ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL SCIENCES

SCHOOL OF GRADAUTE STUDIES

BIOLOGY PROGRAM

IN VITRO PROPAGATION OF KORARIMA (*AFRAMOMUM CORRORIMA)* FROM SHOOT EXPLANTS


A Thesis Submitted to School of Graduate Studies, Addis Ababa University, in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

By

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ADDIS ABABA UNIVERSITY

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LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of Variance
CW	Coconut Water
C/N	Carbon to Nitrogen ratio
BA	N-Benzyleadenine
BAP	6-Benzylaminopurine
IAA	Indole -3-acetic acid
IMA	Imazalil
IBA	Indol-3-butyric acid
MS	Murashige and Skoog
KIN	Kinetin
LSD	Least Significance Difference
NAA	α-Naphthalene acetic acid
PGR	Plant Growth Regulator
PPFD	Photosynthetic Photon Flux Density
TDZ	Thidiazuron

ABSTRACT

This study with a title of in vitro propagation of Aframomum corrorima from shoot explant is done by Teferi Mekonen at Addis Ababa University, inistitute of biotechenology. Korarima (Aframomum corrorima (Braun) P.C.M. Jansen) is herbaceous perennial plant that belongs to the family Zingiberaceae. It is an indigenous spice crop to Ethiopia. Lack of a steady supply of quality planting material is one of the bottlenecks in the production of this crop for local consumption and export. Therefore, the objective of this study is to develop protocol for in vitro propagation of A. corrorima, by using shoot tip as explant for mass propagation. Shoot tips were cultured in MS medium supplemented with Thidiazuron (TDZ) in combination with α-Naphthalene acetic acid (NAA) or Indol-3-acetic acid (IBA) to investigate their effect on shoot multiplication. The multiple clums of shoots were then separated as individual shoot and well developed shoots transferred in to 1/2 MS medium supplemented with NAA, IAA and IBA for root induction. Statistical analysis revealed that there was significant difference among treatments applied in both shoot multiplication and rooting experiments. There was also 100% initiation of shoot after first week of culture in all treatment of shoot multiplication. Average Maximum numbers of shoots per explant (2.97 ± 1.43) were obtained on MS medium containing 0.5 mg/l of TDZ in combination with 0.25 mg/l IBA. The highest numbers of mean roots per shoot (7.13 ± 5.21) was obtained on medium containing 1.0 mg/l of NAA and 0.5 mg/l of IAA combinations. After acclimatization, 86% plants survived in greenhouse.

Key words: Growth regulators, Rooting, Shoot initiation, Shoot multiplication,

1. NTRODUCTION

Korarima (*Aframomum corrorima* (Braun) P.C.M. Jansen), which is indigenous to Ethiopia, is one of the renowned spices and medicinal plants of the family Zingiberaceae. It is herbaceous, perennial and aromatic species classified under the monocotyledonous crops. Morphologically, the plant resembles Indian cardamom (*Elettaria cardamomum*) and consists of an underground rhizome, a pseudostem and several broad leaves (Eyob Solomon, 2009). Mature korarima plants can reach a height of 1-2 m and set seeds after 3-5 years of planting, and it continues to bear seeds for several years. Korarima exists as a cultivated crop only in Ethiopia (Eyob Solomon, 2009). Different plant parts of korarima (e.g. seeds, pods, leaves and flowers) are locally used in traditional medicine to treat humans and cattle as well. Since its rhizomes and leaves spread well on the ground, korarima plants could also serve a lot for soil and water conservation, especially by covering and protecting the soil from erosion and drying all year round in the mountainous areas where the crop is commonly found (Eyob Solomon *et al*., 2008).

The production of korarima is continuously decreasing mainly due to absence of expansion of their cultivated areas and destruction of their forest natural habitat, which had been and still is the major source of korarima production. In line with this, results from formal survey carried out in parts of Southern Ethiopia, attempts of the Ethiopian Government to motivate farmers to expand their korarima plantations were not successful due to the associated varied production constraints. This mainly includes lack of improved korarima varieties with high yielding potential and product quality, together with suitable agronomic practices, like best techniques of propagation (Jansen, 2002; Eyob Solomon, 2009). If suitable agronomic practices are applicable, the yield of dried capsules of 500 - 800 kg per ha without fertilizers application can be obtained (Jansen, 2002; Endashaw Bekele, 2007).

Since korarima is shade loving spice, plant shade level management is one of the key agronomic practices in its production and management. As to other plants, korarima has its own cultural practices for its propagation either by seed or by cutting of its clumps, though the latter is the most common method, as it yields earlier and ensures a true-to-type propagation than the former. During *in vivo* propagation of korarima, the suitable propagation technique is by using seed. However, vegetative propagation through cuttings results in the destruction of the productive garden, on top of the commonly associated shortage of planting materials to cover wider areas of land. Consequently, seed propagation of korarima is undertaken to cover large areas of land retaining the mother productive stand intact, however, it is essential to give the utmost care while preparing the seeds. Therefore, well ripened korarima capsules are selected and collected during the peak harvest season and its seeds are removed and rubbed with ash to facilitate their drying and protect fungal development, as well as to ease their handling during sowing (Endashaw Bekele, 2007).

In the course of drying, the seeds are kept under shade for four to five or more days, prior to sowing in polyethylene bags filled with forest soil or directly on seed beds. Germination and seedling emergence takes from one to two months. Later on, the seedlings will be transplanted to their permanent field at a spacing of 2 x 2 m with depth and width of 30-45 cm after eight to nine months of sowing at the commencement of the main rains (Endashaw Bekele, 2007).

The slow seed germination and growth of the subsequent seedlings are concerns of korarima growers. The germination of korarima seeds faces certain problems due to the presence of some kind of dormancy, possibly associated with its hard seed coat like that reported about seeds of *Elettaria* species (Sulikeri and Kololgi, 1977). The presence of low food reserve in the seed endosperm might be a reason for the very slow growth of korarima seedlings.

Therefore, enhancement of korarima seed germination is essential in propagation and breeding program, as well as for testing and using available germplasm. To make the hard seed coat of korarima permeable to water and/or gases, and hence facilitate its germination, some forms of seed treatments were applicable. Among these, soaking korarima seeds in 50% sulfuric acid (H_2SO4) for 60 min followed by a 24 h treatment with 250 mg/l Gibberillic acid ($GA3$); and soaking in 50% H_2SO4 for 60 min are stated effective to break the dormancy on Mume cultivar of korarima (Eyob Solomon, 2009).

Vegetative propagation involving rhizome splits with one old and another young sucker, is the conventional technique used in korarima propagation. The method shortens the juvenile phase of the stand and also enables propagation of true –to- type plants of a desired clone. However, this particular technique is always accompanied with shortage of planting material to cover large areas of land and involves sacrifice of potential productive stands.

Development of micropropagation methodologies does not only enable production of sufficient amounts of planting material of a desired clone , but also the basis for future improvement through genetic engineering , as well as modern germplasm conservation tasks. Among the prominent members of the family Zingiberaceae, *in vitro* micropropagation has so far been successfully carried out on cadamon, large cardamon, ginger and turmeric. However, unlike its close relatives, no *in vitro* propagation method has been developed for korarima (Wondyifraw Tefera and Wannakrairoj, 2004).

2. LITERATURE REVIEW

2.1 Botanical Description of Korarima

The genus *Aframomum* comprises about 50 species and is widely distributed in the wetter parts of tropical Africa. It is closely related to *Aframomum* from tropical Asia and was formerly included in it. *Aframomum zambesiacan* (Baker) K. Schum occurs in similar habitats as *Aframomum corrorima*. The seeds of the former species, however, are not used, and in Ethiopia it is called 'monkey's korarima'. The two major differences with the real korarima are that its leaves are less aromatic upon crushing, and its inflorescences bear 25-50 flowers (korarima only up to 5) (Jansen, 1981).

