In Vitro Micropropagation Protocol Optimization of Garlic (*Allium sativum.L*) "Holetta Local" and "Kuriftu" Varieties Using Shoot Tip Culture

By

Misgana Chala Gemachu

A Thesis Submitted to Department of Applied Biology, School of Applied Natural Science Presented in Partial Fulfillment for the Requirements of Master of Science in Applied Biology (Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

June, 2021

Adama, Ethiopia

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Advisor: Mulugeta Kebede (PhD)

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DECLARATION

I hereby declare that this Master Thesis entitled “In Vitro Micropropagation Protocol Optimization of Garlic (*Allium sativum.L*) “Holetta Local” and “Kuriftu” Varieties Using Shoot Tip Culture” is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

Misgana Chala	_____	_____
Name of the student	Signature	Date

Recommendation

We, the advisors of this thesis, hereby certify that we have read the revised version of the thesis entitled “In Vitro Micropropagation Protocol Optimization of Garlic (*Allium sativum.L*) “Holetta Local” and “Kuriftu” Varieties Using Shoot Tip Culture” prepared under our guidance by Misgana Chala Gemachu submitted in partial fulfillment of the requirements for the degree of Mater’s of Science in Applied Biology (Biotechnology). Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

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Co-advisor	Signature	Date

APPROVAL OF BOARD OF EXAMINERS

We, the advisors of the thesis entitled "In Vitro Micropropagation Protocol Optimization of Garlic (*Allium sativum.L*) "Holetta Local" and "Kuriftu" Varieties Using Shoot Tip Culture" and developed by Misgana Chala, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

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We, the undersigned, members of the Board of Examiners of the thesis by Misgana Chala have read and evaluated the thesis entitled "In Vitro Micropropagation Protocol Optimization of Garlic (*Allium sativum.L*) "Holetta Local" and "Kuriftu" Varieties Using Shoot Tip Culture" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Biology (Biotechnology).

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Final approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

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AMS	Ally Methyl Sulfide
BAP	Benzylaminopurine
BDS	B5 modified by Dunstan and Short
CACC	Central Agricultural Census Commission
CLV	Common Latent Virus
CSA	Central Statistics Authority
CVR	Crop Variety Register
DZARC	Debre Zeit agricultural research center
ELISA	Enzyme Linked Immuno Absorbent Assay
FAO	Food and Agriculture Organization
FAO	Food and Agriculture Organization
FAOSTAT	Food and agricultural organization statistics
GMV	Garlic Mosaic Virus
GYSV	Garlic Yellow Streak Virus
IAA	Indole-3-acetic acid
IgE	Immunoglobulin E
LS	Linsmaier and Skoog
LYSV	Leek Yellow Stripe Virus
MARC	Melkassa agricultural research center
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
OYDV	Onion Yellow Dwarf Virus
P-CPA	Pchlorophenoxyacetic acid
PGRs	Plant growth regulators
PPF	photosynthetic photon flux
SAS	Statistically Analysis System
SLV	Shallot Latent Virus

ABSTRACT

Garlic (Allium sativum L.) is major bulb plant predominantly used for flavoring and serving dishes and also used as a medicinal plant all over the world. However, numerous crop management problems, the nature of propagation, diseases and insect pest limited its production. Virus is a major detriment for reduction of garlic yield and quality. To produce virus-free garlic, shoot tip culture has been widely adopted. However, shoot tip culture require the optimized protocol and necessary cultural conditions. The objective of this study was to optimize an efficient protocol for in vitro micropropagation of selected garlic varieties ('Holeta Local' and 'Kuriftu') through direct organogenesis using shoot tip culture. The garlic cloves were washed with powder soap, sterilized with 70% ethanol and 15% chlorine bleach solution. Shoot tips containing apical meristems 0.5 to 0.8 cm in length were aseptically excised from sprouted cloves and cultured on half strength Murashige and Skoog medium supplemented with 10mg/l Benzyl Amino Purine (BAP) to initiate the growth of shoot tip and shoot induction. For this experiment cytokinin alone was crucial for better direct shoot induction from shoot tip culture. Shoot multiplication was carried out on half strength MS medium containing different concentrations of Benzyl Amino Purine (BAP) in combination with Naphthalene acetic acid (NAA). Half MS medium without PGRs was used as control. Among the treatment, 2.0 mg/l BAP+0.25 mg/l NAA was found to be the best for 'Holeta Local' where a maximum of 5.48±0.28 shoots and for 'Kuriftu' a maximum of shoot 4.86±0.25 was obtained on 1.5 mg/l BAP+0.25 mg/l NAA. The highest shoot length 7.63±0.73 cm and 6.85±0.33 cm were recorded on 1.5 mg/l BAP+0.25 mg/l NAA for 'Holeta Local' and 'Kuriftu' respectively. Root initiation was carried out on half strength MS medium containing different concentrations of NAA. Half MS medium without PGRs was used as control. Among the treatment, the highest number of roots 3.42±0.32 and 3.65±0.51 per explant were recorded on 1.0 mg/l NAA for 'Holeta Local' and 'Kuriftu' respectively. The highest mean length of root per explant 2.08±0.32 cm was obtained on 0.5 mg/l NAA for 'Holetta Local' and 2.46±0.53 cm for 'Kuriftu' on 1.0 mg/l NAA. The highest percentage of root formation 100% was recorded on 1.0 mg/l NAA for both varieties. Of those shoots which were acclimatized in the greenhouse, 78% of ''Holetta Local'' and 84 % of 'Kuriftu' survived.

Keywords: Allium sativum L, Benzyl Amino Purine (BAP), Micropropagation, Naphthalene acetic acid (NAA), shoot tip

1. INTRODUCTION

1.1 Background

Garlic (*Allium sativum* L.) is a monocotyledonous aromatic plant belongings to Alliaceae family and of genus Allium (Osman *et al*., 2007). It is a diploid species of obligated apomixes, therefore its reproduction system is vegetative through its cloves (Figliuolo *et al*., 2001; Ipek *et al*., 2003). The origin of garlic is thought to be in Central Asia (India, Afghanistan, West China, Russia) and spread to other parts of the world through trade and colonization (Tindal, 1986). Garlic is the most important Allium crop and ranks second next to onion in the world (Voigt, 2004).

Garlic is mainly produced in Asia (87%), China and India, being the largest producers, collectively accounting for 78% of the garlic production. Africa accounts for only about 2.8% of the total world production (FAOSTAT, 2011). On the global scale, the top 5 garlic producing countries are China producing 20.06 million tons, India 1.25 million tons, Bangladesh 0.3, Egypt 0.26 and Russia 0.26 million tons per year (FAOSTAT, 2014). Ethiopia ranked 12th in the series of top garlic producing countries (FAOSTAT, 2014) producing about 24,939,965 tons of garlic which accounts for about 0.39% of the total world garlic production. According to CSA (2015/16) report, its production covers 6,789.11 hectares with a total production of 5,781.767 tons and 8.5 ton/ha on average.

Garlic is predominantly used for flavouring and serving dishes and as a medicinal plant all over the world (Wiczkowski, 2011; Nagy *et al*., 2015). It is used for flavouring many types of dishes ranging from vegetable soup, meat, salad, tomato combination, spaghetti, sausages and pickles (Brewster, 1994). Similar to green onions, it is eaten as green and blenched tops in different ways as fresh and cooked as well as immature bulb consumption is common especially in tropics (Rabinowitch & Brewster, 1990). Garlic has also the medicinal value which is well recognized in the control and treatment of hypertension, worms, germs, bacterial and fungal diseases, diabetes, cancer, ulcer, rheumatism etc. (Kilgori *et al*., 2007).

In Ethiopia, It is mainly used for flavouring and seasoning vegetables in different dishes. It also has many medicinal properties (Abreham, M *et al.,* 2014). As a cash crop, it is used to earn foreign currency by exporting to Europe, the Middle East, Africa countries and the USA (Kilgori *et al.*, 2007). Due to it is the cash crop in many parts of the country, increasing its productivity per unit area and production will enable farmers get encouraging returns and contribute its role in achieving the food security goals (Metasebia, M. & Shimelis H, 1998).

Traditional propagation method has low Multiplication rate and high Transmission of pathogens (Walkey, 1990; Nagakubo *et al*., 1993). Because of its vegetative propagation, it could be easily infected by pathogens, which results in yield decrease of up to 70%, and Loss of product quality (Kamenetsky, 2007). Furthermore, vegetative propagation facilitates the transmission of several virus species throughout the plant life cycles (Parrano *et al*., 2012). Many of the elite garlic cultivars are susceptible to diseases caused by viruses, nematodes and fungi and suffer from insect pests (Verbeek *et al*., 1995).

Biotechnological tools such as micropropagation, meristems culture (to obtain virus-free plants), somaclonalvariation and genetic transformation, have contributed high to the propagation, preservation and breeding of garlic (Fiserova *et al*., 2016). Due to the difficulties in flower induction in garlic species, breeding programs have been limited to clonal selection and production of virus-free materials via meristem culture (Mahajan, 2016). Shoot-tip culture has been used for decades for virus removal in vegetatively propagated plants (Ghaemizadeh *et al*., 2014) and from garlic in vitro plants (Pramesh & Baranwal, 2015). Virus-free clones produced through meristem culture showed higher yield with improved quality (Taskin *et al*., 2013). Therefore, the tissue culture techniques have got the high potential for the improvement of garlic in respect of yield and quality (Mukhopadhyay *et al*., 2005).The use of meristems or shoot-tips as explant for micropropagation of multiple bulb formation is more suitable than another source of explants (Roksana *et al*., 2002).

