OPTIMIZATION OF SUCROSE, PLANT HORMONES AND PHOTOPERIOD FOR *IN VITRO* MULTIPLICATION OF LEMON *(C.limon)* AND MACROPHYLLA *(C.macrophylla)*

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OPTIMIZATION OF SUCROSE, PLANT HORMONES AND PHOTOPERIOD FOR *IN VITRO* MULTIPLICATION OF LEMON *(C.limon)* AND MACROPHYLLA *(C.macrophylla)*

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By

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APPROVAL SHEET

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I have incorporated the suggestions and modifications given during the internal defense and got the approval of my advisors. Hence, I hereby kindly request the department to allow me to submit my thesis for external thesis defense.

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We the thesis advisors have verified that the student has incorporated the suggestions and modifications given during the internal thesis defense and the thesis is ready to be submitted hence, we recommended the thesis to be submitted for external defense.
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Decision/Suggestion of Department Graduate Council (DGC)

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DEDICATION

To the Lord Jesus Christ and to my dad Fanta Lambebo, my mom Mulunesh Haniche and to my dear brother Abenezer Fanta with great loves and respects.

STATEMENT OF THE AUTHOR

I declare that this thesis is my original work and that all sources of materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MSc. degree at Jimma University and is deposited at the University library to be made available to borrowers under rules of the library. I 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 was born on August 24, 1989 in Soddo town, Wolaitta Zone, South Nations, Nationalities and Peoples Regional State, Ethiopia. She had attended her primary school education at Soddo Abiyot ChoraPrimary School from 1993 to 1998 and Giorgis Junior School from 1999 to 2000. She attended her secondary and preparatory education at Soddo Comprehensive High School from 2001 to 2005. She joined Jimma University in 2006 and completed her undergraduate studies with BSc. degree in Horticulture in 2009. She employed at Wolaitta Zone, Damot Sore Woreda Agricultural Office from 2010 to 2012. After she served for three years, she joined the School of Graduate Studies of Jimma University to pursue her M.Sc. study in Plant Biotechnology in September 2012.

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LIST OF ABBREVIATIONS

2, 4-D	2, 4-Dichlorophenoxyacetic acid
BAP	6- Benzyl amino purine
DMRT	Duncan's Multiple Range Taste
IAA	Indole acetic acid
IBA	Indole-3- butyric acid
Kin	Kinetin
LSD	Least significant Difference
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
PLFS	*Pseudocercospora* leaf and fruit spot disease of citrus
PGR	Plant Growth Regulator
TCA	Tricarboxylic acid
TDZ	Thidiazuron

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ABSTRACT

Although citrus is an importantfruit in Ethiopia, its production is limited by different factors like shortage of planting material and disease transmission. Therefore, this study was conducted to investigate the combined effect of growth regulators and sucrose concentration for in vitro multiplicationof citrus. In vitro shoot multiplication was carried out for lemon and macrophylla through shoot tip. Seeds extracted from mature fruits. The extracted seeds cultured on hormone free MS media in the light and dark condition to evaluate percent of shoot initiation and days for shoot initiation. Initiated shoots were cultured in MS medium supplemented with four different concentrations of BAP (0, 2.5, 5.0, and 7.5 mg/L) and Sucrose (0, 15, 30 and 45 g/L). The treatments arranged in 2x4x4 factorial experiment laid out in completely randomized design (CRD) with three replications for genotype, concentrations of BAP and sucrose respectively. Micro-shoots were then transferred onto 1/2 MS medium supplemented with different combination of NAA (0, 1, 2, 3, 4, 5 mg/L) for rooting. The cultures kept at optimum temperature of 25 ± 2°C and light intensity produced from cool white fluorescent tubes for 16 h photoperiod. Finally, eighteen well rootedshoots transferred into greenhouse for acclimatization. Data collected were number of days for shoot initiation and percent of shoot initiation (under shoot initiation experiment), shoot number, shoot length and leaf number (under shoot multiplication experiment) and root number and root length (under rooting experiment). Statistical analysis revealed that there was highly significantly difference (P<0.01) among all treatments in shoot initiation, shoot multiplication and rooting experiments. Hundred percent shoot initiation was obtained in light condition for lemon. Maximum number of shoots per explant (10 ±0.55) and (7 ±0.0)was obtained from MS medium containing 2.5 mg/L BAP combined with 30 g/L sucrose for lemon and macrophylla respectively. The lowest shoot number (1.0 ±0.0) was obtained on the growth regulator free medium for macrophylla. The highest number of roots per shoot (20.3±0.6) obtained at 1 mg/L NAA for lemon. Among the rooted plantlets used for acclimatization, (65%) of them survived. According to the result obtained in this study, shoot initiation in light condition, MS medium supplemented with 2.5 mg/L BAP combined with 30 g/L sucrose for shoot multiplication and 1/2 MS medium supplemented with 1 mg/L NAA for rooting were recommended for in vitro propagation of citrus (lemon and macrophylla). However, further optimization of this protocol may be required for mass propagation of citrus as this study is limited to two genotypes.

Key words/Phrases: BAP, NAA, germination, shoot multiplication, rooting.

1. INTRODUCTION

Citrus is a common term and genus (*Citrus*) of flowering plants in the family, Rutaceae. All citrus species have 2n = 18, with very similar karyotypic morphology and size, although a single seedling of *C. sinensis* cv. Berna was triploid (Guerra *et al.,* 1997). Total production of *Citrus* on the global level were 131 million tons of fruit harvested over 8.7 million hectares and are primarily utilized for juice making and fresh fruit consumption (FAOSTAT, 2013).Citrus (*Citrus* spp.) is one of the most economically important fruit crops grown by small holders and commercial farmers in Ethiopia (Kassahun *et al*., 2006 and Seifu, 2003). The total area coverage and the annual production of citrus were estimated at 6,950 ha and 77,087 tons, respectively (FAOSTAT, 2013). In Ethiopia, citrus production has expanded with privates and government farms. Upper Awash Agro-industry is the largest enterprise, which produces different types of citrus.

