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The Effects of Culture Conditions on *In vitro* Propagation of Korarima (*Aframomum corrorima* (Braun) P.C.M. Jansen)

By

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The Effects of Culture Conditions on *In vitro* Propagation of Korarima (*Aframomum corrorima* (Braun) P.C.M. Jansen)

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BAP	6-Benzyl Amino Purine
CRD	Complete Randomized Design
DMRT	Duncan's Multiple Range Test
EDTA	Ethylene Di-amine Tetra acetic Acid
EARI	Ethiopian Agricultural Research Institute
ESC	Ethiopian Sugar Corporation
GA_3	Gibberillic Acid
LSD	Least Significance Difference
MS	Murashige and Skoog
NAA	α-Naphthalene Acetic Acid
PGRs	Plant Growth Regulators
SPSS	Statistical Package for Social Science

ABSTRACT

Aframomum corrorima is one of the important spice and medicinal plants of the family *Zingiberaceae* which can be propagated from its seed and rhizome. Conventionally, propagation of *A.corrorima* by rhizome causes for genetic erosion and it is a time consuming process. Hence, plant tissue culture has played a very important role to clone the endangered plant species with desirable traits urgently. The present study was conducted to investigate the effects of culture conditions on *in vitro* propagation of *A.corrorima* using MS liquid media. Surface sterilization of seeds in 70% ethanol for 3 minutes then followed by dipping in 25% sodium hypochlorite for 25 minutes gave the best disinfected seeds and the percentages of seed germination and shoot initiation obtained on MS media were 93.5% and 96.25% ,respectively. In the sucrose containing MS media, 3% gave the highest number of shoots (3.10±0.15), leaves (8.77±0.13), roots (9.03±0.72) and shoot length (3.39±0.63 cm) per explant/shoot. Out of different pH levels, pH 5.8 gave the best result regarding the number of shoots (3.20±0.13), leaves (11.40±0.27) and length of shoots (4.08±0.13cm) and root number (9.03±0.72) of korarima. In another MS liquid culture media experiment, maximum shoot number (3.03 ±0.21) and shoot length (3.17 ± 0.12 cm) were recorded in full MS strength medium. The best root number (14.47± 0.96) and root length (6.30 ±0.11cm) were obtained on half MS strength medium at pH 5.8, with sucrose supplied at a concentration of 3% and 0.5 mg/l NAA (α-naphthaleneacetic acid) when shoots were rooted. Acclimatization in greenhouse resulted in 94. 3 % with a mixture of forest soil, sand and farmyard in the ratio of 2:1:1. The result of this study can be used in the future protocol optimization and mass propagation of *A. corrorima*.

Keywords/phrases: Acclimatization, *Aframomum corrorima*, *in vitro* propagation, MS salt strength, sucrose concentration

1. INTRODUCTION

The Ethiopian cardamom (Korarima (*Aframomum corrorima*)) is one of the herbaceous, perennial, aromatic spices and medicinal plants which is an endangered indigenous to Ethiopia, but it is little known outside the country and mainly grown in the natural forests dominated by woody species. This indigenous Ethiopian spice, known as korarima, Ethiopian cardamom or false cardamom, is obtained from the plant seeds (usually dried), and is extensively used in Ethiopian and Eritrean cuisine (Eyob *et al*., 2008). The seeds (usually dried, sometimes fresh) are used to flavour all kinds of sauces locally called 'wot', for which they are ground and usually mixed with other spices. Korarima is used to add flavour to local food, bread and butter, is an ingredient of berbere (Chile pepper powder), mitmita (Piri piri) and other spice mixtures, and is also used to flavour coffee. In addition, korarima seeds are widely used medicinally as a tonic, laxative, carminative and purgative drug, and they are added to food for preservative purposes. Although korarima is consumed as a spice, it may also be used as a source of antioxidants, and the arilloid flesh around the seed is edible. Strings of fruits are sometimes used as an ornament or as rosaries (by the Arabs), and in the past the fruits have been used as money for bartering system in Ethiopia. In addition, korarima is an important plant for soil conservation since the rhizomes and leaves spread over the ground covering and protecting the soil from erosion in hilly areas. It is primarily the red fruits that are used, but other parts of the plant can also be used. The taste of korarima is similar to that of Indian cardamom (*Ellettaria cardamomum*), and it has been used as a substitute for this spice (Tefera and Wannakrairoj, 2004; Eyob *et al.*, 2009).

Korarima plants are propagated both by seeds and rhizome parts and complete their juvenile phase and reach maturity after three to five years of planting to first harvest and have more than seven years of economic life time (Eyob, 2009). During *in vitro* propagation of korarima, the suitable propagation technique is by using seed. However, vegetative propagation through cuttings results in the destruction of the productive garden, on top of the commonly associated shortage of planting materials to cover wider areas of land. Consequently, seed propagation of korarima is undertaken to cover large areas of land retaining the mother productive stand intact. However, it is essential to give the utmost care while preparing the seeds. Therefore, well ripened korarima capsules will be selected and collected during the peak harvest season and its seeds are removed and rubbed with ash to

facilitate their drying and protect fungal development, as well as to ease their handling during sowing (Endashaw, 2007).

Tissue culture is an *in vitro* technique of culturing cells, tissues, organs which are separated from organisms, under aseptic and controlled nutritional and environmental conditions often to produce the clone of organisms (Getnet, 2017). Micropropagation is one of the principal techniques of tissue culture in plant propagation with the objective of producing large numbers of genetically identical plantlets with better quality, screening of useful variants which have high yielding genotypes with greater biotic/abiotic resistance and stress tolerance capacities without limitation of seasons in year round (Abu *et al.*, 2014).The advantages of plant tissue culture over conventional vegetative propagation include; it is rapid and utilizes small space, require less labour cost, can be carried out independent of the seasons, the propagules are free from any diseases and it requires only small explants. *In vitro* propagation or micropropagation protocols have been optimized for several economically important, industrial, spice and medicinal crop varieties in Ethiopia (Woldegiorgis *et al.*, 2016). This study was carried out to analyze the effects of different sucrose concentrations, MS salt strengths and pH ranges essentially used in liquid culture medium with the aim of developing a reliable protocol for *in vitro* propagation of shoot proliferation and root induction of *Aframomum corrorima*.

2. LITERATURE REVIEW

2.1. Origin and Geographic Distribution of Korarima

Ethiopia is a land of diverse climate and soil types that enable prolific growth of several indigenous herbal and medicinal species of plants. Korarima is one of the different crop species that Ethiopia is known to be the center of origin and/or diversity which is an indigenous spice to Ethiopia (Hailemichael *et al.*). Korarima plant is native to the tropical as well as subtropical regions across the globe. It is widely distributed in the natural forests of southern and western parts of Ethiopia (Provinces of Kefa, Gamo Gofa, South Omo, Sidamo, Jimma, Illubabor and Wollega) as well as it is cultivated in the areas of Lake Tana and around Gelemso (Jansen, 1981). Korarima is also native to some parts of western part of South Sudan, western Uganda, Tanzania and Eritrea (Tefera and Wannakrairoj, 2004).

2.2. Botanical Description of Korarima

Korarima (*Aframomum corrorima*) or Ethiopian cardamom ("false cardamom") is herbaceous, perennial, aromatic spice and medicinal crop of the species in the monocotyledonous ginger family *Zingiberaceae* and the genus *Aframomum* which is native to Ethiopia. It is a shade loving plant that grows wild in moist and open woodlands, in the same climate areas as wild coffee, but may also be planted and cultivated (Jansen, 1981).

2. 3. Growth, Development& Ecological Requirements of Korarima

Korarima plants are propagated through asexual and sexual means and complete their juvenile phase and reach maturity after 3 - 5 years of planting to first harvest and have more than seven years of economic life time (Eyob, 2009). In Ethiopia, the main flowering period is June-July, with fruiting in September-October. It is primarily harvested in October and November, but sometimes also bears fruits starting from June. Fruits mature about 2-3 months after flowering (Hailemichael *et al.*, 2016).

Korarima thrives well on acidic soils (i.e. 5.5 - 6.5 pH), deep to medium soils (50-150 cm), high to moderate organic matter and fertility status, constantly moist with high water holding capacity, but not waterlogged situations are considered favorable for this crop and recently well performed in South west part of Ethiopia particularly in Kafa, Gamo-Gofa, Bench-Mahag, Majangs zones (Eyob *et al*., 2008). Korarima grows in altitude 1000 – 2300 meters

above sea level, but is highly favoured in altitudes ranges from 1700 - 2300 meters above sea level and it thrives best and is mostly cultivated near the banks of the water stream with natural forest shade (Getasetegn and Tefera, 2016).

Korarima plants have a preference of an average rainfall ranging between 1300 mm to more than 2000 mm, but with no distinct dry season. However, the crop thrives well where the main rainy season lies in the months of June - August, where 50–60% of the total rainfall is availed to the plant. Korarima grows most in places where the annual day time temperatures vary between 16ºC and 24ºC. However, these plants possess the aptitude to endure the temperature ranges between 7ºC and 35ºC, a moderate shade of 55-63% and a photoperiod with <12 hours of light, i.e. a short day plant (Girma *et al.*, 2008).

2.4. Morphological Description of Korarima

The plant consists of an underground rhizome, a pseudostem, and several broad leaves and resembles *Elettaria cardamomum* species morphologically. Its rhizome reaches up to 1 cm diameter and has leafy stems 1-2 m long with aromatic pungent. The stem is unbranched and formed by the leaf sheathes subterete up to 1 cm in diameter but at the base it is thickened and reaches to 3 cm in diameter. The leaves are arranged in alternate form and make shield to each other. Its inflorescence is a shortly stalked head arisen from the rhizomes near the base of a leafy stem sometimes situated at the end of a rhizomatous runner, up to 5 flowers and peducle up to 7 cm long (Jansen, 1981). The flowers are covered by imbricate, purplish-brown, subovate scales of 2.5 cm × 1.5 cm and each flower is surrounded by a scarious, suboblong bract up to 6 cm × 2 cm, bi dentate, ciliate. The fruit is an indehiscent and subconical berry up to 6 cm × 3.5 cm in size usually it shows 3 longitudinal furrows, but sometimes it gives more shiny green when immature, turning bright red at maturity, with three cells containing 45–65 seeds for each. Seeds are subglobose in outline but usually somewhat angular from 2–5 mm in diameter. The seed testa is finely lined, glossy brown and its hilum is circular, whitish, aril thin and a bit fleshy (Jansen, 1981). Mature korarima can reach a height of 1-2 m. It sets seed after 3-5 years of planting depending on the planting materials used and it continue to bear seeds for a number of decades (Eyob, 2009).