In Ethiopia, garlic production faces a lot challenges like numerous crop management problems, the nature of propagation, diseases and insect pest limited the supply of production as required (Getachew, T., & Asfaw, Z., 2000). According to Lot *et al.* (1998) Virus infection was shown to reduce the bulb yield by 20% to 60% and up to 80% in case of mixed depending on cultivar and stage of infection. Shoot-tip culture is a perfect and beneficial tool for producing virus-free garlic. However, *in vitro* shoot tip require standardized protocol. To this end, no efficient standardized protocol had been availed so far for the shoot tip culture of Garlic (*Allium sativum L*.) clone in Ethiopia. Therefore, the main objective of this study was to standardize the necessary cultural conditions of ("Holetta Local" and "Kuriftu") varieties. It also aimed at exploring the variability of in vitro response among two selected Garlic (*Allium sativum L*.) varieties through shoot tip culture in various combination and concentration of growth hormone.

1.2 Statement of the problem

Garlic is one of the most important and widely cultivated Allium crops worldwide, which is utilized for food and medicinal purposes.

Because of its vegetative propagation, it could be easily infected by pathogens (fungi, viruses, nematodes) which results in yield decrease of up to 70%, and Loss of product quality (Kamenetsky, 2007). Furthermore, vegetative propagation facilitates the transmission of several virus species throughout the plant life cycles (Parrano *et al*., 2012). Many of the elite garlic cultivars are susceptible to diseases caused by viruses, nematodes and fungi and suffer from insect pests (Verbeek *et al*., 1995). Virus infection was shown to reduce the bulb yield by 20% to 60% and up to 80% in case of mixed depending on cultivar and stage of infection (Lot *et al*., 1998).

Therefore, the present production of garlic cannot meet up the increasing demand; the area and production of garlic have not been improved at the desired level (Haque *et al*., 2013). Plant tissue culture techniques have provided many solutions to basic questions and practical difficulties in plant biology. Plant tissue culture techniques require optimized Protocol. However, tissue culture protocols are influenced by genotype and explant used. So, it is necessary to optimize culturing conditions for different species (Barandiaran *et al*., 1999). For effective exploitation of shoot tip culture technique, Protocol optimization at all stages process that suit the specific species and clone at hand is required. Protocol optimization which is necessary for cultural conditions of Garlic (*Allium sativum L*.) by shoot tip culture technique is the major limitation in Ethiopia. Thus, Protocol optimization is extremely fundamental to exploit effectively for the rapid mass propagation, dissemination of Garlic clone and breeding program. Despite its impact, no optimized protocol had been availed so far for garlic propagation. Thus, this research was intended to optimize culturing conditions for micropropagation of Ethiopian garlic (“Holetta Local” and “Kuriftu”).

1.3 Significance of the study

The results of this study is an important for production of free-disease, uniform high quality and quantity, and rapid mass propagation of selected Garlic (*Allium sativum.L*) varieties through optimized protocols. It is also important to bring more efficient and stable regenerated garlic cultivars, fasten the rate of multiplication, induce and develop the strong root, and preserve the garlic cultivars through the necessary optimized culturing conditions. In addition to this, for students who are interesting and involved in garlic research helpful, for academic institutions who are engaged in garlic used as secondary data and useful for government institution like ministry of Agriculture and Other institutions such as international Organization and Agro-business companies.

1.4 Objectives

1.4.1 General objective

- To optimize a protocol for in vitro mass propagation of selected Garlic (*Allium sativum.L*) varieties ("Holetta Local" and "Kuriftu").

1.4.2 Specific objectives

- To optimize protocols for in vitro shoot initiation of "Holetta Local" and "Kuriftu" varieties.
- To optimize protocols for shoot multiplication of "Holetta Local" and "Kuriftu" varieties.
- To optimize protocols for rooting and rapid mass propagation using shoot tip culture for the improvement of the quantity and quality of garlic under greenhouse conditions.
- To compare the two varieties for in vitro shoot initiation, shoot multiplication, root initiation and acclimatization.

2. REVIEW OF LITERATURE

2.1. Garlic (*Allium sativum L.*)

2.1.1. Taxonomy and General Description of Garlic (*Allium sativum L.*)

The genus *Allium*, which belongings to the family Alliaceae, is diverse and comprises about 750 species; but only seven of them are widely cultivated in different parts of the world. Of these, the species important in Ethiopia are onion (*Allium cepa L.*), shallot (*Allium cepa* var. *ascalonicum L.*), garlic (*Allium sativum L.*) and leek (*Allium ampeloprasum L.*) (Jones, 1990; Currah & Rabinowitch, 2002). Garlic is one of the main *Allium* vegetable crops known worldwide for its production and economic value. It is one of the oldest cultivated vegetables and the second most widely produced *Allium* next to onion (Rubatzky & Yamaguchi, 1997; Hamma *et al.*, 2013; Hassan, 2015). It is a diploid species of obligated apomixes, therefore its reproduction system is vegetative through its cloves (Figliuolo *et al*., 2001; Ipek *et al*., 2003).

Garlic is propagated asexually, but shows a high morphological diversity among cultivars. These cultivars have a wide range of adaptation to different environments. Like an onion, garlic plants have thin tape-shaped leaves about 30 cm long. Roots reach up to 50 cm depth or a little more. Heads or bulbs are white-skinned, divided into sections called cloves. Each head could have from 6 to 12 cloves, which are covered with a white or reddish papery layer or “skin” (Hector *et al*., 2012).

Sexual propagation in garlic is expected to facilitate the exchange of genetic traits from one genotype to another and to improve garlic cultivars through classical breeding. Garlic does not produce true seed but it is propagated by planting cloves. Each bulb usually contains a dozen or more cloves and planted separately. Select only larger outer cloves of the best garlic bulbs for planting because larger cloves yield larger size and mature bulbs at harvest (Kamenetsky *et al.*, 2001).

2.1.2. Ecology Requirements and Production of Garlic

The ecological requirements of garlic are mild winter regions which have some rainfall followed by a sunny dry summer, are good for maturity and harvesting of the bulbs production (Brewster, 1994). Very high or Excessive humidity and rainfall are unfavorable to vegetative growth, bulb formation and reduced production. The garlic plant is easily stressed by insufficient moisture and water logging during its growing period (Rubatzky & Yamaguchi, 1997). To attain the maximum yield, the top upper surface should be moist and maintained close to field capacity for most of the growing season (Brewster, 1994). The geographical areas providing mean monthly temperature ranging from 12-24°C is best for garlic growing. High temperature is required for optimum bulb development but a

cooler condition in the early stages favour vegetative growth. Elevations less than 2000 m above sea level is not suitable for garlic growth and production (Nonnecke, 1989).

Anonymous (2004) indicated that the term 'seed' in the garlic referred to individual cloves separated from a bulb. The 'seed' needed per hectare are very variable as the cloves of different species vary greatly in size. When planting stock is used, the planting stock bulbs later than main crop should be harvested, as very mature bulbs are harvested increases the ease of clove separation, otherwise smaller bulbs will be produced. The upper 15-20 cm of the soil surface should be always moist, but not wet, because most of the root system will be grown in this depth.

Yield and quality vary with climate, region, altitude, soil and pH, cultural practices, and the variety of garlic. The term "biological elasticity" describes garlic's ability to acclimate to these factors over time. No one practice is best suited for every situation. Conversation with local growers who have experience growing garlic and experiment with different cultural practices and varieties is advisable (Siktberg *et al*., 2006). Lighter soils need more frequent water applications, but less water applied per application. Water stress during the growing season can cause bulbs to be smaller and is thought to also cause a multiple stem disorder (Anonymous, 2004).

The origin of garlic is thought to be in Central Asia (India, Afghanistan, West China, Russia) and spread to other parts of the world through trade and colonization (Tindal, 1986).Today, garlic is grown in temperate and tropical regions all over the world, and many varieties have been developed to suit different climates. The area covered by garlic exceeds 1,142,000 ha and its average productivity is estimated to be 12t ha-1 in the world (FAO, 2003). It has a wide area of adaptation and cultivation throughout the world. The majority of the garlic is produced in Asia (87%), China and India, being the largest producers, collectively accounting for 78% of the production but in Africa accounting only 2.8% of the total world production (FAOSTAT, 2011). On the global scale, the top 5 producing countries are: China producing, 20.06 million tons India 1.25 million tons. Bangladesh, 0.3 Egypt, 0.26 and Russia, 0.26 million tons per year (FAOSTAT, 2014).