Citrus fruits are considered as the number one fruits of the world due to their high nutritional value, great production potential and preparation of large number of fruit products from them. Citrus species are cultivated in most tropical and subtropical regions of the world. They are very attractive due to their distinctive fruits, colors, and attractive smell, unique from other plants. Containing high amounts of vitamin C, they can be consumedraw or extracted for production of highly nutritious beverages. Citrus species can also be used as traditional medicine, whereby the smell of citrus leaves and fruits can overcome headache and nausea (Fazle *et al.,* 2015). They are widely used to prevent flu and colds and support the immune system (Dhanavade *et al.,* 2011). Citrus fruits also used for patients susceptible to health problems such as gastritis, fever and arterial sclerosis. The juice of lemon used in the pharmaceutical industry since it contains a high quantity of citric acid and essential oils (Bansode *et al.,* 2012). There are also reports about positive effects of lemon fruits against cancer of gastrointestinal and upper respiratory tracts (Foschi *et al.,* 2010).

Citrus can be propagated by both sexual and asexual methods; generally, rootstocks are propagated through seeds, while most of the commercial varieties are propagated through various asexual methods. Conventional methods for citrus propagation are based on bud wood

selection and grafting for scion varieties. Rooted cuttings, or more frequently nucellar seed propagation is used for preparation of rootstocks (Barlass and Skene, 1986). The importance of the citrus industry and the continuous introduction of new improved genotypes emphasize the use of modern methods used to rapidly propagate new and promising plant material (Moore, 1986).

Conventional vegetative propagation of citrus plant is time consuming and mainly dependent on season and availability of plant material which restricts the faster adoption and replacement of new varieties (Rathore *et al.,* 2007). Currently, in Ethiopia citrus production is declining (quality and quantity) due to different factors. Disease is one of the serious problems on citrus production in Ethiopia. For example, *Pseudocercospora* leaf and fruit spot disease of citrus (PLFS) which is identified in Jimma area (Asmare *et al.,* 2014 and Mohammed, 2007). Jimma University is now working to get disease resistant varieties of citrus and started nursery establishment for this purpose. In the conventional method, production of disease resistance planting material takes a long period.

Shortage of planting material is another serious problem in Ethiopia and it is the main issue of this research. Propagation of citrus trees is done mostly by bud grafting, i.e., the insertion of buds of a desired variety onto a stock grown from seed of another variety**.** Planting material density varies from 200 to 800 trees/ha (FAO, 2001). Ethiopia planned to cover 86,875 ha of land by citrus in 2020. (Ethiopian Investment Agency, 2012and Anonymous, 2012). The planting material required to cover this area is 43,437,500 cuttings, which is difficult to meet this need by conventional methods of propagation. *In vitro* propagation is a technique that can solve such problems. It can produce planting material on a relatively large scale compared to conventional methods (Savita *et al.* 2011).

In vitro culture eliminates infections and is faster than conventional propagation methods (Savita *et al.,* 2011). Tissue culture and micropropagation protocols have been described for a number of citrusspecies and explant sources (Barlass and Skene, 1982; Usman *et al.,* 2005; Sharma *et al.,* 2009; Pe´rez-Tornero *et al*., 2010).

Priyanka *et al*., (2012) studied i*n vitro* shoot regeneration on Kinnow mandarin (*Citrus reticulate* Blanco) through shoot tip explants obtained from *in vitro* germinated seedlings. The medium supplemented with 2.5 mg/L BAP and 30 g/L sucrose supported maximum shoot proliferation (2.45 shoots per explant).

Explants of *C.limon* were cultured on 16 different media supplemented with various combinations of plant growth regulators, both auxins and cytokinins, such as BAP, NAA, 2, 4-D and kinetin. The best shoot induction was obtained when the leaf explants were cultured on Murashige and Tucker media supplemented with 3 mg/L BAP alone (Anna *et al*., 2015).

BAP is the most commonly used cytokinin in tissue culture for the genus citrus, but the optimum concentration for maximum proliferation varies among species. For instance, shoot tips of *C.mitis* require4.44-µM BAP, *C.grand* 1.8-µM BAP and *C. depressa, C. jambhiri* and *C. reshni* 4.44-µM BAP for maximum shoot proliferation (Sharma *et al*., 2009).

Several researchers have used different genotypes and plant growth hormones for *in vitro* propagation of citrus but there is no enough information on such work done in Ethiopia. Therefore, the present study was conducted to develop a protocol for *in vitro* propagation of citrus using different genotypes and hormone combinations.

General objective

- To optimize *in vitro* propagation protocols for lemon (*C. limon)* and macrophylla *(C. macrophylla)*

Specific objectives

- To determine the effects of photoperiod on shoot initiation.
- To determine the combined effects of BAP, sucrose, and genotype on shoot multiplication.
- To determine the combined effects of NAA and genotype on *rooting*.

2. LITERATURE REVIEW

2.1. Origin and distribution of citrus

Citrus is native to subtropical and tropical areas of Asia, originating in certain parts of Southeast Asia including China, India, and the Malay Archipelago. Citrus was documented during the reign of Ta Yu (around 2205 to 2197 BC) when citrus fruits, particularly mandarins and pummelos, were considered highly prized tributes and were only available for the imperial court (Nagy, 1980). Lemon was originally grown in India and sweet oranges and mandarins are indigenous to China. Research suggests that, whereas some commercial species such as oranges, mandarins, and lemons originally came from Southeast Asia, the true origins of citrus fruit are Australia, New Caledonia, and New Guinea (Anitei, 2007).

2.2. Botany of citrus

Citrus taxonomy is very complex. Cultivated citrus were derived from various forms of old citrus species found in the wild. Some are only selections of the original wild types, while others are hybrids between two or more ancestors. Citrus plants hybridize easily between species with completely different morphologies, and similar looking citrus fruits may have quite different ancestries. Some differ only in disease resistance. Interbreeding seems possible between all citrus plants, and between citrus plants and some plants that may or may not be categorized as citrus. The ability of citrus hybrids to self-pollinate and reproduce sexually also helps create new varieties. The four core ancestral citrus taxa are citron (*C. medica*), pummelo (*C. maxima*), mandarin (*C. reticulata*), and papeda (*C. micrantha)* (Darren *et al.,* 2014).

2.3. Importance of citrus

Majority of citrus fruits are preferably eaten fresh oranges, mandarins, grape fruits, clementines and tangerines. Orange and grapefruit produce very palatable juice and hence make for nutritious and popular breakfast. Bulk of the total produce of oranges and mandarins goes into juice making. Lemons and limes made into lemonades and pickles their juices can be added to various food preparations to enhance flavor. Delicious marmalades made out of oranges. Essential oils obtained from citrus leaves have recently been found to harbor insecticidal property. Lemon oil

obtained by cold pressing of lemon peels is extensively used in furniture polish. Bergamot, a variety of sour orange is used in making perfumes and massage oils (Talon and Gimitter, 2008).

