

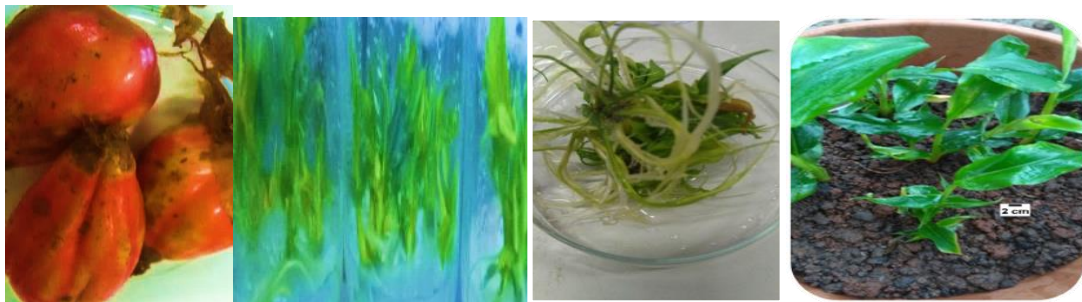
ADDIS ABABA UNIVERSITY
COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES
DEPARTMENT OF ZOOLOGICAL SCIENCE
GENERAL BIOLOGY PROGRAM



Effects of Some Culture Conditions on *In Vitro* Propagation of *Aframomum corrorima*
(Braun) P.C.M. Jansen)

By

ABRHAM SISAY BEYENE



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

March, 2020

Addis Ababa, Ethiopia

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This is to certify that the thesis prepared by Abrham Sisay entitled Effects of Some Culture Conditions on *In-Vitro* Propagation of *Aframomum corrorima* (Braun) P.C.M. Jansen) and submitted in partial fulfillment of the requirement for degree of Masters of Science in Biology complies with regulation of the university and meets the accepted standard with respect to originality and quality.

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Name of advisor: _____ Signature: _____ Date: _____

Board of Examiners (BoE):

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I the under signed, declare that this thesis is my original work and has not been presented for a degree in any university and that all source of materials used for this thesis have been dully acknowledged.

Abrham Sisay Signature _____ Date _____

This thesis has been submitted for examination with my approval as an advisor:

Tileye Feyissa (PhD) Signature _____ Date _____

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LIST OF ABBRIVATIONS

ANOVA	Analysis of Variance
BAP	6-Benzyl Amino Purine
GA3	Gibberellic Acid
IAA	Indol-3-Acetic Acid
IBA	Indol-3-Butyric Acid
LSD	List significant difference
MS	Morishige and Skoog basal medium
NAA	α -Naphthalene acetic acid
PGR	Plant Growth Regulator
TDZ	Thidiazuron

ABSTRACT

Aframomum corrorima is one of the common native crops of Ethiopia which is used as spice and for medicinal purpose. It is an important cash crop having good export potentials and its seeds are used as carminative, purgative and tonic. Major production limitation of *A. corrorima* is lack of high yielding improved varieties with improved agronomic practices like multiplication and the slowest seed germination. The objective of this study is to investigate the effect of medium volume, density of explants and some nutrient concentration in in vitro propagation of *A. corrorima*. Capsules of *A. corrorima* were collected in Oromia region from Jimma zone Goma Woreda at Chocho Keble. The seed were washed by tap water, then sterilized with 70% ethanol for one minute followed by 5% Sodium hypo chlorite for 25-minute and washed three times with sterilized distilled water and cultured on semi solid MS culture medium. Seeds started to germinate at 5th weeks and completed at 11th weeks. This difference is might be happened due to maturation difference of seeds in different capsules and also might be an equal effect of KH₂PO₄ on seeds when soaked it. The maximum mean shoot number recorded during initiation stage was (1.55 ± 0.11), obtained on full strength medium containing 1.5 ml/l BAP. The maximum mean shoot number (3.80 ± 0.23) was obtained on the medium containing two explants per culture vessel. In all treatments the growth regulator hormone used was 1.5 ml/l BAP. The highest mean root number (14.50 ± 0.34) was obtained on one third salt strength supplemented with 1.5 ml/l IBA. Up on acclimatization 90 % were survived in the green house. It can be concluded that *A. corrorima* can be successfully micro propagated from seeds by minimizing transportation and area consumption cost.

Key words: *A. corrorima*, culture vessels, Explant, *In Vitro* propagation, Medium

1. INTRODUCTION

In Ethiopia there are about 60 to 100 different plant species which are used as spices, for example korerima, black cumin, white cumin, coriander, long pepper and ginger. Spice is mostly used as a major raw material in local food preparations. Uses of spice sub-sector have increase from time to time throughout the world due to different opportunities and its long history of cultivations(EIAR, 2000).

Spices are a vegetable products used for flavoring, seasoning and providing aroma in foods (FAO, 2007). In addition to the above, spices are also considered to possess several medicinal and pharmacological properties. So they are important in the preparation of a number of medicines (Risch, 1997).Nearly 50 different spice and herb plants have a trade importance in the world and many other spices and herb crops are used in traditional cooking, health care or other applications in particular regions and also traded locally. Plants used as spices are always grown as shrubs, trees perennials and annuals, wild and cultivated forms. Plant parts used as spices includes: plant leaves, stems, flowers, buds, roots, rhizomes, seeds, fruits, bark and resins. All of these can be commercialized in various forms like: sold as fresh, frozen, dried, whole or ground, distilled into oils or solvent extracted into oleoresins. Spices have aroma due to the complex mixtures of volatile compounds, like monoterpenes, sesquiterpenes and their oxygenated derivatives, which usually occur in low concentrations(Umar *et al.*, 2014).

Spices obtained from turmeric are widely used in Ethiopia and even in the world for food coloring (López *et al.*, 2008).Turmeric is also used for industrial purpose such as in garment and coloring of pharmaceutical products due to its coloring power. Capsules, Fruits, branches and leaves of coriander are very common medicinal plant used as fresh vegetables. The vegetative part of fenugreek and flour of the fenugreek seeds are used as the main protein source for human being in the form of food and as food flavoring. In Ethiopia most of these spices are commonly used for food flavoring and beverages (Jansen, 1981).Korarima, ginger, turmeric, black pepper, pepper, black cumin, coriander and fenugreek are exported either in their dried forms or as oleo resin or essential oil extracts(Roukens *et al.*, 2005).

Good quality seeds of any preferred varieties of spices, aromatic and medicinal plants are the basis of improved productivities, because they satisfy the need of farmers in terms of use of production and household benefits (Delmer, 2005).

Aframomum corrorima is one of the common native crops to Ethiopia which is used as spice and medicinal purpose. It also called Ethiopian cardamom or false cardamom and sometimes called Ethiopian corrorima. With regards to its origin, *A.corrorima* is endemic to Ethiopia (Purseglove *et al.*, 1981). It is endangered and an indigenous plant species for Ethiopia (Jansen, 1981). On the base of its medicinal attributes, *A.corrorima* seeds are used as carminative, purgative, as well as tonic(Jansen, 1981). *A. corrorima* is herbaceous, perennial and aromatic species which are classified under monocotyledonous crops. It is the most important plant for soil conservation via their rhizomes and leaves spread on the ground (Eyob *et al.*, 2008).

Production limitation of *A .corrorima* are lack of high yielding improved varieties with improved agronomic practices like multiplication (propagation) methods (Chombe and Bekele, 2018). Propagation of *A. corrorima* from seed is used to cover large areas of land portions and the mother plant stand undamaged. Well matured *A. corrorima* 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. The germination of *A. corrorima* seeds takes from one to two months. *A. corrorima* is an important cash crop having a good export potential(Eshete *et al.*, 2016).

In vitro propagation is the production of a large number of individual plants from a small piece of plant tissue cultured in a nutrient medium. Advantage of In vitro Propagation of plants are: large scale multiplication of important genotypes, production of disease free plants, growing season independent plants, production of secondary metabolites, prerequisite for crop improvement through genetic transformations, conservation of endangered medicinal plants and facilitating easy exchange of germplasm(Singh *et al.*, 2011).The interest of using this technique is constantly increased for rapid and large-scale propagation of medicinal and aromatic plants (Sahoo and Chand, 1998).

Some researchers like(Tefera and Wannakrairoj, 2004, 2006,Mekonnen and Feyissa, 2016,Eyobet *al.*, 2009,Hagos, 2011,Getie and Feyissa, 2017) conducted their studies on *A. corrorima*. But when considering their economic and medicinal importance's, there is no adequate studies conducted on the effect of different MS salt strength, effects of Potassium dihydrogen phosphate, media volume and density of explants per culture vessel on in vitro propagation of *A. corrorima*. Therefore, the present study was aimed at investigating the effect of different salt strength, concentration of potassium dihydrogen phosphate, medium volume and the effect of density of explants on *in vitro* propagation of *A. corrorima*.

2. LITRETURE REVIEW

2.1. Botanical description of *A. corrorima*

Aframomum corrorima is classified under the family *Zingiberaceae* and one of the members of monocotyledonous family. Its chromosome number is not decided. *A. corrorima* has different botanical synonymous like, *Amomum corrorima*, Braun (1848), *Amomum corrorima*, J. Pereira (1850), *Aframomum corrorima* J. Pereira 1850) K. Schum, Ex Engl (1908), *Aframomum usambarense*, Lock (1976). Different vernacular names are given to *A. corrorima*, like: Ethiopian cardamom, false cardamom, cardamom d'Ethiopie, poivre d'Ethiopie. In different parts of Ethiopia *A. corrorima* also named by different local names like, Korerima in Amharic, ogi'o in Oromifa, korarima in Tigrigna and Offio in Kaffigna (Chombe and Bekele, 2018). *A. corrorima* is endangered and indigenous species to Ethiopia which is used as a medicine and spices (Jansen, 1981); (Demissew, 1993). The plant *A. corrorima* has relatively wider adaptation, better tolerance in moisture stress and productivity than Indian Cardomom (*Elettaria cardamomum*)(EIAR, 2000).

There are about 50 different species which are grouped under the genes of *Aframomum*. They are widely distributed in the wetter parts of tropical Africa. Under the genus *Aframomum* and *Amomum* the well-known commercial species are *A. corrorima* and *Aframomum melegueta* which are found in the Eastern and Western parts of Africa, respectively. In addition to these, *Aframomum zambesiicum* and *K. Schum* also occurs in similar habitats with that of *A. corrorima* in Ethiopia, but their seeds are not commonly used in Ethiopia. They are commonly known as 'monkey'skorarima'. Nevertheless, leaves of *Aframomum zambesiicum* are less aromatic up to crushing and its inflorescences (flower cluster) consist of 25–50 flowers. But in the case of the real korarima, it is highly aromatic and consists of 5 flowers in its flower cluster (Jansen, 1981).

2.2. Morphology and Development of *A. corrorima*

Aframomum corrorima is herbaceous, monoecious and perennial plants. Usually the rhizome of *Aframomum corrorima* grown up to 1cm in diameter and has leafy stems 1-2 m long with aromatic pungent. The stems of *A. corrorima* has no branch and made by the leaf sheathe substrates up to 1 cm in diameter. But at the base, it is thickened and reaches up to 3 cm in diameter. Its leaves are arranged in alternative forms which are shield to each other. *A. corrorima* is a bisexual type of flowering plants. Its flower cluster is a shortly stalked head arising from the rhizome near the base of a leafy stem sometimes located at the end of a rhizomatous runner which are up to 5-flowered and

peduncle up to 7 cm long. Its flowers are covered by snarl, purplish-brown, sub oval scales of 2.5 cm × 1.5 cm and each flower is surrounded by sub oblong bract up to 6 cm × 2 cm, ciliate, tooth like projection. The fruit of *A. corrorima* not break open when mature and subconical berry up to 6 cm × 3.5 cm in size. Mostly it points out 3 longitudinal furrows, and on certain occasions it gives shinier green when immature, but it turns into bright red at maturity. It contains 45-65 seeds within three cells. Seeds are somewhat angular from 2–5 mm in diameter, but nearly globular in outline. The testa of the seed is smooth brown and its hilum is circular, whitish, aril thin and nearly fleshy (Jansen, 1981).

Aframomum corrorima bears strong fibrous scaly rhizomes. Its rhizome is largely branched, reddish-brown in color, covered with thin sub ovate scales. It has fibrous, pale brown and thin roots. The seeds of *A. corrorima* less pungent and have a milder and sweeter taste as compared to other members of *Aframomum species* such as *Aframomum melegueta* (Baser and Kurkcuoglu, 2001). Due to the inconsistency of flowering, the flowers and red ripe fruits of *A. corrorima* cannot be found at the same time in the field. It is so common like cardamom. The capsules of *A. corrorima* is green at the early stage, but when it mature, it become bright red and dark brown after drying (Abraha and Mekbib, 2011). *A. corrorima* is normally flowering between June and July. Its fruit is mature after 2–3 months of flowering and then harvesting between September and October. The stigma of *A. corrorima* plant is situated below or against the base of the theca of anthers. But there is no any scientific evidence regarding the pollination behavior of *Aframomum corrorima*. Based on their flower position the plant *A. corrorima* has the possibility of self-pollination. However, in many other *Aframomum* species, the stigma is situated at the top of theca of the anther and cross-pollination caused by insects is the rule, but the plants are mostly self-fertile and animals such as monkeys eat the aril around the seeds and certainly contribute to natural dispersal of the seeds (Chombe and Bekele, 2018). In addition, the presence of large nectaries at the top of their ovaries also leads to increase the possibility pollination in *A. corrorima* plants by insects. The plant *A. corrorima* are multiplied via asexual and sexual means and complete their juvenile stage and reach maturity between three to five years of planting to first harvest and has more than seven years of economic life. The crop *A. corrorima* is commonly found as undergrowth within the natural forest canopy in the Southwestern parts of Ethiopia. But also, in small scale it can be grown by farmers as cultivated crops or in natural habitat (FAO, 2007).