2.1.3 Growth and Development

Garlic is a cool-season plant; it makes all vitality and leaf growth while the temperatures are cool and the day is short. As the temperature becomes warm and the day is lengthen, the plant stops making leaves and begins to form bulbs. Cloves or young plants exposed to temperatures of between 0°C and 10 °C for one to two months hastens subsequent bulbing under long days (Moore & Gough, 2010). Garlic is adapted to cool climates and should not generally be planted at altitude below 2000 m above sea level. Amount of rainfall during the growing period (4 to 6 months) should be 600 mm to 700

mm. The optimum temperature for growing garlic lies between 12°C and 24°C. Garlic withstands moderate frost on well-drained soils, rain fed crops may be planted on flat beds; but on heavy soils, which are poorly drained during the rains; it is advisable to plant on ridges as for irrigated crops. It is essential to select land with high fertility or to apply considerable quantities of manure or fertilizers to obtain good yields (CVR, 2009).

A study in the Netherlands conducted by (Messiaen & Rouamba, 2004) during the life cycle of plant under go successive stages of growth and development, the dormancy of mature cloves, induced by the temperature of 25-30 °C is eliminated most quickly at 6-7 °C vegetative growth is optimal at 18-20 °C. When 12-14 leaves have been produced, bulb swelling is induced at temperature below 20 °C. There is considerable physiological variability amongst garlic cultivars. The total growing period varies from 4 months to about 9 months.

The matured garlic cloves planted in the fall go through a dormant period. Garlic cloves require a period of 6-8 weeks of cool weather after planting (below 4.4 °C to undergo vernalization inducement to bulb) by low winter temperatures. During the fall and winter, cloves develop their root systems and initiate some top growth. The clove swell considerably, forming a globular bulb with many fine roots. A pair of intertwined leaves emerge from the terminal end of the bulb and eventually break through the soil, depending on the weather and location. Leaf development accelerate with flat, dark green leaves on stems reaching a height of 30 cm or more. Proper bulbing is a function of adequate growth, vernalization, and subsequent growth under longer days. As temperatures rise and day length increases, bulb formation begins. Results show the following development stages in garlic: Sprouting: from sowing to 20-30 days, adventitious roots, leaf emergence and total soluble carbohydrate assimilation in seed cloves are observed. Shoot growth: from the end of sprouting until 140 days after sowing (McLaurin, 2012).

For proper growth, garlic needs a period of cold followed by a period of light and heat. Although garlic requires low temperatures in preparation for bulb development, increased day length and heat are necessary for bulbs to begin forming (Siktberg *et al*., 2006). Garlic is a species of vegetative propagation, showing high morphological diversity. Besides, its clones have specific adaptations to different agro-climatic regions (Paredes *et al.*, 2007). Garlic shows wide morphological and agronomic variations in characteristics such as color and size of the bulb, plant height, number and size of the cloves, days to harvesting, resistance to storage capacity, dormancy and adaptation to agro-climatic conditions (Figliuolo *et al*., 2001).

2.1.4. Importance of Garlic (*Allium sativum L.*)

Garlic is predominantly used for flavoring and serving dishes and as a medicinal plant all over the world (Wiczkowski, 2011; Nagy *et al*., 2015). Garlic is a basic flavoring in many types of dishes ranging from vegetable soup, meat, salad, tomato combination, spaghetti, sausages and pickles (Brewster, 1994). Similar to green onions, it is eaten as green and blenched tops in different ways as fresh and cooked as well as immature bulb consumption is common especially in tropics (Rabinowitch & Brewster, 1990). It is balancing with onion, tomato, or ginger, and be also taken raw, boiled or mixing with honey, meat, cheese, butter and with milk or with coffee. Garlic the length of with cinnamons is used as fish and beef additive, and display antimicrobial material goods at temperature as high as 120°c; the amalgamation can also be second-hand to safeguard fried and cavernous fried food (Ranjan *et al*, 2012). The garlic plant's bulb is the largest part regularly basis worn part, which is divided into numerous fleshy sections call cloves, and are second-hand for consumption as raw or cooked. It has a trait strong, spicy flavor that mellows and sweetens significantly with cooking (Katzer, 2009). They are milder in taste than the bulbs and are often consumed while immature and still tender (Thompson, 1995).

Garlic has also medicinal value which is well recognized in the control and treatment of hypertension, worms, germs, bacterial and fungal diseases, diabetes, cancer, ulcer, rheumatism etc. (Kilgori *et al*., 2007). It has high medicinal properties which a secret armory consists of more than 33 active sulfur-containing substances that do battle with enemies such as bacteria, viruses, and fungi. *Alliin* (S-allylcysteine sulfoxide) the most abundant sulfur compound in garlic which is present at 10 mg/g unsullied and 30 mg/g in dry garlic. It is primary odourless, and a precursor of *allicin* (Stoll and Seebeck, 1948). At the time fresh garlic is chop or crushed, the enzyme *alinase* converts *alliin* into *allicin* (Kouroundaki & Rekka, 1991). *Allicin* is a greasy, somewhat yellow fluid that gives garlic its only one of its kind scent. The *allicin* generated is very unstable and quickly changes into series of another sulfur-containing compound such as diallyl disulfides and this break down occurs within hours at room hotness and throughout food preparation (Blania & Spangenberg, 1991). It decomposes in stomach acids to release diallyl sulphide and diallyl disulphide (Mohamed *et al,* 2003). Allicin has a wide range of biological and pharmacological activities, such as anticoagulation, antihypertensive, antimicrobial, antibiotic, antiparasitic, antimycotic, antiviral, antitumoral, anti-oxidant, anti-aging, antiplatelet, detoxifies heavy metals, fibrinolysis, hypolipidaemic (lipid-lowering) and immune enhancer and modulator (Munchberg *et al.*, 2007).

2.1.5. Composition and Uses

According to Volk & Stern (2009) bulb elemental analysis in Northern Colorado, bulbs were significantly different was performed for the elements on freeze-boron, magnesium, phosphorus, potassium, and nitrate-nitrogen. They also reported that Nevada bulbs were high in potassium, sulfur, and zinc and were high in sodium. But the growth and yield of garlic is influenced by different nutrition management and other factors during their production in the field (Diriba, Sh *et al*., 2013).

Its flavor is due to a group of sulfur-containing compounds and lachrymatory effect of garlic is due to the high proportion of 2-PECSCO (2-propenyl L-cysteine sulphoxide) content. The bulb contains about 1.4% of the fresh weight as alliin (Brewster, 1994). Garlic is rich in sugar, protein, fat, calcium, potassium, phosphorus, sulfur, iodine, fiber and silicon, in addition to vitamins. Its pungent flavor makes it used mainly as a spice, seasoning and flavoring for food stuff involving both green tops and bulbs (Kilgori *et al*, 2007). Moreover, it contains considerable amounts of Ca, P and K and its leaves are sources of protein, vitamin A and C (Mahmood, 2000; Samavatean *et al*., 2011). Garlic has higher nutritive value than other bulb crops: 30–35% dry matter, 6–7% protein, 0.2% lipid, 23–28% carbohydrate, 0.7–0.9% fiber, 1.1–1.4% ash matter and vitamins, especially B1, B2, B6 and C. Garlic also contains antibiotics *garlicin* and *allistatin*, many enzymes, amino acids, universal substances, including trace elements (Maly *et al.,* 1998).

Hector *et al*. (2012) also reported that bulbs are consumed fresh, totally or partially dried, and pickled. Although bulb consumption is more common, tender shoots sometimes are a delicatessen for sophisticated cuisine. These shoots may be prepared like asparagus. Each clove is capable to develop a new plant, since they have an apical shoot bud that can elongate even though they are not sown. This shoot is apparent after three months of the harvest, depending on the genotype and conservation conditions. These stems produce a strong odor from two compounds: alliin and diallylsulfide.

2.1.6. Garlic Production and Productivity in Ethiopia

In Ethiopia, garlic is one of the important bulb crops produced for home consumption and is a sources of income to many peasant farmers in many parts of the country (Metasebia, M. & Shimelis, H, 1998; Getachew, T. & Asfaw, Z., 2000). It is mainly used for flavoring and seasoning vegetables in different dishes. It also has many medicinal properties (Abreham, M *et al.,* 2014). As a cash crop, it is used to earn foreign currency by exporting to Europe, the Middle East, Africa countries and the USA (Kilgori *et al.,* 2007). Due to it is the cash crop in many parts of the country, increasing its productivity per unit area and production will enable farmers to get encouraging returns and contribute its role in achieving the food security goals (Metasebia, M. & Shimelis H, 1998). Out of the total production,

greater than 64% was used for household consumption and 22% was for market. Dehydrated garlic and extracts are also fast replacing fresh bulbs for industrial and home usage in the production of drugs, insecticides and explosives (Pursglove, 1992; kilgori *et al*., 2007).

