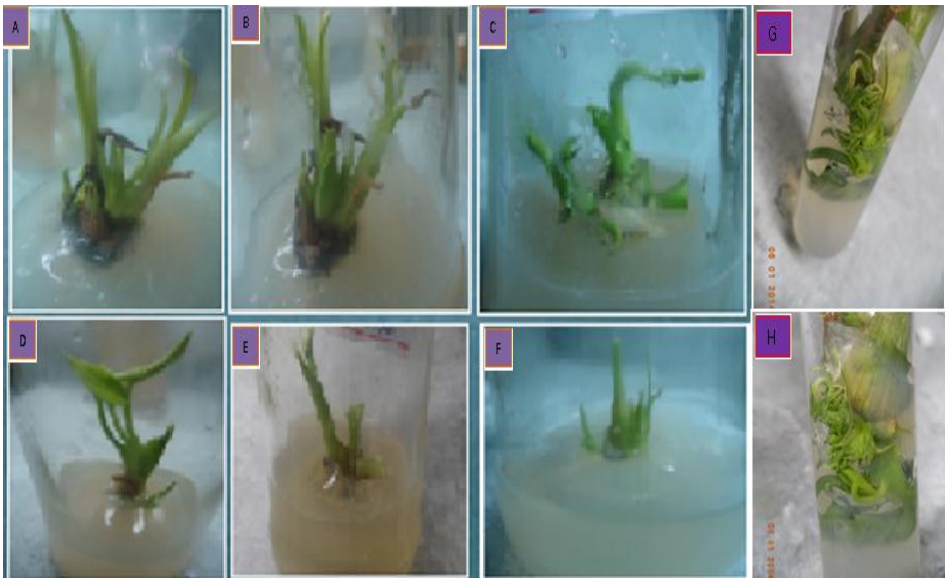

Figure 4: *In-vitro* shoot multiplication of *C. esculenta.* cv. Bolosso I (A, B, C and D) shoots multiplied on 8 mg/l of BAP and 3 mg/l IAA from corm base culture; (E and F) shoots multiplied from sprout tip culture and (G and H) callusing and stunted shoots at the base of explants grown on 12 mg/l BAP and 3mg/l IAA.

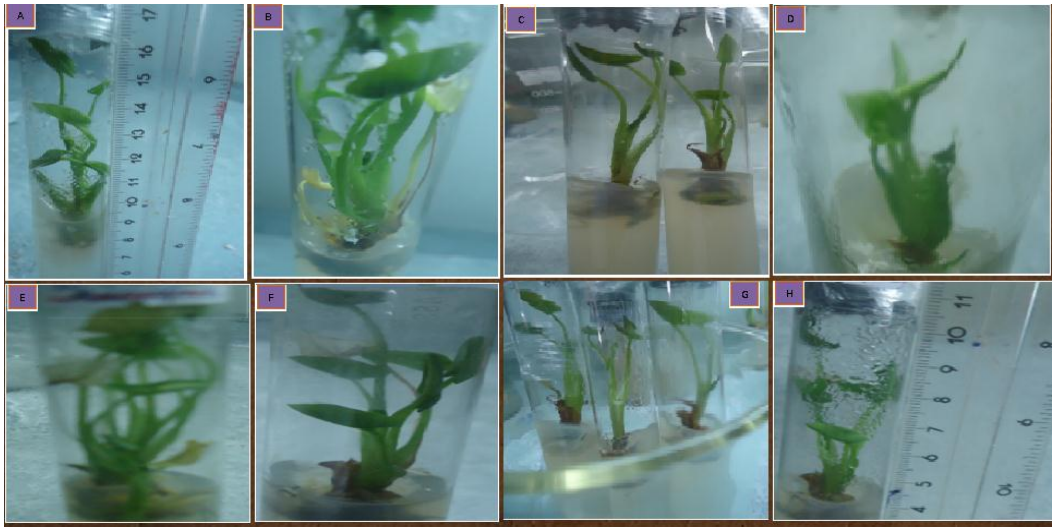

Figure 5 *In-vitro* shoots elongation of *C. esculenta* cv. Boloso I on 8 BAP + 3 IAA (A- F) from corm explant and (G and H) from sprout tip explant.

4. 4. Effect of different concentrations of IBA, IAA and NAA on root regeneration of *C. esculenta*

The analysis of variance indicated that a very highly significant effect of half strength MS media with different growth regulators (1/2MS*IAA, P<0.0001; 1/2MS*NAA, P<0.0001; 1/2MS*IBA, P<0.0001) on *in-vitro* rooting of Taro (*C. esculenta*) (Appendix IV).