2.3. Distribution of *A. corrorima*

Aframomum corrorima is widely distributed in western part of Ethiopia (like Kefa, Sidamo, Illubabor and Wollega), western part of Uganda, Tanzania (Usambara Mountains), and western part of South Sudan. In Ethiopia *A. corrorima* sometimes cultivated outside its natural area, like around Lake Tana and Gelemso and it is also cultivated in Eritrea (Chombe and Bekele, 2018). According to the botanical survey of Ethiopia Gamo Gofa, Debub Omo and Kaffa are the three main areas where *A. corrorima* plant commonly grows (Berhanu *et al.*, 1994).

Aframomum corrorima naturally grows in partially shaded rain forest sites of 1700 and 2000m above sea level. The crop *A. corrorima* mainly grows well in places having mean annual rainfall of 1,300 mm to more than 2,000 mm, but with no distinct dry season. However, it grows well during the main rainy season between June to August, where 50–60% of the total rainfall is available to the plant. The annual average temperature of the season for successful growth and development is shown at 20°C. Commonly found in the wild habitats where wild Coffee Arabica is growing. It usually requires an average temperature ranging between 16–24°C, with an average shade of 55–63% (Girma *et al.*, 2008) and its photoperiod is less than 12 hours of light, this indicates that the plant *A. corrorima* is classified under short day plants. *A. corrorima* is best on acidic soils (5.5–6.5 pH), high to moderate organic matter, deep to medium soils (50–150 cm), and the fertility status of the soil is well drained but with high water holding capacity. In short, the plant *A. corrorima* does better as undergrowth within the forest habitat, where the soil is so permeable and fertile without any compact due to high accumulation of humus. Cropping system of *A. corrorima* during cultivation and harvesting requires much man power (FAO, 2007).

Aframomum corrorima is the most important spice due to its presence of aromatic seeds. But large-scale cultivation for its seeds production is not enough as good and not yet in direct danger of extinction. There are no serious diseases or insect pests known for it *as commonly speaking* except the natural animal enemies like monkey, ape and mice. But in the recent report the rust *Puccinia Aframomum* has been observed on the leaf (Jansen and 2008). In Ethiopia there are about 60 to 100 different plant species which are used as spices, for example korerima, black cumin, white cumin, coriander, long pepper and ginger. Spices in Ethiopia are mostly used as a major raw material in local food preparations. Due to different opportunities and its long history of cultivations, the uses of the spice sub-sector have increased from time to time throughout the world (EIAR, 2000).

2.4. Uses of *A. corrorima*

Most traditional medicine in Ethiopia is made from herbal plants. These herbal plants are also used as ingredients and food flavoring. Traditional medical practitioners mostly use herbs or their spices for bone-setting, spiritual healing, treating different diseases and minor surgical procedures. In Ethiopia traditional medicine is so much complex and diverse among different ethnic groups. For instance, garlic and ginger are commonly used to treat common cold and fever which is a widespread practice in Ethiopia. Similarly, turmeric and coriander are also used for varied culinary medicinal purposes, to treat stomach ache and colic(Eshete *et al.*, 2016)

The use of *A. corrorima* is commonly known in Ethiopia and Eritrea, but in a recent time other country are also giving attention its utilization for different purposes. In most consumer countries, it used as an aromatic ingredient for flavoring liqueurs and processed foods and pharmaceuticals as well as cosmetics (Khan and Abourashed, 2011). In addition to this, the rilloid flesh around the seed is used as a food for human being and the strings of fruits are sometimes used as rosaries or an ornamental plants which are common in Arabs(Jansen, 1981). It is believe that *A. corrorima* is one of the fewest spices and is an important culinary and medicinal plant species which is native to Ethiopia (Abraha and Mekbib, 2011)The seeds of *A. corrorima* are mainly used as a spice and condiment in different traditional Ethiopian dishes and mostly used as a source of economy to the growers as they have high prices both at export and local markets. On the top of this, rhizomes, flowers pods, and leaves of *A. corrorima* are mainly used in traditional medicines, as a spice and condiment in different parts of the country (Eyob *et al.*, 2008). In Ethiopia, the seed of *A. corrorima* is commonly used to serve as a carminative, tonic ,and purgative or laxative and to treat the digestive tract, upper respiratory infections and to cure common colds(Woldeyes, 2011).

Aframomum corrorima seeds have a pleasant, sweet flavor and is less peppery or pungent than other related species seed like *Aframomum melegueta*. It contains essential oils with typical odor and occasionally called ‘nutmeg-cardamom’. When a dried comminuted fruits are distilled ,about 3-3.5% of a pale yellow volatile oil with a flat cineolic odor is produce (Ravindran and Babu, 2016).

The seed of *A. corrorima* had long been part of the daily Ethiopian dishes, in the preparation of curry powder for culinary purposes and others. It also use to flavor all kinds of sauces locally called ‘wot’ for which they are ground and usually mixed with other spices (Etissa, 2006). The dried *A. corrorima* fruits had long been marketed throughout the country. However, its fresh fruits also sold

at local markets in the main cultivating areas. It is relatively expensive when compare with other spices and were mainly used as a currency during local transactions (Jansen, 1981).

2.5. Plant Tissue Culture Techniques and Its Application

The main use of tissue culture as a tool for plant propagation could be specifically relevant for vegetative propagated crop plants that resist common asexual propagation or when wants to make mass propagation from a single plant. Micro propagation is defined as a collection of methods used to grow plant cells, tissues and organs *in vitro*, under highly controlled environment. Different explants are used in the tissue culture such as, axillary bud, shoot tips, meristem tips, and root tips (Hiremath, 2006). A plant tissue culture technique creates many opportunities for sustainable food security and improvement the qualities and quantities of crops (Oggema *et al.*, 2007). In tissue culture 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 technique plays a great role in propagation program of different trees with respect to improving their efficiency and quality (Thomas, 1997). Plant tissue culture technique is not only used plant buds, stem tips, root tips leaves, but also used for the growing of plant parts like; cells, tissue and organs in manmade nutrient medium in a sterile environmental condition. On the fact that, many plant cells have the ability to regenerate in to whole plants (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves (less common in root) often used to generate a new plant on culture media in the given required nutrients and plant hormones (Idowu *et al.*, 2009).

Now a day in a modern laboratory, plant tissue culture is carried under aseptic conditions and in filtered air. Plant materials brought from the natural environments are naturally contaminated on their surfaces and sometimes interiors with microorganisms, due to this, surface sterilization is possible by chemicals solutions like, alcohol, sodium hypo chloride mercuric chloride etc. Now a day's mercuric chloride (HgCl) is not advisable to use for surface sterilization because of its carcinogenic nature. Explants are usually placed on the surface of a solid culture medium and also sometimes placed directly into a liquid medium, specifically when cell suspension cultures are desired. Both solid and liquid media are composed of inorganic salts, a few organic nutrients, vitamins and plant hormones. Solid media are prepared from liquid media with the addition of a gelling agent, like agar and phytigel (Georgiev, 2009).

Propagation of plants from single cell, tissue and organ through in vitro propagation is the application of tissue culture technique under sterile and controlled environmental condition (Hartmann *et al.*, 1990b). When compare other conventional propagation techniques, in vitro propagation enables multiplication of single explants in to several thousands of plants in less than a year, without influencing the growth or productivity of the original plants. Furthermore ,once formed, it will be continuously divide and serve as a continuous source of micro cutting that can result in plant production under greenhouse conditions with no any seasonal influence (Teisson and Alvard, 1995).

As a general a distinct of techniques in plant tissue culture may give a certain values over traditional techniques of plant propagation like production of exact copies of plants which provide specified good fruits, flowers in the absence of seeds or different pollinators from plant cells that have been genetically modified (totipotency) (George and Hall, 2008).

The selection of explant material decided if the plantlets obtained through tissue culture are either diploid or haploid. The chance of increasing microbial contamination is high when inappropriate explants are taken. So, an appropriate choice of explant has great priority in the tissue culture application. The regeneration potential of different organs and explants has various explanations on the base of the specific difference of the plant. The common explants used in the tissue culture is taken from the meristematic ends of the plants like the shoot tip, root tip, rhizome buds and auxiliary bud tips. Since these tissues have high rates of cell division with different amount of growth regulating substances like auxin and cytokinin (Georgiev, 2009). Uncontaminated explants are usually taken from seedlings which are aseptically grown from surface-sterilized seeds. However, this is not always perfect because of the hard surface of the seed coat which is less permeable to penetration of harsh surface sterilizing agents, like sodium hypochlorite, so that, the acceptable conditions of sterilization used.

2.6. Propagation of *A. corrorima*

Micro propagation maintains a mass production of elite clones from hybrid or specific parental lines and provides healthy seedlings with required characteristics (Hiremath *et al.*, 2006) *Aframomum corrorima* propagated from both their seeds and rhizomes. However, propagation by using rhizome has its own draw backs such as large space requirements, high transportation cost and slow multiplication rate. To overcome some of such like problems, micro propagation of *A. corrorima*

cultivator's used rhizome buds. Propagation by using rhizome is probably easier and quicker than propagation by using seed. The development of micro propagation method is the base for the future improvement of the crop through tissue culture, genetic engineering as well as for modern germplasm conservation. The most common prominent members of the family *Zingiberaceae* are cardamom, large cardamom, turmeric and ginger which have been so far successfully grown through micro propagations (Eyob, 2009).

2.6.1. Propagation by Seed

Propagation by seed is quite difficult than propagation by rhizomes because of its hard seed coats. The germination process of *A.corrorima* is affected by a variety of both exogenous and endogenous dormancies (Bewley and Black, 1994). In addition to this, germination process of *A.corrorima* is hindered due to the present of morphological seed dormancy or germination inhibitors in the capsules or endosperm (Hilhorst *et al.*, 2006).Seed dormancy in some seeds can be detected by a close evaluation of seeds, particularly for exogenous seed dormancy. Seed dormancy is a failure of a viable seeds to germinate when conditions are fully favorable.

Soaking of seeds in 50% sulfuric acid (H_2SO_4) for 60 min followed by soaking in 250 mg/l gibberellic acid (GA_3) for 24 hrs.is the best effective treatments to increase the chance of germination of *A.corrorima* seeds.(Eyob, 2009) Therefore, H_2SO_4 is important to break seed dormancy of *A.corrorima*. The thick gluey substance (mucilage) found around the seed is inhibit the diffusion of oxygen to embryo as a result, it prevents germination. Thus, H_2SO_4 pretreatment is required for removing mucilage and then soaking in GA_3 or NAA in order to allow the diffusion of oxygen from the surrounding to the embryos and enhance the chance of seed germination (Soyler and Khawar, 2007).

2.6.2. Propagation by Rhizomes

The conventional technique of propagation of *A. corrorima* is through the splitting of rhizome of one year old and another young sucker parts. Propagation by rhizome is used to shortens the juvenile stage of the stand plants, but in some case, it is always come with shortage of planting materials to cover large areas of land (Tefera and Wannakrairoj, 2004) for seeds can be much more strict than for vegetative materials(Georgiev, 2009).

2.7. Media Composition

The media of plant tissue culture should generally contain, macronutrients, micronutrients, vitamins, amino acid, source of carbon, growth regulators and solidifying agents. Based on the international association for plant physiology, the nutrients in concentrations greater than 0.5mM/l are defined as macro elements and those required in concentrations less than 0.5mM/l as microelements (De Fossard, 1976). However, the optimum concentration of each nutrient for achieving maximum growth rates varies among species.

2.7.1. Macronutrients

Besides Carbon, Hydrogen and Oxygen, macro nutrients in plant tissue culture media includes: nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and Sulphur(S) for satisfactory growth and morphogenesis. Plant culture media should contain at least 25-60 mM of inorganic nitrogen for satisfactory plant cell growth. Potassium is required for cell growth of most plant species. Most media contains K in the form of nitrate, chloride salts at concentrations ranging between 20 and 30 mM, the optimum concentrations of P, Mg, S and Ca ranges from 1-3 mM (Ngomuo *et al.*, 2014).

Selective omission of phosphate, potassium, nitrate, iron and chloride resulted in a significant reduction in the growth of explants. The omission of other minerals, such as magnesium, sulfate, ammonium, sodium, calcium, and several micronutrients did not significantly affect explant growth. The mean number of leaves produced by nodal cultures is also affected by mineral omissions. Omission of nitrate, phosphate, and potassium has significant effect to reduce leaf number, whereas the omission of ammonium, calcium, sulfate, and magnesium had no significant effect (Lee and de Fossard, 1977).