2. 4. Propagation of citrus

Plant propagation is the art and science of reproducing plants while preserving the unique characteristics of a plant from one generation to the next. Citrus is generally propagated through grafting, cutting, or layering. Therefore, conventional propagation is limited to the period when buds are available (Rathore *et al.,* 2007).

2. 4.1. Seed propagation of citrus

Seeds are important starting materials for tree propagation because many rootstocks often raised from seeds (seedlings). This is because seedlings easily develop normal and functional roots, and hence seedlings possess good root development (Mng'omba 2007). Citrus seeds are quite suitable, however, for growing rootstock plants on which the desired cultivar may be budded. Seed from common, edible citrus fruits (sweet orange, grapefruit or mandarin) can be used for growing rootstock plants at home, but this would not be recommended for commercial purposes (Williamson and Jackson, 1994).

2. 4.2. Vegetative propagation

There are several vegetative propagation methods for citrus. Layerage, Cuttage and Graftage are the common propagation methods by which roots were induced to develop from stems while they are still attached to the tree.

2.4.2.1. Layerage and cuttage

Layerage and cuttage are a means of propagation whereby parts of a plant are induced to develop roots. Layering is commonly used on larger branches and rooting occurs while the branch is still attached to the plant. Both techniques can be used with citrus, but the resulting plants grow on their own roots without using the advantages offered by certain rootstocks. A rooted cutting or layer can be used as a rootstock for desirable scion varieties, but this is not commonly done (Jean-Baptiste *et al.,* 2009).

2.4.2.2. Graftage

Graftage refers to any process of inserting a part of one plant into or onto another plant in such a way that they will unite and grow as a single unit. Grafting involves the use of a scion having two or more buds. There are numerous types of grafts including whip, cleft, bridge, inarch, stump, inlay bark, side approach and others (Williston, 2015). Grafting is most commonly used to repair existing trees, to top-work existing trees, to change the variety and to produce new plants.

2. 5. *In vitro* propagation of citrus

Micropropagation is one of the most common techniques in tissue culture. It is defined as *in vitro* regeneration of plants from organs, tissues, cells or protoplasts and the true-to-type propagation of selected genotypes using *in vitro* culture techniques (Anna *et al*., 2015). True-to-type propagation has important benefits for highly heterozygous plants. It also provides a means of maintaining of germplasms as free of disease (Beversdorf *et al.,* 1990).

Tissue culture technique could be used for propagation of citrus rootstocks and thus, the number of plants produced would not be limited by their seed supply, rather more uniform disease free and quality plant populations produced. Hence, plant tissue culture techniques can be applied as a helpful tool to reduce the time for improvement of citrus through soma clonal variation (Chandler *et al.*, 1996). Techniques like *in vitro*culture made it easy to improve citrus against different abiotic stresses, low yield and conserve important citrus genotypes though exploiting soma clonal variations.

2. 5. 1. *In vitro* seed germination

Priyanka *et al*., (2012) reported that best successful seed germination were obtained after a mean of 10.23 days, when incubated in complete dark conditions while incubation in a photoperiod of 16 h, took an average of 16.48 days for initiation of germination. Seed germination was higher 93.33±4.6 under dark conditions compared to under16 h photoperiod culture 83.33 ± 6.92.

2.5.2. Shoot multiplication

The interaction effects of sucrose and BAP concentration in combination were reported by many researchers. For example Jamilah *et al.,* (2014) reported that optimum shoot and root regeneration were obtained from 2.5 mg/L BAP with g/L 30 sucroseand 1 mg/L NAA respectively on rough lemon.Sucrose, plant growth hormone concentration and genotype combinations may have different results.

Cytokinin and auxin are naturally interacting on shoot regeneration. But cytokinin has better result on shoot regeneration. The effect of BAP, kinetin alone or in combination was studied by many authors. BAP was recorded to be better than kinetin in terms of number of days taken to bud break on citrus species. BAP was reported to be highly genotype dependent. Begum *et al.,* (2004) reported that , in *C. grandis*, maximum (7.8 shoots per explant were induced with 1 mg /L BAP and *C. halimii* produced (3.7 shoots per explant) with 2 mg/L BAP. Goswami *et al*., (2013) reported that mmaximum shoot regeneration was observed on low level of BAP 0.1 mg/L or kinetin 0.5 mg/L. The maximum number of shoots per explant was observed on 0.1 mg/L BAP and 0.5 mg/L kinetin. Shoot, proliferation decreased with increasing concentration of BAP alone, but in case of a combination of BAP and NAA 0.1 mg/L each, it increased with increasing concentration of BAP up to 10.0 mg/L. In the case of a combination of BAP + kinetin + IBA, the maximum (5.5 shoots per explants) proliferation was observed on MS medium containing 1.0 mg/L BAP + 0.5 mg/L kinetin + 0.5 mg/L IBA or 0.25 mg/L BAP + 1.0 mg/L kinetin + 1.0 mg/L IBA. In *C. limon,* shoot induction from nodal explants was observed on the MS medium with 2 mg/L BAP and 2 mg/L GA3, while the highest number of shoots were obtained using 2 mg/L BAP (Sarma *et al.,* 2011).

Initiation of adventitious buds takes place directly from the cambial region at the cut surface of explants. It has been shown that regeneration of shoots from the apical end of epicotyl explants inserted longitudinally in semisolid culture medium follows direct regeneration pathway, whereas shoot development at the basal end follows indirect organogenesis after callus formation (Garcia-Luis *et al.,* 1999) and regeneration of adventitious buds relies heavily on BAP and IAA in Troyer citrange.

2. 5.3. *In vitro* rooting

Positive effects of NAA alone or in combinations with another auxin on rooting *in vitro* observed by many researchers. In species like *C. limonia*, C. *sinensis,* and C. *jambhiri,* other auxins added to MS, IBA or IAA cause rooting (Almeida *et al.,* 2002). Rooting of *in vitro* regenerated *C. reticulata* and *C. limon* were also observed using a combination of IBA and NAA (Singh *et al.,* 1994). For rooting of *in vitro* shoots, half-strength MS medium with 1 mg/L IBA was employed which has yielded a large number of rooted plantlets (Al-Khayri and Al-Bahrany, 2001). NAA 1 mg/L reported to give the maximum rooting (75.00%), number of roots per shoot (3.19 per shoot) and length of longest root (4.08 cm) for C. *reticulat* (Rahman *et al.*, 1996). Between two plant growth regulators tried for rooting, NAA was found to give better response (71%) as compared to IBA for troyer citrange (Chakravarty and Goswami, 1999). Other reports which showed NAA to be better rooting hormone for *Citrus* spp. included Pena *et al*. (1995); Normah *et al.* (1997); Usman *et al.* (2005).