2.5. Shade Requirement and Management

Korarima is an obligate shade loving plant and the shade level management is one of the key agronomic practices in korarima production. A study report by Jimma Agricultural Research Center (JARC) identified that the shade level of 55 - 63% is suitable for korarima production under which better growth and yielding performance of this spices was found. Shades create important microclimates that regulate moisture and temperature for korarima plantations. Thus, creating a microclimate for optimum vegetative growth is essential. The microclimate prevailing in shade gardens create a favorable environment for root development, particularly when korarima rhizomes produce very shallow roots at each node. As soon as the soil is directly exposed to direct sun light, soil moisture and soil temperature fluctuate considerably, therefore the root development of shallow rooting plants will be limited if protective shade covering is missing or cut down (Etissa, 1998).The protective influence of shade in maintenance of soil fertility, moisture reserve, facilitating decay of organic matter, weed depressing is fundamental. Therefore, digging, tillage, mulching, weeding requirement may be of little importance in korarima plantation (Menge *et al.*, 2016).

2.6. Harvest and Processing of Korarima

Korarima plants require a minimum of three years to be harvested for the first time and each plant has an economic viability for about seven years from the date of first harvest. The fruits of these plants become mature and are ready for harvesting about 2 - 3 months after flowering. Maturity and harvesting time of korarima varies in different areas of Ethiopia depending on the rain fall quantity, distribution, temperature, etc. but generally the plant flowers in June-July and harvesting is done from October-November. At the early stage, the colour of korarima capsules is green, which turns bright red upon maturity and dark brown after drying (Girma *et al.*, 2008; Eyob *et al.*, 2009).

To get quality product of korarima, the capsules should be red ripe and when the seeds are removed from the capsule, should be dark brown that have pungent and appreciable taste when crushed by teeth. There should be great care while harvesting the capsules of korarima not to create any opening on the capsules since through this opening important quality components (aroma and flavor) will be lost and it will serve as entrance for microorganisms

such as fungi. After harvest, the capsules should be dried in the sun either on cemented surface or on raised bed, which is clean from any contamination (Agize and Zouwen, 2016).

2.7. Chemical Components of Korarima Essential Oils

After hydrodistillation of dried comminuted fruits for 8 hours, 3-3.5% of a pale yellow volatile oil with a flat cineolic odour can be obtained. In addition to the seeds of korarima, the leaves, rhizomes and pods can also provide essential oils of korarima. The oil composition of korarima is qualitatively similar to that of the Indian cardamom (*Ellettaria cardamomum*) oil except for the absence of α- terpinylacetate, which is the major component in the true cardamom oil (Demissew, 1993). The seed oil of *Aframomum corrorima* contained higher levels of the monoterpenes 1, 8-cineole (44.3%) and sabinene (17.3%), whereas the sesquiterpenic compounds (E)-nerolidol (17.2%),β-caryophyllene (9.7%) and caryophyllene oxide (6.9%) dominated the composition of the husk oil. The major component of the oil of the leaf dominated by β-caryophyllene (60.7%) while γ-terpinene (21.8%) and β-pinene (17.6%) dominated the oil of rhizome (Baser and Kürkçüoglu, 2001).

2.8. Importance of Korarima Oils

Korarima is one of the aromatic medicinal plants used in traditional medicine by the people of southern and south western Ethiopia. The seeds, leaves, rhizomes and pods of korarima can provide essential aromatic oils with very complex nature known for their antimicrobial, antioxidant and medicinal properties (Eyob *et al*., 2008).There is also growing interest, both in industry and in scientific research, for spices and medicinal herbs because of their antimicrobial activities (Soler-Rivas *et al.*, 2000).

The antioxidant chemical properties are attributed to a variety of active phytochemicals including phenolics, vitamins, carotenoids and terpenoids (Liu and Ng, 2000), compounds that are considered to have the ability to reduce oxidative damage associated with diseases like cancer, cardiovascular diseases, atherosclerosis, diabetes, asthma, hepatitis, liver injury, arthritis and ageing (Harman, 1995; Lee *et al.*, 2000; Middleton *et al.*, 2000).The ability of phenolic substances including flavonoids and phenolic acids to act as antioxidants has been reported (Rice-Evans *et al.*, 1996; Espín *et al.*, 2000; Liu *et al.*, 2003). As a potent antioxidant, ascorbic acid has the capacity to eliminate several different reactive oxygen species (Arrigoni and De Tullio, 2002).

2.9. Uses of Korarima in Ethiopia

Korarima is one of the spices used in a daily Ethiopian dishes. The seeds are used to flavour coffee, local food, special kinds of bread, butter and all kinds of sauces. It is also an ingredient of 'berbere' (Chile pepper powder), 'mitmita'(Piri piri), and other spice mixtures. Korarima seeds are ground and usually mixed with other spices before they are used to flavor all kinds of sauces locally called 'wot'. Although korarima is consumed as a spice, it may also be used as a source of the arilloid flesh around the seed is edible (Eyob *et al.*, 2009; Mekassa and Chandravanshi, 2015; Mitiku *et al.*, 2015).

According to Eyob *et al*. (2008), korarima seeds, pods, leaves, rhizomes and flowers are used in southern Ethiopia as traditional medicine for human and animal ailments caused by unknown agents; and particularly used to treat any part of the animal body upon swelling. In addition, korarima seeds are widely used medicinally as a tonic, laxative, carminative and purgative drug, and they are added to food for preservative purposes. Different plant parts and seed oils extracts showed the highest anti-fungal activity followed by pod extracts. However, the inhibitory effect of leaf and rhizome is lower and has no activity towards tested organisms at the lowest concentrations. The study on antimicrobial (antifungal) properties in korarima partly supports the use of this medicinal plant as traditional remedies for different aliments (Eyob *et al*., 2008).

Dried capsule of *A. corrorima* has highly significant economic importance for local and as export commodity in addition to various uses. Previously, Ethiopia was well-known for its considerable exports of *A. corrorima* capsules to the world market, mainly as a substitute for the Indian cardamom (Tefera and Wannakrairoj, 2004; Eyob, 2009). Ethiopia exported dried pods of korarima to Sudan, Egypt, Arabia, Iran, India, and the Scandinavian markets (Jansen, 1981; Edwards *et al*., 1997). The great potential of this plant has however, encountered different production problems. In the last few decades, yields, areas of production and biodiversity have declined both from farmers' field and natural forest of southern Ethiopia (Amsalu and De Graaff, 2007) due to this production of korarima (*Aframomum corrorima)* is declining mainly due to destruction of plant's natural habitats (Jansen, 1981; Eyob *et al*., 2008) and other limiting factors such as non-improved variety, non-adapted agronomic practices and lose caused due to biotic and a biotic stress are very important (Etissa, 1998; Girma *et al*., 2008).

Korarima (*A. corrorima*), is one of the cherished spices that occurs in the natural ecosystems together with natural forest and is also collected from the forests. As korarima requires a shady environment and moisture soil for its growth, the production of *A. corrorima* can be an incentive to preserve the endangered natural forests in the South Ethiopian region (Girma *et al.*, 2008). Similarly, *Aframomum corrorima* covers the ground and protects the soil from erosion in hilly areas (Mekassa and Chandravanshi, 2015). According to Woldeyes (2011), korarima production has a role in conservation of landscape elements. It promotes forest management since farmers avoid cutting trees which involve *A. corrorima*. In addition, korarima has conservation benefit that goes beyond the garden. When it is cultivated on wetland, it forms a dense cover on the ground thereby avoiding excess evaporation and consequent drying of streams. Hence, korarima appeared to be an important crop to keep the natural environment and at the same time obtain additional form of income (Agize and Zouwen, 2016).

2.10. Propagation Methods of Korarima

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

2.10.1. Propagation of korarima by rhizomes/clumps

The vegetative propagation of korarima through splitting of rhizome with one year old and another young sucker is the conventional technique used for its propagation. Even if propagation by using rhizome shortens the juvenile stage of the stand plant and also enables to produce true-to-type ones, it always comes with shortage of planting materials to cover large areas of land and it involves sacrifice of potentially productive stands (Tefera and Wannakrairoj, 2004; Eyob *et al.*, 2009).

2.10.2. Propagation of korarima by seeds

Propagation of korarima by seed can be defined as the emergence of the embryo from the seed, but the germination process is hindered by a variety of both exogenous (external hard seed coat) and endogenous dormancies. The latter could be due to germination inhibitors present in the endosperm (Iralu *et al.,* 2019), while exogenous seed dormancy (i.e. outside the embryo) is caused by physiological factors (Yang *et al.,* 2007) due to the presence of hard seed coat (Bopp, 1995) often seed dormancy in some seeds can be established by a close examination of seeds, especially for exogenous seed dormancy. Seed dormancy could be defined as the failure of a viable seeds to germinate when conditions are favorable for seed germination. *In vitro* propagation of many woody tree species and some herbaceous plants are difficult due to low regeneration capacity, especially explants from mature plant tissues (Mng'omba *et al.,* 2007).

Currently, there was a study to break korarima seed dormancy due to its impermeability of hard seed coat. One of these studies was to explore the effect of different treatments on germination and seedling growth attributes of high-land korarima cultivar namely called 'Mume'. According to Eyob (2009), soaking of the seeds in 50% H_2SO_4 for 60 minutes followed by soaking in 250 mg/l GA_3 for 24 hours had the best effective treatments for enhancing germination. Thus, H_2SO_4 is important for breaking of seed dormancy. For example, in seed germination of caper (*Capparis ovata var. herbacea*) the mucilage surrounding the seed is supposed to inhibit diffusion of oxygen to the embryos and prevents germination. Therefore, sulphuric acid pretreatment to remove mucilage and soaking in either of NAA or GA_3 was found effective to allow penetration of oxygen from the surroundings to the embryos and increased germination of seeds (Soyler and Khawar, 2007).

2.11. Plant Tissue Culture Techniques and Its Application

Tissue culture is an *in vitro* technique of culturing cells, tissues, organs which are separated from organisms, under aseptic and controlled nutritional and environmental conditions often to produce the clone of organisms. Plant tissue culture is currently a powerful tool that plays a major role in fast commercial production and propagation of new large scale plants which are genetically uniform and free from any disease through *in vitro* technique on continuous year rounded basis. Apart from its use as a tool of research, plant tissue culture becomes major industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites (Hussain *et al.*, 2012; Tolera *et al.*, 2014).

