In Vitro Protocol Optimization for Micropropagation of Promising Ginger (*Zingiber officinale* Rosc.) Accession 52/86 from Sprouted Rhizome Buds

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I have completed my thesis research work as per the approved proposal and it has been evaluated and accepted by my advisors. Hence, I hereby kindly request the Department to allow me to present the findings of my work and submit the thesis.

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We, the thesis advisers, have evaluated the content of the thesis and found to be satisfactory executed according to the approved proposal, written according to the standard and format of the University and is ready to be submitted. Hence, we recommend the thesis to be submitted.

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DEDICATION

I dedicate this thesis to my family for their commitment for the success of my life.

STATEMENT OF THE AUTHOR

I, Solomon Nigusu, hereby declare that the work is an authentic record of my own work, and that all sources of materials used for this thesis have been duly acknowledged. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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BIOGRAPHICAL SKETCH

The author, Solomon Nigusu was born on October 16, 1994 in Jidda district, North Shoa/Selale/ Zone, Oromia Regional State of Ethiopia. He attended elementary school at Dega Gora Elementary School from 2001-2008, high school at Shambel Abebe Bikila Senior Secondary School from 2009-2010 and his preparatory education at General Tadessa Birru Comprehensive Senior Secondary and Preparatory School from 2012-2013. Then, he joined Mettu University, Gambella College in 2014 and graduated with BSc degree in Plant Science in 2016. After his graduation, he joined Gambella University as graduate assistant and served there from 2016-2017. In September 2017 he joined Jimma University to pursue his graduate studies leading to MSc in Plant Biotechnology.

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ABBREVIATIONS AND ACRONYM

ANOVA	Analysis of variance
BAP	6-Benzylo Amino-Purine
CRD	Completely Randomized Design
FAOSTAT	Food and Agricultural Organization Statistics
IBA	Indole-3- Butyric Acid
IBC	Institute of Biodiversity and Conservation
KIN	Kinetin
JARC	Jimma Agricultural Research Center
LSD	Least Significance Difference
MS	Murashige and Skoog
NAA	α-Naphthalene Acetic Acid
PVP	Poly Vinyl Pyrrolidone
SNNP	South Nations, Nationalities and People
USDA	United States Department of Agriculture

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ABSTRACT

Ginger is one of the important cash crops in Ethiopia. However, unavailability of disease free planting material (rhizomes), poor flowering and seed sets are the major problems in ginger propagation. Tissue culture is the best option to overcome these problems, since it is propagated under aseptic area to reduce disease transmission. Therefore, this study was initiated to optimize protocol for micropropagation of ginger accession 52/86 under in vitro conditions. Murashige and Skoog (MS) basal medium was used in this study. For culture initiation, sterilized sprouted rhizome bud was used and data were collected on number of initiated shoots. In vitro shoot multiplication, five different concentrations of 6-benzylamino-purin (BAP) and four concentrations of kinetin (KN) in a 5 x 4 factorial combination treated with three replications and five explants per jar were used in completely randomized design (CRD). Data were collected on number of shoot per ex-plant, shoot length and number of leaves per shoot. In rooting experiment, five different concentrations of α-Naphthalene acetic acid (NAA) and four concentrations of Indole-3-butyric acid in a 5 x 4 factorial combination treated with three replications and five explants per jar were used in CRD. Data were collected on root number per shoot and root length. Acclimatization was done on six different soil media combined as treatments with 10 plantlets per pot with three replications in greenhouse using CRD. Data were collected on numbers of survived plantlets in greenhouse from each soil media. The results showed that from well sterilized sprouted rhizome buds up to 91.3 % initiated shoots were achieved. On shoot multiplication, maximum shoots per ex-plant (9.34±0.55) were obtained on a medium containing 2.5 mg/L BAP + 1.0 mg/L KN. The largest shoot length (5.10 ± 0.17 cm) was obtained on MS medium containing 2.5 mg/L BAP + 0.5 mg/L KN growth regulators. The highest mean number of leaves per shoot (5.67± 0.06) was obtained on a medium containing 3.0 mg/L BAP+0.5 mg/L KN. The highest mean number of root per shoot (12.60±0.17) was achieved on a medium supplemented with 2.5 mg/L NAA + 1.0 mg/L IBA and largest root length (6.30±0.10 cm) was achieved on a medium containing 1.0 mg/L NAA + 1.0 mg/L IBA hormones. Among soil combined for the plantlets acclimatization, the highest survival percentage (98 %) was recorded on top soil (TS): sandy soil (SS): coffee husk (CH) (1:1:1). Thus, MS + 2.5 mg/L BAP + 1.0 mg/L KN and half MS+ 2.5 mg/L NAA + 1.0 mg/L IBA were the best hormone combination for shoot multiplication and in vitro rooting, respectively. At greenhouse, media combination of TS: SS: CH (1:1:1) ratio was found to be optimal for plantlets acclimatization. Therefore, the optimized protocol can be used for rapid multiplication of the clean planting materials for ginger accession 52/86.

Keywords: *Acclimatization, Aseptic, Ginger, Sprouted Rhizome Bud, Tissue Culture*

1. INTRODUCTION

Ginger belongs to the family *Zingiberaceae,* in the natural order Scitamineae *Zingiberales* (Cronquist and Takhtadzhian, 1981). It is native to Southeast Asia, which is originated from India, where it was introduced to Africa and Caribbean countries (Purseglove, 1972; Sathyagowri and Seran, 2011; Wubshet, 2018). The widely reported chromosome number of ginger is $2n = 2x = 22$ and the basic number of the genus is suggested as $x = 11$ (Morinaga *et al*., 1929; Ramakrishnan, 2016).

Ginger is a high value horticultural crop that has been used for centuries as spice, flavoring agent, food additive in the preparation of meals and medicinal use throughout world history, especially in Chinese, Indian and Japanese medicinal care (Bartley and Jacobs, 2000). It has been credited with a multitude of phytotherapeutical activities like curing muscular aches and pains, congestion, coughs and sinusitis as well as exhibiting anti-inflammatory, antitumor, ant-diabetic and anticancer effects (Ravindran and Babu, 2005). The characteristic components of ginger include essential oil and oleoresin, which are responsible for its fragrant and pungent behavior, respectively (An *et al*., 2016). Its' rhizomes are commonly used in foods and beverages for their characteristic pungency and piquant flavor (Srinivasan, 2017). It is used in numerous forms, including fresh, dried, pickled, preserved, crystallized, candied and powdered or grounded. The powdered rhizome contains 3-6 % fatty oil, 9 % protein, 60-70 % carbohydrates, 3-8 % crude fiber, about 8 % ash, 9-12 % water and 2-3 % volatile oil (Zadeh and Kor, 2014; Mekuriya and Mekibib, 2018).

The main ginger growing countries in the world are: India, Nigeria, China, Indonesia, Brazil, Costa Rica, Ghana, Japan, Malaysia, Bangladesh, Philippines, Thailand, Trinidad and Tobago, Uganda, Hawaii, Guatemala and many Pacific Ocean islands (Ezra *et al*.,2017; Wubshet, 2018). Cultivation of ginger in Ethiopia was started during 13th century when Arabs introduced it from India to East Africa in Ethiopia (Jansen, 1981; Girma *et al*., 2008) and second most widely cultivated spice next to chilies (Ayenew *et al*., 2012). There are more than 45 ginger accessions and varieties collected in collaboration with the expertise from Ethiopian Institute of Biodiversity and Conservation (IBC) and introduced are being maintained in research plots at Tepi

Agricultural Research Center (Girma *et al*., 2008; Zakir *et al*., 2018). Its production was mostly limited in the wetter regions of Southern Nation's Nationalities and People's (SNNP) regional states, but currently, in addition to the southern part of the country, it is widely cultivated in Amhara, Oromia, Gambella and BenishangulGumuz regional states (Wubsh *et al*., 2018; Zakir *et al.,* 2018).

In Ethiopia, production and productivity of ginger in the ginger production areas are constrained by lots of problems. These are low quality varieties, shortage of planting materials, lack of awareness to determine the optimum seed rhizome size or seed sets and post-harvest deterioration due to fungal invasion (Zakir *et al*., 2018). The reproducing part (rhizome) is also the economically used part of the ginger plant, which restricts the availability of ginger seeds (Abbas *et al.*, 2011; Mekuria, 2015). Habetewold *et al*. (2015) studied that, ginger bacterial wilt caused by *Ralstonia solanacearu* is becoming the threat to ginger production and result 80-100% incidence in the area where it produced. The risk of disease transmission through division by sectioning of the rhizomes has hampered propagation by conventional means. Conventionally ginger is always propagated by portion of the rhizomes known as seed pieces or seed sets. Seed sets size especially, in rhizome crops such as ginger and turmeric, is a critical factor that can affect the growth and yield of the plant. Girma and Kinde (2008) recommended that, using 2.5 to 5 cm seed size at least with one bud and seed rate of 2.5 tons of fresh ginger rhizomes per hectare for optimum production in Ethiopia. This is so bulky and difficult to obtain when one require establishing a new farm or expanding its area of cultivation. The use of *in vitro* propagation techniques becomes imperative to alleviate the shortages of planting materials and to producing the disease free planting materials. Therefore, *in vitro* technique is considered the best alternatives method that may supply a large number of planting materials for commercial planting and further studies (Hamirah *et al.,* 2010).

Since micro propagation and other tissue culture techniques are most efficient to produce the disease free planting materials, it will be the best option to produce disease free planting material of ginger also. High speed mass production and distribution of the planting materials will increase the production and productivity of ginger. Earlier, Hosoki and Sagawa (1977) were the first to report their success in the production of an average of 6 shoots per bud from *in vitro* gingers culture. From that time onwards, several workers had succeeded in their *in vitro* culture

of ginger following the organogenesis pathway, indirect somatic embryogenesis pathway, successfully produced disease free ginger micro rhizomes *in vitro*, have also used successfully the *in vitro* technique for conservation of germplasm (Ravindran and Babu, 2016). In Ethiopia protocol optimization for ginger cultivars, Yali (180/73) and Boziab (37/79) have been done by Ayenew*et al*. (2012) and according to this study averages of 7.3 shoots per explants in Yali and 6.67 in Boziab successfully obtained by treating 2.0 mg/L BA with combination of 1.0 mg/L kinetin on MS medium. Recently, Mengs (2018) reported that the procedures of explants surface sterilization for both Yali and Boziab cultivars in Ethiopia.

So far there is no report available on an efficient and reproducible protocol that can enable the *in vitro* rapid multiplication of Ethiopian accession 52/86 of ginger from sprouted rhizomes bud explants. Even thought, few works have been reported on ginger tissue culture, there are limited information on acclimatization condition, using sprouted rhizome buds only as explants for micro propagation in ginger. In addition, no protocol was developed for ginger accession 52/86 in Ethiopia. Therefore, this study was initiated to optimize protocol for *in vitro* propagation of Ethiopian ginger accession 52/86 using sprouted rhizome buds explants.