2.6. Media preparation and compositions

Preparation of culture media is preferred to be performed in an equipped for this purpose compartment. This compartment should beconstructed so as to maintain ease in cleaning and reducing possibility of contamination. Supplies of both tap and distilled water and gas should be provided. Appropriate systems for water sterilization or deionization are also important (Ahloowali and Prakas, 2002). Certain devices are required for better performance such as a refrigerator, freezer, hot plate, stirrer, pH meter; electric balances with different weighing ranges, heater, Bunsen burner inaddition to glassware and chemicals (Brown and Thorpe, 1984). It is well known now that mistakes which occur in tissue culture process most frequently originate from inaccurate media preparation that is why clean glassware, high quality water, pure chemicals and careful measurement of media components shouldbe facilitated.

Growth and morphogenesis of plant tissues *in vitro* largely governed by the composition of the culture media. Although the basic requirements of cultured plant tissues are similar to those of whole plants, in practice nutritional components promoting optimal growth of a tissue under laboratory conditions may vary with respect to the particular species and the type of tissue under consideration (George *et al.,* 2008).Considerable progress had been made in the past few decades

on the development of media formulations for growing plant cells, tissues and/or organs aseptically *in vitro*. Murashige and Skoog (1962) made a significant contribution to formulation of a defined growth medium suitable for a wide range of applications.

Several media formulations are commonly used for cell and tissue culture works for the regeneration of specific plant species from different explant types. Among those, the most frequently used media formulations in tissue culture include Murashige and Skoog (1962), Murashige and Tucker (1969) and Gamboge (1967). Culture media used for *in vitro* cultivation of plant cells are composed of some or all of the following basic components: macronutrients, micronutrients, organic supplements, when deemed necessary, a carbon source, gelling agents, and plant growth regulators.

2.6. 1. Macronutrients

Novak *et al*. (1983) studied the macronutrients provide the six major elements: nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S) required for plant cell or tissue growth. All are essential for plant cell and tissue growth. The authors also showed culture media should contain at least 25 m/mole nitrates and potassium.

However, considerably better results obtained when both nitrates and ammonium or any other reduced nitrogen source contributes the source of nitrogen in media. Hall,(1999) pointed out that in case only ammonium is used, there is a need to add one or more tricarboxylic acid cycle acids (e.g., citrate, succinate, or malate) so that any deleterious effect due to ammonium concentrations in excess of 8 m/mole/L in the medium is diluted. When nitrate and ammonia ions are present together in the culture medium, the latter issued more rapidly.

2.6. 2. Micronutrients

These elements are required in trace amounts for plant growth and development. Manganese (Mn), iodine (I), copper (Cu), cobalt (Co), boron (B), molybdenum (Mo), iron (Fe) and zinc (Zn) are regarded as microelements. Chelated forms of iron are commonly used in preparing culture media. These compounds are difficult to dissolve and frequently precipitate after media are

prepared. Murashige and Skoog (1962) used an ethylene diamineter acetic acid (EDTA) iron chelate to bypass this problem (George, 1996).

2.6. 3. Organic supplements

Growth and morphogenesis of plant tissue cultures can be improved by small amounts of some organic nutrients. These are mainly vitamins, amino acids and certain additive supplements. The amount of these substances required for successful culture varies with the species and genotype, and is probably a reflection of the synthetic capacity of the explant (George *et al.,* 2008).

2.6.3.1. Vitamins

Plants synthesize vitamins endogenously and these are used as catalysts in various metabolic processes. According to Larosa *et al.* (1981), the vitamins most frequently used in cell and tissue culture media include thiamine (B1), nicotinic acid (B3), pyridoxine (B6) and myoinositol. The requirements of cells for added vitamins vary according to the nature of the plant and the type of culture.

2.6.3.2. Amino acids

Cultured tissues are normally capable of synthesizing the amino acids necessary for various metabolic processes. In spite of this, the addition of amino acids to media is important for stimulating cell growth in protoplast cultures and for establishing cell lines. Amino acids provide plant cells with an immediately available source of nitrogen, and uptake can be much more rapid than that of inorganic nitrogen in the same medium (Thom *et al.,* 1981).

2.6.3.3. Other organic supplements

Culture media are often supplemented with a variety of organic extracts, which have constituents of an undefined nature. These include protein (casein) hydrolysates, coconut milk, yeast and malt extracts, ground banana, orange juice, and tomato juice. Yeast extract has been shown to have some unusual properties, which may relate to its amino acid content (Ayabe *et al.,* 1988).

2.6. 4. Carbon and energy source

The most preferred carbon source in plant tissue culture is sucrose. Glucose supports equally good growth while fructose is less efficient. Sucrose, while autoclaving the medium, is converted into glucose and fructose. In the process, first glucose is used and then fructose. Other carbohydrates, such as lactose, galactose, maltose, generally yield inferior results. Autotrophic cells are capable of fully supplying their own carbohydrate needs by carbon dioxide assimilation during photosynthesis. Plant cells and tissues in the culture medium lack autotrophic ability and therefore need external carbon for energy (Larosa *et al.,* 1981).

2.6.5. Plant growth regulators (PGRs)

Some chemicals occurring naturally within plant tissues, i.e. endogenously, have a regulatory, rather than a nutritional role in growth and development. These compounds, which are generally active at very low concentrations, are known as plant hormones or plant growth substances. Five broad classes of growth regulators, namely auxins, cytokinins, gibberellins, abscisic acid, and ethylene, are considered important in tissue culture, as the growth, differentiation and organogenesis of tissues become feasible only with the addition of one or more of these classes of chemicals to the culture medium. Skoog and Miller (1957) were the first to report the critical effects of the ratio of auxins to cytokinin in determining the type and extent of regeneration in plant cell culture. In general, the ratio of hormones required for root or shoot induction varies considerably with the tissue, which seems directly correlated to the quantum of hormones synthesized at endogenous levels within the cells.