The precipitation of minerals during autoclaving was due to the present of Fe-EDTA and KH_2PO_4 . During autoclaving, Fe-EDTA reacted with micronutrients and KH_2PO_4 react with calcium to form insoluble mineral complexes that were no longer available to the plant for growth and development (Schenk *et al.*, 1991). Potassium is not become a part of the chemical structure of plants. But it plays many important regulatory roles in the development of plants and to increases quality crop yields. It is required for a numerous plant growth process. Potassium “activates” at least 60 different types of enzymes involved in plant growth and changes the physical shape of the enzyme molecules and maintains appropriate chemical active sites for reactions. Potassium also

neutralizes various organic anions and other compounds within the plant, helping to stabilize pH between 7 and 8. Potassium present in the cell determines how many of the enzymes can be activated and the rates at which chemical reactions can proceed. Therefore, the rate of a given reaction is controlled by the rate at which K enters the cell (Van Brunt and Sultenfuss, 1998).

From seed germination to seed production, potassium is one of the most important macronutrients, along with nitrogen (N) and phosphorous (P) (Fageria, 2016). It requires for various biochemical and physiological processes that are responsible for plant growth and development. Potassium takes part in protein synthesis, carbohydrate metabolism, and enzyme activation. It is important in the cat ion-anion balance, osmoregulation, water movement, energy transfer, and many other processes. It also plays a mitigating role in various abiotic stresses such as drought, salinity, and metal toxicity, high or chilling temperatures. When a given plants experience potassium deficiency, they exhibit stunted growth and yellowing in their leaf margins, poor root system, lodging, and yield reductions. A lack of K fertilizer increases plant susceptibility to various diseases and pest infestation and makes plants exposed to damage under various stress conditions (Wang *et al.*, 2013). On the top this, potassium also have ultimate significant roles in numerous plant physiological processes for seed germination and emergence, stomata regulation, phloem transport, protein synthesis, photosynthesis, energy transfer ,enzyme activation, nutrient balance, and stress resistance(Prajapati and Modi, 2012).Potassium salts have been thoroughly studied as good catalysts for improving seed germination and the emergence rate. Most common forms of potassium salts used in seed priming are potassium nitrate(KNO₃), potassium chloride(KCl) and potassium dihydrogen phosphate(Tang *et al.*, 2015). In addition to the above uses, potassium has a key role in plant growth and development processes.

The most common signs of potassium deficiency are yellow scorching or firing (chlorosis) along the leaf margin. In most cases of potassium deficiency, the fired margin of the leaf may fall out. But in the case of broadleaf crops, such as cotton and soybeans, the entire leaf may shed resulting in premature defoliation of the crop. Potassium deficient crops grow slowly and have poorly developed root systems, weak Stalks and lodging of cereal crops such as corn and small grain is common. Seeds from potassium deficient plants are small, shriveled, and more susceptible to diseases. Fruit from potassium deficient is often lacking in normal coloration and is low in sugar content (Ashley *et al.*, 2005).

Appropriate amount of phosphorus nutrition is important for all explant growth and morphogenesis (Lee and DeFossard, 1977, (Ramage, 1999).It rapidly consumed by plant suspension cultures (Kato *et al.*, 1977)and it may be the limiting mineral in many plant tissue cultures (Lumsden *et al.*, 1990). If the concentrations phosphate is high it may have limited the availability of other mineral ions like Fe^{3+} and Ca^{2+} (Loneragan and Asher, 1967).

2.7.2. Micronutrients

Essential micronutrients for plant cell and tissue culture media includes: zinc (Zn), boron (B), copper (Cu) iron (Fe), manganese (Mn), and molybdenum (Mo). From these micro elements iron is the most critical once. These elements are used as either citrate or tartrates salts in culture media. But there exist some problems with these compounds for their difficulty to dissolve and precipitate after media preparation. However, there has been try to solve this problem by using ethylene diamine tetra acetic acid (EDTA)-iron chelate (FeEDTA)(Murashige and Skoog, 1962). A method for preparing an iron chelate solution that does not precipitate has been also developed (Steiner and van Winden, 1970).Both cobalt (Co) and iodine (I) may be added to certain media, but their requirements for cell growth has not been exactly established. Sodium (Na) and chlorine (Cl) are also used in some media, in spite of reports, they are not essential for growth. Iron and molybdenum are added at to culture media at concentration of $1\mu M$, iodine at $5\mu M$, zinc at $5-30\mu M$, manganese at $20-90\mu M$, boron at $25-100\mu M$, Copper and cobalt are at $0.1\mu M$, (Johns, 2019).

2.7.3. Carbon and Energy Sources

Frequently use of carbon source in the tissue culture media includes: Sucrose, lactose, galactose, maltose and starch. All lactose, galactose, maltose and starch were reported to be less effective than either sucrose or glucose. As frequently demonstrated autoclaved sucrose was better for growth than filter sterilized sucrose. Autoclaving seems to hydrolyze sucrose into more efficiently utilizable sugars such as fructose. Sucrose acts as morphogenetic trigger in the formation of auxiliary buds and branching of adventitious roots (Vinterhalter and Vinterhalter, 1997).

In addition to the above carbon sources culture media supplements of sugar cane molasses, banana extract and coconut water to basal media can be a good alternative for reducing medium costs. They are also act as a source of vitamins and inorganic ions required for plant growth (Vinterhalter and Vinterhalter, 1997).

2.7.4. Vitamins

Vitamins are required by plants as catalysts in various metabolic processes. They usually act as limiting factors for cell growth and differentiation. Common vitamins used in the cell and tissue culture media includes: thiamin (B1), nicotinic acid and pyridoxine (B6). From these vitamins, thiamin is necessarily required by all cells for growth (Ohira *et al.*, 1976). It used at concentrations ranging from 0.1 to 10 mg/l. Both nicotinic acid and pyridoxine, may not essential for cell growth of many species, they are usually added to culture media (Williams, 1995).The concentration ranges of nicotinic acid is from 0.1-5 mg/l and also pyridoxine is used at a range between 0.1-10 mg/l. Other vitamins like biotin, folic acid, ascorbic acid, pantothenic acid, tocopherol (vitamin E), riboflavin, p-amino-benzoic acid are used in some cell culture media. But they are not growth limiting factors. It was recommended that other vitamins should be added to culture media only when the concentration of thiamin is below the desired level (Murashige and Skoog, 1962). Myo-inositol is also requiring in small quantities to initiate cell growth of most plant species (Vasil and Thorpe, 2013). However, myo-inositol is believed to play a role in cell division due to its ability to breakdown into ascorbic acid and pectin then incorporation into phosphors rinositides and phosphatidyl-inositol. It usually used in plant cells and tissue culture media at concentrations of 50-5000 mg/l.

2.7.5. Solidifying Agents

There are a number of gelling agents such as agar, agarose and gellan gum to harden the culture media. Agar is a polysaccharide obtained from sea weeds which are universal as a gelling agent for preparing semi-solid and solid plant tissue culture media. It has many advantages over other gelling agents like mixed with water, easily melts in a temperature range between 60-100°C and also solidifies at approximately 45°C then it forms a gel stable at all feasible incubation temperatures. Gels of agar do not react with media constituents and are not digested by plant enzymes. The common concentrations of agar usually range between 0.8-1.0 percent. Preparations of pure agar have great importance especially in experiments dealing with tissue metabolisms. A common agar contains calcium, magnesium and other trace elements on comparing different agar brands (Pierik, 1997).The cost of agar in culture media is reduce by more than 70% when gelling agent prepared from the combination of laundry starch, potato starch and semolina in a ratio of 2:1:1(Prakash *et al.*, 1999).

2.7.6. Growth Regulators

One of the important components in plant tissue culture is plant growth regulators. It includes: auxin, cytokinin, gibberellins and abscisic acid. The type and extent of organogenesis in plant tissue cultures is determined by the appropriate proportion of auxins to cytokinin hormones (Skoog and Miller, 1957).

The most common auxins used in plant tissue culture media includes: indole-3- acetic acid (IAA), indole-3- butyric acid (IBA), 2, 4-dichlorophenoxy-acetic acid (2, 4-D) and naphthalene acetic acid (NAA). Among these, IAA is the only natural auxin occurring in plant tissues. All the above hormones are differing in their physiological activities and in the extent to which they translocate through tissue and their metabolized activities. In plant tissue culture, auxins are usually used to stimulate callus production and cell growth, to initiate shoots and rooting, to induce somatic embryogenesis, to stimulate growth from shoot apices and shoot stem culture. NAA and 2, 4-D is considered to be stable and can be stored at 4°C for several months (Gamborg *et al.*, 1981). If they last for longer periods their solution can be stored for several months in a refrigerator at -20°C. However, the solution of IAA can be stored in an amber bottle at 4°C for about a week. So, it is better to prepare fresh IAA solutions each time during medium preparation. IAA and 2, 4-D are dissolved in a small volume of 95% (Gamborg *et al.*, 1976).

The common types of cytokinin hormone used in the plant tissue culture are: BAP (6-benzylaminopurine), kinetin(KN) Zeatin (6-4-hydroxy-3-methyl-trans-2-butenylaminopurine) and TDZ (thiazuron-N-phenyl-N-1, 2,3thiadiazol-5ylurea). Cytokinins are relatively stable compounds in culture media and stored at -20°C. As frequently reported, cytokinin is difficult to dissolve in distilled water. But addition of few drops of 1N HCl or 1N NaOH facilitate their dissolution. It also dissolved in small amounts of dimethyl sulfur oxide (DMSO) without injury to the plant tissue (Schmitz *et al.*, 1972).

2.7.7. Media Preparation

A convenient method for preparation of culture media is to make concentrated stock solutions and immediately diluted to preferred concentration before use. Solutions of macronutrients are better to be prepared as stock solutions of 10 times the strength of the final operative medium and stored in a refrigerator at 2- 4°C. Micronutrients stock solutions are made up at 100 times of the final concentration of the working medium and also stored in a refrigerator or a freezer until needed. Iron

stock solution also 100 times concentrated than the final working medium and stored in a refrigerator. Vitamins are prepared as either 100 or 1000 times concentrated stock solutions and stored in a freezer (-20°C) until used (Gamborg *et al.*, 1981). Stock solutions of growth regulators are usually prepared at 100-1000 times the final desired concentrations.

Concentrations of inorganic and organic components of media are generally expressed in mass values (mg/l) in tissue culture literature. The International Association for plant Physiology has recommended the use of mole values (Bhojwani SS, 1983).

2.7.8. Media sterilization and Media selection

Media contamination could be controlled by adding antimicrobial agents, acidification or by filtration through micro porous filters (Levin and Thnny, 2002). Sterilization rooms should have the least number of openings to minimize the possibilities of contamination. Both media preparation and sterilization should be performed in separate compartments. Area of sterilization should have walls and floor to overcome moisture, heat and steam (Ahloowali BS, 2002).

Media sterilization is performed by autoclaving at the temperature between 115°C – 135°C. Autoclaving has its own advantages and disadvantages. The advantage of autoclaving is related to quickness and its simplicity, whereas the disadvantages of autoclaving are: the pH of the media changes and some components may decompose and so to lose their effectiveness. For example, autoclaving mixtures of fructose, glucose and sucrose resulted in a drop in the agar gelling capacity and affecting pH of the culture medium through the formation of furfural derivatives due to sucrose hydrolysis. Additives of antimicrobial agents are less commonly applied in plant tissue culture media because limitation for their use was reported and attributed to harm imposed on plants as well (Savangikar, 2004).

2.8. Effects of Explant Density and Media Volume on Shoot Multiplication.

Density of the explants does have a clear effect on the proliferation of plants. Density of explants per magenta has invers proportional to multiplication and growth rate of shoots. The effect of density of explant per magenta also has ultimate effects on the survival of plants in the green house. This suggests that, at low density, the explants benefit from a better nutrition and a lower quality of exudates released by shoots (Boullani *et al.*, 2017). Number of shoots was maximal for 3 to 4 explants per magenta and showed a significant decrease when this density was greater than six. This

suggests that, at low density, the explants benefit from a better nutrition and a lower quality of exudates released by shoots. A high number of shoots per glass jar reduced their growth. This is probably due to the competition for nutrients between shoots in the jar (Thomson, 2009). Explants under in vitro conditions compete with each other for a constant amount of water and nutrients in growth medium (Yildiz, 2012). The effect of media volume and densities of plantlets per vessel for shoot multiplication of turmeric plants were also demonstrated by (Halloran and Adelberg, 2011). Based on their experiment results maximum shoot multiplication was observed at low plantlet density and at high media volume. In addition to this, their experiment also explains the effect of media volume for the survival of plantlets in the greenhouse. According to their results and their explanations, plantlets taken from high media volume has greater chance to survive in the green house.