General objective

To optimize *in vitro* protocol for micropropagation of ginger accession 52/86 from sprouted rhizome buds.

Specific objectives

- To determine the optimum concentration of BAP and KIN combination for shoot multiplications
- To determine the optimum concentration of NAA and IBA combination for rooting and
- To identify the best combination of soil/media mix for acclimatization of ginger plantlets in greenhouse.

2. LITERATURE REVIEW

2.1. Description of Ginger

Ginger belongs to the family *Zingiberaceae* and found in the natural order Scitamineae *Zingiberales* (Cronquist and Takhtadzhi͡an, 1981). The genus Zingiber of the family *Zingiberaceae* is distributed in tropical and subtropical Asia and Far East Asia and consists of about 150 species (Park and Rizzuto, 2002). It was introduced to many parts of the globe and has been cultivated for thousands of years as a spice and for medicinal purposes (Park and Rizzuto, 2002). Ginger is native to Southeast Asia, which is originated from India, where it was introduced to Africa and Caribbean countries. However, no definite information on the primary center of domestication of ginger is available (Prabhakaran, 2013). The widely reported chromosome number of ginger is $2n = 2x = 22$ and the basic number of the genus is suggested as $x = 11$ (Morinaga *et al*., 1929; Ramakrishnan, 2016). According to Das *et al. (*2017) most cultivated varieties of gingers are infertile and vary in karyotype.

The main ginger growing countries in the world are: India, China, Jamaica, Taiwan, Sierra Leone, Nigeria, Fiji, Mauritius, Indonesia, Brazil, Costa Rica, Ghana, Japan, Malaysia, Bangladesh, Philippines, Sri Lanka, Solomon Islands, Thailand, Trinidad and Tobago, Uganda, Hawaii, Guatemala and many Pacific Ocean islands (Ezra *et al*.,2017; Wubshet, 2018).

In Ethiopia, ginger is cultivated in Amahra (Bahir Dar, Dejen, DebereMarkos, Kola Dega Damot, Metekel and AgewMider), Oromia (Ilubabuor, Jimma, Bale and Wollega), SNNP (Wolaita, Kembata-Tembaro, Kaffa, GamoGofa, Galeb and HamerBako, Gofa and KuloKonta, Sidama and Arero), Benishangul Gumuz and Gambella regions. The production has been expanding to most parts of the country and is grown under varied climatic conditions where there is no frost problem (Girma *et al*., 2008).

2.2. Uses of Ginger

2.2.1. Medicinal value of ginger

Ginger is one of the well-known medicinal herbs that are used both by traditional healers and in some modern treatment modalities. Research outputs suggest that the nutraceutical compounds of ginger can have values as a complementary treatment for several forms of cancer and ailments of both human and animals. Nutraceutical compounds usually claimed to have medicinal value includes gingerols, shogaol, gingerdiols, Ingenol, paradols and zingerone. Of which, gingerols are thought to be the most pharmacologically active components. Owing to its different active ingredients, ginger is considered as a safe medicinal plant with only few and insignificant adverse effects (Toader, 2014; Mekuriya and Mekibib, 2018). Therefore, ginger is a strong anti-oxidant substance and may either mitigate or prevent generation of free radicals. Figure 1 summarizes the pharmacological values of ginger.

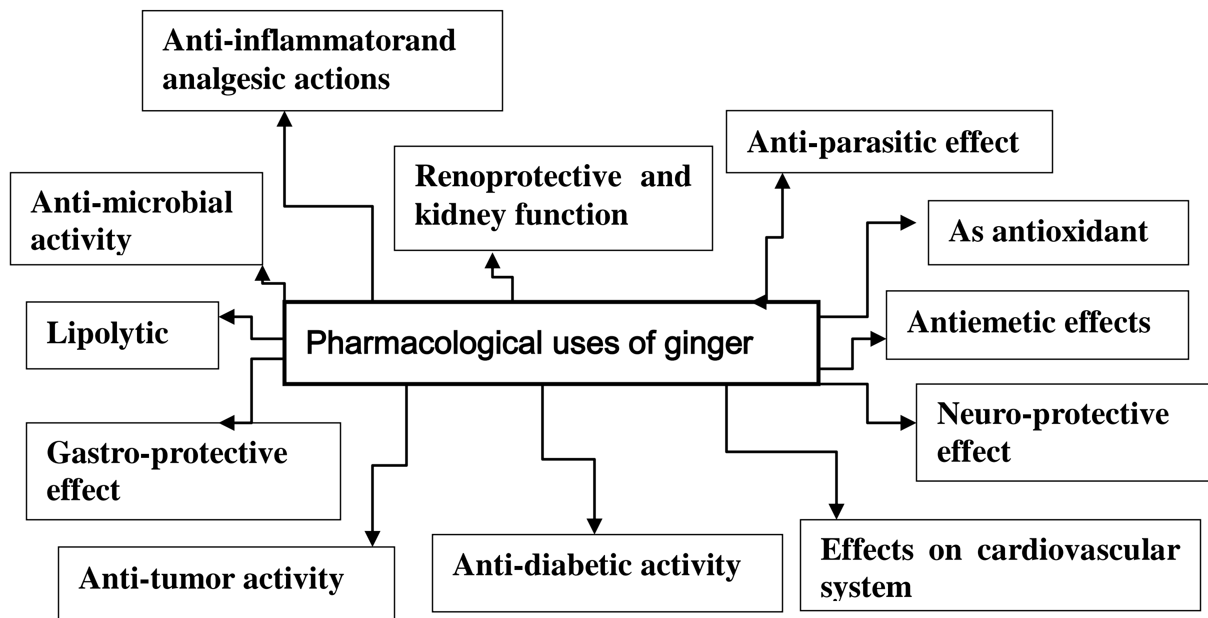

Figure 1. Pharmacological activities of ginger

2.2.2. Nutritional values of ginger

Ginger is used in numerous forms including; fresh, dried, pickled, preserved, crystallized, candied and powdered or grounded. The flavor is somewhat peppery and slightly sweet, with a strong and spicy aroma. Foods that contain ginger include ginger bread, cookies, ginger snaps, ginger ale and a wide variety of savory recipes. Ginger provides a variety of vitamins and minerals (Mishra, 2009; Wagesho and Chandravanshi, 2015). According to the USDA National Nutrient Database: in 100 grams (g) of fresh ginger rhizomes, there are: 79 calories, 17.86 g of carbohydrate, 3.6 g of dietary fiber, 3.57 g of protein, 14 mg of sodium, 1.15 g of iron, 7.7 mg of vitamin C and 33 mg of potassium are found. Other nutrients found in ginger are: vitamin B6, magnesium, phosphorus, zinc, riboflavin and niacin. Fresh or dried ginger can be used to flavor foods and drinks without adding unnecessary salt or sugar (Bode and Dong, 2011).

2.3. Plant Tissue Culture

Plant biotechnology has become increasingly important at the global level, as it offers opportunities to increase sustainability, profitability and international competitiveness in agriculture and forestry. One of the most commercially exploited components of plant biotechnology has been the rapid clonal multiplication/micro propagation of selected genotypes in diverse groups of plant species through plant tissue culture (Rani and Raina, 2000).

Plant tissue culture refers to the set of techniques designed for the growth and multiplication of cells, tissues and organs using nutrient solutions in an aseptic and controlled environment by isolating them from the mother plant, this is because cells are totipotent in nature (Loyola-Vargas and Vázques-Flota, 2006). Other practical application of biotechnology includes; somatic embryogenesis, production of virus free plantlets, embryo rescue, and production of haploid plants, gene transfer and production of transgenic plants (Rao, 1996; Razdon, 2005).

Use of micro propagation has many advantages over conventional methods of plant propagation of which the major once are: (1) it is possible to generate pathogen-free plants, even from explants of infected mother plants; (2) it is important in terms of multiplying plants throughout the year, with control over most facets of production; (3) it is possible to produce haploid organisms; (4) it can be used for further studies in genetic transformation (5) it enables the

production of a large number of plants in a short time from a selected number of genotypes, where the traditional methods of multiplication are either not available or are ineffective in large scale multiplication systems (Garton and Mosses, 1985; Rani *et al.*, 1995; Ravindran and Babu, 2016).

2.4. Ginger Tissue Culture

Ginger is no exception, more so because the conventional breeding programs are hampered due to lack of fertility and natural seed set. Rhizome rot caused by *Pythium*spp. and bacterial wilt caused by *Ralstonia solanacearum* are the major diseases affecting ginger production and spread primarily through infected rhizomes. These problems can be overcome using tissue culture. In ginger, micro-propagation studies were carried out by many researchers using different explants and media compositions; Table 1 summarizes some of the earlier tissue culture studies conducted in ginger.

Table 1. Earlier works done on ginger tissue culture

Trial	Types of tissue culture	Sources of explants	References
1	Organ culture	buds of ginger	Sharma and Singh., 1997
2	Somatic embryogenesis	Young leaf segments	Kackar*et al.,* 1993
3	Organogenesis	Immature inflorescences	Babu*et al.,* 1992
4	protoplast culture	leaf mesophyll tissue	Geetha*et al*., 2000
5	Microrhizome production	Rhizome	Zheng*et al*., 2008
6	Organ culture	auxiliary buds and shoot tips	Ayenew*et al*., 2012
7	Rhizome buds cultured	rhizomes with buds	Sathyagowri and Seran, 2011
8	Germplasm preservation	adventitious shoot primordia	Dekkers*et al*., 1991
9	Micropropagation ginger	Root	Nel, 1985
10	Micropropagation ginger	shoot meristems	Hosoki and Sagawa, 1977

Ginger seed production also suggested by Uozumi *et al.*, (1994) by *in vitro* propagation methods, however this method was not efficient, but expensive and did not meet ginger germplasm conservation requirements. In all, vegetative bud is the most commonly used ex-plant in ginger tissue culture.

Contamination by microbes has been a persistent problem for *in vitro* propagation of ginger and major problems facing plant tissue culture. A serious limitation in ginger micro-propagation is

oxidation of poly-phenols exuded from cut surfaces of explants and their release into the culture media causing browning and necrosis of explants (Mengs, 2018). Sprout bud which are considered as etiolated tissue, since they are raised from rhizomes of ginger, should show less browning than rhizomes. Wounded surface in sprout buds emit higher exudates than rhizomes stem, as the cut surface of explants exude poly-phenols that are easily oxidized and cause explants necrosis and medium darkening (Abdelwahd *et al*., 2008). The phenol of excised plant tissues and nutrient media occurs frequently and remains a major basis for recalcitrance *in vitro*. The severity of browning has varied according to species, tissue or organ, developmental phase of plant, age of tissue or organ, nutrient medium and other tissue culture variables (Huang *et al*., 2002).