However, at this time application of plant tissue culture techniques is widely used throughout the country. In this regard, both the federal and regional agricultural research institutes and some national universities have great role in adopting and expanding tissue culture application in Ethiopia. In addition, private enterprises such as Tigray Biotechnology Center and Narus Biotechnology and Agro-industry also have role in commercialization and dissemination of research outputs to the stakeholders (Kamski, 2016).

The advantages of plant tissue culture over conventional vegetative propagation include; it is rapid and utilizes small space, require less labour cost, screening of useful variants which have high yielding genotypes with greater biotic or abiotic resistance and stress tolerance capacities, can be carried out independent of the seasons, the propagules are free from any diseases and it requires only small explants (Ibrahim *et al*., 2016; Getnet, 2017).

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

Therefore, *in vitro* propagation offers several distinct advantages that are not possible with the conventional propagation techniques. Among others; it enables multiplication of single explant in to several thousands of even some times millions of true-to-type plants in less than a year, without affecting the growth and/or productivity of the mother plant. Moreover, once established, it will be actively dividing *in vitro* culture also serve as a continuous source of micro-cutting, which can result in plant production under greenhouse conditions without any season interruption (Teisson and Alvard, 1995). The most commonly used tissue culture explants are the meristematic ends of the plants like the shoot tip, auxiliary bud tip, rhizome buds and root tip. These tissues have high rates of cell division and either concentrate or produce required growth regulating substances including auxins and cytokinins (Georgiev *et al.*, 2009; Idowu *et al.*, 2009).

Plant tissue culture is used widely in plant science even at molecular levels. It has also a number of commercial applications all over the world's agriculture sectors. Some of the applications are; micropropagation that is widely used for fruits, vegetables, root and tubers,

spices, forestry and floriculture to conserve rare or endangered plant species. A plant breeder may also use tissue culture to screen cells rather than plants for advantageous characters, e.g. yield quality and quantity, herbicide resistance/tolerance and other adverse environmental condition (Georgiev *et al.*, 2009; Idowu *et al.*, 2009).

During *in vitro* culture of higher plants, growth regulators especially auxins and cytokinins are very indispensable. It can be said that *in vitro* culture is impossible without growth regulators. Whether an auxins and/or cytokinins are added to nutrient medium to obtain cell extension and/or cell division are/is completely depends on the type of explants taken and the plant species (Scholten and Pierik, 1998; Idowu *et al.*, 2009).

2.12. Environmental Control of *In Vitro* Cultured Plants

Optimization of the micro environment is a key step in micropropagation and ensures the production of good quality plantlets that have high chances of surviving in the *ex vitro* conditions and ultimately the natural environment. The culture vessel is a miniature greenhouse or growth chamber with tightly controlled conditions. The characteristics of the conventional *in vitro* environment include; constant temperature, high relative humidity, low photosynthetic photon flux density (PPFD), optimized concentrations of sugars, salts and plant growth regulators in the growth medium, aseptic conditions, but also accumulation of secondary metabolites that may be toxic (Kozai *et al.*, 1997). Light is used as a source of energy during photosynthesis. It is an important factor for plant growth and development in plant tissue culture. In particular, light intensity and photoperiod in the microenvironment affect plantlet physiological processes and growth. However, light may be required to a lesser degree in plant tissue culture systems for the regulation of photosynthesis and photo-morphogenesis (Infante *et al.*, 1989). The biophysical and biochemical process of photosynthesis is temperature dependent and is a major determinant of the rate of growth of plants. In nature the perception of ambient temperature allows for the maintenance of plant homeostasis, thereby buffering against potential disruptive effects on cellular stability (Franklin, 2009).

2.13. The Components of Plant Tissue Culture Media

Growth and morphogenesis of plant tissues *in vitro* are largely governed by the composition of the nutrient medium. Nutrient media for plant tissue culture are designed to allow plant tissues to grow and maintain their life in artificial environment. Major constituents of the nutrient media are inorganic nutrients (macro salts, micro salts and iron salts), organic nutrients (vitamins, essential and nonessential amino acids) and supplements like sugars, plant growth regulators, etc. Nutrient media are essential for the growth and development of the plant, hence without water and mineral nutrient a plant cannot live *in vitro* or *in vivo* (Scholten and Pierik, 1998). Accordingly, ingredients of the culture medium may vary with the type of plant and the preparation stage at which one is working. However, certain standard mixture is used for most plant species, but exact formulation may need to be established by testing (Al Ghasheem *et al.*, 2018).

2.14. Liquid Culture Media

In liquid culture, the close contact between plant tissue and culture medium may improve the uptake of nutrients and hormones, which enhances shoot and root growth compared to solid culture. As a result of shaking, apical dominance is reduced, axillary bud regeneration is induced, and the rate of shoot growth increases (Mehrotra *et al.*, 2007). Liquid culture is also effective for reducing costs, because it is easy to change the medium and no agar is used. Explants can be extracted from the culture vessel with minimal damage (Pati *et al.*, 2005).

Liquid culture has been shown repeatedly to improve proliferation rates and quality of shoots, somatic embryos, microtubers and bulblets, but growth of plantlets in such system can sometimes be retarded and development affected by oxygen deprivation and hyperhydration (vitrification).The oxygen concentration of liquid media is often insufficient to meet the respiratory requirements of submerged cells and tissues. It can be increased either by raising the oxygen concentration of the medium or placing cells or tissues in direct contact with air. If the water potential of the medium is greater (less negative) than a cell, water flows into the cell and the vacuole becomes distended. Cells and tissues affected in this way are described as hyperhydric. Hyperhydricity, which is due to high water potential, causes severe disorders and requires control, such as using gyratory shakers, bioreactors (with a capacity of 1 L or more) oraddition of growth retardants (solutes) to the culture medium (Preil, 2005).Hyperhydricity is more common in plants grown on liquid medium, most likely

because the tissues are kept submerged and undergo marked oxidative stress, in addition to high concentrations of reactive oxygen species associated with changes in the activities of antioxidant enzymes (Ziv, 2005).

2.15. Acclimatization of *In Vitro* Rooted Plantlets to Soil

One of the major obstacles in the application of tissue culture methods for plant propagation has been the difficulty in successful transfer of plantlets from the laboratory to the field (Wardle *et al.*, 1983). The reasons for such a difficulty appear to be related to the dramatic change in the environmental conditions. The environment of the culture vessel is one of low light intensity, with very high humidity (generally 100%) and poor root growth, while the greenhouse and/or field conditions are typified by very high light intensity, low humidity and microflora (Desjardins *et al.*, 1987).

3. OBJECTIVES OF THE STUDY

3.1. General Objective

- To investigate the effects of culture conditions on *in vitro* propagation of *Aframomum corrorima* in liquid MS culture media.

3.2. Specific Objectives

- To optimize the effect of different sucrose concentrations on *in vitro* shoot multiplication and rooting of *A. corrorima*
- To investigate the effect of different MS salt strength in liquid culture media on *in vitro* propagation of *A. corrorima*
- To examine the effect of different pH values on morphological parameters of *A. corrorima* cultured on *in vitro*
- To acclimatize the *in vitro* micropropagated korarima plantlets

4. MATERIALS AND METHODS

4.1. Plant Material Source and Experimental Area

Capsules of korarima were collected from the Jimma Agricultural Research Center (JARC), Department of Horticulture, Southwest Ethiopia. Fresh capsules of Jimma local cultivars were obtained at the peak harvestable stage of capsules and were collected from apparent disease free growing parents during November of 2018 cropping season. All the laboratory activities and experiments were carried out in plant tissue culture laboratory, Institute of Biotechnology at Addis Ababa University, College of Natural Sciences.

4.2. Preparation of Stock Solutions and Growth Media

4.2.1. MS stock solution preparation

Murashige and Skoog (MS) medium was used for all the experiments. The stock solution of macronutrients, micronutrients, vitamins and Na_2 EDTA and $FeSO_4.7H_2O$ were prepared separately by weighing the required amount of nutrient in gram per liter and dissolved in distilled water using magnetic stirrer. After all the required components were completely dissolved the solution was poured into plastic bottles and finally stored them in refrigerator at a temperature of -20°C. The prepared stock solutions were used for a month.

4.2.2. Preparation of PGR stock solution

In this study, plant growth regulators (PGRs) namely 6-benzyl amino purine (BAP) and α-naphthalene acetic acid (NAA) were used. Each of these growth regulators was prepared at a concentration of 1.0 mg/ml using precision balance. To enhance the dissolution, 1.0M NaOH and 1.0M HCl were consistently used for auxins (NAA) and cytokinins (BAP), respectively and adjusted to final volume by adding distilled water. After complete dissolution, the PGR solutions were poured into plastic bottles and stored them in refrigerator at +4°C for a maximum of one month.

4.2.3. Liquid culture media preparation

A full strength MS basal medium was prepared by mixing 50ml/l macronutrient, 5ml/l (micronutrient, vitamin and Na_2EDTA & $FeSO_4.7H_2O$), 3% sucrose and for shoot initiation, multiplication and root induction, 1.5 mg/l BAP and 0.5 mg/l NAA of PGRs were added separately into full strength MS medium and all treatment media except those for different

levels of pH were adjusted to pH 5.8 using 1.0 N NaOH and 1.0 N HCl and 7g agar was added (*only for seed germination and shoot initiation*), culture medium was dispensed into culture vessels and finally the medium was autoclaved for 15 min at 121°C and 105 kPa pressures.

4.3. Seed Surface Sterilization and *In vitro* Seed Germination of Korarima

Capsules of korarima were cut open to release seeds and then washed five times immediately with tap water at least for 15 minutes and then seeds were soaked in 50% H_2SO_4 for 60 minutes to remove dead seed coat. To break the dormancy of seeds, the seeds immediately were soaked in 250 mg/l GA_3 for 24 hours. Finally, they were washed with distilled water for 20 minutes and more scales were then removed, followed by washing with detergents.