2.9. In Vitro Shoot Multiplication and Root Induction of *A. corrorima* Related Species

2.9.1. In Vitro Shoot Multiplication

Plant tissue culture has initiation stage, shoot multiplication stage, rooting stage and acclimatization stage. In shoot multiplication stage, each explant has a number of micro shoots. A cluster of micro shoots are divided into a separate micro shoot, that transplanted into new culture medium. The medium used during shoot multiplication stage is always depends on the plant species and type of culture. Stabilization characteristics of different plants are different, for instance most annuals and herbaceous plants stabilized quickly within a few subcultures, but this is not true in woody plants. The combination of phytohormones often determines the morphogenesis, embryogenesis or organogenesis of plants. (Hartmann *et al.*, 1990a). A minimum mass of tissue culture is necessary to produce rapid and uniform multiplication for the next transfers. Multiplication may be repeated many times in order to have enough explants for the purpose of the next tasks. Due to the lack of stabilization of the culture medium, micro shoots sometimes became worsen with time, lose their leaves, develop tip-burn, fail to grow and lose potentiality to regenerate (Pierik, 1997).

The addition of NAA with all the concentration of BAP did not maintain any meaning full effect on number of shoots in three tested varieties of turmeric (*Faisalabad*, *Kasur* and *Bannun*) (Naz *et al.*, 2009). On the top of this, higher amount of BAP results to decrease the rate of multiplication. But the

use of BAP alone in lower concentration in the medium resulted poor regeneration response and produced single shoots (Plants and Short, 2012). Combining use of BA and KN) in tissue culture has negative effect to improve shoot multiplication (Hussain *et al.*, 2008).

Selvakkumar *et al.* (2007) reported that, *Alpinia officinarum* (lesser galangal) has multiple shoots that originated the rhizome's bud were cultured on Morishige and Skoog (MS) medium supplemented with different growth regulators and maximum shoot multiplications were observed on a medium containing 3.0 mg/l kinetin (KN), 1.0 mg/l Naphthalene Acetic Acid (NAA) and 30g/l sucrose. An average of 11 shoots per explant were rise in each rhizome buds.

2.9.2. In Vitro Root Induction

During root formation auxins influenced the mobilization of nitrogen to the zone of rooting (Basak *et al.*, 1995). Enough amount of rooting was formed in a medium having half strength MS media supplemented with 0.5 mg/l of IBA in *Alpinia officinarum* and *Alpinia zerumbet* (Selvakkumar *et al.*, 2007). During the rooting culture of ginger, MS media supplemented with 0.5mg/l and 1.0 mg/l NAA were more effective and provides the maximum number of roots per shoot respectively ((Jagadev *et al.*, 2006). IBA has least effective than NAA in root formation as days needs to rooting (IBA requires only 6-8 days to produce root but NAA requires about 10 -15 days) in ginger (Kambaska *et al.*, 2009).

Geetha *et al.* (1997) reported that, IBA alone did not give a favorable response on rooting. The root produced during this time was generally poor as they were very slender and not vigorous. Their report also includes the combining effect of NAA and IBA for the induction of rooting in the tissue culture.

The *in vitro* propagation and rooting of shoot lets in case of turmeric were also obtained maximum number of rooting and *multiple* shoots on half strength of MS medium supplemented with 0.5 mg/l and 2.0 mg/l of NAA (Behera *et al.*, 2010). During micro propagation of turmeric, the highest rooting was stimulated by sub culturing of the proliferated shoots on half strength MS media with 0.3 mg/l of NAA (Udomdee *et al.*, 2003). Root formation on ginger was potentially assessed by testing on five different combinations of plant hormones. From these tested genotypes, the most effective result was observed in half strength MS media supplemented with 2.0 mg/l IBA and 2.0 mg/l NAA (Hossain *et al.*, 2010).

2.10. Environmental Control of In Vitro Culture Plants

The key steps in micro propagation is optimization of microenvironment that ensure to produce quality plantlets which have high chances of surviving in the in vitro conditions and finally in the natural environment. A conventional in vitro environment have: low photosynthetic photon flux density (PPFD), optimized concentrations of sugars, salts and plant growth regulators in the growth medium, constant temperature, high relative humidity, aseptic conditions and accumulation of secondary metabolites (Kozai *et al.*, 1997)Both the biochemical and biophysical process of photosynthesis is depend on temperature(Gomes Laranjo *et al.* (2006)and it is a major factor which determine the rate of the growth of plants (Samach and Wigge, 2005).The perception of ambient temperature permits for the maintenance of plant homeostasis, thereby buffering against potential disruptive effects on cellular stability (Franklin, 2009).

One of the critical environmental factors which control the plant developmental processes such as bud dormancy, leaf growth, and flowering and stem elongation is photo period. In addition this photoperiod is important for hormone synthesis, pigment accumulation ,ion influx changes and gene expression (Kuhnet *et al.*, 2009).Plant contains sensory photo receptors which detect changes in the ambient light environment (Karpinski *et al.*, 2003)Photoperiod and light intensity in the microenvironment affect the physiological processes of plantlet and their growths. The amount of light requires for photosynthesis in the in vitro cultured plants is less importance as compared to in vivo planted plants (Economou and Read, 1987).Light is used to regulates plant development through the influences of photo period, spectral quality and intensity(Da-Silva and 1997).The energy of light for photosynthesis is measured by photosynthetic photon flux density (PPFD) on the growing plantlet (Ibaraki *et al.*, 2005).

2.11. Acclimatization of In Vitro Rooted plantlets

Acclimatization is the climatic adaptation of an organism that has been moved to a new environment. It is a process that hardens off the plantlets after they had been growing in controlled conditions in the tissue culture laboratory and ready to them for establishment in the open field. It is also defined as the transfer of plantlets from the growth room atmosphere to the outside atmosphere in soil under greenhouse conditions. (Tripathiet *al.*, 2018). This is carried out in a greenhouse and

the plantlets are planted in plastic box with holes filled with soil. It is the prime measure of any tissue cultured plantlets. During acclimatization, agar is washed off completely to remove a potential source of contamination, and the micro plants are transplanted into a standard pasteurized soil mixes in a small pots or cell in a more or less conventional manner. At the beginning the micro plants should be give protection from desiccation in a shaded, high relative humidity tent or under mist fog. Moderate temperature and relatively high humidity were greatly increase the chance of initial survival of potted and field grown plantlets (Tripathiet *al.*, 2018). The “best” conditions for in vitro production of plantlets may not be necessarily best for greenhouse acclimatization (Premkumar *et al.*, 2001).

Hardening of in vitro raised plantlets is essential for better survival and successful establishment. Tissue culture raised plantlets develop a distinct morphology in response to the in vitro environment (Hiren *et al.*, 2004).The most difficulties associated with the survival and growth of tissue cultured plants after transplanting are to the poor control of water loss from the plant and their necessity to transfer from heterotrophic to autotrophic nutrition(Lavanya *et al.*, 2009).They also have very low photosynthesis ratio with the hill reaction. Carbon dioxide (CO₂) uptake and net CO₂exchange in light and dark stage was also low despite the green appearance of the plantlets. This being that CO₂ release in respiration was greater than that taken up by photosynthesis (Grout and Aston, 1977).This physiological stage can only achieved until about 14days after transplanting which accounts for the period of acclimatization where direct sunlight and total exposure to other harsh environmental and physiological activities are still in check, gradually initiating the plantlets to sensing the phototrophic environment. When growth retardant, are added to the rooting medium it usually triggers shoots having fewer and smaller leaves. for citrus this reduction in plant growth makes them more wilt tolerant and increases the survival of plantlets following transfer to the greenhouse (Hazariki *et al.*, 2002).

3. OBJECTIVES

3.1. General Objective

The general objective of this study is to investigate the effect of medium strength, potassium dihydrogen phosphate, medium volume, and density of explants per culture vessel in *in vitro* propagation of *A. corrorima*.

3.2. Specific Objectives

- ✓ To assess the effect of different medium strength in *in vitro* propagation of *Aframomum corrorima*.
- ✓ To investigate the effect of potassium dihydrogen phosphate in *in vitro* propagation of *Aframomum corrorima*.
- ✓ To study the effect of volume of medium on shoots multiplication and rooting of *Aframomum corrorima*.
- ✓ To evaluate the survival rate of plantlets in *ex vitro* environments.

4. MATERIALS AND METHODS

All the experiments of this study were conducted at the tissue culture laboratory of Institute of Biotechnology in Addis Ababa University.

4.1 Preparation of Stock Solution and Growth Media

4.1.1. Preparation of MS Stock Solution

Full strength Morishige and Skoog (1962) stock solutions of macronutrients, micronutrients, vitamins and Na₂ EDTA and FeSO₄ .7H₂O were separately prepared. The required amount of each nutrient was weighed in grams per liter and dissolved in distilled water step by step in such a way that the next nutrient was added after the first one was completely dissolved. After all the required components were completely dissolved using magnetic stirrer, the solution was poured into plastic bottles and stored in refrigerator at -20°C until used.

4.1.2. Preparation of Plant Growth Regulator Stock Solutions

The plant growth regulators (PGRs) used for this study were 6-benzyl amino purine (BAP) and indol-3-butyric acid (IBA). All required growth regulator stock solutions were prepared by weighing and dissolving the powder in distilled water at the concentration of 1.0 mg/ml. Auxins were initially dissolved by 3-4 drops of 1N NaOH, whereas cytokinin were dissolved by 1N HCl and then, the volume was adjusted by adding distilled water. At the end, growth regulator stock solutions were stored at 4°C until used for a maximum of one month.

4.1.3. Culture Media Preparation

MS basal medium was prepared using 50 ml/l macronutrients, 5 ml/l micronutrient, 5 ml/l vitamin, 5 ml/l Fe-Na-EDTA and FeSO₄ mixture, 30 g/l sucrose. The required and appropriate amount of PGR was added and then the pH was adjusted to 5.7 using 1 N HCl and 1 N NaOH and heated on a stirring hot plate, then 7.0 g/l of agar was added and melted. After the agar is dissolved on a stirring hot plate and became clear solution, 50 ml medium was dispensed into Magenta culture vessels and autoclaved at a temperature of 121°C and 105 KPa pressure for about 15-17 minutes.

4.2. Plant Material Collection and Surface Sterilization

Capsules of *A. corrorima* were collected in Oromia Region from Jimma Zone Goma Woreda at Chocho Keble during the month of November. Capsules of *Aframomum corrorima* were cut open to

release seeds and dried in shade for 2- 3 days and the seed was soaked in tap water for about 2 days and 50% H₂SO₄ for 60 minutes. The seeds were then washed with detergent and rinsed in running tap water for 3-5 times. Then the seeds were sterilized in 70% alcohol for 1minute and rinsed 3 times with sterile distilled water and also further sterilized with 5% Clorox for 25 minutes followed by 4-5 rinses with sterile distilled water. The surface sterilized seeds were cultured in Magenta culture vessels containing growth regulators free medium. Fourteen seeds per culture vessels were used for planting in each 20 culture vessels.

4.3. Shoot Initiation

One to two-centimeter-long shoot tips were taken from *in vitro* germinated seedlings and were cultured on MS medium supplemented with 1.5 mg/l BAP. For each treatment, 20 culture vessels containing three explants were used, which is a total of 60 explants per treatment. Each culture vessel with cultured explants were sealed properly with Para film and labeled, then placed in growth room chamber with 16 h photoperiod at a temperature of 25 ± 2°C. Even the normal days for recording is not exceed four weeks, the researcher recorded mean shoot number length and mean leaf number after six weeks because of slow growth nature of the plant. Shoots were cultured on full, half, one third and one fourth salt strength semi solid MS medium.

4.4. Shoot Multiplication

Healthy and young micro shoots were cultured on shoot multiplication medium after six weeks of growth on the culture initiation medium. Healthy shoots were aseptically, carefully cut off and cultured on shoot multiplication medium. The shoot multiplication was carried out in full medium strength, half medium strength, one third medium strength, one fourth medium strength, half concentration of dipotassium hydrogen phosphate, one third concentration of dipotassium hydrogen phosphate, one fourth concentration of dipotassium hydrogen phosphate, different volume of MS medium like (20 ml, 30 ml, 40 ml, 50 ml and 60ml) and in the standard medium volume(50 ml) having different density of explants like(1,2,3,4,5,6,and 7 explants per culture vessel). In all case 1.5 mg/l BAP was used based on the recommendation of Getie (2017). To investigate the effect of both medium strength and concentration of potassium dihydrogen phosphate, 60 explants per treatment, and three explants per culture vessel were used. To investigate the effect of medium volume on shoot multiplication,4 explants per culture vessel and 5 replications for each treatment were used. Finally, to study the effect of density of explants on shoot multiplication, 1, 2, 3, 4, 5, 6 and 7

explants per culture vessel were used in 5 replications for each treatment. In all treatments, MS medium containing 1.5 mg/l BAP was used. Each culture vessel was sealed and labeled properly and placed in the growth room chambers with the same culture conditions as for shoot initiation. All treatments were carried out in semisolid MS medium. To maximize the number of explants, sub culturing was carried out at three weeks interval by transferring the newly multiplied micro shoots to fresh medium and in every four weeks, means shoot number, length per explant and numbers of leaves per explant were recorded.