The effect of phenol oxidation can be minimized by applying antioxidants like activated charcoal, poly vinyl pyrrolidone (PVP), ascorbic acid, citric acid and L-cystine in tissue culture media. Phenol oxidation enzymes could be influenced by environmental factors such as light and high temperature raise browning rate by increasing the enzyme activity (Dobránszki and Teixeira, 2010).

2.5. Basal Media Composition for Tissue Culture

The appropriate components of artificial nutrients media and appropriate concentration and combination are deciding for the successful of ginger *in vitro* culture in addition to plant growth regulators.

2.5.1. Medium composition for ginger tissue culture

The composition of the media is a determining factor for growth (Gamborg*et al*., 1976). Plant tissue culture media should generally contain some or all of the following components: macronutrients, micronutrients, vitamins, amino acids or nitrogen supplements, source(s) of carbon, undefined organic supplements, growth regulators and solidifying agents. It should be considered that the optimum concentration of each nutrient for achieving maximum growth rates varies among species. Regeneration capacity of plants shows a wide range among families, species and even within genotypes from the same species. The growth medium is selected for the

purpose of tissue culture and for the plant species (Fossard, 1976; Pierik, 1997; Leva and Rinaldi, 2012).

Most of scientists have done ginger *in vitro* micro propagation by using Murashige and Skoog's (MS) basal media composition. Villamor (2010) recommended that, for rapid multiplication rate of planting materials of ginger, the MS basal nutrient media with KNO3, as source of nitrogen should be used. Kambaska and Santilata (2009) studied that *in vitro*shoot formation in rhizome sprouting of gingers (cv- Suprava and Suruchi) cultured on semisolid MS medium. Nkere and Mbanaso(2010) used MS (Murashige and Skoog, 1962) basal culture medium containing 3% sucrose and supplemented with 100 mg/L myo-inositol and 100 mg/L ascorbic acid in order to optimizing concentrations of growth regulators for *in vitro* ginger propagation. Mohamed *et al.* (2011) was used both MS basal media and B_5 for ginger *in vitro* micro propagation to shoot multiplication and to rooting respectively. From these a few evidence there is a conclusion of media composition for ginger *in vitro* micro propagation contain all, macronutrients, micronutrients, vitamins, amino acids nitrogen supplements, 30 g/L sucrose as a source(s) of carbon, undefined organic supplements, growth regulators, 7-8 g/L of agar as a solidifying agent and the pH meters adjusted 5.70- 5.80 was a basal media of MS that used for *in vitro* ginger tissue culture and mass propagation almost. Therefore, MS medium was found to be best for micro-propagation of ginger and mainly used cytokinins were BAP, Kinetin, TDZ and auxins were, NAA, IAA and IBA.

2.5.2. Growth conditions for ginger tissue culture

In vitro conditions are the conditions that facilitate the growth of ginger under controlled and growth room in tissue culture areas. These are average temperature from 25 ± 2 ^{O}C, relative humidity range 60-70% and intensity of light a 16 hours photoperiod from cool white 40 W florescent bulbs supplying 2000 to 3000 lux. Available of growth shelf and growth chamber are major facilities at plant tissue culture room which are similarly required for ginger tissue culture. These concepts was supported by Sera (2013) review culture establishments condition for ginger tissue culture and Ayenew*et al.* (2012) was used these growth conditions for culture establishments of Boziab and Yali ginger cultivars in Ethiopia.

2.6. Plant Growth Regulators for Tissue Culture

The developmental pathway of plant cells and tissues in culture medium is determined by the type and composition of plant growth regulators (PGRs). Plant growth regulators (PGRs) can be defined as naturally occurring or synthetic compounds that affect developmental or metabolic processes in higher plants, mostly at low dosages. They do not possess a nutritive value and typically, are not phytotoxic (Rademacher, 2015). They are generally classified into the following groups; auxins, cytokinins, gibberellins, abscisic acid and ethylene. The hormone type and concentration mainly depend on the genotype of plants and the type of explants used for culture. Moreover, proportion of auxins to cytokinins determines the type and extent of organogenesis in plant cell cultures (Skoog and Miller, 1957).

From different types of plant growth hormones cytokines and auxin are the most used in plant tissue culture and most of the scientists were recommend these. The same is true for ginger tissue culture most of the scientists were done the *in vitro* propagation of ginger by using different concentration and combination as well as alone of cytokines and auxin levels of ratios. Studies on *in vitro* propagation of ginger directly from rhizome sprouted buds with best culture media was reviewed and deeply stated by Seran (2013). According to Zuraida *et al.* (2016) MS medium supplemented with BAP concentrations between 3.0-5.0 mg/L provided best results *in vitro* propagation of gingers. BAP at highest dosage is more stimulatory to shoot growth than the lowest dosage in the culture medium (Zuraida *et al*., 2011). Though, BAP and kinetin are commonly used cytokinins on micro propagation, TDZ has also been tested for shoot proliferation and shoot elongation. However, TDZ inhibits shoot proliferation especially in solid medium (Huetteman and Preece, 1993; Amutha *et al*., 2006). These all BAP, kinetin and TDZ are types of cytokinins that were scientists used for *in vitro* micro propagation for gingers tissue culture frequently. Auxins such as NAA, IBA and the IAA are the hormones commonly used for gingers *in vitro* propagation especially in combination with each other to studies rooting effects and root length as well as they support for shoot multiplication within combination of cytokinins stated above.

2.7. Stage of Ginger Tissue Culture

There are five stage of ginger tissue culture which was adopted from the concept of plant tissue culture stage. Under each stage, maximum caution must take place since plant tissue culture is too tedious and need technically skilled human power. In additions, care must be careful taken in selection of mother plants, the source of explant, type of explant, developmental stage and size of explants, sterilization of explants, plant growth regulators, properly combined composition of medium and a well maintained culture conditions for successful application of *in vitro* technology (Seran, 2013).

2.7.1. Healthy mother plant selection

Mother plant is the source of explants to be used for further multiplication. The genotypes may have been selected deliberately from a ginger that has demonstrated desirable traits (good yield, disease resistance, oil contents, rhizome size and nutrient compositions) and propagated as explants taken from that mother ginger (Robinson, 2006; Srinivasan, 2017). In order to get suitable explants at a time, appropriate establishment of mother plant is the priority consideration for every successful mass propagation program. To reduce the level of contamination, rhizome explants could be obtained from ginger plants grown under glasshouse conditions or grown in dark room on sterilized cotton in the laboratory from contamination. Explants from healthy, vigorous mother plants are more suitable to successful initiate *in vitro* cultures.

Explant size is the critical factor next to and formulation of the culture medium which determines the success and efficiency of *in vitro* regeneration. Accordingly, the best size of explants fall between 3-2 cm which finally trimmed to appropriate size (1 cm) for inoculation to the initiation medium (Setargie *et al*., 2015). The size of rhizome explants has an influence on shoot bud initiation and morphogenic response under *in vitro* conditions. Shoot tips of 0.2-0.9 mm long is optimum for *in vitro* propagation (Huang, 1995). This is in agreement with the findings of Sathyagowri and Seran (2011) that the best size of the rhizome buds is 0.5 cm long for *in vitro* culture initiation and shoot multiplication of ginger among the different sizes (0.5, 1.0 and 2 cm long) of explants tested. Ayenew *et al*. (2012) was used Axillary bud and shoot tips of

about 15 mm length for both Boziab and Yali ginger cultivars in vitro micro propagation in Ethiopia.

Explants source: explants can be taken from different parts of a plant such as shoots, leaves, stems, flowers, roots and from many types of mature cells provided they are able to de-differentiate into totipotent cells. The explants taken from active shoot tips and auxiliary buds are mostly used on micro propagation of plant species (Xiao *et al.*, 1997; Seran, 2013). Physiologically younger tissue is more responsive *in vitro*, usually the newest formed and is easier to surface disinfect and establish clean cultures.

Explants types: meristem, auxiliary buds, shoot tips and aerial pseudo stems are the different types of explants used in micro propagation of ginger and other related species. Seran (2013) reviewed that as several scientists able to produce plantlets easily using sprouting bud explants of ginger and turmeric rhizomes are suitable materials for vegetative propagation under *in vitro* conditions as compared with dormant buds which have to force for initiation. There are also different factors affecting explants tissue culture response are in addition to the mentioned above, such as genotype, physiological stage of donor plant, explants age, explants position in donor plant and explants density (Yildiz, 2012).

Aseptic conditions: There are several possible sources of contamination of the medium; these are the culture vessel, the medium itself, the explants, the environment of the transfer area, instruments used to handle plant material during culturing and subculture, the environment of the culture room and the operator. Living plant materials from the environment are naturally contaminated on their surfaces and sometimes interiors with microorganisms. The most important treatment prior to culture initiation is perhaps surface-sterilization of these sources of contaminations as well as explants and using aseptic tissues as source of explants is highly recommended (Yildiz *et al.,* 1997). Surface sterilization of starting materials (explants) in chemical solutions (disinfectants) such as ethanol, sodium hypochlorite (NaOCl), and Tween-20 for wetting agent of NaOCl is required (Aishwarya and Robinson, 2013) to control the growth of fungi and bacteria on the growth media (Singh *et al.*, 2011). Most tissue culture procedures are conducted in sterile operations, such as laminar flow cabinet. Besides the special design of gentle flow of sterile air in cabinet, aseptic cabinet is also equipped with germicidal lamp emitting ultra

violet light. This type of radiation is useful in eliminating air borne contaminants and for surface disinfection. Glassware and all the tools used for tissue culture process canal so cause contamination. It is extremely necessary to autoclave all the material before using it, so that all the microbial contaminants are destroyed (Reed and Tanprasert, 1995).

Sterilization agents: - are the chemicals that are toxic to microorganisms but non-toxic to plant materials. Surface-sterilization process aims to eliminate all microorganisms that can easily grow under *in vitro* conditions; on the other hand, it should guarantee the explants viability and regeneration capacity. A wide range of sterilization agents are ethanol, hydrogen peroxide, bromine water, mercuric chloride, silver nitrate, antibiotics and sodium hypochlorite (NaOCl).The type, concentration and exposure time of disinfectants depend on the tissue type, age and the nature of the explants used for micro propagation (Seran, 2013; Wee *et al.*, 2015). Increasing the exposure time and sterilant concentration had reduced the contamination rate but high number of damage explants resulted.