In the present study, the rooting response in terms of mean root number, root length and regenerated shoot heights in different rooting media was not the same (Table 5). Shoots inoculated on hormone free (medium lacking growth regulators) had no rooting response. MS medium containing IBA at higher concentration (1.5 mg/l) resulted lowest root initiation percentage (38.75 ±4.78%). From all root inducing hormone (auxins), IAA at lower concentration (0.5 mg/l) showed highest rooting response (86.25±2.5%) with mean number of 6.90±0.09 roots per plantlets (Table 4). The second highest response (78.75±2.5%) was recorded from ½MS media supplemented with 1mg/l of NAA with an average root numbers of 5.80±0.23 per cultured explant.

In case of root length and shoot heights, the longest root (11.25±0.5 cm) were obtained from a medium supplemented with 1.5 mg/l IAA followed by 1mg IAA with average root length of 9.63±0.48 cm. Smallest mean root length (2.30±0.26 cm) was obtained from 1.5 mg/l of IBA.

For height of the regenerated shoots on the rooting media, ½ MS augmented with 0.5 mg/l IAA with mean height of 6.20±0.16 cm followed with 1mg/l of IAA with an average shoot height of 5.50±0.18cm were recorded per regenerated plantlet after a month of culture.

Table 4. Mean root number, root length and shoot height produced per Taro plantlet in different auxins on half - strength MS rooting media.

Conc. of PGRs (mg/l)			Rooting percent (Mean ± SD)	Shoot height (cm) (Mean ± SD)	Root number (Mean ± SD)	Root length(cm) (Mean ± SD)
IBA	NAA	IAA				
0	0	0	0.00^{h}±0.00	2.2^{g}±0.28	0.00^{g}±0.00	0.00^{j}±0.00
0	0	0.5	86.25^{a}±2.5	6.20^{a}±0.16	6.90^{a}±0.09	6.93^{c}±0.09
0	0	1	70.00^{c}±4.08	5.50^{b}±0.18	5.40^{cb}±0.16	9.63^{b}±0. 48
0	0	1.5	57.50^{e}±2.88	5.10^{cb}±0.24	3.85^{d}±0.19	11.25^{a}±0.5
0	0.5	0	61.25^{de}±2.5	4.15^{e}±0.18	4.95^{c}±0.19	5.90^{d}±0.19
0	1	0	78.75^{b}±2.5	4.25^{ed}±0.14	5.80^{b}±0.23	5.20^{e}±0.16
0	1.5	0	67.5^{dc}±2.89	4.70^{cd}±0.25	5.30^{c}±0.35	4.20^{f}±0.18
0.5	0	0	47.59^{f}±6.45	4.40^{ed}±0.26	3.40^{e}±0.33	3.6^{g}±0.32
1	0	0	45.00^{gf}±4.08	3.50^{f}±0.24	3.15^{e}±0.29	3.10^{h}±0.20
1.5	0	0	38.75^{g}±4.78	3.30^{f}±0.27	2.60^{f}±0.26	2.30^{j}±0.26
CV			6.6	4.95	5.46	5.38

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 - Gabriel - Welsch Multiple Range Test (REGWQ)

As clearly indicated in Table 4 above, root initiation of cultured shoot of *C. esculenta* were influenced by type of auxins and concentration. IAA at lower level of concentration vigorous root looking with highest shoot number was given. Root initiation percent as well as number of roots per culture increased with increase IAA levels zero up to 0.5 mg/l IAA and then ceased. Low level of IAA (0.5 mg/l) initiated the highest percentage of root (86.25±2.5%) as compared to higher level of IAA (57.50±2.88%). The maximum average number (6.90±0.09) of roots was produced at 0.5 mg/I IAA (Figure 6 A).