4.5. Rooting

Rooting of multiplied shoots of *Aframomum corrorima* was conducted on different medium strength like (full, half one third and one fourth medium strength), on standard, half, one third, and one fourth concentrations of potassium dihydrogen phosphate, and on different medium volumes like (20 ml,30 ml,40ml,50ml and 60 ml)in the given culture medium supplemented with 1.5 mg/l IBA. To investigate the effect of both medium strength and concentration of dipotassium hydrogen phosphate, three explants per culture vessel and a total of 60 explants per treatment were used. To investigate the effect of medium volume, 4 explants per culture vessel in 5 replications were used for each treatment. Mean root number and mean root length was recorded after a month.

4.6. Acclimatization

The roots of well-developed plantlets were washed with tap water and transferred to pots containing sand, field soil and compost in a ratio of 1:2:1. Each plastic pot was covered with polyethylene bag to ensure high humidity, and then kept in the greenhouse. The polyethylene bag was removed after two weeks. Forty-five plantlets were acclimatized.

4.7. Experimental Design and Statistical Data Analysis

A complete randomized design (CRD) was used and the data were analyzed using the statistical data analysis software SPSS version 26.0 by using one-way ANOVA. The significant differences among treatments were compared using Least Significance Difference (LSD) and Duncan for the one-way ANOVA at 5% significant level.

5. RESULTS

5.1. In vitro Seed Germination

The cultured seeds started to germinate in the 5th week and completed at two and half months. Only 1.8% of seeds germinated at the start to germinate at 5th weeks and completed at about two and half months. Most seeds germinated between 7th and 9th weeks. But after 11 weeks no new germinated seeds were observed. The germination time difference of the seed might be because of healthiness of seeds and dormancy difference of seed to seed. From 280 planted seeds during seed culture, only 213 (76%) were germinated within 11 weeks (Figure 1).

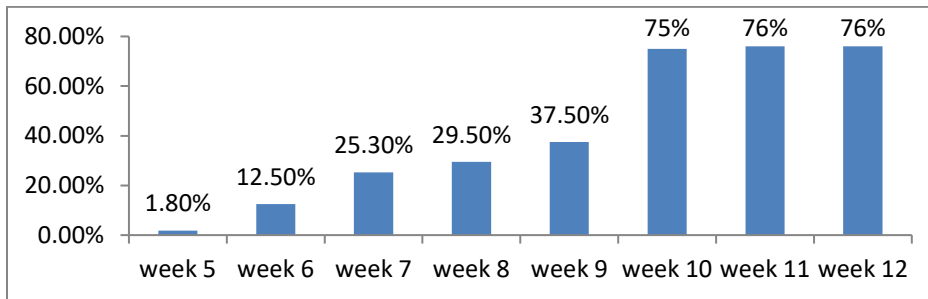


Figure 1. Seed germination percentage of *A. corrorima* in different weeks.

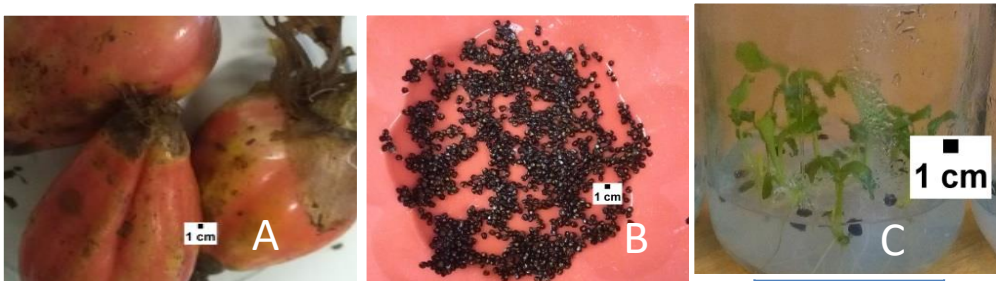


Figure 2. Seed germination on plant growth regulator free MS medium (A) = Fresh capsule of *A. corrorima*, (B) = Dried seeds of *A. corrorima* and (C) = Seedlings after three months of cultured seeds germination.

5.2. Culture Initiation

5.2.1. Effect of Medium Strength on Shoot Initiation

After two weeks of culturing, shoot tips started to develop leaves and at the end six weeks of growth on the initiation medium, only 85%, 81%, 76% and 71% of explants were survived on the medium containing full, half, one third and one fourth medium strength, respectively. Mean shoot number,

length and leaf number varied on the medium containing different salt strength (Table 1 and Figure 3). The highest mean shoot number (1.55 ± 0.11) was recorded on full strength medium. There was no significant difference in mean number of shoots per explant between one third and one fourth salt strength media. The highest mean shoot length (2.47 ± 0.17 cm) and mean leaf number (3.40 ± 0.11) were obtained on full strength medium. Mean shoot length on the medium containing 1/2 and 1/3 salt strength media did not show significant differences. The shortest mean shoot length (2.05 ± 0.78 cm) and the least mean leaf number (2.90 ± 0.69) were obtained on 1/4 salt strength medium. Mean leaf numbers (3.10 ± 0.19 and 3.00 ± 0.18) were recorded on the medium containing 1/3 and 1/4 medium strength respectively. No significant difference was observed between these two salt strengths.

Table 1. Effects of medium strength on shoot initiation of *A. corrorima*. Value indicated as \pm SE

MS strength	Mean no	Mean shoot	Mean no	
of survival	of shoot	length (cm)	of leaf	% of survival
Full MS	1.55 ± 0.11^a	2.47 ± 0.17^a	3.40 ± 0.11^a	85
1/2 MS	1.30 ± 0.10^{ab}	2.29 ± 0.09^{ab}	3.10 ± 0.19^{ab}	81
1/3 MS	1.25 ± 0.09^b	2.15 ± 0.97^{ab}	3.00 ± 0.81^{ab}	76
1/4 MS	1.15 ± 0.82^b	2.05 ± 0.78^b	2.90 ± 0.69^b	71

Means within columns having different letters in superscript are significantly different at $p < 0.05$

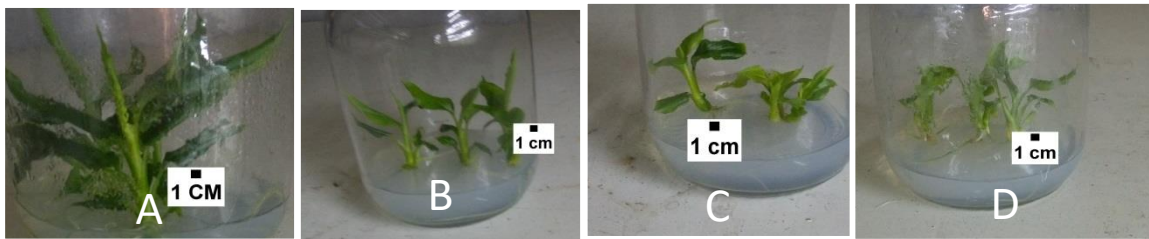


Figure 3. Shoot initiation of *A. corrorima*(A) =full (50 ml/l) salt strength, (B) =1/2 salt strength (c) =1/3 salt strength (D) =1/4 salt strength

5.3. Shoot Multiplication

5.3.1. Effect of Medium strength on Shoot Multiplication of *A. corrorima*

Mean shoot number, shoot length and leaf number of plantlets were different on the medium containing different salt strength medium (Table 2 and Figure 4). The highest mean shoot number per explant (3.20 ± 0.14) was recorded on full salt strength medium. However, there was no significant difference in mean shoot number between full and 1/2 salt strength medium. The highest mean shoot length (3.50 ± 0.13 and 3.40 ± 0.11 cm) were recorded on 1/2 and full salt strength, respectively. The difference was not significant. Mean shoot length (2.80 ± 0.147 and 3.10 ± 0.123 cm) were obtained on 1/4 and 1/3 salt strength, which showed no significant difference. The highest mean leaf number per explant (7.80 ± 0.138) was obtained on full strength medium. The second highest mean leaf number per explant (7.30 ± 0.14 and 6.90 ± 0.16) was recorded on 1/2 and 1/3 salt strength media, respectively. There was no significant difference between these media. The least mean value of leaf number per explant (6.10 ± 0.16) was obtained on 1/4 salt strength medium.

Table 2. Effect of salt strength on shoot multiplication of *A. corrorima*. Value indicated as \pm SE

MS strength	Mean no of shoot	Mean shoot length(cm)	Mean no of leaf
Full MS	3.20 ± 0.14^a	3.40 ± 0.11^{ab}	7.80 ± 0.14^a
1/2	3.00 ± 0.14^{ab}	$3.50a \pm 0.11^a$	7.30 ± 0.15^b
1/3	2.80 ± 0.14^b	2.80 ± 0.14^c	6.90 ± 0.16^b
1/4	2.70 ± 0.10^b	$3.10b \pm 0.12b^c$	6.10 ± 0.16^c

Means within columns having different letters in superscript are significantly different at $p < 0.05$

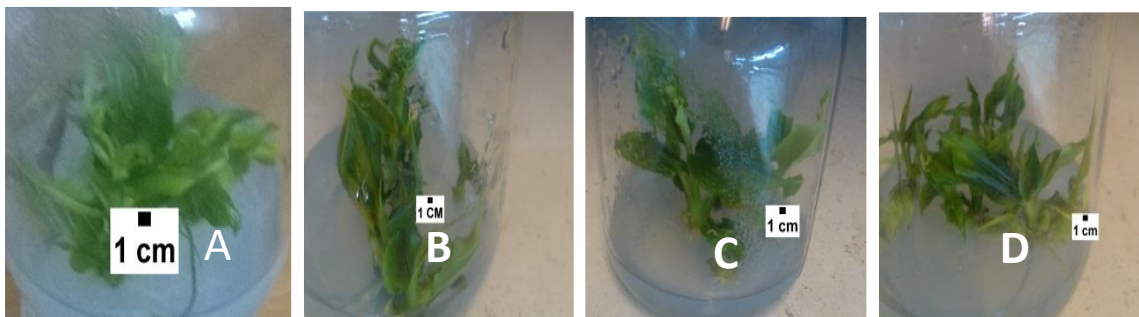


Figure 4. Shoot multiplications of *A. corrorima* on different salt strength media (A) = Full (50 ml/l) MS strength, (B) = $\frac{1}{2}$ MS strength (C) = $\frac{1}{3}$ MS strength (D) = $\frac{1}{4}$ MS strength.

5.3.2. Effects of Potassium di hydrogen phosphate (KH_2PO_4) on Shoot Multiplication of *A. corrorima*

Medium containing different concentrations of potassium dihydrogen phosphate resulted in different response of mean shoot number, shoot length and leaf number per explant (Table 3 and Figure 5). The highest mean shoot number per explant (3.20 ± 0.138) was obtained on medium containing standard MS concentration of potassium dihydrogen phosphate. The next highest mean shoot number per explant (2.50 ± 0.115) was obtained on medium containing $\frac{1}{2}$ concentration of potassium dihydrogen phosphate. There was no significant difference in mean shoot number per explant between media containing $\frac{1}{3}$ and $\frac{1}{4}$ potassium dihydrogen phosphate. Medium lacking potassium dihydrogen phosphate showed the least mean number of shoots. The maximum mean shoot length (3.40 ± 0.11 and $3.33 \pm 0.14\text{cm}$) were recorded on the medium containing standard and $\frac{1}{2}$ potassium dihydrogen phosphate, respectively. The least mean shoot length ($2.550 \pm 0.09\text{ cm}$) was obtained on the medium lacking potassium dihydrogen phosphate. The highest mean leaf number per explant (7.80 ± 0.14) was recorded on medium containing standard MS potassium dihydrogen phosphate. The next highest mean leaf number per explant was (5.80 ± 0.225) was obtained on medium containing half concentration of potassium dihydrogen phosphate.

Table 3. Effect of potassium dihydrogen phosphate on shoot multiplication of *A. corrorima*. Value indicated as \pm SE

Conc. of KH ₂ PO ₄	Mean shoot	Mean shoot	Mean leaf
	number	length(cm)	number
MS Standard	3.20 \pm 0.138 ^a	3.40 \pm 0.11 ^a	7.80 \pm 0.14 ^a
1/2	2.50 \pm 0.12 ^b	3.33 \pm 0.15 ^a	5.80 \pm 0.22 ^b
1/3	1.60 \pm 0.13 ^c	3.05 \pm 0.09 ^{ab}	5.10 \pm 0.19 ^c
1/4	1.90 \pm 0.11 ^{bc}	2.79 \pm 0.08 ^c	3.60 \pm 0.21 ^{cd}
no	1.2 \pm 0.09 ^d	2.55 \pm 0.09 ^d	2.50 \pm 0.18 ^d

Means within columns having different letters in superscript are significantly different at $p < 0.05$

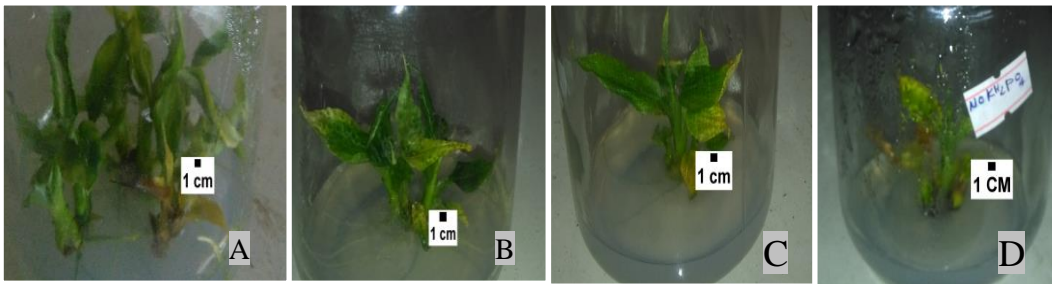


Figure 5. Shoot multiplication of *A. corrorima* on different concentration of KH₂PO₄(A) = 1/2 concentration of KH₂PO₄, (B) = 1/3 concentration of KH₂PO₄ (C) = 1/4 concentration of KH₂PO₄, (D) = Without KH₂PO₄ in the medium.