Sprouted rhizome buds of gingers sterilization: different surface sterilizing agents were used at different concentrations and duration to determine the most efficient procedure for initiation of tissue culture of gingerusing rhizome bud as explants. The use of Mercuric (II) chloride ($HgCl_2$), Sodium hypochlorite (NaOCl), Hydrogen peroxide (H_2O_2) and Bavistin for surface sterilization of sprouted rhizome buds of ginger was studied by Khatun *et al.* (2016). According to this report using Mercuric (II) chloride treatments (0.1 %) for 15 minutes, sodium hypochloride (3 %) for 10 minutes, hydrogen peroxide (5 %) for 10 min and Bavistin (3 %) for 10 minutes were an effective treatment to remove microbes from sprouted rhizome buds of ginger. Sodium hypochlorite has been reported to be very effective against different types of bacterial strains; even micro molar concentrations are enough to significantly reduce bacterial populations (Nakagawara *et al.*, 1998). Recently Mengs (2018) reported using 3 % (w/v) copper sulphate ($CuSO_4$), 0.1 % (w/v) PVP, 0.1 % (w/v) ascorbic acid and 0.1 % (w/v) citric acid for 6 hrs were the best eliminating contamination from sprouted rhizome buds of Ethiopian ginger Boziab and Yali varieties. Ayenew *et al.* (2012) used 70 % ethanol for 1 minute and 30 % (v/v) sodium hypochlorite (5 % active chlorine concentration) local bleach (Berekina) for 15 minutes under aseptic condition and achieved 85 % clean initiated explants from both Boziab and Yali Ethiopian ginger cultivars.

In all there were procedures of sprouted rhizomes buds surface sterilization following these steps. The sprouted rhizome buds were collected in conical flask and kept under running tap water. Wash these explants by running tap water twice to remove soils and another contamination. Wash explants with sterilized water by adding liquid soap plus 2-3 drops of Tween-20 for 25 minutes. Antifungal treatment with Curzate® weight powder 0.25 % or Mecoziab 6 g/L for 20 minutes and washing the explants 2-5 by sterilized water to remove the chemical traces. To remove phenols and bacterial contamination using the copper sulfate 30 g/L for 15 minutes to one hr and washing the explants by sterilized water 3-5 to remove the traces of copper sulfate. Explants surface sterilization by immersing 70 % ethanol for 30 seconds to 2 minutes in laminar air flow cabinet and wash the explants by sterilized water 3-5 to remove the traces of ethanol. Then immersing the explants in the concentration of the sodium hypochlorite of 30 % for 15-20 minutes and to increase efficient of Na_2OCl, add 2-3 a drop of tween-20 per 200 ml solution as wetting agents and wash the explants to remove the traces 3-5 by sterilized water for 5 to 10 minutes and kept in water to avoid desiccation until inoculate. Finally sterilized explants were trimmed suitably to remove sterilizing agent affected parts/brown parts and inoculate on the appropriate medium in laminar air flow cabinet (Ayenew *et al.*, 2012; Wee *et al.*, 2015; Mengs, 2018).

2.7.2. Ginger shoot initiation

The objective this stage is to remove particulate from the tissue and sterilization of explants surface and transferred into nutrient medium. In the shoot proliferation stage, it consists of the establishment *in vitro* of suitable pieces of tissue, free from obvious contamination, and one of most crucial stage of micro propagation method which is recognized by the formation, growth and proliferation of adventitious shoots from the primary explants (George *et al.*, 2008).

If rhizome explants of ginger are less active or dormant it may require different levels of hormones during initial culture establishment to induce sprouting of buds for shoot formation. There were different report about ginger shoot initiation through tissue culture and days of initiations. Sathyagowri and Seran (2011) reported that the minimum days (7.67 days) to first bud initiation was taken. Zuraida *et al.* (2016) reported that based on the explants size days of initiation and capacities of survival of explants varied. According to this report, explants of 0.5 to

one cm took longer days for first bud appearance and scored highest percentage of survival after 45 days where as explants of 2 to 4 cm and over 4 cm took lesser days in the range of 31 to 35 days for first bud appearance and their survival after 45 days significantly decreased to the range of 25 to 35 days. This indicating that the days needed to first bud initiation were increased as size of explants decrease. Ayenew *et al.* (2012) reported 70 % contamination free initiated shoots from both Boziab and Yali Ethiopian ginger varieties after 6 weeks of inoculation by using different sterilizer agents.

2.7.3. Ginger shoot multiplications

The effect of both auxin and cytokinins plant growth regulators hormone concentration and combination alones and together support on ginger *in vitro* shoots multiplication parameters were reported by different authors. Pavallekoodi and Sreeramanan (2016) report that 3.0 mg/L and 5.0 mg/L BAP produced the highest number of micro shoots of ginger var. rubrum(7±1.34 and 6±0.41), respectively. The concentration of 3.0 mg/L kinetin produced multiple micro shoots 4±0.88 and they decided that these concentrations as optimum for shoot multiplication of ginger var. rubrum. The optimum concentration and combination of NAA and BAP for multiple shoot regeneration was obtained from shoot tip explants of ginger on MS medium supplemented with 1.0 mg/L NAA + 3.0 mg/L BAP according to Bhojwani and Dantu (2013) report. Kasilingam *et al*. (2018) reviewed that increasing the concentrations of benzyl-amino-purine from 0 to a maximum level of 3.0 mg/L responsible for high shoot multiplication. Sathyagowri and Seran (2011) reported that an optimum concentration of 5.0 mg/L BAP with 0.5 mg/L NAA for shoot multiplication with average numbers of shoots 5.33 per explant. Das *et al.* (2013) produced high number of shoot per explant *in vitro* culture both *Zingiber moran* and *Zingiber zerumbet* on medium holds MS + 2.0 mg/L BAP +2.0 mg/L KN. Mohamed *et al* .(2011) reported that shoot lets multiplication of ginger after 4 weeks of cultivation with supplied 4.5 mg/L BAP number of shoot per ex-plant 8.00, number of leaves per shoot 15.50 and shoot length 4.10cm on MS medium. The highest number (5.33) of shoots produces on MS with 5.0 mg/L BA and 0.5 mg/L NAA after 5 weeks of culture and this medium gives good response for shoot proliferation. According to Zuraida *et al.* (2016), MS medium supplemented with 3.0 mg/L BAP and 0.5 mg/L NAA produced the highest number of shoots while 0-0.5 mg/L BAP enhanced shoot length. However, shoot multiplication decreases with increasing dosage of BAP from 6.0 mg/L to 8.0

mg/L in ginger (Sathyagowri and Seran, 2011). In Ethiopia, protocol optimization both for ginger cultivars Yali (180/73) and Boziab (37/79) have been done by Ayenew *et al*. (2012). According to this report the best combination cytokinin for shoot multiplication of the two ginger cultivars was 2.0 mg/L BA combined with 1.0mg/L kinetin with average of 7.3 shoots per explants in Yali and 6.67 in Boziab successfully obtained. TDZ at 0.5 mg/L induces higher number (8) of shoots among the cytokinins even at lower concentration (Amutha *et al*., 2006; Hamirah *et al*., 2007).

2.7.4. Ginger rooting on nutrient media

Induction of roots in excised shoots and subsequent survival of plantlets in the soil are the prior to acclimation in which, individual shoot or shoots in clumps are transferred to a nutrient medium supplemented with auxins and ingredients that do not encourage further shoot proliferation and which promote rooting (George *et al*., 2008). The role of auxins in root development is well established and has been reviewed by Scott (1972). Whilst root induction of any plant tissue is dependable on the proper amount and combination of rooting hormones (Doods and Roberts, 1985), there is considerable evidence that other factors, including carbohydrate supply, are important in determining ultimate success (Thompson and Thorpe, 1987).

Rooting of micro-rhizome is generally spontaneous in ginger but auxins either NAA or IBA or IAA may be used for root formation. High concentration of NAA and low to moderate concentrations of IAA increased of root length but high concentration of IAA depressed of root length. Abbas *et al*. (2011) reported that 13.5 maximum number of roots per shoot of ginger on the MS basal medium with supplying 1mg/L of NAA and 6.37 cm length of the roots while one mg/L of IAA gives 3.67 number of root per shoot and 1.97 cm roots length which was optimum for ginger in vitro rooting according to this report. This indicating that the NAA is more prefer for ginger *in vitro* rooting than the IAA hormones. David *et al.* (2016) studied the effect of NAA on ginger rooting by treating 2.0 mg/L induced up to 34.40 roots per ex-plant with an average length of 4.52 ± 0.20 cm after 10 weeks of culture. Idris *et al*. (2015) was reported 28.63 number of roots per ex-plant by treating 1.6 mg/L NAA on MS basal media containing and 5.85 in cm root length on 0.8 mg/L NAA. Ayenew *et al*. (2012) reported that the optimum auxins for root

formation was one mg/L NAA alone resulted in 8.75 roots per shoot with 2.95 cm length on average in gingers cultivars Yali (180/73) and Boziab (37/79).

2.8. Acclimatization

Acclimatization of micro propagated plants to a greenhouse or field environment is essential because there is a significant difference between the micro propagated environment and the greenhouse or field environment. Acclimatization is defined as the climatic or environmental adaptation of an organism, especially a plant, to a new environment (Conover and Poole, 1984). Acclimatization is the process in which an individual organism adjusts to a change in its environment (such as a change in altitude, temperature, humidity, photoperiod, nutrients medium or pH), allowing it to maintain performance across a range of environmental conditions. A substantial number of micro propagated plants do not survive transfer from in vitro conditions to greenhouse or field environments due to the delicate nature of plants raised in vitro. Compared to *in vitro* conditions, the *ex-situ* atmosphere causes stress to the micro propagated plants which has substantially lower relative humidity, higher light, and septic environment. Various factors such as development of epicuticular wax, functional stomata, root system, and increased photosynthetic ability influence the acclimatization. The survival of *in vitro* regenerated plants to *ex vitro* conditions depends on the conditions during transfer of *in vitro* plantlets and soil substrates. Most plant species grown in vitro require a gradual acclimatization and hardening for survival and growth in a natural environment (Preece and Sutter, 1991; George, 1996).

Nirmal (1997) reported that ginger plantlets micro propagated as well as callus regenerated plants were hardened and transplanted in a porous soil mixture of vermiculite, sand, and garden soil in equal proportions with 57 to 85 percent success depending upon the origin of explants. Preece and Sutter (1991) who reported that, plantlets growing under *in vitro* conditions exhibit no or reduced photosynthetic capacity and during acclimatization there is a need for rapid transition from the heterotrophic to the photoautotrophic state for survival. High humidity was maintained for the initial 20 to 30 days by keeping them in a humid chamber. Kambaska and Santilata (2009) observed ginger plantlets after three weeks were transplanted to earthen pots containing mixture of soil + sand + manure in 1:1:1 ratio 95 % was survived. Ayenew *et al.* (2012) observed good survival rate of *in vitro* grown ginger plantlets at 85 % for Boziab and 82

% for Yali cultivars on mixed of soil, sandy soil and well decomposed coffee husk (1:1:1) ratio in green house. David *et al.* (2016) reported plantlets of ginger Tambunan' that planted in poly-bags containing sand and clay at the ratio of 1:4 found to grow healthily with 64 % of survival rate.

3. MATERIALS AND METHODS

3.1. Planting Materials

Ginger accession 52/86, a promising and potential line for release, was used for the study. The rhizomes were obtained from JARC. The accession was chosen because it's potential for high yield, content of oil and stress tolerance (unpublished progress report JARC, 2018).