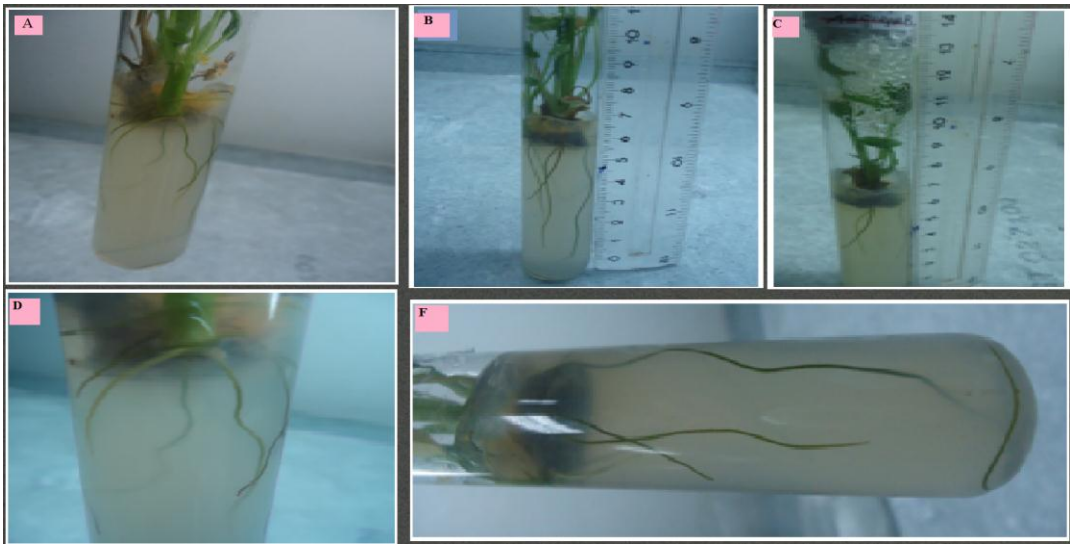

Figure 6. *In-vitro* rooted micro shoots of *C. esculenta.* L. cv. Bolosso I after four weeks of culture on the rooting media (A) ½ MS + 0.5 mg/l IAA (B) longest root on ½ MS + 1.5 IAA (C) low root number on ½ MS + 1.5mg/l IBA (D) near view of (A) and (F) bottom view of (B).

Rooting response in terms of root percent and average root number from ½ MS+0.5 mg/l IAA media agrees with the work of Bhuiyan *et al.* (2011) in *C. esculenta,* who reported that low level of IAA (0.5 mg/l) initiated the highest percentage of root (49.71%) as compared to higher level IAA (34.98%) with maximum number (3.63) of roots per shoot lets. The result in root initiation and average root numbers per plantlets were higher in this study as compare to the earlier report. This variation in figure might be due to the duration of culture stayed on the rooting media. In previous study, rooting status has been recorded after 10 days of culture while here in this study rooting response was recorded after a month of culture on the rooting media. An increasing in root numbers and length per plantlet with duration of time on rooting media has been indicated in micropropagation work of Yambo and Tileye in *Coccinia abyssinica* (2013).

Verma and Cho (2010) also similarly reported for IAA as effective growth hormone for *in-vitro* root regeneration of *C. esculenta* with 100% rooting on MS media contained 0.35mg/l IAA with mean number of 10±5 roots and 8-15 cm root length per plantlets. The result of this study also near to the study on other Coccame species, *X. sagittifolium* by Bari and Paul (2007) reported 90% root proliferation with an average root number 7.9±0.37 and root length of 6.9±0.22cm per plantlets was obtained from MS media containing 0.4 mg/l IAA.

The effect of different levels of NAA on root initiation for shoot of *C. esculenta* cv. Bolosso I also presented in Table 4. Like IAA, NAA also enhanced rooting in *in-vitro* cultures. Table 5 showed that root initiation percent was increased with an increase of NAA level from zero up to 1.0 mg/l, and then it was declined. The highest root initiation (78.75±2.5%) was given by the treatment of 1.0 mg/l NAA, whereas, 1.5 mg/l NAA gave lower percentage of roots (67.50±2.89%) (Table 5). It is clear that auxins is responsible for root initiation, auxins up to a certain level enhanced rooting but excess auxins might work as anti-auxins activity and reduced root initiation percent (Hartmann *et al*., 2002; Hailu *et al*., 2013).

Similarly, number of roots increased with an increase of NAA level up to 1.0 mg/l. The highest average number of 5.80±0.23 roots/culture was obtained from ½ MS medium containing 1.0 mg/l NAA. The highest level of NAA (1.5 mg/l) reduced the number of roots/culture (5.30±0.35). This might be due to anti-auxin effect of excess auxin (Hartmann *et al*., 2002). The effects of NAA on rooting status (both rooting percent and number of roots per culture) increased with level of NAA up to 1mg/l is in accordance the micropropagation work of Bhuiyan *et al*. (2011) in *C. esculenta* var. globulifera. But, it is slightly against the study of Kambaska and Sahoo (2008) in *C. esculenta* cv. Jhankhar, who reported higher level of NAA (2 mg/l) showed highest result (95% rooting response) and average roots of 6.4±0.30 per culture. This deviation might be as a result of the particular genotype of the stock plant used (Aggarwal and Barna, 2004).