Inside the laminar air-flow cabinet, the seeds were then rinsed with 70% ethanol for 3 minutes; followed by one-step surface sterilization using 25% sodium hypochlorite for 25 minutes. Then, the seeds were washed five times using sterile distilled water and further trimmed to remove dead seed coat and sulphuric acid affected scales. Before culturing, the seeds were further soaked in sterile distilled water for 20 minutes. The surface sterilized seeds were cultured on solid MS medium. After culture, each culture vessel was sealed with Parafilm, clearly labeled with the date of culture and placed in growth room chamber with 16 hours photoperiod and at a temperature of 25 ± 2°C. After three months, aseptic and sufficient amount of seedlings were obtained and they were used as explants in the subsequent experiments.

4.4. Shoot Initiation

After seed germination, the shoot tips of korarima were excised from *in vitro* germinated seedlings and were cultured on full MS salt strength medium supplemented with 1.5 mg/l BAP. After four weeks of culture, the percentage of initiated shoots was computed.

4.5. Shoot Multiplication

To investigate the effect of different sucrose concentrations on *in vitro* shoot multiplication, the young *A. corrorima* shoots obtained from culture initiation medium were used as explants and the shoots were excised and eventually cultured onto shoot multiplication MS basal medium containing 1 %, 1.5 %, 2 % and 3% sucrose. Similarly, to investigate the effect of different salt strengths, the regenerated plantlets were maintained on shoot multiplication

medium containing full, half, one third and one-fourth MS salt strengths basal media. Different salt strengths were used to compare their effects on shoot multiplication of korarima.

In another experiment, the initiated microshootlets of korarima were cultured on medium whose pH was adjusted to 5.0, 5.4, 5.8, 6.2 and 6.6 separately prior to autoclaving in order to evaluate the effect of different pH levels on shoot multiplication of korarima.

Each culture medium was supplemented with 1.5 mg/l BAP and adjusted to pH 5.8 (except for pH treatment) using 1N NaOH and 1 N HCl prior to autoclaving at 121°C and105 kPa for 15 minutes. For each treatment, thirty replications with a single explant per culture vessel (25X800 mm) were used and the culture vessels properly sealed, labeled and randomly placed in the growth room chambers with the same culture conditions (temperature, photoperiod and light intensity) as that of the initiation experiment. After four weeks of culture, the average shoot number, shoot length (cm) and leaf number per explant were recorded.

4.6. Rooting

For rooting studies, the proliferated shootlets derived from shoot multiplication MS basal media were cultured on PGRs free MS medium for four weeks before transferring into a liquid rooting media in order to avoid the carry over effect of hormones from the multiplication media on rooting and next the proliferated shoots were independently cultured on rooting media containing different levels of sucrose (1%, 1.5%, 2% and 3%) to investigate the effects of sucrose concentrations on *in vitro* root induction of korarima.

In another experiment, the rooting responses of excised shootlets separately cultured on full, half, one third and one-fourth MS salt strength basal media to identify the aseptic salt strength on *in vitro* root induction of *A. corrorima* explant.

To study the effect of different pH levels, the pH of the rooting medium was set at various pH values i.e. the pH of the media was adjusted to 5.0, 5.4, 5.8, 6.2 and 6.6 before autoclaving. Then after, the shoots were cultured on to full MS rooting media to investigate the effect of pH on *in vitro* root induction of korarima explants.

Each culture medium was supplemented with 0.5 mg/l NAA and adjusted to pH 5.8 (except for pH treatment) using 1N NaOH and 1 N HCl prior to autoclaving at121°C and105 kPa for 15 minutes. For each treatment, thirty replications with a single explant per culture vessel (25X800mm) were used and the culture vessels properly sealed, labeled and randomly placed in the growth room chambers with the same culture conditions (temperature, photoperiod and light intensity) as that of shoot initiation and shoot multiplication experiments. After four weeks of culture, the mean number of roots and root length (cm) per shoot were recorded.

4.7. Acclimatization and Transfer of Plantlets to Soil

For acclimatization process, the *in vitro* plantlets that have well grown roots were obtained from MS medium supplemented with 0.5mg/l NAA and 3% sucrose. Plantlets were taken out of the culture vessels and thoroughly washed with tap water to remove all the traces of the rooting medium. After trimming out excess leaves and roots, the plantlets were transferred to trays containing autoclaved mixture of forest soil, river sand and well decomposed farmyard manure in the proportion of 2:1:1, respectively .The plantlets were watered periodically with tap water and kept for two weeks within the growing room chamber by covering the trays with transparent polyethylene bags for seven days to retain high humidity and the plastic bags were gradually removed from the trays after a week to expose the plantlets for the external environment. Then after, the trays were maintained in greenhouse. Starting from the first week of acclimatization, the percentage/number of surviving shoots was documented on weekly basis until the fourth week of acclimatization period.

4.8. Experimental Design and Statistical Data Analysis

The design used for all the experiments was a complete randomized design (CRD) and the data was analyzed using the statistical data analysis software SPSS (IBM version 26.0) statistics program. One way analysis of variance (ANOVA) was used to calculate statistical significance and the mean ± SE using Duncan's multiple range test at $P < 0.05$ level. Thirty culture vessels (each vessel contained a single explant) per treatment were used and incubated in a controlled growth room chambers. Observations were recorded after four weeks of culture on shoot multiplication and root induction to investigate the effects of culture conditions on *in vitro* propagation of *A. corrorima*.

5. RESULTS

5.1. *In vitro* Seed Germination and Culture Initiation of Korarima

After three months of *in vitro* culture, the percentage of seed germination was 93.5%. Seeds that were washed in detergent, sterilized with70% alcohol for 3 minutes and further sterilized using 25% Clorox (sodium hypochlorite) for 25 minutes were reduced the contamination rate of the seeds.

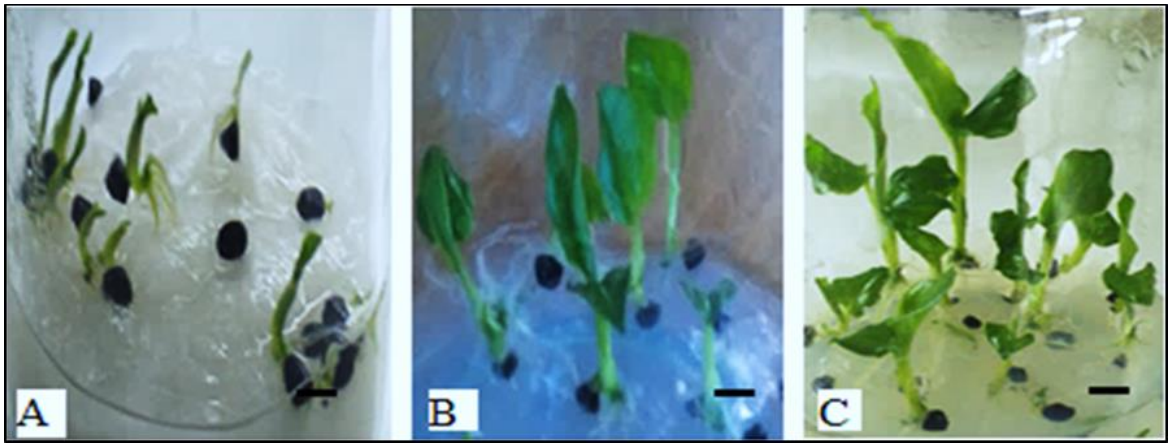

Figure1. *In vitro* seed germination of korarima on plant growth regulator free MS medium (A) After 45 days, (B) After two months of germination, (C) After 3months of germination. Bars = 1 cm.

Among one hundred eighty seven plantlets 180 (96.25%) were survived after one month and the efficient shoot formation achieved from *in vitro* germinated seedling shoot tips in this study will be useful for mass propagation.

Figure 2. Shoot tip explants of korarima on initiation culture medium containing 1.5 mg/ l BAP after four weeks of *in vitro* culture. Bars = 1 cm.

5.2. Effect of Different Sucrose Concentrations on Shoot Multiplication

Number of shoots

As shown in Table 1 and Fig.3, there was a significant difference ($p<0.05$) among the number of shoots per explant. The highest mean number of shoot (3.10 ± 0.15) was recorded in MS medium containing 3% sucrose concentration. The second highest mean number of shoot (2.60 ±0.09) was also noticed from MS medium containing 2% sucrose concentration then followed by 1.5% and 1% sucrose concentrations with mean number of shoots per explant (2.13 ± 0.09 and 1.60 ±0.09), respectively. Therefore, MS medium supplemented with 3% sucrose yielded the maximum number of shoots/explant (3.10 ± 0.15) whereas the least number of shoot/per explant (1.60 ±0.09) was recorded on MS medium supplemented with 1% sucrose. Thus, it can be concluded that increasing concentration of sucrose from 1% to 3% had a positive effect in increasing shoot number per explant of *A. corrorima*.

Shoot Length

The highest mean shoot length (3.39± 0.63 cm) was recorded on MS medium containing 3% sucrose, while the shortest one (2.36 ±0.70 cm) was noticed on 1% concentration of sucrose. In the case of 1.5 and 2% sucrose concentration supplemented MS media, the average shoot lengths were 2.64 ±0.29 and 3.04 ±0.23 cm, respectively. Thus it can be concluded that decreasing the concentration of sucrose from 3% to 1% significantly decreased the average shoot length per explant.

Number of Leaves

As shown in Table 1, the highest average leaf number (8.77 ± 0.13) was obtained when explants grown on MS medium supplemented with 3% sucrose. The data indicated that increasing of the concentrations of sucrose from 1- 3% resulted higher average number of leaves; in contrast 1% sucrose concentration significantly reduced the number of leaves as compared to 3, 2 or 1.5% sucrose concentrations. Thus, it can be concluded that the best treatment to increase the number of leaf was 3% sucrose concentration.

Table1. Effect of different sucrose concentrations on shoot multiplication (Means ± SE) of *A. corrorima* after four weeks of *in vitro* culture.