3.2. Experimental Procedures

3.2.1. Preparation of MS stock solutions

The Murashige and Skoog (1962) nutrient medium was used in this study. The stock solutions of macro-nutrients, micro-nutrients, vitamins, iron EDTA and calcium hypo chloride ($NaOCl_2$) were prepared by dissolving the required quantity of chemicals in double distilled water separately (Appendix 1). The stock solutions of nutrients were prepared fresh every month while that of vitamins every two weeks. For preparation of iron stock solution, $FeSO_4.7H_2O$ and Na2.EDTA were dissolved in hot double distilled water separately. After dissolving, iron sulphate solution was added to Na2.EDTA solution in half liter beaker to avoid the problem of precipitation. EDTA makes iron available at wide range of pH. The volume was adjusted by distilled water and dissolved very well using magnetic stirrer. Iron stock solution was protected from light by storing the solution in bottles wrapped with aluminum foil. Then, the stock solutions were labeled with date of preparation, name of stock solutions, quantities later used and stored in a refrigerator at 4 ^{O}C temperatures.

3.2.2. Plant growth regulators preparation

All of the plant growth regulators stock solutions, used in this experiment such as 6-benzyl amino-purine (BAP), kinetin (KN), α-Naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA) were prepared, separately. The powder of these hormones were weighed based on the quantity required and dissolved by 1N NaOH and/or 1N HCl. Then, the hormones were dissolved in double distilled water at a concentration of 1mg/ml. The volume was adjusted by

adding double distilled water. These growth regulators stock solutions were labeled with the date of preparation and name of plant growth hormones and stored in a refrigerator at $4^{O}C$ until use.

3.2.3. Culture medium preparation

Culture medium was prepared by taking 20 ml/L macronutrient, 5 ml/L micronutrient, 5 ml/L vitamin, 10 ml/L Iron EDTA and 10 ml/L calcium hypo chloride from stock solutions of MS medium (Appendix 3). During initiation stage only 1.0 mg/L of BAP was used alone to enhancing growth of well sterilized sprouted rhizome buds for initiation. Both for shoot multiplication and rooting experiments different plant growth regulators concentration and combination were used according to the treatments. During initiation and shoot multiplication stages, full MS medium was used, while half MS medium was used for rooting experiment. Then, 30 g/L of table sugar was added as a carbon source and dissolved in sterilized water (unpublished laboratory manual JARC, 2018). Then, the volume was adjusted by adding sterilized water. The pH of the culture medium was adjusted to 5.80 using 1N NaOH and/or 1N HCl before adding agar. After pH was adjusted, agar (7 g/L) was added and heated by micro oven till the agar melts properly (Mengs, 2018). After agar was melted, about 25 ml of the medium was dispensed in each test-tube of 75 ml volume for shoot initiation and about 50 ml of the medium was dispensed in culture jar of 250 ml volume for multiplication and rooting experiments. Then, the media was autoclaved at a temperature of 121 ^{O}C with a pressure of 105 Kpa for 15 minutes. Finally the autoclaved media was kept in medium storage room for 3 to 5 days.

3.2.4. Mother plant preparation

The healthy fresh rhizomes of ginger accession 52/86 was selected carefully (Figure 2.A). The selected fresh rhizomes of the ginger accession 52/86 was thoroughly washed with tap water. This was followed by double distilled water plus common liquid soap to get rid of the contamination properly. The cleaned ginger rhizomes were kept on sterilized cotton and in darkroom with room temperature of 20 ^{O}C to 27 ^{O}C until rhizome bud sprouted. The rhizomes were watered regularly using distilled water for 30 to 35 days. Sprouted buds from these rhizomes were used as a source of explants for culture establishments.

3.2.5. Sprouted rhizome buds surface sterilization

The sprouted rhizome buds (explants) were collected and washed in double distilled water and liquid soap (Figure 2.B). The explants were rinsed in distilled water to remove soap properly. Then the explants were washed by sterilized water with liquid soap plus 2-3 drops of Tween-20 for 20 minutes with manual agitation and the residual of soaps and Tween-20 was removed by rinsing using sterilized water. The washed explants were treated by mancozeb 80 % weight powder (WP) 6 g/L for 20 minutes with gentle manual shaking to reduce contamination from fungi. After the explants were treated, the residue of mancozeb was removed properly by sterilized water. This was followed by treating the explants with copper sulfate 30 g/L for 15 minutes to reduce the brown and contamination from bacteria (Figure 2.C). The traces of copper sulfate were removed by washing the explants five times with sterilized water. In the laminar airflow cabinet the explants were surface sterilized by immersing in 70 % (v/v) ethanol for one minute, then rinsed five times in sterile water to remove the trace of ethanol. Finally the explants were treated with 33.33 % (v/v) sodium hypochlorite (NaOCl) (5 % active ingredient of chlorine) of locally produced bleach (Berekina) for 20 minutes. To increase the efficiency of NaOCl, 2-3 drop of Tween-20 per one L solution was added as wetting agent. After decanting the sterilizing solutions under safe condition, the explants were washed five times each for three minutes with sterilized water to remove traces of NaOCl. At last the explants were transferred in autoclaved culture vessels contained sterilized water under laminar airflow cabinet to overcome the problem of desiccation of explants until culture took place. The sterilized explants were pilled to remove the injured leaf during surface sterilization by autoclaved forceps and surgery blades on Petri-dish under laminar airflow cabinet. The cleaned/pilled/ explants were cultured in test-tube medium for shoot initiation. After vertically culturing the explants in culture test-tube, the mouth of test-tube was quickly flamed, covered by aluminum foils and sealed by parafilm (Figure 2.D). The culture tube with explant was properly labeled with cultured date and code of treatment. These test-tubes were transferred to growth room. The growth shelf was disinfected by 70 % (v/v) alcohol and cleaned properly to put the test-tubes holder on growth shelf in the growth room.

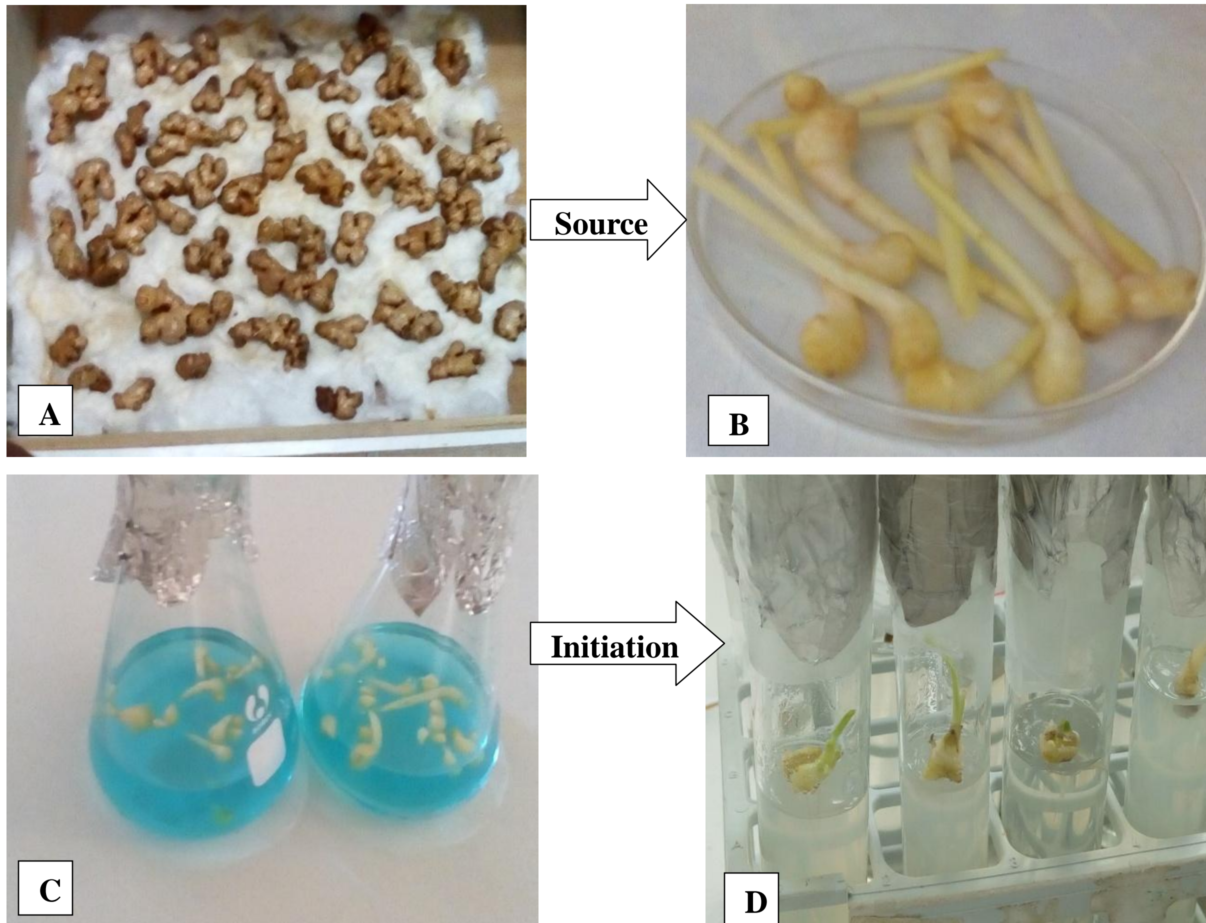

Figure 2. Mother plant selection and sterilization of explants (A) mother plant, (B) explants from sprouted rhizome buds (C) explants sterilization in Cu_2SO_4 (D) initiated explants

3.3. Culture Conditions

All cultures were kept under 60 to 70 % relative humidity, average temperature of 25 ± 2^{O}C and 16 hours photoperiod at a photosynthetic flux of 2000 to 3000 μmol/m^2/s^2 provided by cool fluorescent lambs in growth room.

3.4. Treatment Arrangement and Experimental Design

During stage one ex-plant per culture tube was used for initiation. This experiment was done twice with the same procedures of sterilization, explants size (1.5 cm), MS media composition and 1.0 mg/L BAP hormone. In order to achieve excess explants for next experiment multiplication, this experiment was cultured on MS basal media contained 1.0 mg/L BAP for 30

days. At this stage data were collected on number of clean initiated shoots after 30 days of culturing and this is converted into percent of initiated shoot.