Korarima **(***Aframomum corrorima* (Braun) P.C.M. Jansen) is grouped under the family Zingiberaceae with a chromosome number is not determined. There are other synonymous botanical names of korarima such as *Aframomum corrorima* Braun (1848), *Aframomum korarima* J. Pereira (1850), *Aframomum korarima* (J. Pereira) K. Schum. Ex Engl.(1908), *Aframomum usambarense* Lock (1976). On top of these, Jansen (2002) had also reviewed the different vernacular names of korarima, including Ethiopian cardamom, false cardamom (En) and korarima, cardamome d'Ethiopie, poivre d'Ethiopie (Fr). Locally, the plant is also known by different local names in different parts of the country, like ogi'o in Oromifa, korerima in Amharic, Offio in Kaffigna and korarima in Tigrigna.

2.2 Growth and Development of Korarima

In Ethiopia, korarima flowers between September and January. Fruits mature about 2-3 months after flowering. In Kefa province of Ethiopia, the main flowering period is June-July, with fruiting in September-October. Most probably the flowers are open for only one day. The position of the stigma in the flower, below the base of the thecae of the anthers, hints at

self pollination but there is neither observational nor experimental evidence. The presence of large nectaries at the top of the ovary makes insect visits probable. In many other *Aframomum* species, the stigma is situated at the top of the anther thecae and cross-pollination caused by insect is the rule, although the plants are self-fertile too. Animals such as monkey eat the aril around the seeds and certainly contribute to natural dispersal of the seeds (Jansen, 1981).

Korarima is a perennial, monocious, herbaceous and its rhizome reaches up to 1 cm diameter and has leafy stems 1-2 m long with aromatic pungent. The stem is unbranched and formed by the leaf sheathes subterete up to 1 cm in diameter but at the base it is thickened and reaches to 3 cm in diameter. The leaves are arranged in alternative form shield to each other. Its inflorescence is a shortly stalked head arisen from the rhizomes near the base of a leafy stem sometimes situated at the end of a rhizomatous runner, up to 5 flowers and peducle up to 7 cm long (Jansen, 1981). The flowers are covered by imbricate, purplish-brown, subovate scales of 2.5 cm × 1.5 cm and each flower is surrounded by a scarious, suboblong bract up to 6 cm × 2 cm, bidentate, ciliate. The fruit is an indehiscent and subconical berry up to 6 cm × 3.5 cm in size usually it shows 3 longitudinal furrows, but sometimes it gives more shiny green when immature, turning bright red at maturity, with three cells containing 45–65 seeds for each. Seeds are subglobose in outline but usually somewhat angular from 2–5 mm in diameter. The seed testa is finely lined, glossy brown and its hilum is circular, whitish, aril thin and a bit fleshy (Jansen, 1981).

Korarima has strong fibrous subterranean scaly rhizomes. Its rhizomes are subterete, profusely branched, red-brown in color, covered with thin, subovate scales of about 6×4 cm size, and bearing thin, fibrous, pale brown roots. As compared to other members of the *Aframomum* spp., such as *Aframomum melegueta*, the seeds of korarima are less pungent

and have a milder and sweeter taste (Baser and Kurkcuoglu, 2001). Additionally, korarima flowers and red ripe fruits can be found at the same time in the field, due to the irregularity of flowering, which is so common like in that of cardamom. At the early stage, the color of korarima capsules is green, which turns bright red upon maturity (Girma H/michael *et al.*, 2008), and dark brown after drying. Korarima plants are propagated through asexual and sexual means and complete their juvenile phase and reach maturity after three to five years of planting to first harvest and have more than seven years of economical life. The crop is mainly found as undergrowth within the natural forest canopy in the Southwestern parts of Ethiopia, but it is also grown in small scale farms as cultivated crop or in natural habitat/forest (FAO, 2007).

2.3 Origin and Geographical Distribution of Korarima

Aframomum corrorima is widely distributed in western Ethiopia (provinces of Kefa, Sidamo, Illubabor and Wollega), Sudan (south- western, Aloma plateau), Uganda (western) and Tanzania (Usambara Mountians). In Ethiopia it is occasionally cultivated outside its natural area (around lake Tana and Gelemso) and it is cultivated in Eritrea (Jansen, 2002).

2.4 Ecological Requirement of Korarima

Korarima grows naturally at 1700-2000 m.a.s.l altitude in slightly shaded, more or less open sites in higher altitude rain forest. Annual rain falls varies from 1300 mm to more than 2000 mm; with no distinct dry season but usually most rain falls in June-August (50-60%). The annual average temperature requirement for its growth is about 20°C. In Ethiopia, korarima grows in almost the same habitats as wild coffee species (Girma H/michael et al., 2008).

Korarima thrives well on acidic soils (*i.e.* 5.5 - 6.5 pH), deep to medium soils (50-150 cm), high to moderate organic matter and fertility status, well drained but with high water

holding capacity. In general, the crop does better as undergrowth within the forest habitat, where the soil is so porous and fertile without any compaction due to high accumulation of humus. Moreover, the level of cropping system of korarima during its cultivation and harvesting takes much man power (FAO, 2007). But it may also be possible to bring under domestication by providing some optimum requirements for its production and productivity.

As korarima will probably remain some of the most important spices due to its presence of aromatic seeds, it will continue to be attractive to customers/consumers. However, till now large-scale cultivation for its seeds production is not expected as good. Although not yet in direct danger of extinction, korarima, like all other *Aframomum* species, deserves to be part of germplasm collections. Germplasm collections for kararima are not known. Germplasm collection and conservation, in situ and ex situ, is recommended because of the rapid degradation and destruction of mountain forest. Unreservedly, there are no serious diseases or insect pests known for korarima except the natural enemies like, ape, monkey and mice, but current reports show that the rust *Puccinia aframomi* has been observed on korarima leaves in Ethiopia (Jansen, 2008).

2.5 Uses of Korarima

The use of korarima is only known from Ethiopia and Eritrea. The seeds (usually dried, sometimes fresh) are used to flavour all kinds of sauces, for which they are ground and usually mixed with other spices; occasionally they are also used to flavour coffee, tea, bread, cake, milk and butter. In Ethiopia, the seeds are used medicinally as a tonic, carminative and purgative. The arilloid flesh around the seed is edible. Strings of fruits are sometimes used as an ornament or as rosaries by the Arabs (Jansen, 1981).

Korarima is used as an aromatic ingredient for flavoring liqueurs and processed foods and in confectionery, cosmetics as well as pharmaceuticals (Zenebe Woldu, 2006). Seeds of korarima are mainly used as a spice and condiment in different traditional Ethiopian dishes. In addition, pods, leaves, rhizomes and/or flowers of korarima are commonly used in traditional medicine and as a spice and condiment in different parts of the country, including Southern Ethiopia (Eyob Solomon *et al.,* 2008). In Ethiopia, korarima seeds are widely used to serve as a tonic, carminative and purgative or laxative. Korarima is said to aid the digestive tract and to cure common colds and upper respiratory infections (Piem, 2010). The seeds are also important ingredients in the preparation of *berbere*, *mitmita*, *awaze*, and other spice mixtures, and are also used to flavor coffee (Demisew Sebsebe, 1993; Jansen, 2008).Although no statistics are available, the amount of korarima fruits (the seeds are sold per fruit) offered for sale in Ethiopia is considerable. Korarima is present at every market, and is sold for a high price compared to other spices. Some korarima is exported from Ethiopia to markets in Sudan, Egypt, the Arabian Peninsula, Iran and India. Export to Europe and the United States is very small, mainly due to the product often being of poor quality. To improve good quality of the product the fruits should be carefully dried. In Ethiopia, they are usually pierced near the rope, strung on a rope and hung to dry in the sun. If the fruits are not properly dried, or are mixed with immature ones, the end product is poor quality. (Jansen,2008).

Korarima seed has a mild, sweet flavour and is less peppery or pungent than seed of *Aframomum melegueta*. The seeds contain essential oil which has a typical odour and is sometimes called ‘nutmeg-cardamom’. After distillation of dried comminuted fruits, 3-3.5% of a pale yellow volatile oil with a flat cineolic odour can be obtained (Reghuath and Bajaj, 1992).

Hydrodistilliation of leaves, rhizomes and pods can also provide korarima essential oils. The chemical composition of korarima essential oil is similar to that of Indian cardamom except for its reduced content of terpinylacetate, which is common on the latter (Demesew Sebsebe, 1993).There are reports on the leaf oils which have high activity against selected microorganisms (antioxidants and antiradical activities) and also used to condiment/seasoning as a source of vitamins and minerals (Jansen, 1981; Demissew Sebsebe, 1993; Eyob Solomon *et al*., 2008). Generally, the use of essential oils of korarima still now is not clearly available for each specific essential oil as cardamom, but there are minor reports on antioxidant and antimicrobial activities of korarima (Eyob Solomon *et al*., 2008).