Garlic is produced mainly in the mid and high lands of the country (Getachew, T. & Asfaw, Z., 2000; CACC, 2002). The bulk of garlic for the domestic market is produced in homestead gardens of subsistence farmers especially in Ambo, Debrework, Adiet, Sinnana and many other areas of Ethiopian highlands, and characterized by low yields of about 11.7 t ha-1 (CSA, 2010) and in East shewa about 11.31 t ha-1 (CSA, 2012). Of the total production of Alliums in the country, Ethiopia, the area coverage of garlic in 2007/2008 was 9,316.90 ha, and total production was about 103,541.68 tonnes of bulbs, which was produced by 1,490,681 landholders (CSA, 2008). In 2009/2010, CSA (2010) reported that national garlic cultivated land was 15,361 ha owned by 2.079 million landholders. According to CSA (2011) the number of holders practicing garlic farming is considered to be 1,411,151 farmers which are much less than that of grains or cereals crops. In the same year the area under garlic production was estimated 10,690.41hectars and the production obtained from these hectares were about 1,284,409.36 quintals as well as the average production predicted 120.15 quintal yield per hectare. In Ethiopia, 16,411.19 ha of land was covered under garlic cultivation with a production of about 159,093.58 tones (CSA, 2014). Out of world total production, 24,939,965 ton Ethiopia shares 0.39% of the world total production and it occupies 12th in the series of top-producing countries (FAOSTAT, 2014). According to CSA (2015/16) report, its production covers 6,789.11 hectare with a total production of 5,781.767 tons and 8.5 ton/ha on average.

2.1.7. Garlic (*Allium sativum L.)* Propagation and its challenges

Garlic is mainly propagated by vegetative methods and its improvement through breeding programs is limited due to difficulties in flower induction (Haider *et al*., 2015). As garlic bulbs cannot be stored for more than 6-8 months, maintenance is usually done by planting them in the field every year. However, infection with different pathogens and pests makes the maintenance of the field a costly affair (Mishra *et al*., 2014). Prolonged conservation in the field leads to decreased yields and sometimes, to their total destruction by the accumulation of viruses in the bulbs (Asha Devi *et al*., 2007). Systemic viral infection of garlic plants causes significant losses in crop production around the world (McDonald *et a*l., 2004; Perotto *et al*., 2010). Furthermore, vegetative propagation facilitates the transmission of several virus species throughout the plant life cycles (Parrano *et al*., 2012). vegetative propagation has various constraints for the crop: A low multiplication rate, Expensive and short-term storage that requires wide spaces, Transmission of phytopathogens (fungi, viruses, nematodes) through generations which can cause a yield decrease of up to 70%, and Loss of

product quality (Kamenetsky, 2007). Virus infection was shown to reduce the bulb yield by 20% to 60% and up to 80% in case of mixed depending on cultivar and stage of infection (Lot *et al*., 1998). Many of the elite garlic cultivars are susceptible to diseases caused by viruses, nematodes and fungi and suffer from insect pests (Verbeek *et al*., 1995).

The viruses that tend to cause it severe damages are potyviruses, such as Leek Yellow Stripe Virus (LYSV), Garlic Yellow Streak Virus (GYSV) and Onion Yellow Dwarf Virus (OYDV). Some carla-viruses, like Common Latent Virus (GCLV) and Shallot Latent Virus (SLV) can also infect the garlic plant (Messiaen *et al.,* 2004).

One of the most widely spread diseases in garlic producing countries is white rot, caused by the fungus *Sclerotium cepivorum,* which provokes wilting of the plant and rotting of the bulb. Its sclerotia can survive in the soil for up to 20 years, therefore limiting garlic production (Delgadillo-Sánchez, 2000). Various bacteria (*Bacillus* spp., *Erwinia* spp., *Pseudomonas* spp.) can also cause damages on bulbs upon storage. Garlic can also be affected by pests like thrips (*Thrips tabaci*), which are insects that infest plants from early developmental stages and cause severe foliage damages (Bujanos-Muñiz & Marín-Jarillo, 2000).

2.2. Tissue culture of garlic (*Allium sativum L*)

Biotechnological tools such as micropropagation, meristems culture (to obtain virus-free plants), somaclonalvariation, and genetic transformation, have contributed high to the propagation, preservation and breeding of garlic (Fiserova *et al*., 2016).

2.2.1 Micro Propagation

The micropropagation technology has a vast potential to produce plants of superior quality, isolation of useful variants in well-adapted high yielding genotypes with better disease resistance and stress tolerance capacities (Brown & Thorpe, 1995). Studies related to the application of tissue culture techniques such as micropropagation for garlic production started in 1970. This technique proved to be advantageous over clove reproduction, as it only requires cells or small tissue fragments to generate a high number of plants. Micropropagation can be carried out via two morphogenetic ways: organogenesis, which results in the formation of organs (shoots or roots), and somatic embryogenesis, which leads to the formation of structures having a similar or equal morphology to that of a zygotic embryo. Both processes can involve (indirect) or not (direct) a previous callus phase. Morphogenetic ability in garlic decreases as the callus grows older and the emergence of abnormal plants increases (Novak, 1990).

2.2.2 Shoot tip (meristems) culture

Due to the difficulties in flower induction in garlic species, breeding programs have been limited to clonal selection and production of virus-free materials via meristem culture (Mahajan, 2016). Shoot-tip culture has been used for decades for virus removal in vegetatively propagated plants (Ghaemizadeh *et al*., 2014) and from garlic in-vitro plants (Pramesh & Baranwal, 2015). Virus-free clones produced through meristem culture showed higher yield with improved quality (Taskin *et al*., 2013). Therefore, the tissue culture techniques have got a high potential for the improvement of garlic in respect of yield and quality (Mukhopadhyay *et al*., 2005). The use of meristems or shoot-tips as an explant for micropropagation of multiple bulb formation is more suitable than another source of explants (Roksana *et al*., 2002).

Early studies reported *in vitro* plant regeneration of garlic through somatic embryogenesis using shoot tips as explants (Abo El-Nil, 1977; Kehr & Schaeffer, 1976; Koul *et al*., 1994; Novak, 1980). (Nagakubo *et al*., 1993) developed a micropropagation system of garlic by the combination of initial shoot-tip culture, shoot multiplication and *in vitro* bulblet formation. Garlic shoot tips were cultured on LS medium (Linsmaier *et al*., 1965) containing 1 μM indole- 3-acetic acid (IAA) and 1 μM 6-benzyladenine (BA) to regenerate proliferative shoots.

Meristem culture is a technique used for obtaining virus-free plants, and also for micropropagation. (Messiaen et al., 1970) were the first in regenerating garlic plants from meristems. Shoot or bud formation from callus was achieved using a combination of 6-furfurylaminopurine (kinetin), indol-3-acetic acid (IAA) and 2, 4-diclorophenoxiacetic acid (2, 4-D) (9.28μM, 11.4μM and 4.5μM, respectively). Likewise, (Havránek & Novak, 1973) obtained numerous growth areas on calli produced from meristems on a culture medium with 2, 4-D. The subculture of calli to a medium containing IAA (11.4μM) and kinetin (46.5μM) induced the formation of adventitious shoots.

In a different work, calli obtained from meristems of three garlic varieties (*Rose de Lautrec*, *California Early* and *California Late*) cultivated in a medium with 2, 4-D (4.5μM) and IAA (5.7μM) produced adventitious shoots when transferred to a medium with IAA (5.7μM) and kinetin (4.6μM) (Kehr & Shaeffer, 1976). For his part, (Abo-El-Nil, 1977) started cultures from meristems, stems and leaf discs of the variety *Extra Early White*, on a medium with pchlorophenoxyacetic acid (p-CPA) (10μM), 2,4-D (2μM) and kinetin (0.5μM), from which callus formation was achieved, which in turn resulted in the formation of adventitious shoots in the presence of kinetin (10μM) and IAA (10μM). In other works, meristems were cultivated on B5 medium (Gamborg *et al*., 1968) with 2.5μM 2-isopentenyladenine (2ip) and 0.55μM naphtalenacetic acid (NAA) (Bhojwani, 1980), or in Linsmaier

and Skoog (LS) (Linsmaier & Skoog, 1965) medium with 9µM N6-benzyladenine (BA) alone or with 11.1µM NAA, and multiple shoots were obtained (Osawa *et al*., 1981).

(Nagakubo *et al*., 1993) developed a micropropagation protocol for six varieties (*Isshuwase*, *Isshu-gokuwase*, *Shanhai*, *Santo*, *Furano* and *Howaito-roppen*) starting from shoot-tips which were cultivated in a medium supplemented with NAA (1µM) and BA (1µM). Regenerated adventitious shoots were subcultured for four generations in presence of NAA (5µM) and BA (10µM) for their multiplication. The application of this protocol enables the production of 256 plants from one shoot-tip in 10 months. A novel regeneration protocol was developed by dissecting and sectioning longitudinally the shoots developed from cloves of the cultivar *Extra Select Sets*. These shoots were cultivated on a medium with BA (8µM) and NAA (0.1µM), and after five weeks they produced eight more shoots compared to the ones that had been kept intact (Mohamed-Yassen *et al*., 1994).