In this study, root regeneration performance for *C. esculenta* cv. Bolosso I using IBA also tested. In all case, IBA was not effective and showed lowest result (47.59±6.45%) on initiation as compared to IAA and NAA. Better effect of both IAA and NAA reported for different cultivars of Taro in different authors (Kambaska and Sahoo, 2008; Bhuiyan *et al*., 2011; Verma and Cho, 2010 and Paul and Bari, 2007). In the present study also IAA, NAA at lower concentration (0.5 up to 1 mg/l) showed optimum result whereas concentrations above 1mg/l is supra-optimal for *in-vitro* root regeneration for micropropagation of Taro (*C. esculenta* cv. Bolosso I). Such protocol in *in-vitro* rooting is important for the successful establishment of regenerated Taro plantlets in the soil and finally useful for the improvement of other related root crops through modern biotechnology.

4. 5 Acclimatization of Taro plantlets

The results indicated that plantlets could be survived on all tested media with good overall percent of survival (76.67%). Although the survived plantlet performance is good looking in all case, the different ratio of those soil types showed various survival percentages. Soil media containing sand, red soil and coffee husk in the ratio of 2: 1: 2 showed highest survival rate (80%) as compared to other ratio of the same soil type (Table 5 and Figure 7C).

Table 5. Effect of soil media combination on the survival rate of Taro plantlets during acclimatization.

Potting soil media	Soil media ratio	Survival plantlets (%)
Sand + Red +Coffee husk	2:1:2	80
	1: 2: 2	75
	1: 1: 1	75
Total %		76.67

Survival plantlet (%) = (Survival plantlets /Total plantlets) x 100.

Figure 7. Acclimatization of *in-vitro* grown Taro plantlets; plantlets ready for acclimatize (A) plantlets covered in polyethylene bag (B); survived plantlets after three week (C) and plantlets after six week of acclimatization (D).

The above result might be a good indication that Taro needs fertile soil as it is cultivated closer to residence and naturally grown to riverside areas where different compost and sandy soil are available. The requirement of fertile soil (soil with dung) and aerated soil such as sandy soil for hardening of Taro during acclimatization is also stated by Chien-Ying *et al*. (2008). Fujimoto (2009) also reported that the need of such soil combination during cultivation of Taro under natural field condition.

CHAPTER FIVE

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In the present study, optimum micropropagation protocol for sterilization, shoot initiation, multiplication, root induction and acclimatization for Taro (*C. esculenta* cv. Bolosso I) were developed. According to the findings of the present experiments the following conclusions are drawn from the study;

- Surface sterilization with NaOCl (2.0% for 15-minutes) with 70% alcohol dip was best for surface sterilization of the Taro explants.
- Best shoot proliferation 81.33±0.59 % for corm and 76.67±0.48 % for sprout tip culture was achieved on MS medium containing BAP 8.0 mg/l alone.
- The combination media of BAP and Kinetin was lower than BAP alone in shoot induction of Taro.
- Corm explants gave better initiation performance than sprout tips culture, thus better to use corm explant during *in vitro* multiplication of Taro.
- The highest number of shoots (8.53± 0.07) and (5.80±0.39) was observed on an MS medium supplemented with 8 mg/l BAP and 3 mg/l IAA from corm and sprout tip explants, respectively.
- For both corm and sprout tip explants, MS basal media containing 8 mg/l BAP and 3 mg/l IAA were found to be optimum media for *in-vitro* shoot multiplication of Taro.
- Best rooting status (rooting %, root number and shoot heights were achieved on half strength MS basal media containing 0.5 mg/l IAA with followed by the same strength MS basal media containing 1 mg/l NAA
- From all rooting media half MS media containing 1.5 mg/l IAA gave longest mean root length (11.25±0.5 cm).
- Both auxin types (IAA and NAA) gave better response interms of root percent, number, and length than MS media containing IBA for *in-vitro* rooting of Taro.

- Those plantlets well performed *in-vitro* showed 80 % survival efficiency after hardening and acclimatization on soil media mix ratio of 2:1:2, sand, red soil and coffee husk, respectively.
- Green house acclimatized Taro plants were found to be morphologically similar to the mother plant (control plant) based on visual observation.