2.6 Propagation Methods of Korarima

Korarima can be propagated by seed and clumps/vegetative methods. Propagation of korarima by seed is quite difficult by traditional breeding methods, but planting rhizome parts is probably easier and quicker than seeds. Propagation by seed is difficult due to the presence of seed dormancy, which needs a means of breaking to improve the situation other than *in vitro* propagation. The development of micropropagation methods does not only enable production of sufficient amounts of planting material of a desired clone for commercial productions, local cultivation and others but it is the base for the future improvement of the crop through tissue culture, genetic engineering as well as for modern germplasm conservation tasks (Lincy , 2010).

2.6.1 Propagation by rhizomes/clumps

The vegetative propagation of korarima through splitting of rhizome with one year old and another young sucker is the conventional technique used for its propagation. Even if propagation by using rhizome shortens the juvenile stage of the stand plant and also enables

to produce true-to-type ones, it always comes with shortage of planting materials to cover large areas of land and it involves sacrifice of potentially productive stands (Wondyifraw Tefera and Wannakrairoj, 2004; Eyob Solomon, 2009).

2.6.2 Propagation by seed

Propagation of korarima by seed can be defined as the emergence of the embryo from the seed, but the germination process is hindered by a variety of both exogenous (external hard seed coat) and endogenous dormancies. The latter could be due to germination inhibitors present in the endosperm (Hilhorst *et al*., 2006), while exogenous seed dormancy (*i.e.* outside the embryo) is caused by physiological factors (Yang *et al.*, 2007) due to the presence of hard seed coat (Bewley and Black, 1994).

Often seed dormancy in some seeds can be established by a close examination of seeds, especially for exogenous seed dormancy. Seed dormancy could be defined as the failure of a viable seeds to germinate when conditions are favorable for seed germination. *In vitro* propagation of many woody tree species and some herbaceous plants are difficult due to low regeneration capacity, especially explants from mature plant tissues (Mng'omba *et al.,* 2007).

In korarima, propagation by seed is quite difficult by traditional methods. But currently, there was a study to break korarima seed dormancy due to its impermeability of hard seed coat. One of these studies was to explore the effect of different treatments on germination and seedling growth attributes of high-land korarima cultivar namely called 'Mume'. According to Eyob Solomon (2009), soaking of the seeds in 50% H_2SO_4 for 60 min followed by soaking in 250 mg/l GA_3 for 24 h and soaking in 50% H_2SO_4 for 60 min had the best effective treatments for enhancing germination. Thus, H_2SO_4 is important for

breaking of seed dormancy. For example, in seed germination of caper (*Capparis ovata* var. *herbacea)* the mucilage surrounding the seed is supposed to inhibit diffusion of oxygen to the embryos and prevents germination. Therefore, sulphuric acid pretreatment to remove mucilage and soaking in either of NAA or GA3 was found effective to allow penetration of xygen from the surroundings to the embryos and increased germination of seeds (Soyler and Khawar, 2007).

2.7 Plant Tissue Culture Techniques and its Application

Tissue culture is a method based on vegetative propagation of planting materials (Oggema et al*.,* 2007). It involves *in vitro* multiplication of plant material under aseptic and controlled environmental conditions. Elementary methods of tissue culture were developed in the 1950´s (Haapala, 2004). It assists the storage of disease free collections and facilitates easier maintenance and distribution of germplasm.

Tissue culture techniques open up many possibilities for sustainable food security and improvement of crops (Oggema *et al.,* 2007b). The systems are capable of creating genetic variability and producing plants with novel characters (Roca, 1979), which could be more favorable than the existing crop varieties.

Plant tissue culture allows production of large number of planting materials within short period of time and small space, but with high capital and much attention as compared to conventional/traditional method of propagation. The use of tissue culture as a tool for plant propagation could be particularly relevant for vegetatively propagated crop plants that resist conventional asexual propagation or when a fast method for mass propagation of a single plant is required. The different explants such as axillary bud, shoot tips, meristem tips, root tips are commonly used (Hiremath, 2006).

Tissue culture techniques have been applied to a wide range of tree species, shrubs, fruits, vegetables, ornamental plants, herbs and spice, etc. It is evident that there is a strong and complex interaction between the explants, plant growth regulators/phytohormones, culture conditions and type of genotype. It is a very useful tool and can play an important role in forest tree breeding programs with respect to improving their efficiency and the quality of the trees that are produced (Hartmann *et al.,* 1990).

Plant tissue culture is also the technique of growing plant parts like; cells, tissues and organs in an artificially prepared nutrient medium in a sterile environmental condition. It relies on the fact that many plant cells have the ability to regenerate in to a whole plant (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, or (less commonly) roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones (Akin-Idowu *et al*., 2009).

Therefore, *in vitro* propagation offers several distinct advantages that are not possible with the conventional propagation techniques. Among others; it enables multiplication of single explant in to several thousands of even some times millions of true-to-type plants in less than a year, without affecting the growth and/or productivity of the mother plant. Moreover, once established it will be actively dividing *in vitro* culture also serve as a continuous source of micro-cutting, which can result in plant production under greenhouse conditions without any season interruption (Teisson and Alvard, 1994).

Generally, different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation. These include production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits; quickly produce mature plants and production of multiples of plants in the absence of seeds or

necessary pollinators to produce seeds and regeneration of whole plants from plant cells that have been genetically modified (totipotency) (George *et al*, 2008). The specific differences in the regeneration potential of different organs and explants have various explanations. The significant factors include differences in the stage of the cells in the cell cycle, the availability of or ability to transport endogenous growth regulators, and the metabolic capabilities of the cells. The most commonly used tissue culture explants are the meristematic ends of the plants like the shoot tip, auxiliary bud tip, rhizome buds and root tip. These tissues have high rates of cell division and either concentrate or produce required growth regulating substances including auxins and cytokinins (Akin-Idowu *et al*., 2009; Georgiev *et al*., 2009).

Plant tissue culture is used widely in plant science even at molecular levels. It has also a number of commercial applications all over the world's agriculture sectors. Some of the applications are; micropropagation that is widely used for fruits, vegetables, root and tubers, spices, forestry and floriculture to conserve rare or endangered plant species. A plant breeder may also use tissue culture to screen cells rather than plants for advantageous characters, *e.g*. yield quality and quantity, herbicide resistance/tolerance and other adverse environmental condition (Akin-Idowu *et al*., 2009; Georgiev *et al*., 2009).

During *in vitro* culture of higher plants, growth regulators especially auxins and cytokinins are very indispensable. It can be said that *in vitro* culture is impossible without growth regulators. Whether an auxins and/or cytokinins are added to nutrient medium to obtain cell extension and/or cell division are/is completely depends on the type of explants taken and the plant species (Pierik, 1997; Akin-Idowu *et al*., 2009).

Generally, there are four broad classes of growth regulators important in plant tissue culture. These includes; auxins, cytokinins, gibberellins and abscisic acid. Skoog and Miller (1957) was the first to report that the ratio of auxin to cytokinin determined the type and extent of organogenesis in plant cell cultures. Both an auxin and cytokinin are usually added to culture media in order to obtain morphogenesis, although the ratio of hormones required for root and shoot induction is not universally the same. Considerable variability exists among genera, species and even cultivars in the type and amount of auxin and cytokinin required for induction of morphogenesis (Hartmann *et al*., 1990).

2.7.1 Media composition and culture condition

Growth culture conditions are a complete medium with growth supplements already added to basal medium which contains essential and nonessential amino acids, vitamins, inorganic salts, organic compounds, and trace elements.Growth culture is ready for use and it is the most popular cell medium format. Each growth supplement is optimized for its specific cell type. When the growth supplements are added to the basal medium, it forms the growth culture (Pierik, 1997). Accordingly, ingredients of the culture medium may vary with the type of plant and the preparation stage at which one is working. However, certain standard mixture is used for most plant species, but exact formulation may need to be established by testing (Hartmann *et al*., 1990).