Concentrations of sucrose(%)	Mean No. of Shoots per explant	Mean Shoot Length (cm)	Mean No. of leaves per explant
1	1.60 ±0.09[d]	2.36 ±0.70[d]	5.07 ±0.16[d]
1.5	2.13 ± 0.09[c]	2.64 ±0.29[c]	6.17 ±0.13[c]
2	2.60 ±0.09[b]	3.04 ±0.23[b]	7.37 ±0.26[b]
3	3.10 ± 0.15[a]	3.39± 0.63[a]	8.77 ±0.13[a]

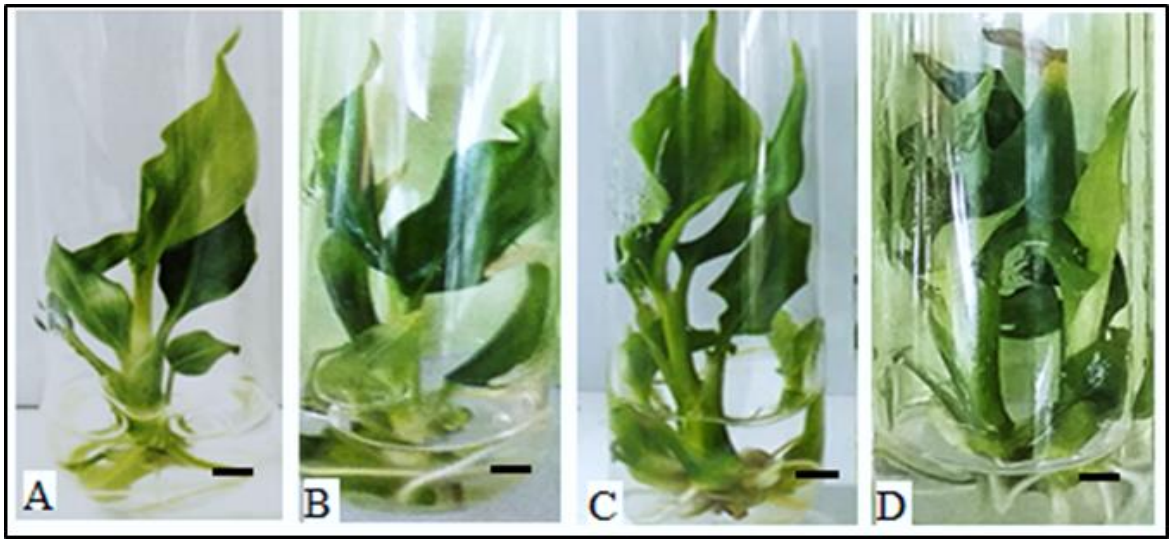

Figure 3.Shoot multiplication of korarima with different sucrose concentrations (A) = 1% sucrose, (B) = 1.5% sucrose, (C) =2%sucrose, (D) =3%sucrose. Bars = 1cm

5.3. Effect of Different MS Salt Strength on Shoot Multiplication

Number of Shoot

As indicated in Table 2 and Fig.4 Different salt strength MS media (full, half, one third and one fourth) had a significant effect on shoot multiplication of *A. corrorima in vitro* culture. The highest mean number of shoot (3.03 ±0.21) per explant was evolved from full salt strength medium as compared with half (2.60 ±0.14), one-third (2.47 ±0.17) and one fourth salt strength (2.07 ±0.08) media. Therefore, maximum (3.03 ±0.21) and minimum (2.07 ±0.08) mean shoot numbers were recorded on full and one fourth salt strength MS media.

Shoot Length

As shown in table 2 that the longest average shoot length /explant was cultivated on full (3.17±0.12 cm) salt strength and then followed by half (2.89±0.16cm), one third (2.26±0.03 cm) and one fourth (2.04 ±0.05cm) salt strength MS basal media. Therefore, MS medium that contained full salt strength was best treatment for shoot length.

Number of Leaves

There was no significant difference for average number of leaf/explant cultured on half and full MS salt strength media but the lowest average number of leaf (4.40 ±0.22) was noticed on one quarter salt strength. Thus, it can be asserted that full and half salt strength MS media were the best treatments to increase the number of leaf/explant.

Table 2. Effect of different MS salt strength on shoot multiplication (Means ± SE) of *A.corrorima* after four weeks of *in vitro* culture.

Medium salt Strength	Mean No. of Shoots per explant	Mean Shoot Length (cm)	Mean No. of Leaves per explant
One fourth MS	2.07 ±0.08^{c}	2.04 ±0.05^{d}	4.40 ±0.22^{b*}
One third MS	2.47 ±0.17^{bc}	2.26±0.03^{c}	5.60±0.18^{b*}
Half MS	2.60 ±0.14^{ab}	2.89±0.16^{b}	9.80 ±0.78^{a*}
Full MS	3.03 ±0.21^{a}	3.17±0.12^{a}	9.40±0.69^{a*}

*Means followed by the same letter (s) within each column are not significantly different at $p < 0.05$, according to Duncan's multiple range tests.

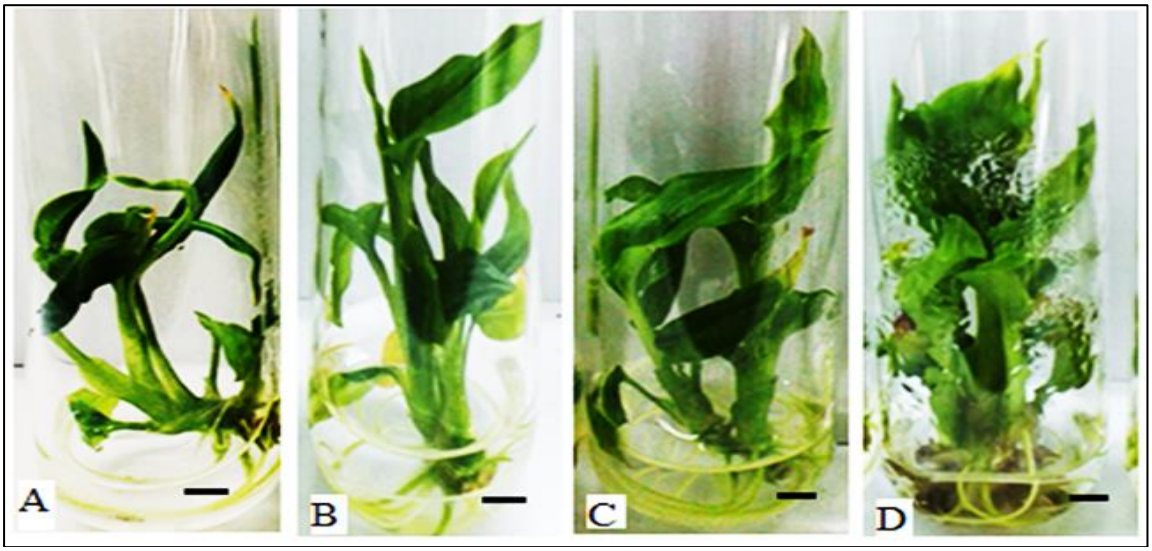

Figure 4. Shoot multiplications on MS medium containing different salt strength, (A) =1/4MS, (B) =1/3MS, (C) =1/2MS, (D) =Full MS. Bars = 1cm.

5.4. Effect of different pH Levels on Shoot Multiplication

Number of Shoots

Different data on medium pH was affected the number of shoots produced. As a result, the highest mean shoot number (3.20 ±0.13) was recorded at pH 5.8. The second maximum mean

number of shoot (2.87±0.15) / explant was documented at pH 6.2. The culture media pH 5.4, 6.6 and pH 5.0 were produced the mean shoot number of 2.60 ±0.22, 2.20 ±0.21 and 2.13 ±0.11, respectively. Thus it can be concluded that pH 5.8 was the best treatment to increase the number of shoot /explant.

Shoot Length

As shown in table 3, there was a significant effect of pH on shoot length of korarima. The longest average shoot length (4.08±0.13 cm) was recorded at pH 5.8. The second longest shoot (3.76 ±0.07 cm) was also produced at pH 6.2 followed by pH5.4, pH5.0 and pH 6.6 with mean shoot length of 3.46±0.11, 3.40±0.09 and 3.30 ±0.06 cm. The results revealed that, reduced mean shoot lengths were noticed below and above pH 5.8 extremes. Therefore, the best shoot length was produced at pH 5.8.

Number of Leaves

Data in table 3 indicated that the highest (11.40 ±0.27) and minimum (7.00±0.45) average number of leaves were recorded at pH 5.8 and 6.6, respectively. To acquire maximum numbers of shoots, leaves and shoot lengths from *in vitro* culture, the culture medium should be adjusted to pH 5.8 before culturing the explant.

Table 3.Effectof different pH ranges on shoot multiplication (Means±SE) of *A. corrorima* after four weeks of *in vitro* culture.

pH values	Mean No. of Shoots per explant	Mean Shoot Length (cm)	Mean No. of Leaves per explant
pH5.0	2.13 ±0.11^{c}	3.40±0.09^{c*}	9.20 ±0.31^{bc}
pH5.4	2.60 ±0.22^{bc}	3.46±0.11^{c*}	8.20 ±0.46^{c}
pH5.8	3.20 ±0.13^{a}	4.08±0.13^{a}	11.40 ±0.27^{a}
pH6.2	2.87±0.15^{ab}	3.76 ±0.07^{b}	10.00 ±0.26^{b}
pH6.6	2.20 ±0.21^{c}	3.30 ±0.06^{c*}	7.00±0.45^{d}

*Means followed by the same letter (s) within each column are not significantly different at $p < 0.05$, according to Duncan's multiple range tests.

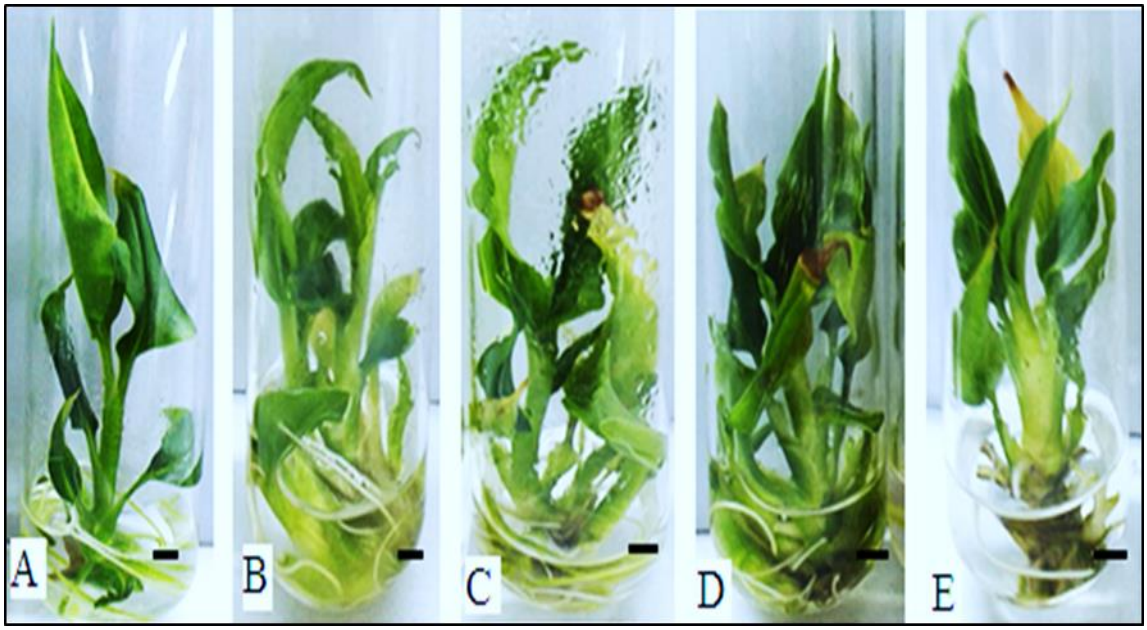

Figure 5. Shoot multiplications on MS medium containing different pH levels (A) =pH 5.0, (B) =pH 5.4, (C) =pH 5.8, (D) =pH 6.2, (E) =pH 6.6. Bars = 1cm.