The roots developed from adventitious shoots obtained *in vitro* also allowed garlic micropropagation when cultivated on a medium that induced callus formation and then transferred to a medium with BA (13.3µM) and 4-amino-3,5,6-trichloro-picolinic acid (picloram) (1.4µM). This method enables the regeneration of 5.4 shoots per explant (Myers &Simon, 1998). A protocol named one-step was developed when the same type of explants was cultivated on a modified B5 medium supplemented with 0.1µM 2,4-D, 11.1µM NAA and 13.6µM BA, under two light conditions (16 hours photoperiod and complete darkness). In general, the root tips cultivated under low light conditions displayed the highest percentage of organogenic calli. The application of this protocol allowed the formation of callus and the regeneration of 250 shoots per gram of callus in the same culture medium and under the same light conditions (Martín-Urdíroz *et al*., 2004). (Zheng *et al*., 2003) also obtained adventitious shoots by using apical and non-apical root fragments, originating from plants generated *in vitro* of four cultivars grown in Europe (*Messidrome*, *Morado de Cuenca*, *Morasol* and *Printanor*). The explants were cultivated on MS medium with 4.5µM 2, 4- D and 0.5µM 2iP to induce calli formation, which was then transferred to a medium containing 4.7µM kinetin to promote shoot differentiation. The highest regeneration rate was obtained when non-apical fragments were used, although the difference was not significant.

In a different work, (Ayabe & Sumi, 1998) used the stem disc (consisting in the apical meristem and the lateral buds of the clove) to regenerate plants of the cultivar *Fukuchihowaito*. When this was cut into various fragments and then cultivated on a medium with BA (0.4µM), 20-25 adventitious shoots were obtained. The same result was observed when protoplasts isolated from shoot primordia were cultured in the presence of NAA (0.5µM) and 2iP (2.4µM), adenine and coconut milk (Ayabe *et al*., 1995). (Barandiaran *et al*.,1999) used immature bulbs of 23 accessions as a source of axillary buds,

which were cultivated during six weeks on B5 medium with 2.5µM 2iP and 0.55µM NAA (establishment phase). Multiplication of regenerated shoots was done on the same culture medium and 20 weeks later shoot clusters were separated to cultivate them individually and to induce bulb formation at a low temperature (4°C). Although plants and bulbs were obtained for all accessions under tested conditions, response depended on genotype (accession). Three months later, 60% of bulbs that were transferred to soil survived and produced shoots. This protocol allowed the use of the same culture medium for all phases of micropropagation (establishment, multiplication and bulb formation) and for all accessions, which enabled the handling of all materials tested at the same time, as only three subcultures were required for seven months.

(Haque *et al*., 2003) developed a protocol for plant regeneration and bulb formation from the shoot and root meristems of the cultivar *Bangladesh Local*. Meristems were cultivated on MS medium without growth regulators or containing various concentrations of BA (1-10µM) and NAA (1-5µM). None of the combinations of regulators tested produced a higher response than the one observed in their absence (95.5%). The presence of these compounds suppressed shoot formation in a directly proportional manner to concentration; 45% of root explants formed adventitious shoots, 60% of which produced bulbs. Although a higher number of buds resulted in shoot formation, the root meristems produced more shoots per explant (20). Bulbs derived from root meristems were smaller than the ones derived from bud meristems.On the other hand, (Luciani *et al*., 2006) tested different explants for micropropagation of variety *069*, which were cultivated on BDS medium (Dustan & Short, 1977), supplemented with picloram, 2, 4-D and BA. The basal plates and meristems resulted in the highest values of shoot regeneration, and 2, 4-D proved to be better than picloram for inducing callus and shoot formation. By using a combination of 0.25µM 2, 4-D and 4.43µM BA, 100% of explants were able to produce calli, which differentiated into both embryos and shoots.

2.3 Factors affecting in vitro propagation

Murashige & Skoog medium (MS medium) is most extensively used for the vegetative propagation of many plant species *in vitro*, because most plants react to it favorably. The pH of the medium is also important that affects both the growth of plants and the activity of plant growth regulators. Most tissue cultures are grown at pH 5.2 - 5.8 with pH adjustments being made before to autoclaving (Skirvin *et al*., 1986). The different factors affecting *in vitro* propagation of plants, i.e. media, type of explant, genotype, source and orientation of explant, mineral nutrition, growth regulators, carbon source, gelling agents and the main ones are presented below.

2.3.1 Source and type of explant

Source and type of explant is one of the important factors in *in vitro* propagation of plants. Type of explants like leaf, petiole, hypocotyl, epicotyl, embryo, internode and root explant and source of explant significantly affect the regeneration of plants (Ali & Mirza, 2006; Kumar *et al*., 2011; Nitish & Reddy, 2011). The most commonly used explants are shoot tips, nodal buds and root tips. Large explants can increase chances of contamination and small explants like merisems can sometimes show less growth (Fowler, 1993; Staba & Seabrook, 1980)**.**

The fact that the source of explant has a different capacity of regeneration are well documented (Tileye, F *et al*., 2005). *In vitro* explant, in general, has a better potential for organogenesis as compared to *in vivo* explant. The difference may be due to the level of endogenous hormones present in the plant explant. The seedling explant is more responsive or meristematic than mature plants (Tileye, F *et al*., 2005) due to different level of plant hormones present in the plants

2.3.2 Type and composition of culture medium

Various basal media like White medium, Nitsch & Nitsch medium, B5 medium and Gamborg medium have been used for micropropagation (Diallo *et al*., 2008). The composition of the culture medium is a major determinant of *in vitro* growth of plants. Plant tissue culture medium contains all the nutrients required for the normal growth and development of plants. It is mainly composed of macronutrients, micronutrients, vitamins, other organic components, plant growth regulators, carbon source and some gelling agents in case of solid medium (Murashige & Skoog, 1962). Selection, strength and combination of media are also one of important parameter for optimizing the regeneration protocol of *in vitro* propagation (Diallo *et al*., 2008).

According to the recommendations of the International Association for Plant Physiologists, the elements required by plants in concentrations greater than 0.5 mmol 1-1 are referred to as macroelements, relatively large amounts of some inorganic elements (the so-called major plant nutrients): ions of nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg) and sulphur (S); and those in concentrations less than 0.5 mmol 1-1 are microelements, small quantities of other elements (minor plant nutrients or trace elements): iron (Fe), nickel (Ni), chlorine (Cl), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo), (De Fossard, 1976).

Sucrose is by far the most used carbon source, for several reasons. It is cheap, readily available, relatively stable to autoclaving, and readily assimilated by plants; is an important component in medium and its addition is essential for *in vitro* growth and development of plants because photosynthesis is insufficient, due to the growth taking place in conditions unsuitable for

photosynthesis or without photosynthesis (Pierik, 1997). Other carbohydrates can be also used, such as glucose, maltose and galactose as well as the sugar-alcohols glycerol and sorbitol (Fowler, 2000). Four vitamins; *myo*-inositol, thiamine, nicotinic acid, and pyridoxine are ingredients of Murashige & Skoog (1962) medium and have been used in varying proportions for the culture of tissues of many plant species . The requirements of cells for added vitamins vary according to the nature of the plant and the type of culture.

2.3.3 Plant Growth Regulators

The highest rate of micropropagation often depends not only on the selection of the most suitable explant, but also on the discovery of the correct combination of growth regulators, and/or the best nutritional composition of the medium (Mathews, 1987) for particular explants. The growth regulators are required in very minute quantities. There are many synthetic substances having growth regulatory activity, with differences in activity and species specificity. It often requires testing of various types, concentrations and mixtures of growth substances during the development of a tissue culture protocol for a new plant species (Bhojwani, 1996).

Auxins (IAA, IBA, NAA or 2, 4-D) are involved in the regulation of several physiological processes, for example, apical dominance and formation of lateral and adventitious roots. These growth regulators generally cause cell elongation and swelling of tissues, cell division, callus formation and the formation of adventitious roots as well as the inhibition of adventitious and axillary shoot formation (Pierik, 1997). Also auxins are often added to the culture medium to promote the growth of callus, cell suspensions or organs, and to regulate morphogenesis, especially in combination with cytokinin (George, 1993).

Cytokinins are derived from adenine (aminopurine) and play an important role in the *in vitro* manipulation of plant cells and tissues (Torres *et al*., 2001). The most common cytokinins used are kinetin, 6-BenzylAminoPurine (BAP), thidiazuron (TDZ) and zeatin and 2iP (Pierik, 1997). These hormones are concerned with cell division, modification of apical dominance, shoot differentiation, etc. In tissue culture media, cytokinins are incorporated mainly to initiate cell division and differentiation of adventitious shoots from callus and organs. These compounds are also used for shoot proliferation by the release of axillary buds from apical dominance (Bhojwani, 1996).

Gibberellins are a group of compounds that are not necessarily used in the *in vitro* culture of higher plants. In some species, these growth regulators are required to enhance and in others to inhibit growth (Razdan, 1993). Gibberellic acid (GA3) is the most common gibberellin used. It induces the elongation of internodes and the growth of meristems or buds *in vitro* (Pierik, 1997).

All kinds of plant tissue cultures produce ethylene, and the rate of production increases under stress conditions. In cultures, ethylene is also produced when the organic constituents of the medium are subjected to heat, oxidation, sunlight or ionizing radiation (Matthys *et al.*, 1995).

Abscisic acid is most often required for the normal growth and development of somatic embryos and only in its presence do they closely resemble zygotic embryos (Ammirato, 1988). It is also known to promote morphogenesis in Begonia cultures. There has been some interest in the application of growth retardants, such as paclobutrazol, during the acclimatization stage of micropropagation to reduce hyperhydricity and regulate leaf growth and function in relation to control of water stress (Smith & Krikorian, 1990).