A significant medium improvement came with the development of White's medium, which included glycine, nicotinic acid, thiamine and pyridoxine. The formulation of Murashige and Skoog (MS) medium (1962) was based on White's medium, but included high concentrations of ammonium, nitrate, phosphate and potassium salts as well as chelated

iron and myo- inositol (Vasil, 2008). Murashige and Skoog medium or (MS0 (MS-zero)) is a plant growth medium used in the laboratories for cultivation of plant cell culture.

The composition of the medium, particularly the plant hormones and the nitrogen source (nitrate versus ammonium salts or amino acids) have profound effects on the morphology of the tissues that grow from the initial explant. For example, an excess of auxin will often result in a proliferation of roots, while an excess of cytokinin may yield shoots. A combination of auxin and cytokinin may produce an unorganized growth of cells, or callus, but the morphology of the outgrowth will depend on the plant species as well as the medium composition (Hartmann *et al.*, 1990).

2.7.2 Acclimatization of *in vitro* rooted plantlets

Acclimatization is the prime measure of any tissue cultured plantlet. Acclimatization is the transfer of plantlets from the growth room atmosphere to the outside atmosphere in soil under greenhouse conditions. This is carried out in a greenhouse and the plantlets are planted in plastic box with holes filled with soil. Acclimatization hardens off the plantlets after they had been growing in controlled conditions in the laboratory and prepares them for establishment in the open field. Rooted and unrooted microshoots are removed from the culture vessel and hence agar is washed off completely to remove a potential source of contamination, and the micro plants are transplanted into a sterilized soil mix in small pots. Initially the micro plants should be protected from desiccation in a shaded, high relative humidity tent or under mist fog (Hartmann *et al.*, 1990).

2.7.3 Environmental Control of *In Vitro* Cultured Plants

Optimization of the microenvironment is a key step in micropropagation and ensures the production of good quality plantlets that have high chances of surviving in the *ex vitro*

conditions and ultimately the natural environment. The culture vessel is a miniature greenhouse or growth chamber with tightly controlled conditions. The characteristics of the conventional *in vitro* environment include; constant temperature, high relative humidity, low photosynthetic photon flux density (PPFD), optimized concentrations of sugars, salts and plant growth regulators in the growth medium, aseptic conditions, but also accumulation of secondary metabolites that may be toxic (Kozai *et al*., 1997).

Light is used as a source of energy during photosynthesis. It is an important factor for plant growth and development in plant tissue culture. In particular, light intensity and photoperiod in the microenvironment affect plantlet physiological processes and growth. However, light may be required to a lesser degree in plant tissue culture systems for the regulation of photosynthesis and photo-morphogenesis (Economou and Read, 1987).

The biophysical and biochemical process of photosynthesis is temperature dependent and is a major determinant of the rate of growth of plants. In nature the perception of ambient temperature allows for the maintenance of plant homeostasis, thereby buffering against potential disruptive effects on cellular stability (Franklin, 2009).

2.7.4 *In vitro* propagation of korarima

Jansen (2008) reported that propagation of korarima by using rhizome has drawbacks. That is, the need for bulk use of rhizome as planting materials requires much attention, space, high transportation cost/manpower and has slow multiplication rate. Moreover, collection of germplasm and breeding programmes (genetic resources) for korarima is not well known still now but it is recommended to conserve the germplasm both *in situ* and *ex situ* because of the rapid degradation and destruction of mountain forests (Eyob Solomon, 2009).

For micropropagation of korarima, MS medium has been proved to be the best for the establishment stage. That is addition of 5% coconut water to the culture medium was found to be effective in enhancing shoot multiplication. The basal medium supplemented with 2.0 mg/l imazalil (IMA) in combination with 0.5 mg/l Thidiazuron (TDZ) was given higher shoot multiplication compared to hormone free medium within eight weeks of culture period (Wondyifraw Tefera and Wannakrairoj, 2004). In addition, shoot proliferation of korarima can also be enhanced by hormonal combination of TDZ at 0.5 mg/l with either 2.0 mg/l of IMA or 3.0 mg/l BA in the culture medium. On the contrary, the presence of TDZ in the culture medium has greatly reduced root number, root length and shoot length of the crop. Not only this but also the use of coconut water to culture medium has a negative effect on root number and length (Wondyifraw Tefera and Wannakrairoj, 2004). The small sized shoots produced from TDZ supplemented medium were easily elongated and produced roots when subcultured on a PGR- free MS medium. In addition, as the concentration of TDZ increases, the shoot length decreases and inclusion of BA to the culture medium had a negative effect on shoot length (Faria, 1995).

Eyob Solomon (2009) reported that, seed pretreatment had positive effects on seedling height, number of leaves, root number, root length and fresh weight. Maximum number of shoots (9.62) were obtained from pretreated seeds of the cultivar 'Mume' cultured on MS medium containing 0.5 mg/l thidiazuron (TDZ) compared to number of shoots (2.67), number of leaves (6.00) and fresh weight (0.25g) in PGR free medium. MS medium with 0.5 mg/l TDZ and 3.0 mg/l 6- benzyladenine (BA) also gave high shoot number (10.00) per explant but shoots were short. Highest percent survival and number of shoots were obtained from *in vitro* seedling shoot tips compared to rhizome buds, but the longest shoots and lowest shoot dry weight were obtained in the PGR free medium. *In vitro* rooting of

korarima was reported by Eyob Solomon (2009) and Wondyifraw Tefera and Wannakrairoj (2004) in which 1.0 mg/l IBA was the best to induce roots from *in vitro* culture of korarima.

3. Objective of the study

3.1 General objective

- To optimize *in vitro* propagation protocol for korarima using shoot tip explants.

3.2 The specific objectives

- To evaluate the combined effects of different cytokinins and auxins on shoot induction and multiplication.
- To evaluate the effects of different concentrations of NAA + IAA and NAA + IBA combinations on root induction, growth and development
- To acclimatize *in vitro* developed plantlets.

4. Materials and Methods

4.1 Preparation of Stock Solution and Growth Media

4.1.1 MS Stock solution preparation

The stock solution of macronutrients, micronutrients and vitamins were prepared separately by weighing the required amount of powder followed by dissolving in distilled water and stored them in refrigerator at a temperature of 4°C. The prepared stock solutions were used within a maximum of one month.

4.1.2 Preparation of PGR stock solution

In this study, different plant growth regulators namely Thidiazuron (TDZ), α-naphthalene acetic acid (NAA), indol-3-acetic acid (IAA), and indol-3-butyric acid (IBA) were used.

Each of these growth regulators was prepared at a concentration of 1.0 mg/ml by weighting 50 mg using precision balance followed by dissolving in few drops of 1.0 M NaOH and adjusted to final volume of 50 ml with distilled water. After complete dissolution, the solution was transferred to 50 ml bottle and stored at 4°C.

4.1.3 Culture Media Preparation

Culture media were prepared by mixing 50ml/l of macro nutrient, 5ml/l of micro nutrient, and 5ml/l of vitamin, 5ml/l of iron EDTL and of 30 g/l sucrose. For shoot multiplication and rooting different concentrations and combinations of plant growth regulators were added separately to the MS medium. The pH of the medium was adjusted to 5.8 with 1.0 N NaOH or 1.0 N HCl solution and then 8g/l agar was added to

the medium. Finally, the culture medium was autoclaved for 15 min at 121°C and 15 kpa pressures.

4.2 Plant materials, explants preparation and sterilization

Capsules of korarima were collected from the Jimma Agricultural Research Center (JARC), Department of Horticulture, Southwest Ethiopia. Fresh capsules (2–5 mm in diameter) of Jimma local cultivars were obtained at the peak harvestable stage of capsules and were collected from apparent disease free growing parents. Seeds were isolated from capsules and then sterilized before cultured. Then seeds were washed five times immediately with tap water at least for 15 min and then soaked in 3.0 g/l Kocid ($Cu(OH)_2$) for 30 min, and rinsed five times with distilled water. The rinsed seeds were also soaked in 50% H_2SO_4 for 60 min. Then the seeds crushed and immediately soaked in 250 mg/l GA_3 for 24 h. Finally, they were washed with distilled water for 20 min and more scales were then removed, followed by washing with detergents. The seeds were then rinsed with 70% ethanol for 3 min; followed by one-step surface sterilization using 25% sodium hypochlorite for 25 min. Then, the seeds were washed five times using sterile distilled water and were further trimmed to remove dead seed coat and sulfuric acid affected scales. Before culturing, the seeds were further soaked in sterile distilled water for 20 min. The surface sterilized seeds were cultured on solid one-fourth MS medium. After three months, aseptic and sufficient amount of seedlings were obtained and they were used as explants in the subsequent experiments.