5.5. Effect of Different Sucrose Concentrations on Rooting

Number of Roots

Data in table 4 indicated that root number was affected by different concentrations of sucrose, thus increasing sucrose concentration had a positive effect on the root number. Minimum mean root number (2.78 ± 0.24) was produced in MS medium supplemented with 1% sucrose and the highest mean root number (9.03±0.72) was recorded on medium containing 3 % sucrose. Thus, it can be concluded that 3% sucrose was the best treatment for induction of maximum number of roots.

Root length

It is quite clear from table 4 that there were no significant differences on the root lengths cultured on3 and 2% sucrose concentrations. The shortest mean root (2.37 ±0.25 cm) was recorded on medium containing 1% sucrose. Therefore, root length decreased proportionally with decrease in sucrose concentration. Thus, it can be asserted that 3 and 2% sucrose concentrations were the best treatments for inductions of the longest roots.

Table 4.Effect of different sucrose concentrations on root induction (Means ± SE) of *A.corrorima* after four weeks of *in vitro* culture.

Concentrations of sucrose (%)	Mean No. of Roots per shoot	Mean Root Length (cm)
1	2.78 ± 0.24[d]	2.37±0.25[c]
1.5	5.37 ±0.42[c]	3.24±0.32[ab]
2	7.26 ± 0.68[b]	3.78±0.18[a*]
3	9.03± 0.72[a]	4.04±0.15[a*]

*Means followed by the same letter (s) within each column are not significantly different at $p < 0.05$, according to Duncan's multiple range tests.

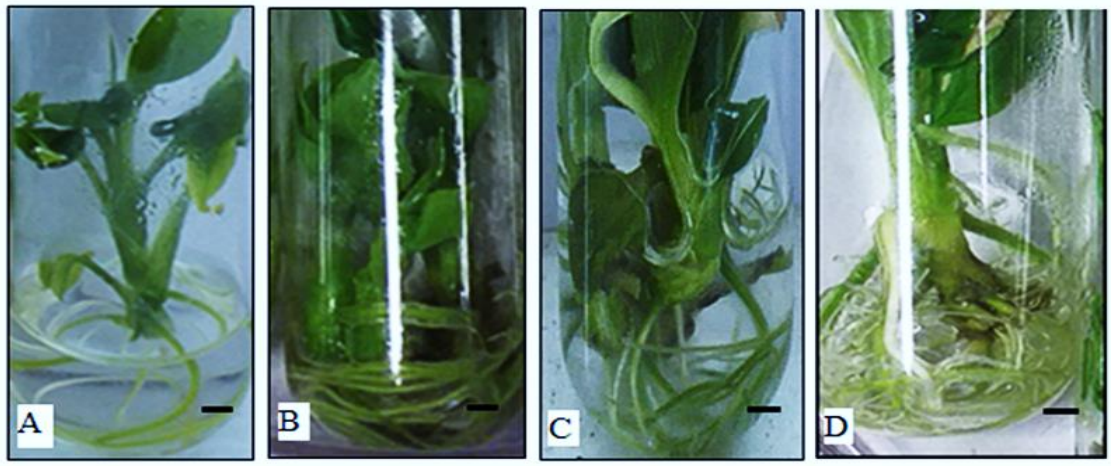

Figure 6. Root induction of korarima in different concentrations of sucrose (A) = 1% sucrose, (B) = 1.5% sucrose, (C) =2%sucrose, (D) =3%sucrose. Bars = 1cm.

5.6. Effect of Different MS Salt Strength on Root Formation

Number of Roots

Table 5 showed that the highest mean number of root (14.47± 0.96) was recorded on half MS salt strength medium as compared to full (12.13 ±0.68), one third (7.87 ± 0.46) and one fourth (7.52±0.45) salt strength MS media.

Root Length

The longest root (6.30 ±0.11cm) was also recorded on half MS salt strength medium (Table 5). Half MS salt strength was significantly differed from full (5.22 ±0.19cm), one third (4.35 ±0.20cm) and one fourth (3.68 ±0.10cm) of salt strength. Thus it can be asserted that, half MS salt strength was the best treatment for root induction.

Table 5.Effect of different MS salt strength on root induction (Means± SE) of *A.corrorima* after four weeks of *in vitro* culture.

Medium salt Strength	Mean No. of Roots per shoot	Mean Root Length (cm)
One fourth MS	7.52 ± 0.45^{c*}	3.68 ±0.10^{d}
One third MS	7.87 ± 0.46^{c*}	4.35 ±0.20^{c}
Half MS	14.47± 0.96^{a}	6.30 ±0.11^{a}
Full MS	12.13 ± 0.68^{b}	5.22 ±0.19^{b}

*Means followed by the same letter (s) within each column are not significantly different at $p < 0.05$, according to Duncan's multiple range tests.

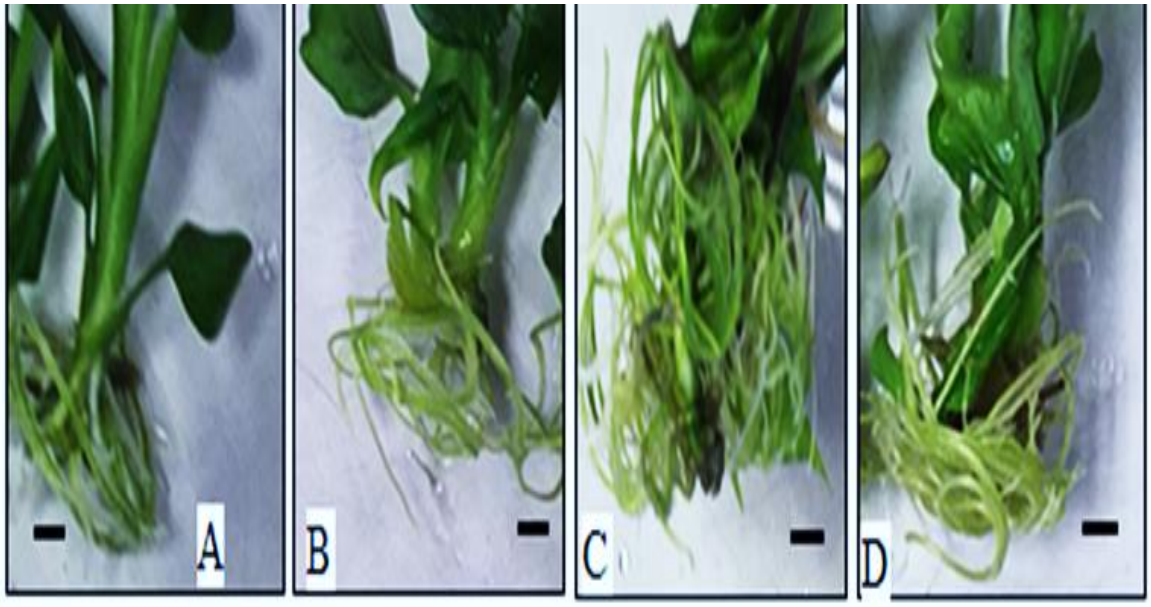

Figure 7. Root inductions on MS medium containing different salt strength (A) =1/4MS, (B) =1/3MS, (C) =1/2MS, (D) =Full MS, Bars = 1cm.

5.7. Effect of Different pH ranges on Root Induction

Number of roots

Maximum average root number (17.00 ±1.10) was cultured on pH 5.8 and then followed by pH 6.2 with mean root number of 14.60±0.73 (Table 6). The least mean root number (3.20 ±0.21) was noticed on pH 6.6.

Root length

Maximum mean root length (6.94±0.60 cm) was recorded at pH 6.2 followed by pH 5.4, 5.8, and 5.0 each with mean root length of (6.12±0.28 cm), (5.48±0.12 cm), (5.34±0.23 cm) and (3.78±0.22 cm), respectively. Therefore, minimum mean root length (3.78±0.22 cm) was recorded at pH 6.6. As a result, root induction of korarima was best treatment between pH5.8 and 6.2.

Table 6.Effect of different pH on root induction (Means± SE) of *A. corrorima* After four weeks of *in vitro* culture.

pH values	Mean No. of Roots per shoot	Mean Root Length (cm)
pH5.0	10.20 ±0.92^{c}	5.34±0.23^{b*}
pH 5.4	13.60 ±1.50^{b}	6.12±0.28^{ab}
pH5.8	17.00 ±1.10^{a}	5.48±0.12^{b*}
pH 6.2	14.60 ±0.73^{ab}	6.94±0.60^{a}
pH 6.6	3.20 ±0.21^{d}	3.78±0.22^{c}

*Means followed by the same letter (s) within each column are not significantly different at $p < 0.05$, according to Duncan's multiple range tests.