3. MATERIALS AND METHODS

3.1 Plant material and culture establishment

The present study was conducted at Melkassa agricultural research center (MARC) on two cultivars of garlic varieties namely “Holetta Local” and “Kuriftu” which was obtained from Debre Zeit agricultural research center (DZARC). Garlic cloves were separated from the compound bulb, peeled manually and washed thoroughly in running tap water with powder soap (omo) several times. It was then washed for three minutes with autoclaved distilled water in the laminar airflow cabinet. The cloves were then rinsed with 70% (v/v) ethanol for 30 s, rinsed five times with sterile distilled water, disinfected with 15% chlorine bleach solution(15ml chlorine bleach + 85ml water + two drops of tween-20) for 15 min followed by rinsing five times in sterile distilled water. Shoot tips of the sprouting plantlets containing the apical meristems of 0.5 to 0.8 cm were aseptically dissected by slitting out the sprouted cloves by using sharp surgical blades and forceps under a laminar airflow hood. Shoot tips were inoculated in sterile test tubes each containing 20 ml of half-strength MS medium (Murashige & Skoog, 1962) for shoot initiation.

3.2. Stock Solutions and medium preparations

3.2.1. MS medium stock solution preparation

Throughout this study, Murashige & Skoog (MS) medium (1962) was used. For this, a full-strength stock solution containing the macronutrients, micronutrients, vitamins, KI, CaCl2 and Fe-EDTA were prepared by weighing and dissolving the powders of each component in distilled water (appendix 1). After all the components were fully dissolved and evenly mixed using a magnetic stirrer, the solution was dispensed into plastic bottles and stored at 4°C until used. Calcium chloride ($CaCl_2$) and potassium iodide (KI) were dissolved separately and then added to the rest solution to avoid precipitation.

3.2.2. Plant growth regulators stock solution preparation

In this study, plant growth regulators such as 6-Benzyl Amino-purine (BAP) alone for shoot induction; BAP and Naphthalene Acetic acid (NAA) for shoot multiplication and NAA alone for rooting were used. The stock solutions for all the plant growth regulators were prepared by weighing the powder in 1mg/1ml (W/V) ratio. The powders were first dissolved by adding 2-3 drops of NaOH and HCl and evenly mixed through stirring. The volume was finally adjusted by adding double distilled water and stored in a refrigerator at 4°C until used.

3.2.3 Culture medium preparation

A liter of MS medium was prepared by taking 12.5ml of macronutrient, 0.5ml of micronutrients, 5.02ml of Fe-EDTA, 0.5ml of KI, 1.56ml of $CaCl_2$ from stock solutions and vitamins; 0.1g of Thiamine, 0.325g pyridoxine and 0.05g myoinositol added sequentially. Then, 15gm of sucrose was added while stirring. After adding the growth hormones, pH was adjusted to 5.8 using 1N NaOH and 1N HCl and adding 7gm of agar powder, the final volume was adjusted to 1000ml with distilled water and autoclaved at 121ºC for 20 min.

3.3 Shoot induction and Shoot Multiplication

To stimulate shoot induction from Shoot tips of the two garlic varieties "Holetta Local" and "Kuriftu", 30 excised shoot tips (one shoot tip per test tube) of each varieties were inoculated on half strength MS medium supplemented with 10 mg/l 6-benzyladenine (BAP) and containing macronutrient, micronutrient, vitamin, Fe-EDTA and FeSO4 mixture, sucrose, and agar per liter as mentioned above.

The cultures were incubated under controlled temperature of 25 ± 2°C with photo-period of 16:8 hours light and dark and photo flux density of 200lux for three weeks. Sub-culturing was done at three weeks intervals. Data of the explants that produced shoot was scored after six weeks of incubation.

Shoot multiplication was also done on half-strength MS medium supplemented with the growth regulators of different concentrations of BAP and NAA. The preparation procedures of medium were as of shoot induction medium. Shoots of ≥1.5 cm long were transferred to the multiplication medium by dissecting and sectioning longitudinally the induced shoots carefully by using a small knife under sterilized conditions. Induced Shoots were used as explants and inoculated onto prepared MS basal medium containing the concentrations of BAP (0.5, 1.0, 1.5 and 2.0 mg/l) in combination with NAA (0.25 mg/l). Growth regulators free MS medium was used as a control. All experiments (total of 10 treatments) for two varieties were performed by using three explants per culture glass jar containing 40 ml MS medium with three replications for Shoot Multiplication for four weeks. Cultures were maintained at 25 ± 2°C with Photo period of 16:8 hours light and dark with photo flux density of 200lux for four weeks. The number of shoots per explant and Shoot length (cm) per explant were recorded after four weeks of culture.

3.4 Root initiation

Shoots of 1.0–1.5 cm length were harvested from multiple plants and then cultured in half-strength MS medium supplemented with different concentrations of NAA (0.5, 1.0, 1.5 and 2.0 mg/l). In

addition, growth regulators free MS basal medium was used as a control. All experiments (a total of 10 treatments) for two varieties were performed by using two explants per culture glass jar containing 40 ml MS medium with three replications for four weeks. The cultures were maintained at 25 ± 2°C with a Photoperiod of 16:8 hours light and dark with a photo-flux density of 200lux. Data of the Root number per explant, Root length (cm) and rooting percentage (%) were recorded after four weeks of culture.

3.5. Acclimatization

For acclimatization, garlic plantlets with well-developed roots were removed from the culture medium and transplanted into unsterilized soil containing red soil, compost and sand at a ratio of 1:1:1 in the greenhouse, at Makassar agricultural research center (MARC). The number of survived plants and the acclimatization percentage (%) among the total of forty-two plantlets of the two varieties were recorded after two weeks

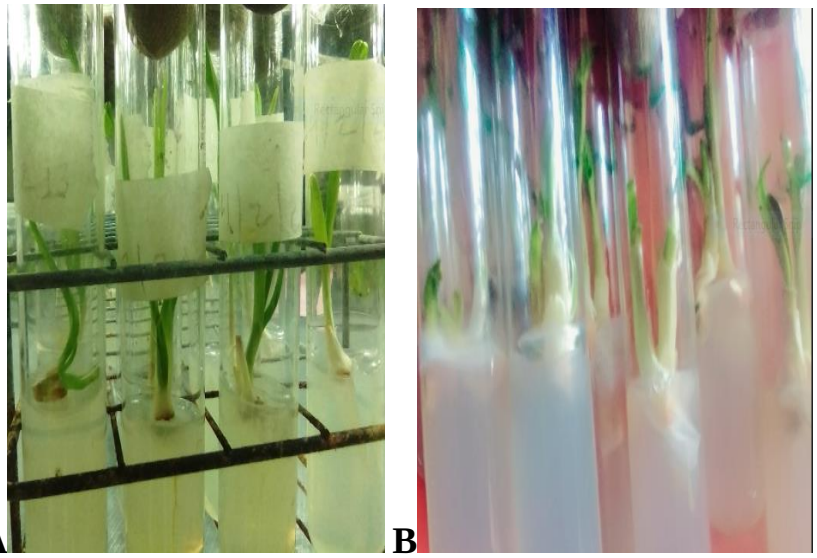
3.6 Data analysis

After recording the number and length of the shoot; the number, length and percentage (%) of root and the number of survived plants and Acclimatization percentage (%), the data were statistically analysed using SAS software of version 8.0.1.

4. RESULT

4.1 Shoot induction

The shoot induction responses of the two plant genotypes cultured on MS medium containing 10mg/l BAP were observed from the shoot tips culture after Six weeks of culturing. While the initiation of direct shoot induction was observed in both cultivars, the response rate was variety-dependent. The maximum shoot initiation was 27 out of 30 (90%) in “Holetta Local” and 24 out of 30 (80%) in “Kuriftu”. The explant without direct shoot induction from shoot tip culture had no growths in “Kuriftu”. In “Holetta Local”, the explant without direct shoot induction had a slight callus on this medium. 85% explant of these two cultivars had growth.



A **B**

Figure 1: Shoot induction from the shoot tip cultures on half MS medium supplemented with 10mg/l BAP alone.

(A) Shoot induced of “Holetta Local” on half MS medium supplemented with 10mg/l BAP after 6 weeks of shoot tip culture, (B) Shoot induced of “Kuriftu” on half MS medium supplemented with 10mg/l BAP after 6 weeks of shoot tip culture.

4.2 Shoot Multiplication

After four weeks of incubation, the shoots induced were responded differently to the difference in multiplication medium varying concentration of BAP with the combination of NAA in medium including control (Table 1 and Figure 2). Among the treatments, 2.0 mg/l BAP + 0.25 mg/l NAA was

found to be the best for “Holetta Local” where the maximum of 5.48±0.28 shoots number was recorded followed by 4.19±0.14 on 1.5 mg/l BAP + 0.25 mg/l NAA combinations. For “Kuriftu”, the maximum of 4.86±0.25 shoot number have been counted on 1.5 mg/l BAP+0.25 mg/l NAA followed by 3.52 ± 0.15 on 2.0 mg/l BAP+0.25 mg/l NAA. The highest shoot length of 7.63±0.73 cm and 6.85±0.33 cm were recorded on 1.5 mg/l BAP + 0.25 mg/l NAA for “Holetta Local” and “Kuriftu” followed by 7.4±0.14 cm and 6.00±0.28 cm on 2.0 mg/l BAP+0.25 mg/l NAA for both varieties respectively. In all combinations of BAP with NAA, with the increase in the concentration of BAP (from 0.5 to 2.0 mg/l) increased the number of shoots but increase of BAP (from 0.5 to 1.5 mg/l) increased the length of shoots and decreased length of shoots on 2.0 mg/l BAP for “Holetta Local”. For “Kuriftu”, the number and length of shoots increased while increasing the concentration of BAP from (0.5 to 1.0, and 1.5) but decreased on 2.0 mg/l. On the other hand, shoot proliferation was not efficient in MS medium free growth regulators.