4.3 Shoot Initiation

After germination, shoot tips were cultured on a medium with 1.0 mg/l BAP alone and 1.0mg/l BAP combined with 0.5mg/l IBA. Data on survival were recorded after a month.

4.4 Shoot multiplication

Shoots were used as explants and cultured onto freshly prepared MS medium containing different concentrations of growth regulators. For shoot multiplication, 30 different concentrations and combinations of growth regulators were used. Growth regulators-free MS basal medium was used as a control.

Shoots excised from aseptically germinated seedlings were cultured on MS media supplemented with different concentrations of TDZ (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/l) in combination with IBA (0.0, 0.25, 0.5 mg/l) and TDZ (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0 mg/l) in combination with NAA (0.0, 0.1, 0.25, 0.5 mg/l) were used. Six shoots were cultured in each Majenta and five culture vessels (30 shoots per treatment) were used. The cultures were maintained in the culture room at a temperature of 25 ± 2°C under a 16 h photoperiod provided by cool-white fluorescent lamps. After four weeks of culture, all explants were survived and data on number of shoots, average shoot length and number of leaves per explant were recorded.

4.5 Rooting

Before rooting of the proliferated shoots, the shoots were cultured on PGRs-free MS medium for one month to protect carry over effect of the previous applied GRs. The shoots were then cultured on half strength MS medium containing IAA (0.0, 0.5, 1.5 mg/l) in combination with

0.1 mg/l of NAA , IAA (0.0, 0.5, 1.5 mg/l) in combination with 0.2 mg/l NAA. In addition to this, IBA (0.5, 1.5mg/l) combined with 1.0 mg/l of NAA, IBA (0.5, 1.5mg/l) combined with 0.5 mg/l NAA, NAA (0.0, 2.0mg/l) combined with 0.5mg/l IBA, 1.0mg/l NAA in combination with 2.0 mg/l IBA, and 2.0 mg/l NAA combined with 1.0 mg/l IBA were applied.

All the media used in this study were supplemented with 3% (w/v) sucrose, the pH adjusted to 5.8 with 1.0 N NaOH and 1.0 N HCl and solidified with 0.8% (w/v) agar. followed by autoclaving at 121°C and 15 kpa for 15 min. The cultures were completely randomized and maintained in growth room at 25 ± 2°C under a 16 h photoperiod provided by cool-white fluorescent lamps. PGRs-free medium was used as control. Number and length of roots were recorded after four weeks.

4.6 Acclimatization

For the purpose of acclimatization, well-developed *in vitro* rooted plantlets were subsequently removed from the rooting medium and carefully washed off with tap water to remove adhering gel and transferred to plastic pots containing a mixture of sterilized forest soil, compost and sand soil in the ratio of 2:1:1. Plantlets were thoroughly acclimatized by covering the pots with polyethylene bags for seven days in greenhouse for four weeks and watered as necessary. After the initiation of new leaves and preliminary hardening, the plantlets were again transferred to a screen-shaded nursery for further growth and development. Data on survival were recorded.

4.7 Data Analysis

For all experiments, mean value of the parameters were subjected to analysis of variance (one-way ANOVA) using the SPSS software packages (SPSS, version 20). The significant differences among treatments were compared using Least Significance Difference (LSD) for the one-way ANOVA at a 5% probability level.

5. RESULTS

5.1 Shoot Initiation

After ten days of culturing, shoot tips started to develop micro leaves. Though they were cultured on a medium with 1.0 mg/l of BAP alone and 1.0mg/l of BAP combined with 0.5mg/l of IBA. After a month of growth on the initiation medium, 96.5% and 100% of the explants survived, respectively.

5.2 Effects of TDZ and NAA on shoot multiplication

As indicated in Table 1, number of shoots was significantly different among some of the treatment combinations of TDZ and NAA. The best medium for shoot multiplication was obtained on 0.5 mg/l TDZ + 0.5 mg/l NAA and 0.5 mg/l + 0.1 mg/l TDZ and NAA and they had a good response to produce highest number of shoots with a mean number of 2.13 ± 1.14 and 1.73 ± 0.74 shoots per explant respectively. The combinations of 1.0 + 0.25, 2.5 + 0.25, 3.0 + 0.25, 5.0 + 0.25, 1.0 + 0.1, 3.0 + 0.1, 5.0 + 0.1, 1.0 + 0.5 and 2.0 + 0.5 mg/l TDZ and NAA were not significantly different in number of shoots. The lowest number of shoot multiplication (1.13 ± 0.43) was observed on medium containing 5.0 mg/l TDZ and 0.5 mg/l NAA and the PGR free MS medium was used as a control also induced 1.23 ± 0.43 shoots per explant. Even though it had no significance difference with other treatment combinations, as the concentration of TDZ increases, the average numbers of shoots per shoot decreases.

Table 1.Effect of TDZ and NAA on shoot multiplication after four weeks of culture

TDZ (mg/l)	NAA (mg/l)	Shoot number per explant	Shoot length per explant (cm)	Leaf number per explant
0.0	0.0	$1.23^{g} \pm 0.43$	$2.10^{a} \pm 0.53$	$1.10^{e} \pm 0.51$
0.5	0.25	$1.20^{g} \pm 0.48$	$1.10^{g} \pm 0.31$	$0.20^{m} \pm 0.61$
1	0.25	$1.30^{f} \pm 0.47$	$0.88^{k} \pm 0.36$	$1.07^{f} \pm 1.14$
1.5	0.25	$1.47^{d} \pm 0.68$	$0.97^{i} \pm 0.59$	$0.60^{j} \pm 1.00$
2	0.25	$1.20^{g} \pm 0.41$	0.90j ± 0.44	$0.80^{h} \pm 1.00$
2.5	0.25	$1.37^{e} \pm 0.61$	$1.20^{f} \pm 0.41$	$0.97^{g} \pm 1.10$
3	0.25	$1.30^{f} \pm 0.53$	$0.85^{k} \pm 0.40$	$0.53^{k} \pm 0.90$
5	0.25	$1.30^{f} \pm 0.70$	$1.37^{e} \pm 0.81$	$1.17^{e} \pm 1.29$
0.5	0.1	$1.73^{b} \pm 0.74$	$1.38^{e} \pm 0.74$	$1.53^{c} \pm 1.55$
1	0.1	$1.33^{e} \pm 0.55$	$1.58^{c} \pm 0.66$	$1.40^{d} \pm 1.50$
1.5	0.1	$1.23^{g} \pm 0.43$	$1.13^{g} \pm 0.35$	$0.20^{m} \pm 0.61$
2	0.1	$1.53^{c} \pm 0.68$	$1.83^{b} \pm 0.65$	$1.60^{b} \pm 1.52$
2.5	0.1	$1.53^{c} \pm 0.68$	$1.13^{g} \pm 0.46$	$1.13^{e} \pm 1.53$
3	0.1	$1.33^{e} \pm 0.55$	$1.42^{d} \pm 0.51$	$0.47^{l} \pm 1.01$
5	0.1	1.37e ± 0.61	$1.10^{g} \pm 0.31$	$0.63^{j} \pm 1.13$
0.5	0.5	$2.13^{a} \pm 1.14$	$1.65^{c} \pm 0.76$	$2.20^{a} \pm 1.92$
1	0.5	$1.33^{e} \pm 0.55$	$1.23^{f} \pm 0.57$	$0.60^{j} \pm 1.04$
1.5	0.5	$1.20^{g} \pm 0.48$	$1.18^{g} \pm 0.38$	$0.13^{n} \pm 0.51$
2	0.5	$1.37^{e} \pm 0.61$	$1.20^{f} \pm 0.41$	$0.73^{i} \pm 1.11$
2.5	0.5	$1.57^{c} \pm 0.68$	$1.13^{g} \pm 0.38$	$0.80^{h} \pm 1.00$
5	0.5	$1.13^{h} \pm 0.43$	$1.07^{h} \pm 0.37$	$0.13^{n} \pm 0.51$

Mean values with the same letter in column are not significantly differentatP≤0.05. The values represent mean ± standard deviation.