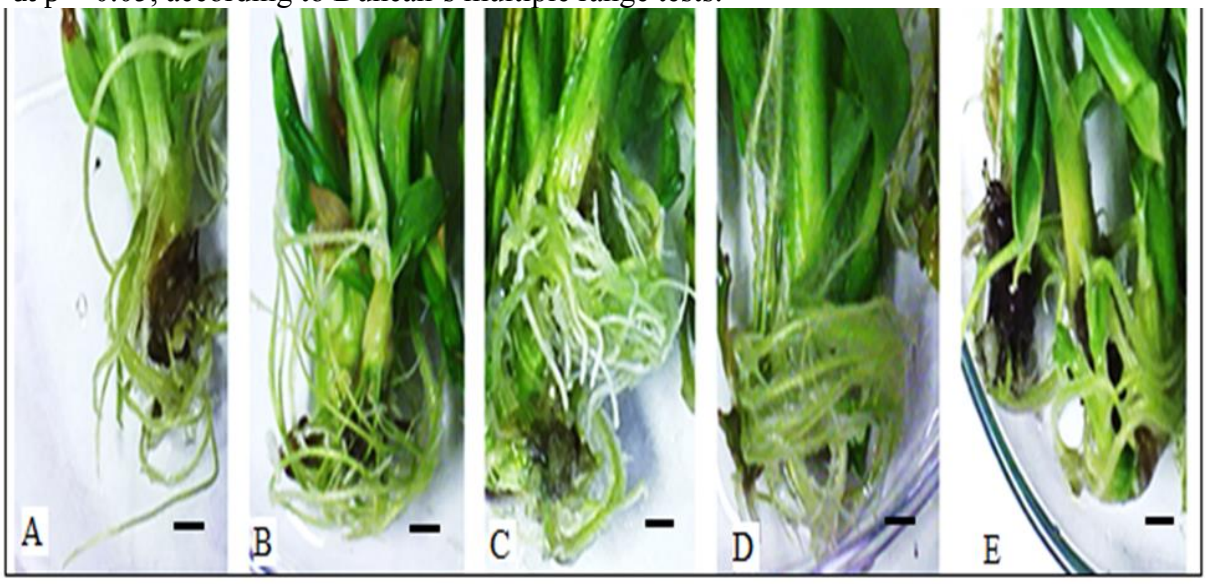

Figure 8. Root inductions on MS medium at different pH levels

(A) = pH 5.0, (B) = pH 5.4, (C) = pH 5.8, (D) = pH 6.2, (E) = pH 6.6. Bars = 1cm.

5.8. Acclimatization

Plantlets that have well grown roots were transferred to the soil for acclimatization. After four weeks of acclimatization, nearly 94.3 % (50) plants were survived among fifty three explants cultivated on garden soil, farmyard (manure) and sand at 2:1:1 ratio, respectively.

Figure 9. Acclimatization of *in vitro* rooted microshoots of *A. corrorima*

(A) Acclimatized plantlets covered with polyethylene bags, (B) Acclimatization after one week, (C) Acclimatization after two weeks and (D) Acclimatized plants after 30 days. Bars = 1cm.

6. DISCUSSION

6.1. *In Vitro* Seed Germination and Culture Initiation of Korarima

Successful surface sterilization is one of the most critical steps of *in vitro* culture. The present result showed that the seeds of *A. corrorima* were washed in detergent, disinfected with 70% alcohol for 3 minutes and further sterilized with 25% Clorox for 25 minutes were successful surface sterilization treatments that reduced seed contamination rate. After three months, the percentage of seed germination was 93.5%.The result was in agreement with the findings of Tefera and Wannakrairoj (2004) who successfully germinated korarima seeds that resulted in 94% germination.

After a week of culturing, shoot tips started to develop microleaves on MS medium supplemented with 1.5 mg/l BAP. After a month of growth on the initiation medium, 96.25% of korarima explants survived. The current result was closly in agreement with the reports of Leweye (2017) who stated that 90 % of korarima explants survived after 30 days of growth on MS culture medium. The efficient shoot formation of korarima from *in vitro* germinated seedling shoot tips in this study is useful for mass propagation.

6.2. Effect of Different Sucrose Concentrations on Shoot Multiplication

In vitro propagation of plants is not fully autotrophic. Under these conditions, plants have low photosynthesis capacity due to insufficient light intensity, high air relative humidity, and lack of gas exchange. Therefore, the addition of external carbon source is necessary for growth and development of micropropagation efficiency. Sucrose in culture media serve as energy, carbon sources, maintain a stable osmotic environment and are signaling molecules to sustain the growth of *in vitro* cultures of plants. This energy available from carbon sources is used in the developmental process (shoot proliferation, root induction and flowering), embryogenesis and organogenesis, which are highly energy demanding processes (Yaseen *et al.*, 2013; Kozai *et al.*, 1997).

Improvements in shoot regeneration efficiency require better understanding of the influence of culture conditions on shoot regeneration. The use of different concentrations of sucrose highly affected the *in vitro* shoot multiplication of *A. corrorima.* Based on the results obtained in the present study, the best response was exhibited by the explants on medium comprising 3% sucrose. Thus increasing sucrose level had a positive effect on shoot

multiplication of korarima up to 3% sucrose concentration. This may be due to the fact that high sucrose concentrations available in the culture medium may speed up cell division thus leading to an increase in the volume and weight of tissues as pointed out by other researchers (Chong *et al.*, 1972).

Ahmed *et al.* (2004) studied the effect of different sucrose concentrations (3%, 2% and 1%) on banana (*Musa spp*.) cv. Grand nine plantlets and they reported that the highest number of leaves (7.00) and length of shoots (6.20 cm) were documented on 3% sucrose concentration and then followed by 2% with 6.33 mean leaf number and shoot length (5.70cm). The least number of leaves (5.66) and shoot lengths (4.90 cm) were also recorded on 1% of sucrose concentration which is in agreement with the present study that leaf number and shoot length increased with increase in sucrose concentration.

In another study, Leweye (2017) investigated the effect of different concentrations of sucrose (1%, 2% and 3% (w/v)) on *in vitro* korarima plants. He reported that the maximum average shoot number (6.67±0.55) and shoot length (2.81± 0.09 cm) were obtained in 3% sucrose containing medium among the rest treatments. From these results, 3% sucrose concentration in the basal medium seems to be sufficient for normal plant growth as recommended by Murashige and Skoog (1962). The supplemental sugar in the *in vitro* medium may assist in water conservation and maintaining the osmotic potential of cells (Hazarika, 2003). In the present study, the need for sucrose increased considerably from 1-3%, perhaps indicated that there was a direct relationship between cell division and sucrose consumption in the cultured tissues may be satisfied the energy requirements as well as in water conservation and for cell differentiation without having any negative osmotic effect on shoot formation.

6.3. Effect of Different MS Salt Strength on Shoot Multiplication

The explants cultivated on growth medium without mineral salts did not display shoot formation, even when exogenous cytokinin was used in the medium. Therefore, the addition of optimum amount of external mineral salt is necessary for shoot formation in *in vitro* culture to provide sufficient nutrient requirements that enhance biochemical metabolism result in cell growth and to prevent toxic effects of media salt (Fadel *et al.*, 2010).

Different mineral salt strength in the culture medium affected the number of shoot and shoot length of korarima. The current results showed that the highest average shoot number (3.03±0.21) and shoot length (3.17±0.12 cm) were recorded in full MS salt strength medium

then followed by half , one third and one fourth salt MS salt strength media. However, there was no significant difference in leaf number between full and half salt strengths. These results agreed with work of Tefera and Wannakrairoj (2004) who reported that in korarima the highest mean shoot number (6.2) per explant and mean shoot length (5.37 cm) were obtained in full MS salt strength medium.

Similar result was reported by Leweye (2017) on korarima in that full strength MS medium resulted in the highest number of shoots per explant, mean length of shoots and the values were (5.27 ± 0.53 shoot/explant and 3.08 ± 0.20 cm), respectively. Thus, increasing the salt strength of MS basal medium resulted in better shoot number and shoot length of *A. corrorima*. Generally, the obtained result indicated that full- MS strength medium improved shoot proliferation of korarima under investigation.

The current results are also in agreement with findings from Taha *et al.* (2013) on *Ficus carica* and Dahab *et al.* (2005) in *Ruscus hypoglossum* who obtained the highest mean shoot number per explant using full salt strength MS medium. Mineral salts regulate the growth and morphology of the plant, providing essential nutrients (Greenway *et al.*, 2012).

6.4. Effect of Different pH on Shoot Multiplication

In the present study, pH 5.8 produced significantly more shoots, leaves and shoot length among the rest of treatments, with mean number of shoots (3.20±0.13), leaves (11.40±0.27) and shoot length (4.08±0.13cm). In addition, shoot length was not significant in other treatments. As the pH level decreased from 5.8 to 5.0 or increased from 5.8 to 6.6, there were significant decreases in the number of shoots and leaves per explant.

In agreement with the current results, Parveen and Shahzad (2014) reported that the optimal value of pH for shoot proliferation of *Senna sophera* (L.) explant proved to be 5.8 where the maximum mean number of shoots was 19.50 ± 0.51 per explant. Below or above the optimal pH (5.8) value showed a decreasing trend on shoot number of *Senna sophera* (L.) explant. It is known that the medium pH has effects not only on the uptake of nutrients but also on chemical reactions especially those catalyzed by enzymes (Thorpe *et al.*, 1991).

Nair and Seeni (2003) also obtained the best shoot multiplication at pH of 5.8 in a medicinal plant, *Calaphyllum apetalum*. Based on the current report, it is hypothesized that the effect of pH 5.8 on shoot multiplication probably regulates the activities of enzymes, growth

regulation, up take of nutrients and the function of the cell membrane as well as the buffered pH of the cytoplasmic activity that enhances cell division and the growth of shoots.

6.5. Effect of Different Sucrose Concentrations on Rooting

Root induction occurred when the media contained sufficient amount of carbon sources. Media devoid of carbon sources did not produce roots indicating the importance of sugar in root formation. According to McCown (1988) *in vitro* root formation does not occur when photosynthesis products are supplied in insufficient quantities. In the current study, numbers of roots were affected with different concentrations of sucrose, as it gave the highest value at 3% with a mean of 9.03±0.72 then followed by 2% (7.26±0.68). As the concentrations of sucrose decreased from 3 to 1%, root number decreased as well as the vigor. Therefore, the number of roots per shoot increased proportionally with sucrose concentration and there were significant differences in all four sucrose treatments. Thompson and Thorpe (1987) also indicated that root initiation and growth were high energy requiring processes that could occur at the expense of available metabolic substrates, which were mainly carbohydrates.

The above results are in agreement with the finding of Ahmed *et al.* (2014) who reported the effect of different concentrations of sucrose (3%, 2% and 1%) on banana (*Musa spp*.) cv. Grand nine plantlets and they found that the highest number of roots (6.00) was documented on 3% sucrose concentration and then followed by 2% sucrose treatment whereas the least number of roots (4.00) and root length (5.66 cm) were also recorded on 1% of sucrose concentration medium. This may be due to the fact that 3% sucrose concentration in *in vitro* rooting culture medium may speed up cell division thus leading to cell expansion that results in increase in the volume and weight of tissues.