A  **B** 

C  **D** 

E  **F** 

Figure 2: Shoot multiplication from shoot tip explants of “Holetta Local” (A, C and E) and “Kuriftu” (B, D and F) garlic varieties on MS medium containing different concentrations of BAP in combination with NAA and no PGR (control).

(A) 2.0 mg/l BAP + 0.25 mg/l NAA, (B) 1.5 mg/l BAP + 0.25 mg/l NAA, (C) 1.5 mg/l BAP + 0.25 mg/l NAA, (D) 2.0 mg/l BAP + 0.25 mg/l NAA, (E) and (F) control

Table 1: Mean number of shoots per explants and Average Shoot length in a different BAP with the combination of NAA medium.

Mean values of shoot number and shoot lengths are indicated as Mean ±SE.

BAP (mg/l)	NAA (mg/l)	Mean No. of shoot/explants Mean ±SE		Average Shoot length(cm) Mean ±SE	
		“Holetta Local”	“Kuriftu”	“Holetta Local”	“Kuriftu”
0.5	0.25	2.62 ±0.27	2.42±0.42	5.75±0.08	4.50±0.28
1.0	0.25	3.93±0.53	2.58±0.43	7.00±0.28	5.67±0.33
1.5	0.25	4.19±0.14	**4.86±0.25**	**7.63±0.73**	**6.85±0.33**
2.0	0.25	**5.48±0.28**	3.52±0.15	7.4±0.14	6.00±0.28
0.0	0.0	1.63±0.64	1.23±0.76	3.83±0.16	3.45±0.33

4.3 Rooting and Acclimatization

For this experiment, shoots with 1.0–1.5 cm length were harvested from multiple shoots and cultured on half-strength MS rooting media supplemented with different NAA concentrations (0.5, 1.0, 1.5 and 2.0 mg/L) including control ((Table 2 and Figure 3).

Among the different treatments, medium supplemented with 1.0 mg/l NAA was found to be the best for both “Holetta Local” and “Kuriftu” with the maximum of 3.42±0.32 and 3.65±0.51 roots respectively. For other treatments; media supplemented with 0.5 mg/l yields 2.88±0.73 and 3.10±0.92 roots for “Holetta Local” and “Kuriftu” respectively. The highest mean root length of 2.08±0.32 cm was recorded per explant when treated with 0.5 mg/l NAA for “Holetta Local” and 2.46±0.53 cm for “Kuriftu” on 1.0 mg/l NAA followed by 1.98±0.32 cm on 1.0 mg/l for “Holetta Local” and 1.75±0.84 cm on 0.5 mg/l NAA for “Kuriftu” respectively. The highest percentage of root 100% was recorded at 1.0 mg/l NAA for both varieties. On the other hand, root number was not efficient in MS medium free growth regulators.

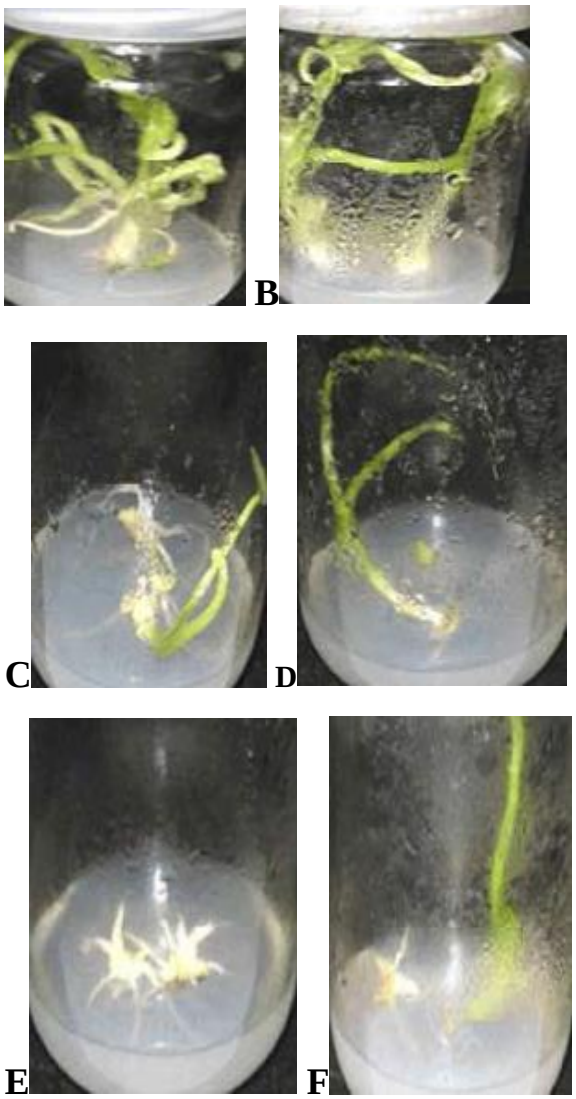


Figure 3: In vitro rooting of garlic “Holetta Local” (A, C and E) and “Kuriftu” (B, D and F) varieties on half strength MS medium containing different concentrations NAA and no PGR (control).

(A and B) 1.0 mg/l NAA, (C and D) 0.5 mg/l NAA and (E and F) control

Table 2: Mean number of root per explants, Average root length and percentage (%) of root in different concentration of NAA medium

Mean values of root number and root lengths are indicated as Mean ±SE

NAA (mg/l)	Mean No. of root/explants Mean ±SE		Average root length(cm) Mean ±SE		percentage (%) of root	
	"Holetta Local"	"Kuriftu"	"Holetta Local"	"Kuriftu"	"Holetta Local"	"Kuriftu"
0.5	2.88±0.73	3.10±0.92	**2.08±0.32**	1.75±0.84	83	83
1.0	**3.42±0.32**	**3.65±0.51**	1.98±0.32	**2.46±0.53**	100	100
1.5	2.06±0.88	1.91±0.43	1.66±0.08	1.22±0.28	67	67
2.0	1.92±0.64	1.33±0.37	1.32±0.10	1.05±0.81	67	50
0.0	1.27±0.37	1.03±0.69	1.84±0.05	1.47±0.15	50	50

After 14 days of acclimatization, the survival rate of total plantlets transferred to greenhouse was found to be 78% and 84% for "Holetta Local" and "Kuriftu" varieties respectively (Figure 4)

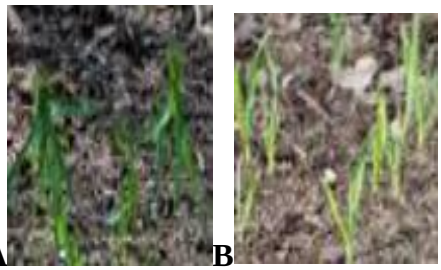


Figure 4: Acclimatization of in vitro rooted shoots of "Holetta Local" and "Kuriftu"

(A) "Holetta Local" Plants covered with plastic plate after 14 days of acclimatization and (B)

(B) "Kuriftu" Plants covered with plastic plate after 14 days of acclimatization.

5. DISCUSSION

5.1. Shoot induction

After passing through standard tissue culture sterilization techniques Shoot tip explant of two cultivars were excised from well-sprouted were grown on half MS medium containing 10mg/l BAP alone. 10mg/l BAP was a suitable for shoot regeneration from shoot tip explants, which resulted in high percentage of shoot formation, producing high number of shoots per explant in both varieties. In this study BAP has been reported to play an important role in shoot induction in garlic tissue culture. Although BAP was able to regenerate shoots from shoot tip explants, a high percentage of explants producing shoots were observed in "Holetta Local". Out of total explant inoculated, 85% explant of these two cultivars had growth.

One variety was found to be less suitable than other for in vitro shoot-tip culture. "Kuriftu" cultivar had initiated shoot less 10% than "Holetta Local". This is probably related to genetic differences among the varieties and not due to specific nutritional requirement. The effects of growth regulators were monitored by scoring the percentage of regenerated shoots. Among the varieties, "Holetta Local" was better than "Kuriftu" for shoot induction from shoot tips which was varied from 80 to 90 % in percentage of shoot regeneration. However, in both cultivars BAP resulted in better for *in-vitro* shoot formation. This suggests that, BAP alone is crucial for better direct shoot induction from shoot tip culture. Gull *et al*. (2014) and Iram *et al*. (2014) indicated that BAP was found as a potent phytohormone for shoot formation in garlic from shoot tip culture. In addition, Roksana et *al.* (2002) also supported our finding as they found better result on MS basal medium with BAP singly. In fact, they also indicated that shoot-tips have great potential to induce shoots when cultured on MS media containing BAP alone which in an agreement with our study.