5.3 Effect of TDZ and IBA on shoot multiplication

The combined effect of TDZ and IBA exhibited more effect compared to combined effect of TDZ and NAA. The maximum mean shoot number obtained on a medium containing 0.5 mg/l of TDZ and 0.25 mg/l IBA was 2.97 ± 1.43 which was greater than mean shoot number of 2.13 ± 1.14 obtained on medium containing 0.5 mg/l TDZ and 0.5 mg/l NAA. The lowest number of shoots per explant (1.30 ± 0.47) was obtained on a medium containing 3.0 mg/l TDZ and 0.5 mg/l IBA.

As the concentration of TDZ increases and the level of IBA was 0.25 mg/l and when the concentration of IBA increased from 0.25 to 0.5 mg/l in combination with 0.5, 1.5, 2.0, 3.0 mg/l TDZ, there was a continuous decrease in number of shoots.

Table 2. Effect of TDZ and IBA on shoot multiplication after four weeks of culture

TDZ (mg/l)	IBA (mg/l)	Shoot number per explant	Shoot length per explant (cm)	Leaf number per explant
0.0	0.0	$1.82^{e} \pm 0.74$	$2.47^{a} \pm 0.92$	$1.13^{f} \pm 1.20$
0.5	0.25	$2.97^{a} \pm 1.43$	$2.13^{b} \pm 0.83$	$3.80^{a} \pm 2.22$
1	0.25	$2.87^{b} \pm 1.19$	$1.43^{c} \pm 0.55$	$3.83^{a} \pm 2.31$
1.5	0.25	$2.67^{c} \pm 1.15$	$2.17^{b} \pm 0.98$	$3.20^{b} \pm 2.22$
2	0.25	$2.43^{d} \pm 1.69$	$1.17^{d} \pm 0.67$	$3.47^{a} \pm 1.59$
2.5	0.25	$2.43^{d} \pm 1.22$	$1.42^{c} \pm 0.99$	$1.23^{e} \pm 1.23$
3	0.25	$2.40^{d} \pm 1.13$	$1.11^{e} \pm 0.49$	$2.67^{d} \pm 1.18$
0.5	0.5	$1.67^{f} \pm 0.88$	$1.40^{c} \pm 0.62$	$3.13^{c} \pm 1.55$
1.5	0.5	$1.67^{f} \pm 0.84$	$1.13^{e} \pm 0.35$	$0.53^{g} \pm 1.04$
2	0.5	$1.57^{g} \pm 0.68$	$1.10^{e} \pm 0.31$	$0.33^{h} \pm 0.76$
3	0.5	$1.30^{h} \pm 0.47$	$1.10^{e} \pm 0.31$	$0.13^{i} \pm 0.51$

Mean values with the same letter in a column are not significantly different at $P \leq 0.05$

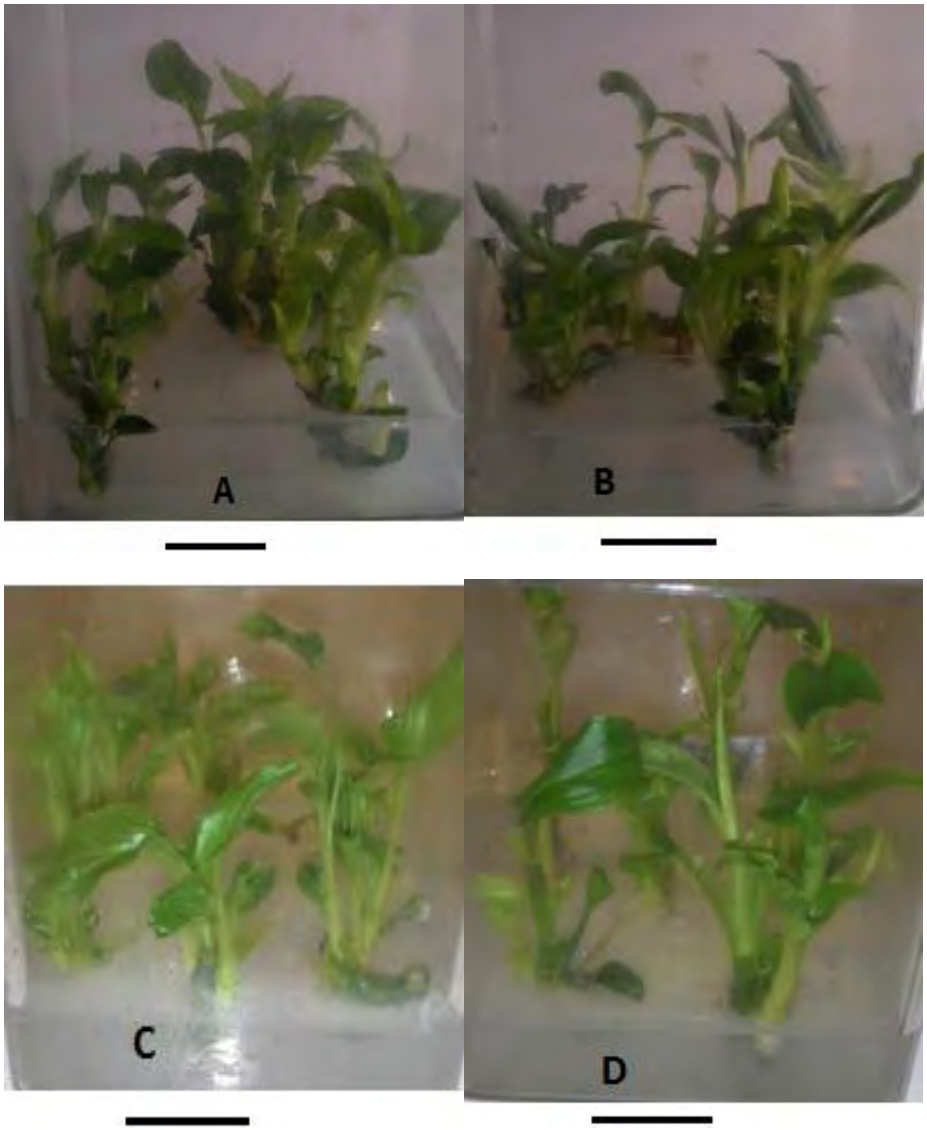

Figure 1. Effect of TDZ, NAA and IBA on shoot multiplication**.** A) 0.5 mg/l TDZ + 0.5 mg/l NAA, B) 1.0 mg/l TDZ + 0.25 mg/l IBA, C) 0.5 mg/l TDZ + 0.25 mg/l IBA, D) PGR free. Each bar represents 5cm.

5.4 Effect of NAA and IAA on root induction

ANOVA indicated that, the different combinations of NAA+ IAA and NAA + IBA on half MS medium strength affects all root growth parameters (mean root number and average root length per explant) with highly significant difference at 1% probability level.

The highest number of roots per explant (7.13 ± 5.21, 7.10 ± 3.44,) were obtained on half strength MS medium containing 1.0 mg/l NAA in combination with 0.5 mg/l IAA, and 1.0 mg/l NAA combined with 1.5 mg/l IAA, respectively. The lowest shoot number per explant (3.17 ± 2.82) was obtained on half strength MS medium containing 2.0 mg/l NAA (Table 3).

Table 3. Effect of NAA and IAA on rooting

NAA Mg/l	IAA mg/l	Root no. per explants	Root length per explants (cm)
0.0	0.0	$6.07^{b} \pm 3.44$	$2.12^{a} \pm 0.64$
1	0.0	$7.07^{a} \pm 2.60$	$1.98^{b} \pm 0.69$
1	0.5	$7.13^{a} \pm 5.21$	$1.20^{c} \pm 0.55$
1	1.5	$7.10^{a} \pm 3.44$	$0.95^{d} \pm 0.44$
2	0.0	$3.17^{d} \pm 2.82$	$0.63^{e} \pm 0.76$
2	1.5	$6.07^{b} \pm 4.88$	$0.92^{d} \pm 0.35$
2	0.5	$3.97^{c} \pm 3.85$	$0.60^{e} \pm 0.37$

Numbers within the same column with different letter (s) are significantly different at $p \leq 0.05$. The values represent mean ± standard deviation.