Our results are similar to those reported by Testa *et al.* (2016) who studied on Elite Sugarcane (*Saccharum officinarum* L.) genotypes and they reported that there was no rooting of micro-shoot recorded on liquid MS medium supplemented with different level of NAA without the presence of sucrose. On the contrary, root induction occurred when the micro-shoots were cultured in liquid MS medium supplemented with various concentrations of sucrose without NAA. These results confirmed the current study that rooting process requires high energy source that occurs at the expense of available sucrose concentration in rooting medium for *A. corrorima* root induction. However, increasing sucrose over the threshold concentration (above 3%) may have resulted in reduction of average root length and number

of roots. Earlier reports also confirmed higher concentrations of sucrose in the medium have a negative impact on overall rooting due to accumulation of rooting inhibitors, excessive carbohydrate and hinder photosynthesis, the reduction of rooting promoters in the medium, and the transformation of added sugars in to insoluble and storage form which eventually impairs cell growth of plant (Cappellades *et al.*, 1991; Ahmed *et al.*, 2004).

6.6. Effect of Different MS Salt Strength media on Rooting

The mineral concentration in the culture medium affects rooting characteristic and several researchers have proposed that reduction of salt strength to half strength improved rooting (Dimassi-Theriou and Economou, 1993; Sarropoulou *et al.*, 2015).

In the current study, *in vitro* root induction of korarima was enhanced when the concentration of salts in the medium was reduced by half. The maximum mean root number (14.47±0.96) and root length (6.30±0.11cm) were associated with 1/2 MS salt strength basal medium than full, 1/3 and ¼ MS salt strength medium. In agreement with current findings, Tefera and Wannakrairoj (2004) reported that the maximum root number (7.3) in half strength MS and root length (4.19 cm) in half nitrogen MS medium. The reason behind increasing rooting rate on half strength culture medium might be due to a disorder in carbohydrate to nitrogen in nutrient medium, which lead to decreasing nitrogen level in shoot and then improving rooting rate, initiating roots, increasing root number and lengths (Fotopoulos and Sotiropoulos, 2005).

Leweye (2017) showed that half strength MS medium resulted in the highest number of roots per shoot, mean length of roots and the values were (18.50 ± 1.15 root/shoot and 4.03 ± 0.15 cm), respectively.

In another study, an increase in the root number of *Zingiberaceae* family species was reported when the concentration of salts in the medium was reduced by half (Abbas *et al.*, 2011; Haque and Ghosh, 2018; de Souza Ferrari *et al.*, 2019). In the earlier experiment, the maximum number of roots and average root length were observed on half MS salt strength medium supplemented with 3% sucrose and 0.5 mg/l NAA. Halving the strength of MS medium resulted in increased rooting of *Mentha arvensis* regenerants (Fadel *et al.*, 2010). Perhaps its reason can be explained that due to decrease in the concentration of mineral salts, reduction in hyperhydricity and low osmotic pressure was

resulted in increasing the number and length of roots. The roots can dehydrate or undergo cellular collapse if the osmotic potential of the medium is very high (da Silva *et al.*, 2017).

6.7. Effect of Different pH on Rooting

Roots are directly in contact with media pH and are potentially the first line to respond to the media environment. At optimal pH, normal roots sharply induced while below or above the optimum pH level the morphological states of roots namely the root cap cells, root tips, root meristems and the epidermal cell might be abnormal. Root injury at high pH levels indicates it as the physical mechanism of growth inhibition under that condition. It is believed that roots are mediator capable of monitoring the changing conditions in the growing media and transmitting the information to the shoot (Mohammad and Shiraishi, 1999).

Korarima plants have a preference for a pH ranging between 5.5 and 6.5. However, they are able to tolerate a pH range of 4.5 to 7.5 (Jansen, 2002). In the present study, maximum number of roots (17.00±1.10) and root length (6.94±0.60 cm) were recorded at pH 5.8 and 6.2 respectively, which were significantly superior to the rest of treatments. The least number of roots (3.20±0.21) and root length (3.37±0.22 cm) were also noticed at pH of 6.6. However, there are few reports on the effect of media pH on the growth of culture. The poor characteristics of pH 5.0 and 6.6 may be due to differential availability of various nutrients (trace elements such as boron) or due to some toxic effect (change in buffer action) attributed by low or high medium pH resulting in root injuries. In guava, Amin and Jaiswal (1989) observed that comparatively less acidic medium was better than more acidic medium for *in vitro* rooting.

6.8. Acclimatization

After four weeks of acclimatization on garden soil, farmyard (manure) and sand (2:1:1) mixtures, 94.3 % plants survived. This result is in agreement with the earlier findings of Leweye (2017) and Tefera and Wannakrairoj (2004) reported similar acclimatization result (90.83%) and (93%) survival rates for *A. corrorima* ,respectively. Hence, it can be concluded that the soil composition of forest soil, compost and sand in 2:1:1 ratio might have well drained with high water holding capacity, good aeration and nutrients present in the mixture that contributed to the survival rates for acclimatization of korarima.

7. CONCLUSION

After three months of *in vitro* culture, the percentage of seed germination was 93.5%. The result may prove to be there was a successful seed surface sterilization protocol that reduced seed contamination rate.

The results of this study also showed that 3% sucrose concentration in culture medium was better for shoot multiplication and root induction. Hence 3% sucrose serves as energy, carbon source, maintain a stable osmotic environment and signaling molecule to sustain the growth of *in vitro* cultures of *A. corrorima.*

It has been observed that maximum shoot numbers (3.03 ±0.21) and shoot length (3.17 ±0.12 cm) were recorded in full MS strength medium. However, the maximum average number of roots (14.47±0.96) and root length (6.30±0.11 cm) were noticed on half MS salt strength medium. Therefore shoot and root multiplication/induction may have different salt strength requirements.

Our results revealed that the highest mean shoot number (3.20 ±0.13), leaf (11.40 ±0.27) and shoot length (4.08±0.13 cm) were recorded at pH 5.8. However maximum root numbers (17.00 ±1.10) and root length (6.94±0.60cm) were recorded at pH 5.8 and 6.2, respectively. This indicated that, the highest mean shoot number, leaf, root and shoot and root length significantly occurred at pH 5.8 and 6.2 among other treatments. Thus it can be concluded that better shoot multiplication and rooting of korarima occurred at high acidic pH range (5.8-6.2) rather than at low alkaline (> 6.2-6.6).

Most species grown *in vitro* require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil. After four weeks of acclimatization, 94.3% plants survived on garden soil, farmyard (manure) and sand at 2:1:1 ratio, respectively. The reasons behind that the soil composition of forest soil, compost and sand in 2:1:1 ratio might have well drained with high water holding capacity, good aeration and nutrients present in the mixture that contributed to the survival rates for acclimatization of korarima.

8. RECOMMENDATIONS

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

- Development of a liquid culture medium *in vitro* micropropagation protocol could be beneficial to increase propagation rates, which will help to reduce time and cost factor because no agar is used as well as explants can be extracted from the culture vessel with minimal damage.

- Research on further optimization of acclimatization should be done in order to ensure the sufficient number of plants survive and grow vigorously when transferred to soil.

- Although no statistics are available, *A.corrorima* is an endangered species due to the rapid degradation and destruction of its natural habitat by human and other natural factors. Therefore, plant tissue culture has made a significant contribution to propagate mass of clonal explants urgently. The results obtained in the current study will help for germplasm collection and conservation of this important species.

- In korarima, propagation by seed is quite difficult by traditional breeding methods. Currently, there was a study in breaking of korarima seed dormancy due to its impermeability of hard seed coat. But cost of *in vitro* seedlings protocol needs to be known for cost benefit analysis.

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APPENDIX

Appendix table, Composition of culture medium, Murashige and Skoog (MS) Medium (1962) and concentrations for MS stock solution

Macronutrients	Molecular Formula	Concentration	
		(g/L)	(ml/L)
Calcium chloride dehydrate	$CaCl_2 \cdot 2H_2O$	8.8	50ml/L
Potassium dihydrogen orthophosphate	KH_2PO_4	3.4	
Potassium nitrate	KNO_3	38	
Magnesium sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	7.4	
Ammonium nitrate	NH_4NO_3	33	

Micronutrients	Molecular Formula	Concentration	
		(g/L)	(ml/L)
Cobalt chloride hexahydrate	$Cocl_2.6H_20$	0.05	5ml/L
Ferrous sulphate heptahydrate	$FeSO_4 \cdot 7H_2O$	5.56	
Boric acid	H_3BO_3	1.124	
Potassium iodide	KI	0.166	
Manganous sulfate monohydrate	$MnSO_4 \cdot H_2O$	0.05	
Sodium molybdenum oxide dehydrate	$NaMoO_4 \cdot 2H_2O$	0.05	
Sodium Ethylene Di-Amine Tetra acetic Acid	$C_{10}H_{14}N_2O_8Na_2H_2ONa_2$ EDTA	7.472	
Zinc sulphate heptahydrate	$ZnSO_4 \cdot 7H_2O$	1.72	

Vitamin Sources	Molecular Formula	Concentration	
		(g/L)	(ml/L)
Glycine	$C_6H_{12}O_6$	0.4	5ml/L
Myo-inositol	$C_2H_5NO_3$	20	
Nicotinic Acid	$C_6H_5NO_2$	0.1	
Thiamine HCl	$C_{12}H_{17}CIN_4OS \cdot HCl$	0.02	
Pyridoxine HCl	$C_8H_{12}N_2O_2 \cdot 2$ HCl	0.1	

- Na_2EDTA and $FeSO_4.7H_2O$ prepared alone

Declaration

I undersigned declare that this MSc thesis entitled; the effects of culture conditions on *in vitro* propagation of korarima (*Aframomum corrorima* (Braun) P.C.M. Jansen) is conducted by me under the advisor of Tileye Feyissa (PhD, Associate Professor) from the institution of biotechnology, school of graduate studies, Addis Ababa University. I further declare that, this MSc thesis is my original work, and it was not submitted to other universities or institutions in the fulfillment of any degree. All sources of materials used in this thesis are duly acknowledged.

Abdela Zeinu Mohammed signature_____ Date_____
(Candidate student)

This thesis has been submitted for examination with my approval as a university advisor.

Tileye Feyissa (PhD, Associate Professor) signature_____ Date_____
(Advisor)