5.2 Shoot multiplication

In the attempt to optimize the concentration in shoot multiplication and compare the potential of the two varieties using a half-strength MS medium supplemented with the combinations of two growth regulators along with control, the maximum number of shoots was obtained on 2.0 mg/l BAP+0.25 mg/l NAA, whereas maximum mean length of shoot was achieved on 1.5 mg/l BAP + 0.25 mg/l NAA for "Holetta Local". For "Kuriftu" the maximum shoot number and length of shoot have been counted on 1.5 mg/l BAP+0.25 mg/l NAA.

With an increase in the concentration of BAP (from 0.5 to 1.0 and 1.5 mg/l) in combination with 0.25 mg/l NAA , the number of shoots per explant increased significantly and then decreased at 2.0 mg/l

BAP for "Kuriftu" variety. For "Holetta Local" as the concentration of BAP increased (from 0.5 to 1.0, 1.5 and 2.0 mg/l) in combination with 0.25 mg/l NAA the number of shoots per explant increased significantly. Our result for "Kuriftu" variety is in agreement with finding of Chadha *et al*. (2018) who reported an increase in the concentration of BAP (from 1.0 to 2.0 mg/l) will decrease the mean number of Tunisian local garlic shoots per shoot tip explants and is in contrast for "Holetta Local" variety.

"Holetta Local" variety is in agreement with the finding of Bekheet (2004) who reported that an increase in the concentration of BAP (from 1.0 to 2.0 mg/l) in combination with 1.0 mg/l NAA, resulted in an increase in shoot proliferation of Balady cultivar and is in contrast for "Kuriftu". This may be related to the genotype of the cultured cultivar and medium composition. The same behaviour of proliferated garlic explant was reported by Asha Devi *et al*. (2007) who indicated that genotype plays a very important role in the response to garlic culture under in vitro conditions. Roksana *et al*. (2002) also noticed that the response of garlic shoot-root proliferation was varying with genotype.

Mohamed-Yassen *et al*. (1994) have counted a maximum of 8 shoots per explants from the shoot tip of cloves of the cultivar *Extra Select Sets* on a medium with BAP (8µM) and NAA (0.1µM) after five weeks. For similar purpose, a medium with BAP (1.5 mg/l) and NAA (0.5 mg/l) was applied by Roksana *et al*. (2002) for garlic multiplication and it leads to a maximum of 7.9 shoots per explants from local cultivars of Bangladesh by Shoot tip culture after three weeks. The difference between the numbers of the shoot by comparing with this findings might be in the response to garlic genotype. Therefore, in this study, the effect of BAP on shoot multiplication process garlic varieties was readily observed, where an optimal cytokinin concentration for shoot multiplication based on garlic varieties.

In the same context, MS free growth regulator medium was not efficient for shoot proliferation since it offered less shoot number for two varieties and it was highly less than the other tested media containing BAP and NAA. However, no single shoot was recorded from plantlets/explant on MS free growth regulator medium for both varieties. Similar observations were recorded by Gad El Hak *et al*. (2011) who found the lowest number of plantlets/explant when explants were grown on the complete MS hormone-free medium. In contrast Roksana *et al*. (2002) noticed that no shoot proliferation was observed in medium without any growth regulators.

The length of multiplied shoots was different in media containing various concentrations of cytokinins in combination with auxin. Shoot number and shoot length of explants in this study had a direct relationship for "Kuriftu". Shoot length also increases with an increase in shoot number. For "Holetta Local" Shoot number and shoot length had an inversely relationship on the concentration of 2.0mg/l

BAP in combination with 0.25 mg/l NAA. For “Kuriftu” this is in agreement with Bekheet (2004) who reported that an increase in the concentration of BAP (from 1.0 to 2.0 mg/l) in combination with 1.0 mg/l NAA, resulted in an increase in shoot proliferation of Balady cultivar and also increase in shoot length after four weeks of culture on the proliferation media. In this investigation, higher concentrations of cytokinin reduced the shoot number as well as shoot length for ‘Kuriftu’.

5.3 Rooting and acclimatization

The effect of half MS strength medium with different NAA concentrations and root induction potential of the two varieties were observed. Root induction potential of plantlets varies with variation in the concentration of NAA was recorded for both varieties. With an increase in the concentration of NAA (from 0.5 to 1.0 mg/l) the number of roots per explant increased and then decreased from 1.5 to 2.0 mg/l NAA for two varieties. For a similar purpose, this hormonal composition was applied by Iram *et al.* (2014) who reported an increase in the concentration of NAA (from 0.5 to 1.0 mg/l) increase the mean number of Pakistan local garlic roots and decrease the mean number of roots from the concentration of NAA (1.5 to 2.0 mg/l). This result reveals that increase the concentration of NAA decreases the growth and formation of roots. In addition to these, similar observations were recorded by Iram *et al.* (2014) who found the highest mean number of root per explant and percentage (%) of the root which in agreement with this findings on 1.0 mg/l NAA but the highest mean number of root per explant was obtained by them as it is compared with this finding.

In other way, Bekheet (2004) recorded a maximum of 1cm root length and 1.7 number of root per explants at 1.0 mg/l NAA which is lower than our study for both varieties. This may be related to the genotype of the cultured cultivar. Roksana *et al*. (2002) noticed that the response of garlic shoot-root proliferation was varying with genotype.

In the same context, MS free growth regulator medium not efficient for root proliferation and percentage (%) of root but it seems efficient for root length as it offered high root length for two varieties and it was found slightly better than the other tested media with 1.5 mg/l NAA and 2.0 mg/l NAA. Our result seems to support the report of Iram *et al.* (2014) who showed root induction was observed in almost all the compositions even in the hormone-free full-strength MS medium and in contrary to Jitendra *et al*. (2013) who reported that full or half strength of MS medium without any PGR was failed to induce rooting of regenerated shoots.

The survival percentage of the two garlic varieties in the green house was found to be 78% and 84% for “Holetta Local” and “Kuriftu” respectively.

6. CONCLUSION

Shoot tip containing meristem cells are enable to increase the multiplication potential of garlic plantlets. According to the findings of the present experiment:

• The establishments of culture medium by adjusting their concentration and maintenance of aseptic conditions are the keys to success for *in vitro* multiplication of garlic.

• BAP potential alone is crucial for better direct shoot induction from shoot tip culture for both varieties.

• For multiplication, 2.0 mg/l BAP+0.25 mg/l NAA was best for number of shoots and 1.5 BAP mg/l +0.25 NAA mg/l for shoot length for “Holetta Local”.

•1.5 mg/l BAP+0.25 mg/l NAA was found to be the best for the number of shoots and shoot length for “Kuriftu”.

• For rooting, the highest mean number of root was found at 1.0 mg/l NAA for both varieties.

• The highest mean length of root was obtained at 0.5 mg/l NAA for “Holetta Local” and at 1.0 mg/l NAA for “Kuriftu”.

• In vitro rooted plantlets were transferred to acclimatization and survived up to 78% and 84% for “Holetta Local” and “Kuriftu” respectively.

• In this investigation a significant difference was observed in the response of the two varieties to different initiation, multiplication medium combinations and rooting medium.

7. RECOMMENDATIONS

• The effect of different concentrations of auxins and cytokinins, and more subculturing on shoot induction and regeneration of shoots should be further studied.

• The effect of different concentrations of auxins and cytokinins, and more subculturing should be done to examine the subculture stages at which maximum shoot multiplication takes place and the stage when the multiplication rate starts to decline.

• The effect of different concentrations of auxins and cytokinins, on root induction also should be further studied.

• It is important to develop shoot tip culture protocol for other garlic varieties.

• One advantage of shoot tip culture is production of virus free plantlets so in the future studies using shoot tip culture virus indexing should be incorporated.

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9. APPENDIX

APPENDIX 1. The main constituents of MS media used in the present study

	Constituents	Concentration (mg/l or g/l)	mL/L or mg/l during media preparation
Macronutrients:			
Ammonium nitrate	NH_4NO_3	1650 mg	
Potassium nitrate	KNO_3	1900 mg	
Magnesium chloride	$MgSO_4.7H_2O$	370 mg	12.5ml
Potassium phosphate	KH_2PO_4	170 mg	
Calcium chloride	$CaCl_2.2H_2O$	53g	1.56ml
Micronutrients:			
Phosphoric acid	H3PO3	6.20 mg	
Manganese sulphate	MnSO4.4H2O	22.30 mg	
Zinc sulphate	ZnSO4.7H2O	8.60 mg	0.5ml
Sodium molibidate	Na2MoO4.2H2O	0.25 mg	
Cupper chloride	CuSO4.7H2O	0.025 mg	
Cobalt chloride	CoCl2.6H2O	0.025 mg	
potassium iodide	KI	0.83g	0.5ml
Na2EDTA		7.472 g	5.02ml
FeSO4.7H2O		5.56 g	
Vitamins:			
Pyridoxine			0.05 g
Thiamine			0.1 g
Myo Inositol			0.325g
Sucrose			15.0 g
Agar			7.0 g

- $CaCl_2.2H_2O$, KI, Na_2EDTA and $FeSO4.7H_2O$ were prepared alone