Thus, this protocol could be useful for large-scale production of highly demanded Taro cultivar true-to-type and provide a possible system towards genetic improvement of the crop using corm, sprout tips and other related explant sources.

5. 2 Recommendations

Based on the objectives and result of this study the following points as recommendations are drawn;

- Low cost and locally available sterilant for surface sterilization such as berekina should be tried.
- Using powder media in tissue culture is costy, other low cost culture media from stock solution should be tried.
- One advantage of plant tissue culture is production of virus free plantlets so; in the future studies on meristem culture should also be given attention and incorporated.
- Proper hardening procedure can be tried to address maximum field establishment in Taro plantlets raised through *in-vitro* technique.
- Using the developed protocol, Taro plantlets should be multiplied and distributed to farmers for large scale production.

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APPENDICES

I Sterilization experiment: SAS out put

Appendix I: Summary of analysis of variance for the interaction effect of NaOCl with Exposure time on percentage of clean alive culture, tissue death and contamination of *C. esculenta* sprout tip and corm base explants

Appendix I A: Summary of ANOVA table for the interaction effect of NaOCl with Exposure time on percentage of clean alive culture from corm explants of *C. esculenta*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Na	3	8343.34	2781.11**	5102.04	<.0001
Dur	3	555.71	185.23**	339.82	<.0001
Na*Dur	9	1153.13	128.12**	235.05	<.0001

R - Square = 0.99 CV % = 1.81, mean =40.77, ** = highly significant at α = 5 %, DF = degree of freedom

**= 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 clean alive culture from sprout tip explants of *C. esculenta*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Na	3	12171.29	4057.09	8585.56	<.0001
Dur	3	155.95	51.98	110.01	<.0001
Na*Dur	9	3417.20	379.68	803.49	<.0001

R - Square = 0.99 CV % = 1.41, mean =48.83, ** = highly significant at α = 5 %, DF = degree of freedom

**= 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 contamination from sprout explants of *C. esculenta*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Na	3	39365.66	13121.89**	49904.3	<.0001
Dur	3	1270.33	423.44**	1610.42	<.0001
Na*Dur	9	619.01	68.78**	261.57	<.0001

R - Square = 0.99 CV % = 1.46, mean =35.09, ** = highly significant at α = 5 %, DF = degree of freedom

**= 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 contamination from corm explants of *C. esculenta*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Na	3	38789.35	12929.78**	52293.3	<.0001
Dur	3	2229.35	743.11**	3005.48	<.0001
Na*Dur	9	350.40	38.93**	157.46	<.0001

R - Square = 0.99 CV % = 1.27, mean =39.03, ** = highly significant at α = 5 %, DF = degree of freedom

**= 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 sprout tip explants of *C. esculenta*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Na	3	17806.25	5935.41**	15774.2	<.0001
Dur	3	1222.91	407.63**	1083.36	<.0001
Na*Dur	9	1135.41	126.15**	335.28	<.0001

R - Square = 0.99 CV % = 3.96, mean =15.49, ** = highly significant at α = 5 %, DF = degree of freedom

**= 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 corm base explants of *C. esculenta*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Na	3	16342.33	5447.44**	13652.1	<.0001
Dur	3	555.66	185.22**	464.19	<.0001
Na*Dur	9	387.0	43.00**	107.76	<.0001

R - Square = 0.99 CV % = 3.13, mean =20.15, ** = highly significant at α = 5 %, DF = degree of freedom

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

II. Shoot initiation experiment SAS output

Appendix II: Effect of different levels of BAP and kinetin on shoot induction of Taro (*C. esculenta*)

Appendix II A: Summary of ANOVA table for the interaction effect of BAP with Kinetin on percentage of shoot initiation from corm explant of Taro (*C. esculenta*)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BAP	4	13647.73248	3411.93312	7894.00	<.0001**
KIN	3	35855.38562	11951.79521	27652.2	<.0001**
BAP*KIN	12	5738.62236	478.21853	1106.43	<.0001**

R - square = 0.99 CV % =1.29, PSINDUC=50.70, ** = highly significant at α = 5 %, DF = degree of freedom, PSINDUC= % shoot initiation, explant = corm and sprout 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 sprout tip explant of Taro (*C. esculenta*)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BAP	4	13651.60783	3412.90196	2263.57	<.0001**
KIN	3	33994.55434	11331.51811	7515.49	<.0001**
BAP*KIN	12	33994.55434	409.54227	271.62	<.0001**