2.6.5.1. Auxins

These are very widely used in plant tissue culture and usually form an integral part of nutrient media. In many cases, the auxins commonly used in tissue culture media are IAA, IBA, NAA

and 2, 4-D (Kefu *et al*., 1991). The most commonly detected natural auxin is IAA but endogenous occurrences of 4-chloro-IAA and of indole-3-butyric acid (IBA) have also been demonstrated. Auxins promote, mainly in combination with cytokinins, the growth of calli, cell suspensions and organs, and regulate the direction of morphogenesis. At the cellular level, auxins control basic processes such as cell division and cell elongation. Since they are capable of initiating cell division, they are involved in the formation of meristems giving rise to either unorganized tissue, or defined organs (Davies, 2004).

2.6.5.2. Cytokinins

Cytokinins added to shoot culture media, these compounds overcome apical dominance and enhance the release of lateral buds that otherwise are set dormant. Except in research works, the natural cytokinins 2-iP and zeatin are not commonly used in commercial laboratories, due to their associated high cost. Fortunately, several chemical analogues of natural cytokinins apart from kinetin have been identified that were found to be highly effective substitutes of these naturally occurring cytokinins (Kaminek *et al.,* 1987). Cytokinins are by large adenine derivatives, are mainly concerned with cell division, modification of apical dominance, and shoot differentiation in the tissue culture. Cytokinins commonly used in culture media include BAP 2iP, kinetin, Zeatin and TDZ (Iwamura *et al*., 1980).

2.6.6. Gelling agents

Gelling or solidifying agents are commonly used for preparing semisolid or solid tissue culture media. In static liquid cultures, the tissue or cells become submerged and die due to lack of oxygen, thus calling upon such gels that would provide a support to the growing tissues under static conditions. The most common of these include agar-agar, as well as others that are commonly known as gelatin**,** alginate, phytagel and gerlite (George *et al.,* 2008).

2.6.6.1. Agar

It is polysaccharide obtained from seaweed called agar, has several advantages over other gelling agents. First, agar gels do not react with media constituents. Secondly, they are not digested by plant enzymes and remain stable at all feasible incubation temperatures. Normally, 0.5 to 1% agar is used in the medium to form a firm gel at the pH typical of plant cell culture

media (Beruto *et al.,* 1995). They also contain phenolic substances and less pure grades may contain long chain fatty acids, inhibitory to the growth of some bacteria. As agar can be the most expensive component of plant media, there is interest in minimizing its concentration (Scherer *et al.,* 1988).

3. MATERIALS AND METHODS

3.1. Plant material

The genotypes used for this study were Lemon and Macrophylla. These genotypes were selected based on their high demand at the local market, less commercial production, high disease resistance and macrophylla is the best rootstock. Macrophylla was obtained from Upper Awash Horizon Plantation and that of Lemon from Jimma local market.

3.2. Media preparation

MS basal medium (Murashige and Skoog, 1962) was used for shoot initiation, shoot multiplication and ½ MS medium was used for rooting. Half MS media were prepared by reducing all nutrients by half from that of full strength media including sucrose concentration. Sucrose was used as a carbon source. Stock solutions of macronutrients, micronutrients, growth regulators and vitamins were prepared separately and stored at 4ºC. Then MS basal media was prepared by mixing the appropriate volumes of stock solutions and finally different concentrations and combinations of growth regulators and sucrose were added accordingly. After dissolving all the components, the pH was adjusted to 5.8 using 1N NaOH and 1N HCl and then 0.8% agar was added. After melting the prepared media by using hot plate magnetic stirrer, the medium was dispensed into each culture jar and autoclaved at 121°C and 15 psi for 15 minutes.

3.3. Surface sterilization and preparation of explants

Seeds were extracted from matured fruits of macrophylla and lemon. The seeds were collected in autoclaved beaker and covered by paraffilm to protect from microorganisms contact and dried at room temperature for three days. The seeds were washed under tap water for 15 minutes to remove mucus and sugar present on the seed coat under aseptic condition. Then the seeds were soaked in sterilized double distilled water for 30 minutes. This was followed by peeling of seed coat under aseptic conditions in laminar airflow cabinet. Peeled seeds were immersed in 70% ethanol for 1 minute and rinsing three times with double distilled water. Subsequently, the seeds were surface sterilized with 50% sodium hypochlorite for 20 minute and thoroughly rinsed with sterilized double distilled water for three times to remove the traces of sodium hypochlorite .

3.4. Culturing and culture condition

All cultures were kept under 16h/8h light and dark photoperiod at optimum photosynthetic flux provided by cool fluorescent lamps and maintained at 25 ± 2°C.

3.5. Experimental Design and Treatments

In this study, three different experiments were carried out using Completely Randomized Design (CRD) with three replications for shoot multiplication and for rooting experiments whereas six replications were used for the shoot initiation experiment.

3.5.1. Experiment 1: Effect of photoperiod on shoot initiation.

The extracted seeds were cultured on MS medium which was prepared without growth hormones and kept under light (on the shelf with white fluorescent) and dark (dark room covered with black curtains) conditions. Numbers of seeds per petridish were five, with six replications. The treatments were dark and light.

3.5.2. Experiment 2: The combined effects of BAP, sucrose and genotypes on shoot multiplication.

The shoots tips initiated at experiment one were separated and the edge were cut and then cultured on MS media supplemented with four different concentrations of BAP (0.0, 2.5, 5, 7.5 mg/L) and four different (0, 15, 30, 45 g/L) concentrations of sucrose. The treatments arranged in a 2x4x4 factorial arrangement in Completely Randomized Design (CRD); for genotypes, concentrations of BAP and concentrations of sucrose respectively with three replications. The data collected in this experiment were shoot number, shoot length and leaf number.

3.5.3. Experiment 3: The effect of NAA on rooting of micro-shoots.

In this experiment, ½ MS basal medium with different concentrations of NAA (0.0, 1.0, 2.0, 3.0, 4.0, 5.0 mg/L) were used. The treatments were arranged in a 2x6 factorial experiment laid in Completely Randomize Design (CRD); for genotypes and NAA concentration levels respectively with three replications. Data collected under this experiment were root number and root length.

3.6. Acclimatization

Eighty plantlets with well-developed shoots and roots were taken out of the culture jars and washed with tap water in order to remove any adhering medium and soaked with anti-fungal to prevent fungus infection. The plantlets were planted into plastic pots containing autoclaved garden soil mixed with compost in the ratio of 1:1. Hardening of potted plantlets was accomplished in culture room set at 26±2 °C, 16h light by covering with plastic cover to maintain high humidity. In the first week of transfer, plantlets were watered with autoclaved water regularly and after that watering frequency was reduced. The plastic cover was removed initially for a short duration 30 minutes daily for about one week. After 15 days gradually, the daily exposure time was increased by 30 min for each day. Plastic cover was completely removed after 20 days. After one month, plantlets were transferred into greenhouse.