5.3.3. Effect of Medium Volume on Shoot Multiplication

Mean number of shoots, shoot length and leaf number showed variation on different medium volume (Table 4 and Figure 6). The highest mean shoot number per explant (3.60 \pm 0.21) was obtained on 50 ml medium volume. The next highest mean shoot number per explant (3.40 \pm 0.10) was obtained on 40 ml medium volume. The minimum mean shoot number was obtained on 60 ml medium volume. However, mean shoot number obtained on 50 ml and 40ml did not show significant difference. The maximum mean shoot length (4.640 \pm 0.61 cm) was recorded on the medium containing 60ml medium volume. The second maximum mean shoot length (4.340 \pm 0.770 and 4.42 \pm

0.78 cm) was obtained on 50 ml and 40 ml medium volume, respectively. There was no significant difference between the two. The least mean shoot length (3.32 ± 0.99 cm) was obtained on 20 ml medium volume. The maximum mean leaf number per explant (8.40 ± 0.10 and 8.20 ± 0.15) was recorded on 40 ml and 50 ml medium volume respectively. There was no significant difference between the two medium volumes. The least mean number leaves per explant (5.60 ± 0.16) was recorded on 20 ml medium volume.

Table 4. Effect of medium volume on shoot multiplication of *A. corrorima*. Value indicated as \pm SE

Medium volume (ml)	Mean no of shoot	Mean shoot length(cm)	Mean no of leaf
20	2.60 ± 0.16^{cd}	3.32 ± 0.99^d	6.00 ± 0.26^{bc}
30	3.00 ± 0.13^{bc}	4.04 ± 0.67^c	6.40 ± 0.38^b
40	3.40 ± 0.10^{ab}	4.34 ± 0.77^b	8.40 ± 0.10^a
50	3.60 ± 0.21^a	4.42 ± 0.78^b	8.20 ± 0.15^a
60	2.40 ± 0.10^d	4.64 ± 0.61^a	5.60 ± 0.16^c

Means within columns having different letters in superscript are significantly different at $p < 0$

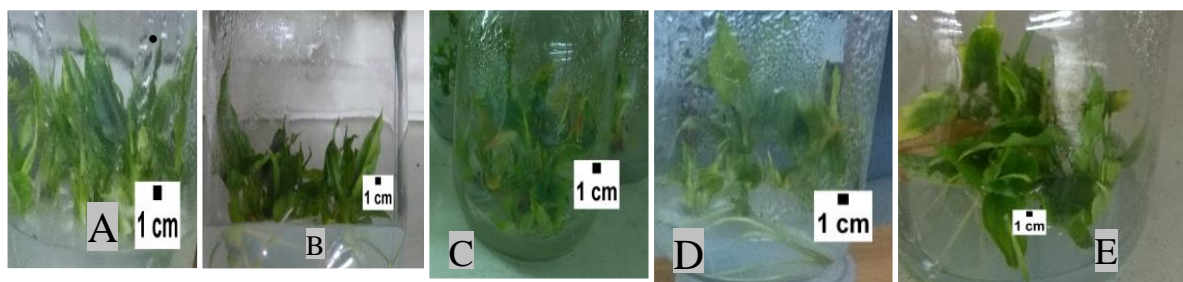


Figure 6:-Shoot multiplication of *A. corrorima* on different medium volumes(A) =50ml, (B) = 40ml, (C) =60 ml, (D) = 30ml and (E) =20ml

5.3.4. Effects of Explant Density on Shoot Multiplication

The mean shoot number, shoot length and mean number of leaves were found to be different in different shoot density per culture vessel (Table 5 and Figure 7). The maximum mean number of shoots per explant (3.80 ± 0.25) was recorded on the medium containing 2 explants per culture

vessel. Mean shoot number per explant of 3.60 ± 0.40 and 3.40 ± 0.21 were also obtained on the medium containing 3 and 1 shoot per culture vessel respectively. There was no significant difference in mean shoot number per explant between media containing one and three shoot explant per culture vessel. The least mean number of shoots per explant were (2.00 ± 0.11 and 1.80 ± 0.74) were obtained from medium containing 7 and 6 explants per culture vessel, respectively. The maximum mean shoot lengths (4.60 ± 0.64 & 4.58 ± 0.71 cm) were recorded on the medium containing 7 and 6 shoot explants per culture vessel, respectively. mean shoot length (4.40 ± 0.76 and 4.38 ± 0.05 cm) were also recorded on the medium containing 5 and 4 explants per culture vessel respectively. There was no significant difference between the two mean shoot lengths. The least mean shoot length (4.06 ± 0.12 cm) was obtained on the medium containing one explant per culture vessel. Mean number of leaves per explant (8.40 ± 0.51 , 8.21 ± 0.39 , 8.00 ± 0.17 and 7.80 ± 0.27) were recorded on the medium containing 1, 2, 3 and 4 explants per culture vessel respectively. There was no significant difference in mean number of leaves per explant among 1, 2, 3 and 4 cultured explants per culture vessel. The lowest mean leaf number per explant (4.60 ± 0.08) was recorded on the medium containing 7 explants per culture vessel. When the numbers of explants per culture vessel increased, the mean leaf numbers per explant decreased (Table 5). When the number of explants per culture vessel increased, results exudate production by shoots resulted in yellow color leaves (Figure 7 F and G).

Table 5:- Effects of explant density on shoot multiplication of *A. corrorima*. Value indicated as \pm SE

No of shoot per culture vessel	Mean shoot number	Mean shoot length(cm)	Mean Leaf number
1	3.60 ± 0.40^{ab}	4.06 ± 0.12^c	8.40 ± 0.51^a
2	3.80 ± 0.23^a	4.14 ± 0.22^{bc}	8.21 ± 0.39^a
3	3.40 ± 0.21^{ab}	4.26 ± 0.06^{bc}	8.00 ± 0.17^a
4	3.20 ± 0.09^b	4.38 ± 0.05^{ab}	7.80 ± 0.27^a
5	2.60 ± 0.10^c	4.40 ± 0.76^{ab}	5.80 ± 0.15^b
6	1.80 ± 0.74^d	4.58 ± 0.71^a	5.20 ± 0.14^{bc}
7	2.00 ± 0.11^d	4.60 ± 0.64^a	4.60 ± 0.08^c

Means within columns having different letters in superscript are significantly different at $p < 0.05$.

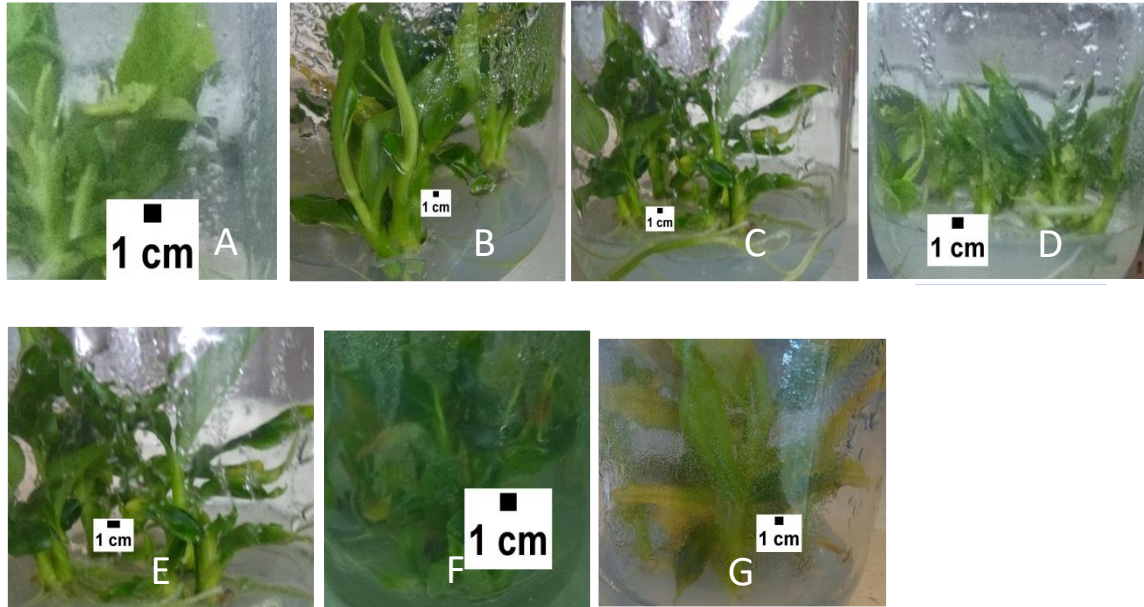


Figure 7. Effect of explant density on shoot multiplication of *A. corrorima*(A) = 1 explant per culture vessel,(B) = 2 explants per culture vessel, (C) = 3 explants per culture vessel, (D) = 4 explants per culture vessel, (E) = 5 explants per culture vessel,(F) = 6 explants per culture vessel,(G) = 7 explants per culture vessel.

5.4. Rooting

5.4.1. The Effect of Medium Strength on Rooting

Mean root number and mean root length of plantlets varied at different medium strength (Table 6 and Figure 8). The maximum mean root number (14.50 ± 0.34 and 13.20 ± 0.54) were recorded on 1/3 and 1/2 medium strength, respectively. There was no significant difference among them. The lowest mean root number per explant (9.05 ± 0.36) was recorded on 1/4 medium strength. The maximum mean root length (2.83 ± 0.19 cm) was obtained on the medium having 1/4 medium strength. The maximum mean root length (2.83 ± 0.19 cm) was obtained on 1/4 medium strength. The second maximum mean root length (2.63 ± 0.24 and 2.46 ± 0.15 cm) was obtained on the medium containing 1/3 and 1/2 medium strength, respectively. There was no significant difference in mean root length between these two media. The least mean root length (2.14 ± 0.18 cm) was obtained on full strength medium. Root length and medium strength were found to show inverse relationship.

Table 6. Effect of medium strength on root number and length of *A. corrorima*. Value indicated as \pm SE

Medium	Mean root number	Mean root length(cm)
Full	11.35 \pm 0.62 ^b	2.14 \pm 0.18 ^b
1/2	13.20 \pm 0.54 ^a	2.46 \pm 0.15 ^{ab}
1/3	14.50 \pm 0.34 ^a	2.63 \pm 0.24 ^{ab}
1/4	9.05 \pm 0.36 ^c	2.83 \pm 0.19 ^a

Means within columns having different letters in superscript are significantly different at $p < 0.05$

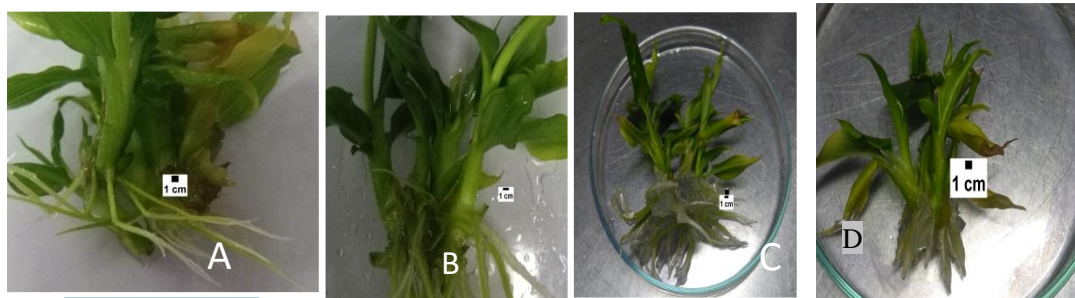


Figure 8 :-Rooting of shoots of *A. corrorima* on different salt strength MS medium(A) = on full (50 ml/l) salt strength (B) = on 1/2 salt strength (C) = on 1/3 salt strength (D) = on 1/4 salt strength.