5.5 Effect of NAA and IBA on rooting

The maximum numbers of roots were obtained from 1.0 mg/l NAA + 0.5 mg/l IBA (5.80 ± 2.67 roots per shoot) and 1.0 mg/l NAA + 1.5 mg/l IBA (5.13 ± 2.66 roots per shootlet) treatment combinations.

The least number of roots per explants (1.60 ± 2.80) were obtained on medium containing 2.0 mg/l NAA + 0.5 mg/l IBA (1.20 ± 2.38).

The combined effect of NAA and IBA on rooting varied according to the concentration of NAA and IBA. When the concentration of NAA was 1.0 mg/l and that of IBA was 0.5, 1.5 and 2.0 mg/l, relatively better result was recorded.

Table 4. Effect of NAA and IBA on rooting

NAA (mg/l)	IBA (mg/l)	Root no. per explant	Root length per explant (cm)
0.0	0.0	$3.20^{e} \pm 3.12$	$2.13^{a} \pm 0.53$
0.0	0.5	$1.60^{h} \pm 2.80$	$0.59^{g} \pm 0.56$
0.0	1	$2.27^{f} \pm 3.12$	$0.36^{h} \pm 0.40$
0.5	0.5	$4.23^{c} \pm 3.32$	$0.96^{d} \pm 0.61$
0.5	1.5	$3.57^{d} \pm 3.22$	$0.61^{f} \pm 0.49$
1	1.5	$5.13^{b} \pm 2.66$	$1.15^{c} \pm 0.40$
1	0.5	$5.80^{a} \pm 2.67$	$1.32^{b} \pm 0.53$
1	2	$5.03^{d} \pm 2.83$	$0.72^{e} \pm 0.26$
2	0.5	$1.20^{i} \pm 2.38$	$0.39^{h} \pm 0.44$
2	1	$1.73^{g} \pm 2.66$	$0.17^{i} \pm 0.32$

Numbers within the same column with different letter (s) are significantly different at $p \leq 0.05$.

The values represent mean ± standard deviation.

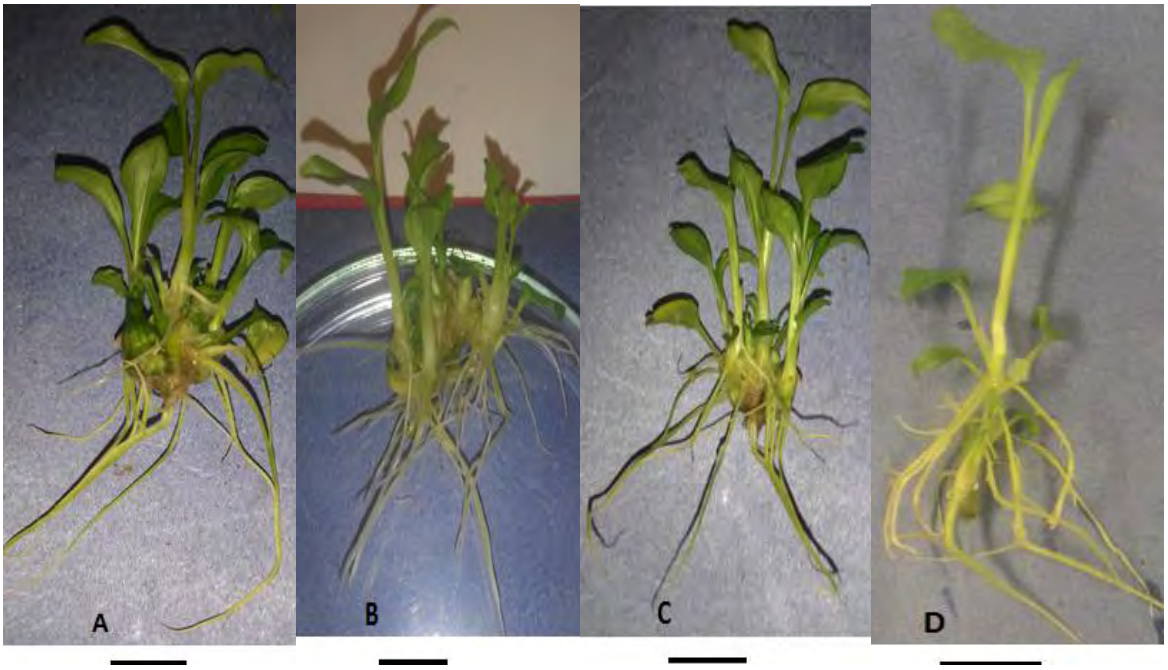

Figure 2. Effect of NAA, IBA and IAA on rooting of shoots. A) 1.0 mg/l NAA + 0.5 mg/l IAA, B) 1.0 mg/l NAA + 0.5 mg/l IBA, C) 1.0 mg/l NAA + 1.5 mg/l IBA, D) PGR free medium. Each bar represents 5cm.

5.6 Acclimatization

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in the soil (acclimatization), thus plantlets that have well grown roots were transferred to the soil for acclimatization. A total of 50 plantlets were acclimatized (10 plantlets per pot) and 43 explants survived after a month. Plantlets were acclimatized at mean temperature of 24°C and a relative humidity of 50-60%. After two weeks of acclimatization, a little increment in plant height was observed. Initially, a few branches, each bearing an average of two to three leaves developed adjacent to the main shoot. Survival rate of 86 % was obtained.

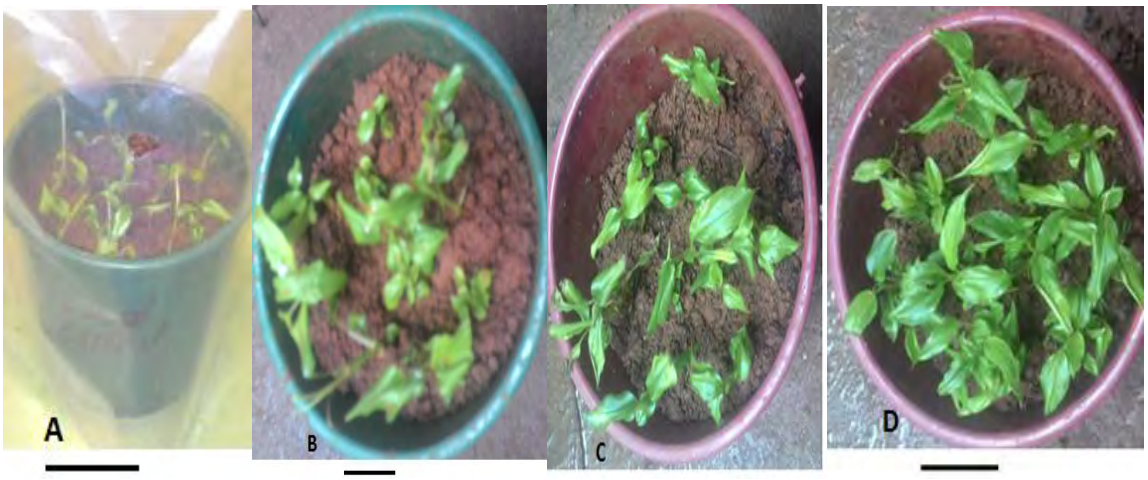

Figure 3**.** Acclimatization; A) During transplanting, B) After two weeks, C) After three weeks, D) After four weeks. Each bar represents 5cm.

6. DISCUSSION

6.1 Shoot Initiation

From this study, it was observed that cultured shoot tips gave best response to both cultured media compositions that contain 1.0mg/l BAP and 1.0mg/l bap + 0.5mg/l IBA. From the observed results, 100% shoots were survived on the initiation medium supplemented with 1.0 mg/l of BAP combined with 0.5 mg/l IBA. These initiated shoots were with healthy morphology and had sufficient number of leaves and shoot height. In agreement with this, Rahiel *Hagos* (2011) reported that 1.0 mg/l BAP combined with 0.5 mg/l IBA is best for *in vitro* culture initiation of *A. corrorima*. This result is obtained due to combined effect of different factors. One of these factors is the growth regulators composition of the medium.