3.7. Data Analysis

The collected data were analyzed using SAS (Version 9.2) software (SAS Institute Inc. 2008). The collected data were subjected to two way and three way analysis of variance (ANOVA) according to the CRD model for factorial experiments and means were compared using Duncan's Multiple Range Tests (DMRT) at 1%.

4. RESULTS AND DISCUSSION

4.1. Effect of photoperiod on shoot initiation.

The analysis of variance indicated that the effect of photoperiod was highly significantly different ($P<0.01$) on initiation date and percentage (Appendix 1; Table 1). Seeds cultured in complete dark conditions showed 10.0±11.1 initiation at the fourth day for lemon, whereas lemon seeds cultured under light condition showed 53.33±20.6 initiation at the third day (Figure1). For macrophylla, seeds cultured under complete dark condition showed 10.0±11.1 initiation at fifth day whereas macrophylla seeds cultured under light condition showed 43.33±11.05 at the third day. At the seventh day, shoot initiation percentage was higher 100±0.0 for lemon than macrophylla 90±16.7 under light conditions.

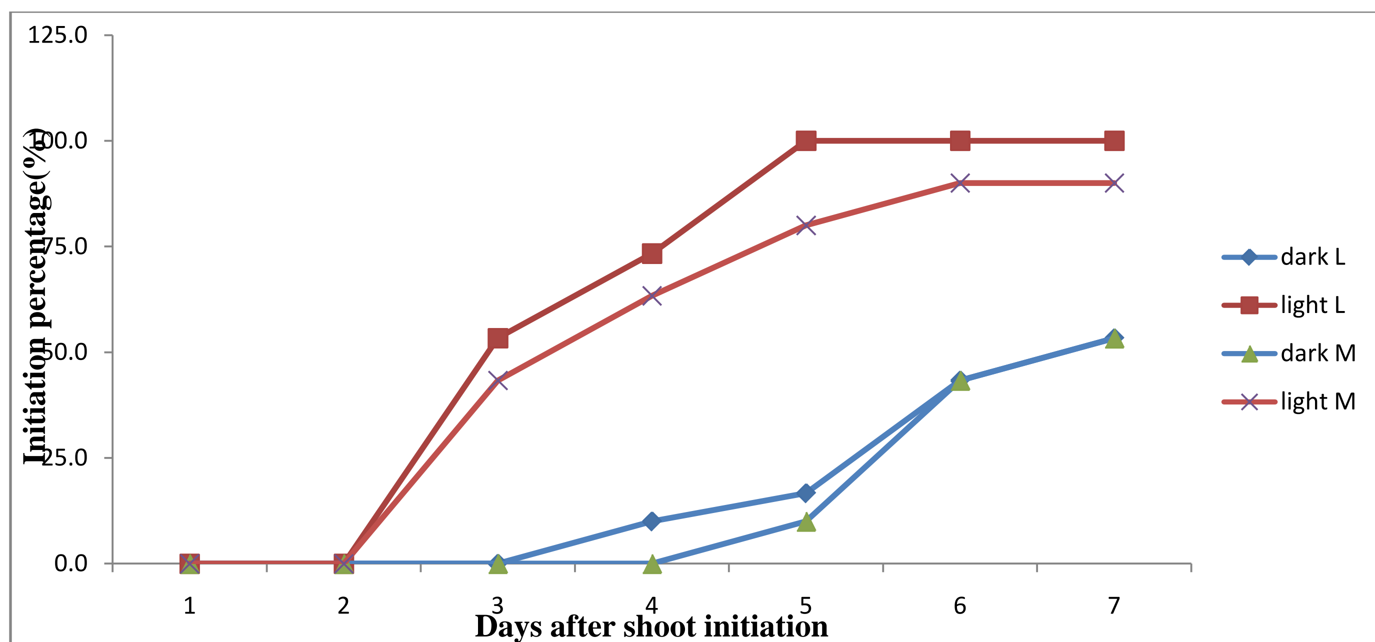

Figure 1: Effects Photoperiod on *in vitro* shoot initiation of Lemon and Macrophylla.

Dark L= lemon cultured under dark condition

Light L=lemon cultured under light condition

Dark M=macrophylla cultured under dark condition

Light M=macrophylla cultured under light condition

The initiation percentage was the same for both genotypes at complete dark condition 53.33±10.32 at the seventh day but a little different on the light condition for Lemon 100±0.0 and 90±16.7 for macrophylla in the same day.

Initiation started on the third day for both genotypes under light condition but it was delayed to an average of 4 and 5 days for lemon and macrophylla in the dark condition, respectively. This is in agreement with Anna, (2015) who found the best result in seed cultured in a chamber with a 16/8 photoperiod (16 hours of light, 8 hours of darkness, 170 μ $molm/s^2$ light intensity) and also with Sharma *et al,*(2009) who reported under 16 h photoperiod culture conditions compared to dark condition. This is perhaps because citrus species need warm conditions than cold environment. Contrary to Priyanka *et al.,* (2012) who found 93.33 ± 4.63 seed germination from seeds cultured under complete dark condition with a mean of 10.23 days and 83.33 ± 6.92 seed germination from seeds cultured under a photoperiod of 16 hours with mean of 16.48 days . This difference is may be because of the seed regeneration media they have used. The media which is they have used were MS media supplemented with different concentrations of KN, GA3 and IBA. It is known that GA3 is better to break dormancy of many fruits including citrus. Citrus seeds have been reported to germinate at a range of temperatures (11-35°C) but 30°C is recommended as a best temperature, with germination usually completed by 4-7 weeks (Soetisna *et al.,* 1985).

4.2. The combined effect of BAP, sucrose and genotype on *in vitro* shoot multiplication

The analysis of variance indicated that the interaction effects of BAP, sucrose and genotype was highly significantly different ($P<0.01$) for shoot number, shoot length and leaf number (Appendix Table2). The best results for shoot number 10.0±0.0 and shoot length 8.0±0.1 were obtained at 2.5 mg/L BAP combined with 30 g/L sucrose for lemon (Table 1). The minimum shoot number obtained1.0±0.0 for macrophylla at hormone free media. When the BAP and sucrose level increased above 2.5 mg/L and 30 g/L respectively, the shoot number and shoot length were decreased in both genotypes. In the same way, a decrease in BAP and sucrose from these levels (2.5 mg/L & 30 g/L) showed a decrease in shoot number and shoot length in both genotypes.