3.4.1 Experiment 1: Effect of both cytokinins combination on shoot multiplication

In this experiment, five different concentrations of BAP (1, 1.5, 2, 2.5, 3) mg/L and four concentrations of KN (0.5, 1, 1.5, 2) mg/L in a 5 x 4 factorial combination with a total of 21 treatments and three replications were used. Basal media without plant growth regulators was included as control. A jar (experimental unit) contained five explants, making a total of 15 explants per treatment. The experiment was laid out in Completely Randomized Design (CRD). The well initiated explants on artificial nutrient media during initiation stage were used for this experiment after the effect of residual BAP hormone used during initiation was avoided by grown the initiated shoots on basal medium free from PGRs for 21 days. Thereafter, the cultures were transferred and randomly placed in growth room. Data were collected on number of shoots per ex-plant, shoot length in cm and number of leaves per shoot after 42 days of experiment established, as well as the mean values of each of these parameters were computed.

3.4.2 Experiment 2: Effect of both Auxis combination on rooting

For rooting experiment, five different concentrations of NAA (1, 1.5, 2, 2.5, 3) mg/L and four concentrations of IBA (0.5, 1, 1.5, 2) mg/L in a 5 x 4 factorial combination with a total of 21 treatments and three replications were used. Basal media without plant growth regulators was included as control. A jar (experimental unit) contained five explants, making a total of 15 explants per treatment. The experiment was laid out in Completely Randomized Design (CRD). The well multiplied shoots were used for this experiment after the effect of residual BAP and KN hormones used during multiplication were avoided by grown the multiplied shoots on basal medium free from PGRs for 30 days. The data were collected after 30 days of experiment established on number of roots per shoot, root length in cm and mean values of each of these parameters were computed.

3.4.3 Plantlet acclimatization

The well rooted plantlets on rooting medium were used for acclimatization. These plantlets were thoroughly washed with sterile water to remove residual agar and artificial nutrients from roots to avoid the growth of microorganism at greenhouse. Media components such as top soil (TS), sandy soil (SS), compost (CT) and well decomposed coffee husk (CH) were sterilized by sun light energy and used for acclimatization in different combinations (Table 4). These media components were moisten by distilled water and mixed very well separately (according to their ratio). Plantlets with a 3.8 cm height were used for acclimatization. After mixing very well, they were collected into pots, six different media combination ratios as treatments and 10 plantlets per pot with three replications were used. The pots were covered by polyethylene plastic sheet and placed randomly in the greenhouse. These planted plantlets in the pots were covered by polyethylene plastic sheet for 14 days to maintain high relative humidity (60-80 %) and average temperature of 20 to 27 ^{O}C in green house. The plantlets were watered by distilled water with required amounts three times in a day. After two weeks, the polyethylene plastic sheet was removed and the plantlets were grown open in green house and watered properly to avoided desiccation. After 42 days of acclimatization, data were collected on percentage of survived plantlets, height of the survived plantlets and number of shoots per plantlets from each pot. The mean values of each parameter were computed.

3.5. Data Analysis

The data were subjected to one way analysis of variance (ANOVA) using the SAS software packages (version 9.3) and significant differences among mean values were compared using Least Significant Difference (LSD) at alpha level of 0.05.

4. RESULTS AND DISCUSSION

4.1. Shoot Initiation

From surface sterilized sprouted rhizome buds, 91.3 % contamination free initiated shoots were recorded (Figure 2.D). The well initiated and survived explants were kept for shoot multiplication experiment.

4.2. Effect of BAP and KN Combination on Shoot Multiplication

The results of analysis of variance showed that BAP and KN combinations had significant (P<0.05) effect on number of shoots per ex-plant, shoot length and number of leaves per shoot (Appendix 1).

The interaction effect of BAP and KN on shoot multiplication showed that the better performance (9.34±0.55) and (9.00±0.00) mean number of shoots per ex-plant on medium fortified by 2.5 mg/L BAP + 1.0 mg/L KN and 3.0 mg/L BAP + 1.0 mg/L KN concentration compared with other treatments, respectively (Table 2, Figure 3.B). Similar finding was reported by Ayenew *et al*. (2012) on Yali and Boziab cultivars of ginger shoot multiplication. Whereas the minimum mean number of shoots per ex-plant (2.10±0.00) was recorded from medium containing higher concentration of 3.0 mg/L BAP+2.0 mg/L KN (Figure 3.C). Increasing BAP hormones concentration increased the number of shoots per ex-plant, while keeping the concentration of KN at 0.5 mg/L (Table 2). As both BAP and KN concentration increased the number of shoot per explant also increased up to the optimum 2.5 mg/L BAP + 1.0 mg/L KN concentration and combination. Hence, medium supplemented with both BAP and KN induced best shoot multiplication, indicating that the effectiveness of each of growth hormones in inducing shoots multiplication was influenced by the presence or absence of the other. Cultures under these combinations were more vigorous and better in shoot proliferation rate as compared to plant growth regulators free medium. This result showed that the culture medium devoid of plant growth regulator (control) produced the least mean number of shoots per explant (1.17±0.29) when compared with medium fortified with growth hormones (Table 2). This study showed that mean number of shoot per explant was affected by mutual 6-Benzylo Amino-Purine

concentrations in association with kinetin. This result is similar to Ayenew *et al.* (2012) who reported optimum concentration for shoot multiplication (MS + 2.0 mg/L BAP + 1.0 mg/L KN) for ginger cultivars Yali and Boziab. However, increasing both BAP and KN concentrations and combinations higher than 2.5 mg/L BAP + 1.0 mg/L KN have exhibited negative effect on shoot proliferation. This is due to plant cells response to the hormones induced synthesis of few minor RNAs and some proteins that are inhibitors of respiration, transcription and translation with inhibit cytokinis mediated cell division as studied by Gottieb and Skoog,(1954).

Augmenting the culture medium with 2.5 mg/L BAP + 0.5 mg/L KN showed the highest shoot height (5.10±0.17 cm) (Table 2, Figure 3.D), followed (4.80±0.10 cm) media containing 3.0 mg/L BAP and 0.5 mg/L KN growth hormones. This result was achieved on a medium containing high concentration of BAP with low concentration of KN and at high concentration of KN shoot height was stunted. Similarly, Balachandran *et al.* (1990) and Ayenew *et al.* (2012) observed such poor growth and development of the plantlets and explained that it is due to high kinetin that inhibits the oxygen uptake of the cells. The shortest shoot length (1.60±0.10) was recorded on media containing 3.0 mg/L BAP + 2.0mg/L KN hormones (Figure 3.C). This means the optimum concentration of growth regulator for this particular parameter was 2.5 mg/L BAP + 0.5 mg/L KN, whereas any concentration above or lower this concentration resulted in poor performance for this character (Table 2). As the levels of BAP increased (>2.5mg/L) with KN (>0.5mg/L) the shoot showed stunted, bushy and distorted growth. This feature was expressed because of high concentration of cytokinin. Shoots grown on basal free medium from growth hormone had larger height than shoots grown on high concentration and combination of both BAP and KN beyond optimum concentration (Table 2).

The maximum mean number of leaves per shoot (5.67±0.06 and 5.50±0.00) were obtained on the medium containing 3.0 mg/L BAP + 0.5 mg/L KN and 2.5 mg/L BAP + 0.5 mg/L KN, respectively (Table 2 and Figure 3.D). The least mean number of leaves per shoot (2.17±0.06) was achieved on a medium supplemented higher concentration and combination of 3.0 mg/L BAP + 2.0 mg/L KN. Thus, combinations of cytokinins resulted in considerable effects on various growth parameters associated with ginger shoot multiplication which is in line with the finding of Islam *et al*. (2004) and Ayenew *et al.* (2012).

Table 2.Mean shoot number, length and leaf number of accession 52/86 ginger on medium supplemented with BAP and KN combination.

Growth Regulator (BAP + KN) mg/L	Means no of shoot Per ex-plant ± SD	Mean no of Shoot length(cm) ±SD	Mean no of leaf per Ex-plant ± SD
0.0 + 0.0	1.17^{n} ±0.29	3.13^{hi}±0.06	3.60^{ij} ± 0.00
1.0 + 0.5	3.00^{kl}±0.00	3.50^{fg}±0.00	3.97^{gh}± 0.06
1.5 + 0.5	3.50^{jk} ±0.00	3.90^{e}±0.00	4.50^{ef}± 0.00
2.0 + 0.5	4.00^{ij} ±0.00	4.50^{c}±0.00	5.20^{bc}± 0.00
2.5 + 0.5	4.50^{hi}±0.00	5.10^{a}±0.17	5.50^{ba}± 0.00
3.0 + 0.5	5.00^{gh}±0.00	4.80^{b}±0.10	5.67^{a} ± 0.06
1.0 + 1.0	5.50^{fg}±0.00	4.20^{d}±0.10	4.00^{gh}± 0.17
1.5 + 1.0	6.00^{ef}±0.00	3.70^{ef}±0.00	4.30^{fg} ± 0.35
2.0 + 1.0	7.67^{b}±0.15	3.10^{hi}±0.10	4.90^{cd}± 0.35
2.5 + 1.0	9.34^{a} ±0.55	2.93^{ij}±0.12	4.80^{de}± 0.26
3.0 + 1.0	9.00^{a}±0.00	2.60^{lm}±0.00	4.20^{fgh}± 0.35
1.0 + 1.5	7.00^{c}±1.00	3.30^{gh} ±0.00	4.43^{f} ± 0.40
1.5 + 1.5	6.67^{cd}±0.58	2.90^{ijk}±0.10	4.30^{fg} ± 0.17
2.0 + 1.5	6.33^{ed} ±0.29	2.67^{jklm}±0.21	3.93^{hi}± 0.38
2.5 + 1.5	5.53^{f}± 0.47	2.43^{mn} ±0.06	3.40^{jk}± 0.35
3.0 + 1.5	4.90^{h}±0.10	2.17^{no} ±0.06	3.30^{jk}± 0.00
1.0 + 2.0	4.83^{h}±0.06	2.73^{jkl} ±0.64	3.20^{kl}± 0.00
1.5 + 2.0	3.90^{j}±0.00	2.63^{klm} ±0.35	2.90^{lm}± 0.00
2.0 + 2.0	3.00^{kl}±0.00	2.00^{op}± 0.00	2.70^{mn}± 0.00
2.5 + 2.0	2.67^{l} ±0.06	1.77^{pq}±0.06	2.37^{no}± 0.12
3.0 + 2.0	2.10^{m} ±0.00	1.60^{q}± 0.10	2.17^{o}± 0.06
LSD	0.52	0.30	0.35
CV%	6.24	5.78	5.33

CV; Coefficient of variation, SD; Standard Deviation, LSD; Least Significant Difference, Means within columns having different letters in superscript are significantly different at $p<0.05$.