6.2 Effect of TDZ, NAA and IBA on shoot multiplication

In vitro propagation of related species was investigated well, but for *A.corrorima*, there are few available reports. Therefore, in the present study efforts have been made to optimize *in vitro* propagation protocol for *A. corrorima.*

The type and concentration of cytokinins used in the shoot multiplication depends upon the type and kind of genotypes and plant species. For instance, in some plant species the reduction of shoot height caused by TDZ could be attributed to its strong cytokinin-like activity. TDZ stimulate shoot proliferation than shoot elongation (Lincy and Sasikumar, 2010). In addition, best regeneration rate in the post-storage cultivation was achieved on MS media with TDZ or KIN at concentrations lower than 2.0 mg/l and higher concentrations of cytokinin

induces shoot number but decreases shoot length but it depends on the type and concentration of cytokinin and type of plant species.

In the present study, full strength MS medium containing different types and concentrations of PGRs have been used to assess the multiplication potential of the initiated shoots. In one of the previous studies Rahiel Hagos (2011) reported that all the shootlets regenerated were survived (100%) without contamination. However, some physiological senescence like drying on the bottom of the shootlets and tip burn in the shoots cultured with more concentration of KIN were observed.

From all the combinations, the best numbers of shoots were obtained on media containing TDZ and IBA. The highest mean shoot number per explant (2.97± 1.42) was obtained on a medium containing 0.5 mg/l TDZ and 0.25 mg/l IBA. Among the shoot multiplication studies conducted, it is shown that TDZ alone was able to regenerate high number of shoots at lower concentration. Wondyifraw Tefera and Wannakrairoj (2003) obtained 15.52 shoots per explants on MS medium containing 0.5 mg/l TDZ. This indicates that, relatively higher numbers of shoots were obtained when small concentration of TDZ was used alone rather than combining it with IBA. The reduced number of shoots in the combination of TDZ and IBA may attribute to the inhibitory effect of IBA for shoot multiplication and genotypic differences of the explants used.

The best medium for shoot multiplication of this study is similar with findings of Kochuthressia *et al*. (2010)) and 0.5 + 0.25 mg/l TDZ and IBA combination and they had a good response to regenerate the largest number of shoots with an average number of 3.01 shoots per shootlet.

As the concentration of TDZ increased, there was a continuous decrease in number of shoots. In fact on one previous study, Rahiel Hagos (2011) reported that high concentration of BAP (6.0 mg/l) produced 10.33 shoots per explant. According to this author, as the concentration of BA increases throughout the hormonal combination of the medium, the number of shoot induced was increased. This indicates that TDZ is effective at low concentration whereas BAP is effective at high concentration. Low number of shoots per explants obtained in the present study as compared to the previous studies is most likely due to genotype difference.

6.3 Rooting

To assess the rooting response of *A. corrorima*, half MS medium containing combinations of NAA+ IAA and NAA + IBA were used. Half MS media is recommended by different authors for rooting (Dalal *et al*., 2006; Hartman *et al*; 2004). In some species of plants, the interaction of IBA and NAA promotes starch hydrolysis better than IAA and IBA during root development and subsequently reduced the C/N ratio and increased the protein-nitrogen activity during root development which suggested that the auxin influenced mobilization of nitrogen to the rooting zone (Basak *et al*., 1995).

The cultured shoots on rooting medium responded differently to different concentrations of PGRs used. PGR free medium was highly significant in terms of short number of days to root induction and longer root length, but not on number of roots. PGR free media had drawbacks during *in vitro* rooting of shootlets on the culture system, where shootlets were difficult for further regeneration of roots due to lack of enough space and roots were tinder and turned over the medium.

Rahiel *Hagos* (2011) obtained 14.67 roots per explant on half strength MS medium containing 0.5 mg/l NAA. In the present study, 5.80 ± 2.67 roots per explants were obtained on half MS medium containing 1.0 mg/l NAA in combination with 1.5 mg/l IBA. These variable responses could be due to different factors including genetic differences, differences in the explant source, the concentration difference of growth regulators and the type and/or age of explants used to establish the cultures.

The present study is not also agreed with the findings of Geetha, *et al*., (1997) where high number of roots (8.6 4) was produced in *Kaempferia galanga* in the medium with NAA at 1.0 mg/l, combined with IBA at 1.5 mg/l. This in agreement is due to species difference of the explants used.

6.4 Acclimatization

After a month of acclimatization, 86 % of survival rate was obtained. Rahiel Hagos reported that 66.67% of survival rate was obtained, after she acclimatized korarima plantlets in the soil composition of coffee husk, forest soil and sand in a ratio of 2:2:1. This disagreement in the survival rate may be due to the difference in the composition and ratio of the soil used. The soil composition of forest soil, compost and sand in 2:1:1 ratio is well dried with high water holding capacity which is suitable for acclimatization of korarima.

7. CONCLUSION

From the present study it was concluded that there was significant difference in the mean number of shoots per explant using different concentrations of TDZ and auxins (NAA or IBA). The application of high concentration of TDZ (5.0 mg/l) combined with 0.5 NAA resulted in significantly lower shoot regeneration as compared to low concentration of TDZ (0.5 mg /l) combined with 0.5 mg/l NAA. The use of 0.5 mg/l TDZ + 0.25 mg/l IBA showed better result than 0.5 mg/l TDZ + 0.5 mg/l NAA for shoot multiplication of korarima in all three growth parameters. Reducing the concentration of TDZ promoted the rate of shoot multiplication. The highest number of shoots per explant was obtained on medium containing 0.5 mg/l TDZ combined with 0.25 mg/l IBA.

From this result, it can be concluded that 1.0 mg/l NAA with small concentration of IAA on half strength MS medium is the most effective in rooting response of korarima. The rooted shoots were also successfully acclimatized with 86% survival rate.

Generally, results of this study indicate that large- scale propagation of *A. corrorima* by tissue culture methods is feasible and several plantlets can be produced in short period of time. Low concentration of TDZ was effective for shoot induction as well as shoot proliferation. MS medium containing 0.5 mg/l TDZ and 0.25 mg/l IBA resulted in highest number of shoots per explant (2.97 ± 1.43).

8. Recommendation

According to the results obtained in this study, MS medium supplemented with 0.5 mg/l TDZ + 0.25 mg/l IBA for shoot multiplication and ½ MS medium supplemented with 1.0 + 0.5 mg/l NAA and IAA for rooting is recommended for *in vitro* propagation of *A. corrorima* from shoot tip explants. However, the number of shoots per explant obtained in this study is relatively low. Therefore, from this study the following are recommended.

- Further optimization of this protocol may be required for mass propagation of this plant.
- The protocol may also require low cost mass propagation of this plant by reducing or substituting the media components.
- Use liquid medium to avoid the cost of agar for mass propagation of *A. Corrorima.*
- Trying different genotypes of the explants.

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10. Appendix

Nutrient composition and concentration of MS basal medium.

Major Nutrients		Micronutrients (g/l)		Organic supplements	
NH_4NO_3	16.5	H_3BO_3	0.62	Myoinositol	1.0
KNO_3	19.0	$MnSO_4.4H_2O$	2.23	Glycin	0.2
$CaCl_2.2H_2O$	4.4	$ZnSO_4.7H_2O$	0.86	Nicotinic acid	0.05
$MgSO_4.7H_2O$	3.7	Kl	0.083	Pyridoxin (B6)	0.05
KH_2PO_4	1.7	$Na_2MoO_4.2H_2O$	0.025	Thiamin (B1)	0.01
		$CuSO_4.5H_2O$	0.0025		
		$CoCl_2.6H_2O$	0.0025		
		Fe-Na.EDTA	4.0		

DECLARATION

I, the undersigned, declare that this thesis is my original work in the department of Biotechnology of Addis Ababa University and has not been presented for any academic degree in any other university. All assistance received from other individuals and organizations has been acknowledged and full reference is made to all published unpublished sources used.

Name: Teferi Mekonen Signature _____Date _____

This Thesis has been submitted for examination with my approval as an advisor.

Tileye Feyissa (PhD)

Signature_____

Date_____