Increasing the concentration level of sucrose from 0 g/L to 45 g /L, keeping BAP at 0.0 mg/L has increased shoot number, shoot length and leaf number. At sugar levels (0, 15, 30, 45 g/L), shoot number has increased from 2.67±0.57 to 6.0±0.0 for lemon. The same sugar levels (0, 15, 30, 45 g/L) increased shoot length from 3.96±0.05 to 5.3±0.0 for lemon. For leaf number, these sugar levels (0, 15, 30, 45 g/L) increased it from 5.8±0.15 to 16.8±0.25 for lemon. For macrophylla, similar levels of sugar increased shoot number from 1.0±00 to 4.0±0.0. Similarly, these sugar levels increased shoot length from 1.9±0.06 to 2.6±0.0. Leaf number also increased from 2.4±0.17 to 8.67±0.57 with the same levels of sugar (0, 15, 30, 45 g/L).

Table1: The Effect of BAP, Sucrose and Genotype on i*n vitro* shoot multiplication.

Levels			**Variables**		
Genotype	BAP (mg/L)	Sucrose (g/L)	Shoot number ±SD	Shoot length ±SD	Leaf number ±SD
Lemon	0.0	0.0	2.6^{i} ±0.6	3.96^{h}±0.1	5.8^{g} ±0.15
	0.0	15.0	3.0^{ih}±0.0	4.9^{g} ±0.1	9.2^{d}±1.2
	0.0	30.0	4.0^{g} ±0.0	5.2^{f} ±0.0	11.3^{c} ±0.35
	0.0	45.0	6.0^{e} ±0.0	5.3^{f} ±0.0	16.8^{a} ±0.2
	2.5	0.0	6.0^{e} ±0.0	5.9^{e}±0.15	1.7^{onp} ±0.1
	2.5	15.0	7.0^{d} ±0.0	7.5^{b} ±0.0	2.0^{moln} ± 0.1
	2.5	30.0	10.0^{a} ±0.0	8.0^{a} ±0.1	2.5^{mjlik} ±0.1
	2.5	45.0	9.0^{b} ±0.0	7.0^{c} ±0.1	12.6^{b}±0.2
	5.0	0.0	8.0^{c} ±0.0	6.8^{c} ±0.0	4.7^{h} ±0.0
	5.0	15.0	8.0^{c} ±0.0	6.5^{d} ±0.0	5.9^{g} ± 0.1
	5.0	30.0	7.0^{d} ±0.0	5.06^{gf} ±0.1	6.4^{g} ±0.1
	5.0	45.0	4.0^{g} ±0.0	4.0^{g} ±0.1	7.6^{f}±0.1
	7.5	0.0	7.0^{d} ±0.0	3.6^{i} ±0.0	5.96^{g} ±0.1
	7.5	15.0	6.0^{e} ±0.0	5.3^{f} ±0.0	8.1^{ef} ±0.0
	7.5	30.0	6.0^{e} ±0.0	6.9^{c} ±0.0	8.8^{ed} ±0.1
	7.5	45.0	3.0^{ih} ±0.0	2.4^{nm}±0.0	8.2^{ef} ±0.1

Table 1: Continued

Macrophylla					
	0.0	0.0	1.0^{k}±0.0	1.9^{qp} ±0.1	2.4^{mjlnk}±0.2
	0.0	15.0	3.0^{ih} ±0.1	2.5^{lm} ±0.0	2.9^{jik} ±0.2
	0.0	30.0	3.0^{ih} ±0.0	2.53^{lm}±0.4	4.1^{h} ±0.2
	0.0	45.0	4.0^{g} ±0.0	2.6^{lkm} ±0.0	8.7^{ed} ±0.6
	2.5	0.0	4.0^{g} ±0.0	1.3^{t}±0.0	2.96^{jik} ±0.12
	2.5	15.0	4.0^{g}±0.0	1.5^{srt} ±0.0	1.3^{op} ±0.1
	2.5	30.0	7.0^{d} ±0.0	3.03^{j}±0.1	2.2^{mlnk} ± 0.1
	2.5	45.0	6.0^{e} ±0.0	2.8^{k} ±0.0	2.3^{mlnk} ±0.05
	5.0	0.0	5.0^{f} ±0.0	2.0^{op} ±0.1	0.93^{p} ±0.1
	5.0	15.0	4.0^{g} ±0.0	2.2^{on} ±0.0	2.2^{mlnk} ±0.6
	5.0	30.0	4.0^{g} ±0.0	2.7^{lk} ±0.0	3.3^{i} ±0.0
	5.0	45.0	3.0^{ih} ±0.0	1.6^{sr} ±0.0	1.83^{mon} ±0.2
	7.5	0.0	2.0^{j} ±0.0	1.9^{qp}±0.1	1.8^{mon} ± 0.3
	7.5	15.0	3.3^{h} ±0.6	1.7^{qr} ±0.0	2.2^{mlnk} ±0.2
	7.5	30.0	3.0^{ih} ±0.0	1.4st ±0.0	2.7^{jlik} ±0.0
	7.5	45.0	2.0^{j} ±0.0	1.0^{u} ±0.0	3.13^{ji} ±0.3
Means			**4.8**	**3.7**	**5.07**
CV(%)			**2.98**	**2.39**	**5.59**

Means followed by different alphabets denote significant differences within column at 1% probability level.

When the BAP level increased from 0 mg/L to 2.5 mg/L, 5 mg/L the shoot number increased from 2.67±0.5, 6.0±0.0 and 8.0±0.0 respectively but it showed a decrease at 7.5 mg/L with the mean value of 7.0 ±0.0 for lemon. With the same BAP levels, the shoot number for macrophylla increased from 1.0±0.0, 4.0±0.0 and 5.0±0.0 respectively but it showed a decrease at 7.5 mg/L with the mean value of 2.0±0.0 for macrophylla. This result is in agreement with (Usman *et al.*,2005) who reported similar results with BAP at different concentrations for the proliferation of citrus shoots and indicated that shoot induction from citrus cultivars was directly proportional to the increase in BAP levels, although the highest concentrations reduced shoot induction.