5.4.2. Effect of Potassium Dihydrogen Phosphate (KH₂PO₄) on Rooting

Different concentration of potassium dihydrogen phosphate on the medium resulted in different mean root number and length (Table 7 and Figure 9). Medium containing standard potassium dihydrogen phosphate provided maximum mean root numbers per explant (11.35 \pm 0.62). Medium lacking di potassium hydrogen phosphate resulted in lowest mean root number (0.50 \pm 0.23). Mean root number obtained on 1/4 concentration of potassium dihydrogen phosphate and medium lacking potassium dihydrogen phosphate have no significant difference all. Medium having standard concentration of potassium dihydrogen phosphate resulted in maximum mean root length (2.14 \pm 0.18 cm). The least mean root length (1.45 \pm 0.29 and 1.05 \pm 0.24 cm) were obtained on the medium containing 1/4 potassium dihydrogen phosphate and mediums lacking it respectively. Mean shoot

number, length and leaf number decreased significantly when the concentration of potassium dihydrogen phosphate decreased in MS medium.

Table 7:- Effects of potassium dihydrogen phosphate on mean root number and length of *A. Corrorima*. Value indicated as \pm SE

Conc. of KH_2PO_4 in the medium	Mean root number	Mean root length(cm)
Standard	11.35 ± 0.625^a	2.14 ± 0.18^a
1/2	5.50 ± 0.23^b	2.04 ± 0.09^b
1/3	2.50 ± 0.47^c	1.87 ± 0.14^{bc}
1/4	1.10 ± 0.28^{ed}	1.45 ± 0.29^c
No	0.50 ± 0.23^e	1.05 ± 0.24^d

Means within columns having different letters in superscript are significantly different at $p < 0.05$

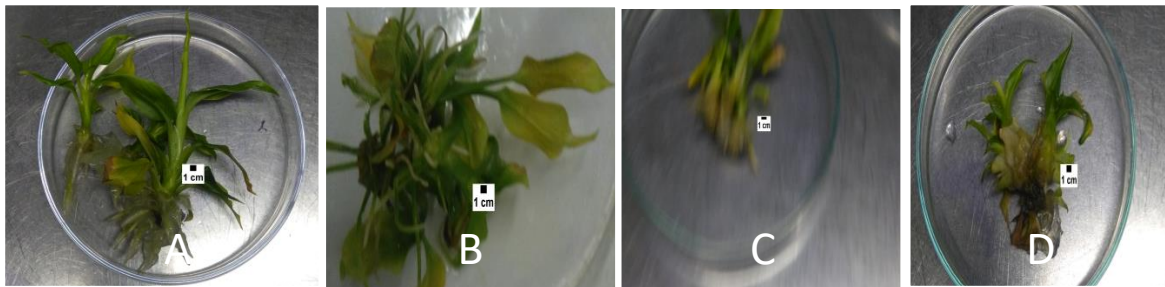


Figure 9.Root induction of *A. corrorima* on medium containing different concentrations of KH_2PO_4 (A) $\frac{1}{2}$ concentration of KH_2PO_4 (B) $=\frac{1}{3}$ concentration of KH_2PO_4 (c) $=\frac{1}{4}$ concentration of (D) = Lacking KH_2PO_4

5.4.3. Effects of Medium Volume for Rooting

Shoots cultured on different medium volumes responded differently in terms of mean root number and length (Table 8 and Figure 10). The highest mean root number per explant (14.05 ± 0.37 and 13.20 ± 0.54) was recorded on 20ml and 30ml medium volume, respectively. There was no significant difference the two. The least mean root number per explant (9.86 ± 0.53) was obtained on 60 ml medium volume. There was significant difference of mean root with the mean root number recorded on 20 ml, 30 ml and 40 ml medium volumes. The highest mean root length (3.46 ± 0.18 cm) was obtained on 20 ml medium. The second highest mean root length (3.24 ± 0.13 and 3.11 ± 0.13 cm) were recorded on the medium having 30 ml and 40 ml medium respectively. There was no significant difference between the two volumes. However, the least mean root lengths (3.02 ± 0.13 and 3.03 ± 0.09 cm) were obtained on 50 ml and 60 ml medium volumes. There was no significant difference between them. But there was significance difference in the mean root length recorded on 30 ml and 40ml medium volumes. There was no significant difference between the two volumes. However, the least mean root lengths (3.02 ± 0.13 and 3.03 ± 0.09 cm) were obtained on 50 ml and 60 ml medium volumes. There was no significant difference between them. But there was significance difference in the mean root length recorded on 30 ml and 40ml medium volumes

Table 8:-Effects of medium volume on mean root number and length of *A. corrorima*. Value indicated as \pm SE

Medium Volume (ml)	Mean number no of root	Mean root length (cm)
20	14.05 ± 0.37^a	3.46 ± 0.18^a
30	13.20 ± 0.54^a	3.24 ± 0.13^{ab}
40	11.42 ± 0.50^b	3.10 ± 0.13^{ab}
50	10.25 ± 0.52^{bc}	3.02 ± 0.12^b
60	9.86 ± 0.53^c	3.03 ± 0.09^b

Means within columns having different letters in superscript are significantly different at $p < 0.05$

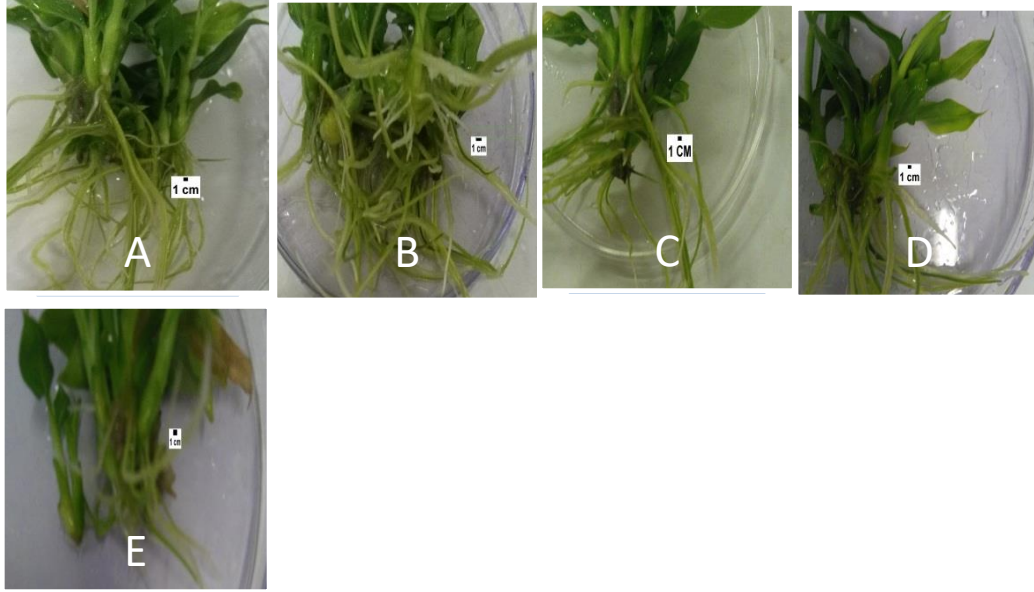


Figure 10:-Rooting of *A. corrorima* on different medium volumes(A) = 50 ml, (B) = 40 ml, (C) =60 ml, (D) = 30ml and (E) =20ml.

5.5. Acclimatization

During acclimatization 30 plantlets were taken from the medium containing 1/2 salt strength and 1/3salt strength and 15 plantlets also were taken from the medium containing 1/2 concentration of potassium dihydrogen phosphate. From 30 plantlets, 90% survived. But no plantlets were survived from 15 plantlets taken from the medium containing 1/2potassium di hydrogen phosphate on the green house after a month.

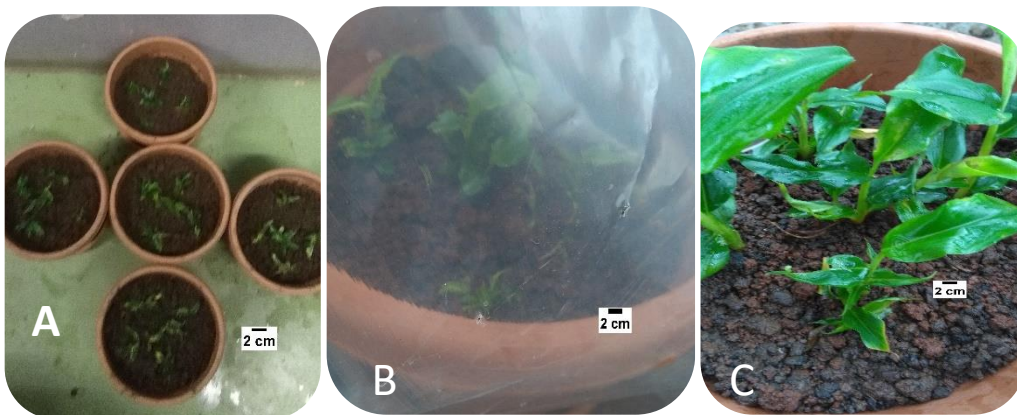


Figure 11:-Response of acclimatization of *A. corrorima*(A) =immediately after transfer of plantlets in to the pot, (B) = Plantlets covered by polyethylene bag (C) = Plants after one month of acclimatization

6. DISCUSSION

6.1. Germination of Seed

In this study the result of germination showed different response at different week intervals. The protocol used for seed germination was taken from previous studies conducted by Getie ,(2017) on the same plant at different treatments. In the present study, the seed of *A. corrorima* was started to germinate at the 5th weeks and stopped its germination at about 11th week. Most of seeds were germinated between the 6th and 8th weeks. The germination of *A. corrorima* seeds takes from one to two months (Eshete *et al.*, 2016).In the finding of Hagos,(2011) the seeds were started to germinate at three weeks and completed at the end of two months, but in case of Getie (2017) findings the seeds were started to germinate at the 6th weeks, However, in the present study the seeds were started to germinate at 5th weeks of cultured seeds .As we understanding here, the duration of seed germinated in this study lies between Hagos, (2011) and Getie (2017) study results. It showed longer germination period than Hagos(2011) findings and shorter germination time than Getie,(2017)findings. This difference is maintained may be due to genotype difference of the plant freshness of the seeds and ways to break down seed dormancy. The reason why some seeds started to germinate early and the other were germinated later might be maturation difference between seeds in different capsule, and might be an equal effect of KH_2PO_4 on seeds during soaking to break down dormancy.

6.2. Shoot Initiation

A number of studies showed different medium strength for optimum shoot multiplication and initiation. In the present study, culture initiation experiment on *A. corrorima* was carried out on the medium containing full, 1/2, 1/3 and 1/4 medium strength supplemented with 1.5 mg/l BAP. Here the best result in terms of shoot number, shoot length and leaf number was recorded on the medium containing full strength medium which gives the highest mean shoot number, length and leaf number (1.55 ± 0.11 , 2.47 ± 0.17 cm, 3.40 ± 0.11), respectively. Mean number of shoots, shoot length and mean leaf number (1.23 ± 0.08 , 1.73 ± 0.13 cm and 2.70 ± 0.16) were reported by Getie, (2017) on the same plants. Mean value of shoot number, length and leaf number (1.43 ± 0.23 , 2.30 cm and 3.12) respectively were also reported by Hagos, (2011) as a maximum result. However ,the present result was disagree with the results reported by both Getie,(2017) and Hagos at the same salt

strength on the same plant. This is might be maintain due to genotype difference and size of explants used during culturing.

6.3 Shoot Multiplication

6.3.1. Effects of Medium Strength on Shoot Multiplication of *A. corrorima*

Like shoot initiation, mean shoot number, shoot length and leaf number of *A. corrorima* explant varied on different media. The result indicated that highest mean number of shoots, shoot length and leaf number were (3.20 ± 0.14 , 3.50 ± 0.11 cm and 7.8 ± 0.14), respectively. The highest mean shoot number and leaf number were recorded on the medium containing full strength medium. The highest mean shoot length was obtained on 1/2 strength medium rather than full strength medium. The least mean shoot number, length and leaf number were recorded on 1/4 strength medium. (Tefera and Wannakrairoj, 2006) obtained (6.2, and 13.5cm and 5.37) mean number of shoots, shoot length and leaf number per explant respectively and Getie , (2017) also obtained (3.00, 3.52 cm and 7.03) mean shoot number, length and leaf number on full strength MS supplemented by the same hormone used in this study (1.5 mg/l BAP). The present result is somewhat similar to the finding of Getie (2017). However, it disagrees with the finding of (Tefera and Wannakrairoj, 2006). This is may be due to genotype difference, and explant densities per culture vessels. When explant densities per culture vessel increase, number of shoots and leaf number were decreased. So, this is might be also reason why the result of the present study is disagree with the result reported by Tefera and Wannakrairoj, (2006).

6.3.2. Effect of Potassium dihydrogen Phosphate on Shoot Multiplication of *A. corrorima*

Potassium is one of the most important macronutrients, along with nitrogen (N) and phosphorous (P) from seed germination to seed production (Fageria, 2016).