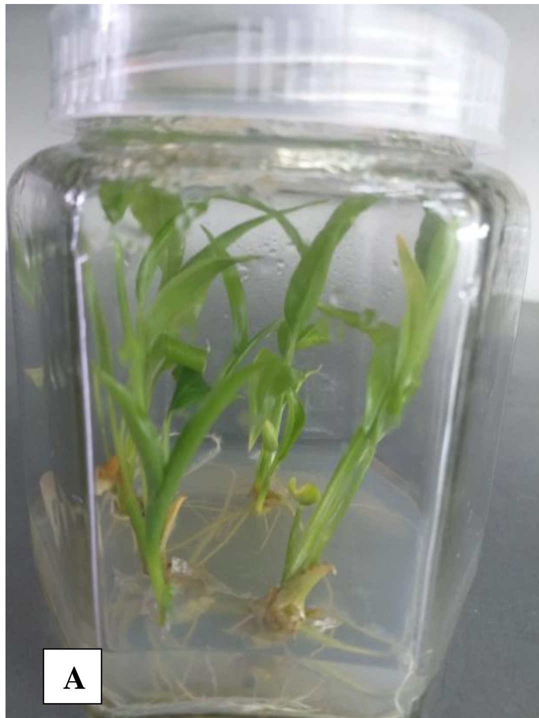

Figure 3. Shoot multiplication on different treatments (A) shoot grown on plant growth regulators free media (B) shoot multiplied of media containing 2.5 mg/L BAP +1.0 mg/L KN hormones (C) shoot multiplied on media containing 3.0 mg/L BAP + 2.0 mg/L KN hormones (D) shoot length and number of leaves per shoot.

4.3. Effect of NAA and IBA on Ginger in Vitro Rooting

The results of analysis of variance showed that NAA and IBA had significant ($P<0.05$) effect on root number per shoot and root length (Appendix 3).The plantlets cultured on rooting medium were able to induce roots in all media supplemented with NAA and IBA. A maximum mean number of roots per shoot (12.60±0.17) was scored on half MS medium supplemented with 2.5 mg/L NAA + 1.0 mg/L IBA (Table 3, Figure 4). This was followed by 12.30±0.20 mean number of roots per shoot on MS medium containing 3.0 mg/L NAA + 1.0 mg/L IBA (Table 3, Figure 4.B). This study did not agree with the findings of Idris *et al.* (2015) and David *et al.* (2016), which may be due to the differences of hormones concentration and genotype. The low mean number of roots per shoot were scored on MS medium containing high concentration of 3.0 mg/L NAA + 2.0 mg/L IBA and low hormones concentration of 1.0 mg/L NAA + 0.5 mg/L IBA when compared with treatments supplemented with different combinations of NAA and IBA (Table 3). As concentration and combination of both NAA and IBA slightly increased, root number per shoot also increased up to optimum 2.5 mg/L NAA + 1.0 mg/L IBA and beyond optimum (>2.5 mg/L NAA + 1.0 mg/L IBA) the root number per shoot start to decline. The medium containing NAA and IBA concentration and combination beyond and below optimum could not provide high number of roots per shoot as compared with each other (Table 3). Plant growth regulators free basal medium produced the lowest mean number of roots per shoot (2.32 ± 0.12) (Figure 4.A).

The highest mean root length (6.30±0.10 cm) was achieved on MS media fortified by 1.0 mg/L NAA + 1.0 mg/L IBA hormones, followed by 6.03±0.23 cm MS medium containing 1.5 mg/L NAA + 1.0 mg/L IBA (Table 3). The shortest roots length (2.40 ± 0.10 cm) was achieved on medium supplemented with 3.0 mg/L NAA + 2.0 mg/L IBA (Table 3). Increasing NAA concentration from 0 to 3.0 mg/L, slightly increased root length while IBA kept constant at 0.5 mg/L. As both concentrations and combination of NAA and IBA increased, the root length also increased up to optimum 1.0 mg/L NAA + 1.0 mg/L IBA. In the present study, increasing the concentrations of NAA and IBA beyond this optimum caused ginger root length decline.

Table 3. Mean root number and root length of accession 52/86 ginger on medium supplemented with NAA and IBA.

Growth regulators hormone (NAA + IBA) mg/L	Mean no of Root Per Shoot ± SD	Mean no Root length in (cm) ± SD
0.0 + 0.0	$2.32^{n} \pm 0.12$	$3.37^{l} \pm 0.16$
1.0 + 0.5	$5.27^{m} \pm 0.21$	$3.87^{j} \pm 0.06$
1.5 + 0.5	$5.97^{kl} \pm 0.12$	$4.03^{hi} \pm 0.06$
2.0 + 0.5	$6.43^{j} \pm 0.12$	$4.50^{f} \pm 0.00$
2.5 + 0.5	$6.80^{gh} \pm 0.10$	$4.70^{e} \pm 0.10$
3.0 + 0.5	$6.67^{hi} \pm 0.06$	$4.43^{fg} \pm 0.06$
1.0 + 1.0	$5.83^{l} \pm 0.16$	$6.30^{a} \pm 0.10$
1.5 + 1.0	$6.66^{hi} \pm 0.06$	$6.03^{b} \pm 0.23$
2.0 + 1.0	$7.80^{d} \pm 0.10$	$5.40^{c} \pm 0.10$
2.5 + 1.0	$12.60^{a} \pm 0.17$	$4.93^{d} \pm 0.06$
3.0 + 1.0	$12.30^{b} \pm 0.20$	$4.50^{f} \pm 0.00$
1.0 + 1.5	$8.60^{c} \pm 0.10$	$4.70^{e} \pm 0.10$
1.5 + 1.5	$8.40^{c} \pm 0.10$	$4.13^{h} \pm 0.06$
2.0 + 1.5	$7.80^{d} \pm 0.10$	$4.33^{g} \pm 0.06$
2.5 + 1.5	$7.43^{e} \pm 0.12$	$3.67^{k} \pm 0.06$
3.0 + 1.5	$7.10^{f} \pm 0.17$	$3.03^{m} \pm 0.06$
1.0 + 2.0	$6.90^{fg} \pm 0.00$	$4.17^{h} \pm 0.05$
1.5 + 2.0	$6.45^{ij} \pm 0.06$	$3.93^{ij} \pm 0.06$
2.0 + 2.0	$6.10^{k} \pm 0.17$	$3.60^{k} \pm 0.10$
2.5 + 2.0	$5.80^{l} \pm 0.10$	$2.83^{n} \pm 0.06$
3.0 + 2.0	$5.23^{m} \pm 0.12$	$2.40^{o} \pm 0.10$
LSD	0.21	0.15
CV%	1.78	2.08

CV; Coefficient of variation, SD; Standard Deviation, LSD; Least Significant Difference, Means within columns having different letters in superscript are significantly different at $p<0.05$.

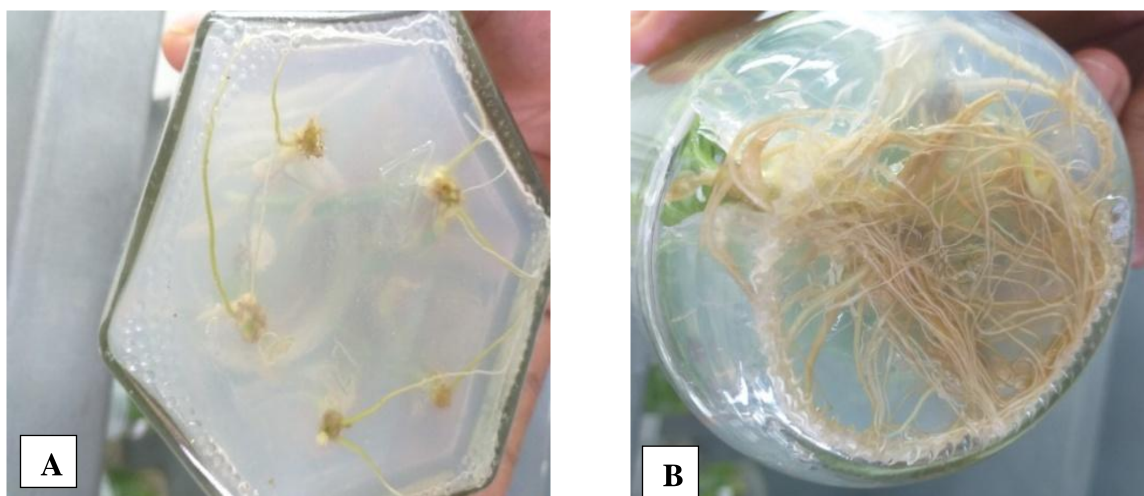

Figure 4. Ginger rooted on rooting medium (A) rooted shoot on hormone free media (B) rooted shoot on media containing 2.5 mg/L NAA + 1.0 mg/L IBA

4.4. Effect of Media Components on Plantlets Acclimatization

The ultimate success of *in vitro* propagation lies on the successful establishment of plants in the soil. Table 4 shows the effect of different soil combination as growth media on plantlet survival percentage of ginger accession 52/86. The growth medium containing TS: SS: CH (1:1:1) gave the highest survival percentage (98 %) of plantlets (Figure 5.C) and followed by the media containing TS: SS: CT (1:1:1) (95 %) of survival plantlets, while low survival rate of plantlets (70 %) were scored on the media containing top soil only (Figure 5.D). Results depicted in Table 4 also indicate that, the various treatments had significant effect on plant height. The tallest plant height (7.17 cm) was scored on a medium containing TS: SS: CT (1:1:1), followed by (6.23 cm) with the treatment of TS: SS: CH (1:1:1) and the least (4.12 cm) was scored on a treatment of top soil. There was no significant effect scored from media components combination of TS, TS: SS, TS: CH and TS: CT on mean number of shoot per plant while TS: SS: CH (1:1:1) and TS: SS: CT (1:1:1) were significantly different from the rest. The highest number of shoot per plantlet (1.23) were achieved on soil media TS: SS: CH (1:1:1) when compared with other.

The plantlets transferred to greenhouse showed good performance in terms of survival percent, plant height and number of shoot per plantlets mainly scored on medium containing a combination of TS:SS:CH (1:1:1). This followed by TS:SS:CT (1:1:1) with the survival

percentage of 95 % , mean of plant height of 7.17 cm and mean number of shoot per plantlets 1.23, which was morphological related with plantlets acclimatized on TS:SS:CH (1:1:1). This media components combination may be used when there is no well decomposed coffee husk.

Table 4. Influence of media components combination on acclimatization of micro propagated plantlets of ginger accession 52/86 under greenhouse conditions after 42 days of acclimatized.

Treatment(T)	Plantlet Survival (%)	Plant height (cm)	Number of shoot Per plant
TS	70	4.12^{d}	1.00^{b}
TS:SS (1:1)	80	5.60^{c}	1.00^{b}
TS:CH (1:1)	88	4.20^{d}	1.00^{b}
TS:CT (1:1)	85	5.60^{c}	1.00^{b}
TS:SS:CH(1:1:1)	98	6.23^{b}	1.22^{a}
TS:SS:CT(1:1:1)	95	7.17^{a}	1.13^{ab}
LSD		0.29	0.10
CV%		3.24	8.83

TS; Top soil, SS; Sandy Soil, CH; Coffee Husk; CT; Compost, CV; Coefficient of variation; LSD; Least Significant Difference. Means within columns having different letters in superscript are significantly different at p<0.05.