R - square = 0.99 CV % = 2.38, PSINDUC =51.42, ** = highly significant at a = 5 %, DF = degree of freedom, PSINDUC = % shoot initiation, explant = corm and sprout tip explants

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

III. Shoot multiplication experiment SAS Output

Appendix III: shoot multiplication

Appendix III A: Summary of ANOVA table for the interaction effect of BAP with IAA on percentage of shoot number from corm explant of Taro (*C. esculenta*)

Source	DF	Type III SS	Mean Square	F -Value	Pr > F
BAP	6	181.68	30.28	1106.10	<.0001**
IAA	3	23.78	7.92	289.56	<.0001**
BAP*IAA	18	17.76	0.98	36.05	<.0001**

R - square = 0.99 CV % = 3.64, mean of shoot number =4.55, ** = highly significant at α = 5 %, DF = degree of freedom, PESHN= % 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 BAP with IAA on percentage of shoot length of from corm explant of Taro (*C. esculenta*)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BAP	6	78.71	13.11	325.75	<.0001**
IAA	3	9.91	3.30	82.10	<.0001**
BAP*IAA	18	10.93	0.60	15.08	<.0001**

R - square = 0.98 CV % =5.54, mean of shoot length =3.62

** = highly significant at α = 5 %, DF = degree of freedom, PESHL= % of shoot length

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

Appendix III C: Summary of ANOVA table for the interaction effect of BAP with IAA on percentage of shoot number of from sprout tip explant of Taro (*C. esculenta*)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BAP	6	83.23	13.87	474.67	<.0001**
IAA	3	11.77	3.92	134.30	<.0001**
BAP*IAA	18	8.18	0.45	15.56	<.0001**

R - square = 0.98 CV % = 5.59, PESHN Mean=3.05

** = highly significant at a = 5 %, DF = degree of freedom, PESHN = % of shoot number

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

Appendix III D: Summary ANOVA table for the interaction effect of BAP with IAA on percentage of shoot length from sprout tip explant of Taro (*C. esculenta*)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BAP	6	85.56	14.26	279.24	<.0001**
IAA	3	7.42	2.47	48.49	<.0001**
BAP*IAA	18	12.12	0.67	13.19	<.0001**

R - square = 0.97 CV % = 6.86, mean of shoot length =3.29, ** = highly significant at α = 5 %, DF = degree of freedom, PESHL= % of shoot length

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

IV. SAS output- root induction experiment

Appendix IV: rooting

Appendix IV A: Summery of ANOVA table for the interaction effect of NAA, IAA and IBA on rooting percentage of *C. esculenta* (Taro)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
NAA	3	14031.25	4677.08	350.78	<.0001***
IAA	3	17517.18	5839.06	437.93	<.0001***
IBA	3	5904.68	1968.22	147.62	<.0001***

R - square = 0.99 CV % = 6.6, mean of rooting percentage =55.25, *** = highly significant at α = 5 %, DF = degree of freedom, PSRINN = percentage of root initiation

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

Appendix VI B: ANOVA table for the interaction effect of NAA, IAA and IBA on root number of Taro (*C. esculenta*)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
NAA	3	87.3275000	29.1091667	570.77	<.0001***
IAA	3	105.5475000	35.1825000	689.85	<.0001***
IBA	3	29.2475000	9.7491667	191.16	<.0001***

R - square = 0.99 CV % = 5.46, mean of root number=4.13, *** = highly significant at α = 5 %, DF = degree of freedom, PESRN = % of root number

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

Appendix IV C: ANOVA table for the interaction effect of NAA, IAA and IBA on root length of Taro (*C. esculenta*)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
NAA	3	84.70	28.23	358.17	<.0001***
IAA	3	295.79	98.59	1250.72	<.0001***
IBA	3	30.78	10.26	130.18	<.0001***

R - square = 0.99 CV % = 5.38, mean of root length = 5.22, *** = highly significant at α = 5 %, DF = degree of freedom, PESRL = % of root length

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

Appendix IV D: ANOVA table for the interaction effect of NAA, IAA and IBA on shoot height of Taro (*C. esculenta*)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
NAA	3	14.77	4.92	107.03	<.0001***
IAA	3	37.16	12.38	269.28	<.0001***
IBA	3	9.80	3.26	71.01	<.0001***

R - square = 0.97 CV % = 4.95, mean of shoot length =4.33 *** = highly significant at α = 5 %, DF = degree of freedom, PESRL= % of shoot length

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