Shoot multiplication is also affected by different concentration of potassium dihydrogen phosphate. The highest mean values of shoot number, shoot height and leaf number of *A. corrorima* was (3.20 ± 0.14 , 3.40 ± 0.11 cm and 7.80 ± 0.14) respectively which recorded on the medium having standard concentration of potassium dihydrogen phosphate. Medium containing ½ potassium dihydrogen phosphate concentrations resulted in the second highest mean value of shoot number, shoot length and leaf number. Similar result was reported by Getie,(2017) on mean shoot number and leaf number when he used standard potassium dihydrogen phosphate. But in case of mean shoot length,

the present study showed difference from Getie , (2017) reports. When the concentration of potassium dihydrogen phosphate decreased, mean shoot number, shoot length and leaf number were significantly decreased. Mineral components in the growth medium are vitally important for the *invitro* regeneration process in plants (Williams, 1995).The concentration of potassium dihydrogen phosphate has ultimate effect on plantlets growth, shoot multiplication and formation of leaves, since potassium dihydrogen phosphate always react with other macro and micro nutrients during plantlet growth starting from seed germination to fruiting (Ashley *et al.*, 2005).

Therefore, if the concentration of potassium hydrogen phosphate in the medium varied from the optimum, it affects the function of both macro and micronutrients for the growth and development of shoots. When plants experience potassium deficiency, they exhibit stunted growth and yellowing in their leaf margins and poor root system (Wang *et al.*, 2013).Omissions of potassium on MS medium resulted in a significant reduction in the growth of explants, shoot number and leaf number of explants. Similar results were reported in different plant species (Wang *et al.*, 2013).; (Ashley *et al.*, 2005).

6.3.3. Effects of Medium Volume on Shoot Multiplication of *A. corrorima*.

In this study the highest mean value of shoot number, shoot length and leaf number were (3.60 ± 0.21 , 4.64 ± 0.61 cm and 8.40 ± 0.10) respectively whereas the least mean value was (2.40 ± 0.10 , 3.52 ± 0.99 cm and 5.60 ± 0.16), respectively. The highest mean shoot number 3.60 ± 0.21 and mean number of leaf (8.40 ± 0.10) were recorded on 50ml medium volume culture vessels.(Getie and Feyissa, 2017)was reported (3.00 ± 0.23 , 3.52 ± 0.15 cm and 7.03) as the highest mean shoot number, shoot length and leaf number respectively. However, the present result was disagreed with Getie's (2017) findings on the same medium volume and plant species. This is may be due to genotype and explant density difference per culture vessels. The highest mean shoot length (4.64 ± 0.61 cm) was obtained on the medium having 60ml medium volume. As we understand from (Table 6) mean length of shoots of plantlets were increase when medium volume increases. This may be due to high nutrient availability on the medium. However; the least mean value of leaf (5.60 ± 0.16) was also recorded on this medium volume. Here we can conclude that mean shoot length was high at maximum medium volume with least mean number of leaves. This was might be due to stress difference of explants on each medium volume. That means there may not nutrient stress when the medium volume is high. To sum up both shoot number and leaf number were maximum when the

medium volume were 50 ml and 40 ml. This indicate that number of shoots and leaf number were not show good result when the medium volume was bellow 40 ml and above 50 ml. if the medium volume is far apart less least to the normal, due to this, nutrient availability might be reduced in the medium. So, number of leaves and shoot number were also reduced.

6.3.4. Effects of Explant Density on Shoot Multiplication of *A. corrorima*

Number of explants per culture vessel had clear impact on mean number of shoots, shoot length and number of leaves. In the present study, 1, 2, 3, 4, 5, 6 and 7 explants per culture vessel were used. The highest mean value of shoot, shoot length and leaf number (3.80 ± 0.25 , 3.60 ± 0.40 , and 3.40 ± 0.21) were obtained on medium containing 2, 1 and 3 explants per culture vessel respectively. There was no significant difference in mean shoot number among the three treatments. The least mean shoot number per explant was also recorded on the medium containing 6 and 7 explants per vessels. The highest mean shoot length (4.60 ± 0.64 and 4.58 ± 0.71 cm) was also obtained on the medium containing 6 & 7 explants per culture vessel. The highest mean leaf number (8.40 ± 0.51) was recorded on the medium containing one explant per culture vessel. But this result has no significant difference to the result obtained from the medium containing 2, 3 and 4 explants per culture vessel. Generally, when the number of explants increased, mean number of shoot and leaf number gradually decreased, but their shoots became long and thin. The effect of media volume and densities of plantlets per vessel for shoot multiplication of turmeric plants were demonstrated by Halloran and Adelberg, (2011).Based on this author's the maximum shoot multiplication was observed at low shoot density and at large medium volume. Density of explants per culture vessel has inverse relationship with shoot multiplications(Halloran and Adelberg, 2011) When plantlets density per culture vessel increased, competition of nutrients between plantlets and exudate production by shoots would directly increase, the release of exudate by shoots in culture vessel. Due to this shoot multiplication and number of leaves were reduce. (Boullani *et al.*, 2017).Similar result was also obtained in the present study except mean shoots length. The difference results showed on shoot length was might be due to plant species difference and number of explants cultured on MS medium.

6.4. Root Induction

6.4.1 Effect of Medium Strength on Root Induction of *A. corrorima*

Different medium salt strength showed clear difference on both mean number of roots and root length. In the present study the maximum mean root number were recorded on the medium

containing 1/3 and 1/2 salt strength. But the maximum mean root length was obtained on the medium containing 1/4 salt strength. The lowest mean root number and the maximum mean root length was recorded on the medium containing 1/4 salt strength. Half salt strength is recommended by Hartman *et al.* (2004) for better root formation, but in the present study, medium containing 1/3 medium strength was more preferable than mediums having 1/2 medium strength. Based on this result, medium salt strength ranging between full MS and one fourth MS was the most preferable for root induction of *A. corrorima*. The mean number of roots obtained on 1/2 salt strength in the present study was similar to the finding of Hagos, (2011) on the same plant species. The reason why root induction was best on the medium containing 1/2 and 1/3 salt strength was might be the presence of nutrient stress on it. But when nutrient stress was too, it may best to root length rather than root number. So, too little and too much salt strength might be not good for root induction.

6.4.2. Effects of Potassium dihydrogen phosphate on Rooting

The mean value of root number (11.35 ± 0.62 , 5.50 ± 0.28 , 2.50 ± 0.47 , 1.10 ± 0.28 and 0.5 ± 0.23) were recorded on the medium containing standard, 1/2, 1/3, 1/4 and zero potassium dihydrogen phosphate respectively. The maximum mean root number was recorded on the medium having standard potassium hydrogen phosphate. The least mean root number was obtained on the medium lacking potassium dihydrogen phosphate. The maximum and the minimum mean root length was also obtained on the same media. Potassium dihydrogen phosphate has ultimate effect on plantlets growth, shoot multiplication, formation of leaf and root induction, since potassium dihydrogen phosphate always react with other macro and micro nutrients during plantlet growth starting from seed germination to fruiting (Ashley *et al.*, 2005). Omissions of potassium on MS medium resulted in a significant reduction in the growth of explants, shoot number, leaf number and root induction of explants (Lee and de Fossard, 1977). This is also true in the present study, as the concentration of potassium dihydrogen phosphate decreased both the number of root and root length. The root strength also decreased with the reduction of potassium dihydrogen phosphate. This was might be due to the effect of potassium di hydrogen phosphate on the function of both other macro and micronutrients.

6.4.3. Effect of Medium volume on Root induction of *A. corrorima*

Mean root number and root length gradually decreased when medium volume increased. The highest mean root number (14.05 ± 0.37) was obtained on the medium containing 20 ml medium volume. It

is somewhat related with the mean number of roots reported by Hagos (2011), but the highest mean root length obtained in the present study was very different from her results. The least mean value of root number and length were 9.86 ± 0.53 and 2.22 ± 0.13 cm, which is obtained on the medium containing 60 ml, respectively. The result of the present study varies from the finding of Hagos, (2011) and Getie,(2017). This might be genotype difference, number of explant per culture vessel, medium volume, culture conditions and season of culturing.

6.5. Acclimatization

Tissue cultured plants are difficult to transplant for two main reasons, firstly, their heterotrophic mode of nutrition and secondly, their poor control of water loss. After four weeks of acclimatization 90% of 30 plantlets survived in the greenhouse. These plantlets were taken from the medium containing 1/2 and 1/3 salt strength. But plantlets taken from the medium containing 1/2 concentrations of potassium dihydrogen phosphate did not survive in the greenhouse at all. This may be due to poor number of roots of plantlets; lack of strength of roots of plantlets obtained from the medium containing little concentration of potassium dihydrogen phosphate and may be due to the nature of potassium concentration to influence the function of both macro and micro nutrients during the growth of plants (Ashley *et al.*, 2005). Mekonnen,(2016) and Getie,(2017) reported slightly lower percentage (86%) of survival. Hagos, (2011) also reported (66.7%) survival rates of plantlets after a month of acclimatization. But in the case of the present study the survival rate of plantlets in the green house was 90%.

7. CONCLUSION AND RECOMMENDATIONS

7.1. Conclusions

The present result showed that seed germination of *A. corrorima* plants started at 5th weeks after culture and completed at the end of 11th weeks. During shoot initiation in different medium strength, mean number of shoots, length and survival of plantlets varied. Using fresh seeds and soaking by tap water for about 2-3 days are best to shorten the germination rate of *A. corrorima* seeds. One third MS medium strength is better to use for root formation during *in vitro* propagation. The highest mean shoot number, length, leaf number (1.55, 2.47 cm, 3.40) were obtained on full strength MS medium. Medium strength significantly affected the mean shoot number, shoot length and number of leaves per explant during shoot multiplication. The highest mean shoot number, shoot length and leaf number per explant (3.20, 3.40 cm, 7.80) were obtained from the medium containing full salt strength medium. Based on these findings, full salt strength medium showed the highest performance for shoot multiplication of *A. corrorima*. On the case of the medium containing different concentration of potassium dihydrogen phosphate, the next maximum mean value of shoot number, shoot length and leaf number (2.50, 3.33cm, and 5.80) were obtained on the medium containing half concentration of potassium dihydrogen phosphate. But the maximum mean value of shoot number, shoot length and leaf number were obtained on the medium containing standard concentration of potassium dihydrogen phosphate as we mentioned from full strength MS medium. Here we can conclude that, shoot number, shoot length and leaf number of plantlets have greatly affected by the concentration of potassium dihydrogen phosphate. The highest mean shoot number (3.60) was obtained on 50 ml medium volume with 5 explant densities per culture vessels. The highest mean number of shoots (3.80) was recorded on the medium having 2 explants per culture vessel. The highest mean shoot length (4.60 cm) was obtained on the medium containing seven explants per culture vessel and the highest mean leaf number (8.40) was also recorded on the medium containing one explant per culture vessel. Based on these findings, the number of explants per culture vessel has direct relationship with their shoot length and inverse relationship with mean leaf number and shoot number. However, shoot number obtained on the medium containing one, two and three explants per culture vessel did not show significant difference among them.

Different medium salt strength, concentration of potassium dihydrogen phosphate and medium volume showed different results on mean root number and length. On medium salt strength, the

highest mean value of root number was 14.50, obtained from the one third MS medium strength and the highest mean root length (2.83 cm) was obtained from the medium containing one fourth medium salt strength. On the base of these results, we can conclude that the favorable medium strength lies between full and one fourth medium strength, but for root length one fourth medium strength was preferable to the rest. The maximum mean root number and root length (11.35 and 2.14 cm) respectively were obtained on the medium containing standard potassium dihydrogen phosphate. When the concentration of potassium dihydrogen phosphate on the medium was decreased, root number and root length also decreased. The highest mean root number and length (14.05 and 2.76 cm) respectively, were obtained from 20ml medium volumes. It was agreed with the result obtained from the medium containing one third medium strength. After four weeks of acclimatization 90% plantlets survived in the greenhouse.

7.2. Recommendations

Based on the results of the present study, the following recommendations are made:

- Using seeds for invitro propagation of *A. corrorima* is recommended to minimize transportation cost keeping the mother plant from damage.
- Government should expand different institutions which used for tissue culture in order to multiply and keep endanger plant species like *A. corrorima*
- Further investigations are required to test the efficiency of survival rate of plantlets in the field condition.
- Further investigations are required to study the effect of plantlet densities for root inductions.
- It is important to establish well defined shade nursery before exposure of plantlets to the green house (field)
- It is necessary that the tissue culture plants be followed up throughout the productive life span of the crop.

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9. APPENDICES

Appendices1. Stock solution for MS (Murashige and Skoog, 1962) medium

Components	Concentration(g/L)	Volume/litter during media preparation
Micronutrients		5 ml/l
ZnSO ₄ .7H ₂ O	1.72	
H ₃ BO ₃	1.124	
MnSO ₄ .H ₂ O	0.05	
KI	0.166	
NaMoO ₄ .2H ₂ O	0.05	
CoCl ₂ .6H ₂ O	0.05	
Na ₂ EDTA	7.472	
FeSO ₄ .7H ₂ O	5.56	
Macronutrients		
NH ₄ NO ₃	33	
KNO ₃	38	
CaCl ₂ .2H ₂ O	8.8	
MgSO ₄ .7H ₂ O	7.4	
KH ₂ PO ₄	3.4	
Vitamins		5 ml/l
Myo-inositol	20	
Glycin (glycocoll)	0.4	
Nicotinic acid	0.1	
Pyridoxin (B6)	0.1	
Thiamin (B1)	0.02	

❖ Na₂EDTA and FeSO₄.7H₂O prepared alone