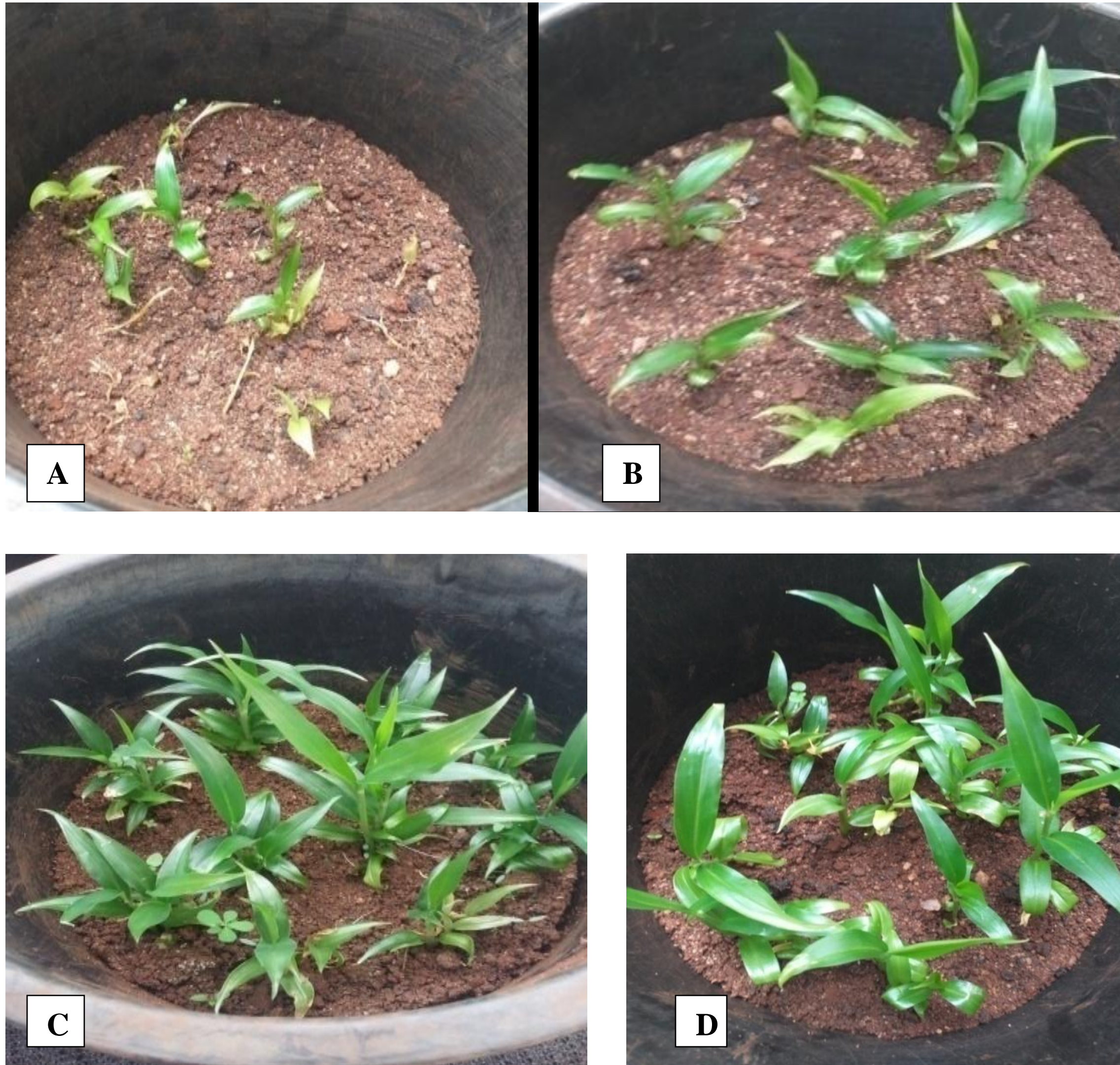

Figure 5. Plantlets acclimatization in greenhouse (A) plantlets survived on the top soil media alone (B) plantlets survived on the top soil: coffee husk (C) plantlets survived on top soil: sandy soil: coffee husk (D) plantlets survived on the top soil: sandy soil: compost

5. SUMMARY AND CONCLUSIONS

Ginger is one of the well-known spices used in several kitchens, as well as a medicine in traditional healers and in some modern treatment modalities. Even though ginger has such economic and social importance, its production and productivity is limited by factors such as lack of enough planting materials, lack of disease free planting materials, due to biotic factors and infertile seeds as well as wilting bacteria. Therefore, using *in vitro* propagation could alleviate these problems. So this study was initiated to *in vitro* propagation of ginger protocol optimization to provide methods of planting materials producing and disseminate to ginger growers in the country.

Ginger accession 52/86 is promising and in pipeline to be promoted to cultivar by JARC. It was secured from this institution and used for the experiment. In this study, the sprouted rhizome bud explants were properly sterilized and used for shoot initiation. The well survived and initiated explants were used for shoot multiplication experiment. In rooting experiment, the shoots from multiplication stage were used and plantlets rooted on half MS medium were acclimatized in greenhouse.

In present study, mancozeb 6g/L for 20 minutes, copper sulfate 30g/L for 15 minutes, 70% (v/v) ethanol for one minute and local bleach (berekin) 33.33%(v/v) for 20 minutes were used for sprouted rhizome buds disinfection with 91.3% success rate of culture initiation. For multiplication experiment the optimum combination and concentration plant growth regulator for ginger accession 52/86 high shoot multiplication was achieved on MS medium harbored 2.5mg/L BAP+1.0mg/L KN when compared with other. The highest shoot length was achieved on the medium with 2.5mg/L BAP+0.5mg/L KN and most mean number of leaves per shoot was obtained from the medium fortified with both 2.5mg/L BAP+0.5mg/L KN and 3.0mg/L BAP+0.5mg/L KN. Hence using 2.5mg/L BAP+0.5mg/L KN more preferred in economic saving since there was no significance difference between these two treatments in terms of mean number of leaves per shoot. In rooting experiment the highest rooted shoot was scored on the MS medium containing 2.5mg/L NAA+ 1.0mg/L IBA. For acclimatization good soil combination for plantlet adaptation was observed on media containing top soil: sandy soil: coffee husk (1:1:1) with 98 % survived plantlets and good morphological performance. Therefore, the optimized

protocol is useful for *in vitro* propagation of this specific ginger accession 52/86 planting material.

Recommendation

- ✓ Even though, there was no statistical difference between MS+2.5mg/L BAP+1.0mg/L KN and MS+3.0mg/L BAP+1.0mg/L KN on shoot multiplication, 2.5mg/L BAP+1.0mg/L KN, could be taken as the best option for shoot multiplication as it reduces the cost of the growth regulator hormones.
- ✓ Half MS+2.5mg/L NAA+1.0mg/L IBA was the best medium for *in vitro* rooting.
- ✓ Since all morphological parameters of acclimatized plantlets showed best performance on top soil: sandy soil: coffee husk 1:1:1 ratio, using this soil combination is the best for ginger acclimatization. Using compost is a second option when decomposed coffee husk not available.

Future line of work:-

- ➢ Since contamination at initiation stage is very problematic standardization of *in vitro* sterilization procedures for micro propagation of ginger needs to be further studied
- ➢ Further studies are needed using combinations of hormones such as BAP with NAA or IBA, KN with NAA or IBA which are potential cytokinins and auxins for culture establishment and shoot proliferation.
- ➢ The effect of sub-culture on the multiplication of shoots should be further studied to determine whether sub-culturing increases or decreases shoot to significant extent.

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7. APPENDIXES

Appendix 1. Analysis of variance summary of the effect of BAP and KIN combination on shoot multiplication growth parameters from sprouted explants of ginger *(Zingiber officinale* Rosc.*).*

		Number of shoot per explants		Shoot length		Numbers of leaves per shoot	
Source of variation	Degree of Freedom	Means square	Pr> F	Mean square	Pr> F	Mean square	Pr> F
BAP	4	0.4468333	0.0051	0.57382540	<.0001	0.25530159	0.0031
KN	3	56.1597222	<.0001	13.50844444	<.0001	14.60577778	<.0001
BAP*KN	12	5.4380556	<.0001	1.04802778	<.0001	1.02688889	<.0001
Error	40	0.1025635	<.0001	0.03427778	<.0001	0.04461111	<.0001

Appendix 2. Analysis of variance summary of the effect of NAA and IBA combination on number of root and root length of in vitro propagated ginger *(Zingiber officinale* Rosc.*)*.

		Number of Root per Shoot		Root Length	
Source of Variation	Degree of Freedom	Mean Square	Pr> F	Mean Square	Pr> F
NAA	4	18.7645000	<.0001	2.53380159	<.0001
IBA	3	29.7023889	<.0001	11.11438889	<.0001
NAA*IBA	12	9.6953056	<.0001	0.79147222	<.0001
Error	40	0.0140000	<.0001	0.00530159	<.0001

Appendix 3.Murashige and Skoog (1962) Medium Stock Solution used for sprouted rhizome buds of ginger tissue culture.

Stock type	Components	Concentration(mg/L)	Stock solution	Amount required in L
I			**X50/1000ml**	20ml/L
	NH_4NO_3	1650	82.5g	
	KNO_3	1900	95g	
	$MgSO_4.7H_2O$	370	18.5g	
	KH_2PO_4	170	8.5g	
			X100/500ml	
II	H_3BO_4	6.1	0.62g	5ml/L
	$MnSO_4.2H_2O$	22.3	2.23g	
	$ZnSO_4.7H_2O$	10.6	1.06g	
	KI	0.83	83mg	
	$NaMoO_4.2H_2O$	0.25	25mg	
	# $CuSO_4.5H_2O$	0.025	2.5ml from ISS	
	# $CoCl_2.6H_2O$	0.025	2.5ml from ISS	
			X50/500ml	
III	$FeSO_4.7H_2O$	27.8	1.39g	10ml/L
	Na_2EDTA	37.3	1.865g	
			X100/500ml	
IV	Myo-inositol	100	10g	5ml/L
	Nicotinic acid	0.5	50mg	
	Pyridoxine-HCl	0.5	50mg	
	Thiamine-HCl	0.1	10mg	
	Glycine	2	200mg	
			X50/500ml	
V	$CaCl_2.2H_2O$	440	22g	10ml/L

Intermediate Stock Solution (ISS)

No	Components	Concentration mg/L	ISS	Amount Required/500ml stock
1	$CuSO_45H_2O$	0.025	100mg/100ml	2.5ml/L
2	$CoCl_2.6H_2O$	0.025	100mg/100ml	2.5ml/L
3	Glycine	0.025	100mg/100ml	The required amount
4	Thiamine-HCl	0.025	100mg/100ml	
5	Cysteine	0.025	100mg/100ml	