The shoot length shows continuous increase from 5.93±0.15 to 8.0±0.1 in lemon and in macrophylla from 1.3±0.0 to 3.03±0.06 at BAP 2.5 mg/L and sucrose levels of 0, 15 and 30 g/L respectively. The longest shoot length 8.0±0.1 found at 2.5 mg/L BAP and 30 g/L sucrose for lemon and minimum shoot length 1.0±0.0 was recorded at BAP 7.5 mg/L and sucrose at 45 g/L for macrophylla.

The reduction in shoot length at high concentration of BAP might be due to the toxic effects of ethylene, produced at high cytokine in concentration. This result is similar with Thomas and Blakesley, (1987) who reported that the production of ethylene by the excessive cytokinins application causing the inhibition of internode elongation and regeneration of tobacco disc. Although cytokinins are necessary to stimulate cell division and promote shoot proliferation, cautions, needed as high levels of cytokinin may inhibit elongation, increasing number of short shoots (Kadota and Niimi, 2003).

The leaf number showed maximum 16.75±0.25 at BAP 0 mg/L and sucrose at 45g/L for lemon and for macrophylla. This is in agreement with Wee *et al*., (2014), who found highest mean number of leaves (5.41 leaves) with medium devoid of BAP on kinnow mandarin. At a low concentration of BAP (0.25 mg/L), more leaves were recorded.

The number of shoots or buds obtained per explant has been found to vary with the genotype (Carimi and De Pasquale 2003). In the current study, maximum shoot number 10.0±0.0

observedfor lemon seeds were cultured on MS supplemented 2.5 mg/L BAP combined with sucrose 30 g/L (Figure 2). This is similar to that of Perez-Tornero *et al*., (2010) who found similar results in three variables; shoot number, shoot length and leaf number in macrophylla and the highest percentage of shoot multiplication was obtained when shoots were cultured on MS media supplemented with 2.0 mg/L of BAP and 30 g/L sucrose in musambi and lemon. The shoot number and leaf length also showed the maximum number on lemon.

Figure 2: The effect of BAP and sucrose on shoot multiplication A) Macrophyllashoot multiplication at BAP 2.5 mg/L and 30 g/L sucrose after 45 days B) Lemon shoot multiplication at BAP 2.5 mg/L and 30 g/L sucrose after 45 days C) Macrophylla shoot multiplication at BAP 0 mg/L and sucrose 0 g/L after 45 days D) Lemon shoot multiplication at BAP 0 mg/L and sucrose 0g/L after 45 days.

The reduction in number of shoots at concentrations above 2.5 mg/L of BAP may be due to the inhibitory effect of higher concentration of BAP. These results are in agreement with those of Kotsias and Roussos, (2001); these authors observed that in explants of lemon seedlings the greatest shoot length was obtained with 2 mg/L.

According to Moreira-Dias *et al.* (2001), the BAP level is variety dependent that BAP at 1.0 mg /L was the best concentration for Natal, Valencia and Hamlin, with averages of 1.59, 1.76 and 2.43 shoots per explant, respectively. For Rangpur lime, 3.0 mg /L of BAP gave the best result. Troyer citrange gave an average of 10.4 shoots per explant, from which only 7.2 developed into plantlets. Each of these authors used a different combination of cultivar, explant type and induction medium.

4.3. The effect of NAA on *in vitro* rooting of micro-shoots

The analysis of variance indicated the interaction effects of NAA and genotypes were significantly different (P<0.01) for root number and root length (Appendix 3).

Table 2: The effect ofNAA and genotype on rooting .

Genotype	NAA(mg/L)	Root number ±SD	Root length ±SD
Lemon	0.0	1.0^{e}±0.0	3.0^{k}±0.1
	1.0	20.3^{a}±0.57	8.3^{a}±0.15
	2.0	13.7^{b}±0.57	4.9^{b}±0.1
	3.0	13.0^{b}±1.0	4.5^{cb}±0.5
	4.0	7.3^{c}±0.58	3.4^{d}±0.1
	5.0	1.0^{e}±0.0	1.4^{g}±0.1
Macrophylla	0.0	1.0^{d}±0.0	1.23^{c}±0.15
	1.0	3.7^{d}±0.58	4.23^{d}±0.32
	2.0	3.13^{ed}±0.23	3.26^{fg}±0.25
	3.0	2.83^{d}±0.28	2.23^{feg}±0.2
	4.0	2.13^{d}±0.23	2.03^{fe}±0.1
	5.0	1.0^{e}±0.0	1.7^{e}±0.15
Means		**5.8**	**3.3**
CV (%)		**7.85**	**6.58**

Means followed by different alphabets denote significant differences within column at 1%.

The highest mean root numbers per shoot for lemon and macrophylla were 20.3±0.57 and 3.7±0.57 respectively and the highest mean root length were 8.3±0.15 and 4.23±0.32 for lemon and macrophylla respectively obtained at 1 mg/L NAA (Table 2). The second highest mean root numbers 13.7±0.57 and 3.13±0.2 and the root length 4.9±0.1 and 4.9±0.1 for lemon and macrophylla respectively were obtained at 2 mg/L NAA.

Increasing NAA from 0 mg/L to 1 mg/L showed a significant increase in the number of roots per shoot from 1.0±0.0 to 20.3±0.57 in lemon (Figure 3) and from 1.0±0.0 to 3.66±0.57 in

macrophylla and mean root length from 3.66±0.57 to 8.3±0.15 in lemon and 1.2±0.15 to 4.23±0.32 in macrophylla. Increasing NAA concentration from 1 mg/L to 2 mg/L showed a significant decrease in the number of roots per shoot from 20.3±0.57 to 13.7±0.57 in lemon and from 3.66±0.57 to 3.13±0.23 in macrophylla and mean root length from 8.3±0.15 to 8.3±0.15 in lemon and from 4.23±0.32 to 3.26±0.25 in macrophylla.

Generally the root parameters showed continuous decreasing when NAA level increased beyond 1 mg/L. This is may be due to the inhibitory effects of high concentration of auxins. These results agree with those of Bordón*et al.* (2000), who obtained maximum 2.5 roots per explant, in a medium with 1 mg/L NAA. Kim *et al.* (2002) reported that MS media supplemented with 1.5 mg /L NAA was most effective for root induction in Yooza mandarin. From the two genotypes, Lemon showed the highest performance compared with macrophylla. In all higher concentration of auxins, lower root number and root length were recorded. In this study, the optimum concentration was found to be 1 mg/L NAA. Mukhtar *et al*., (2005) reported that the results regarding root formation percentage under different concentrations of NAA revealed that highest rooting percentage in musambi was obtained at concentration of 1.5 mg /L NAA.

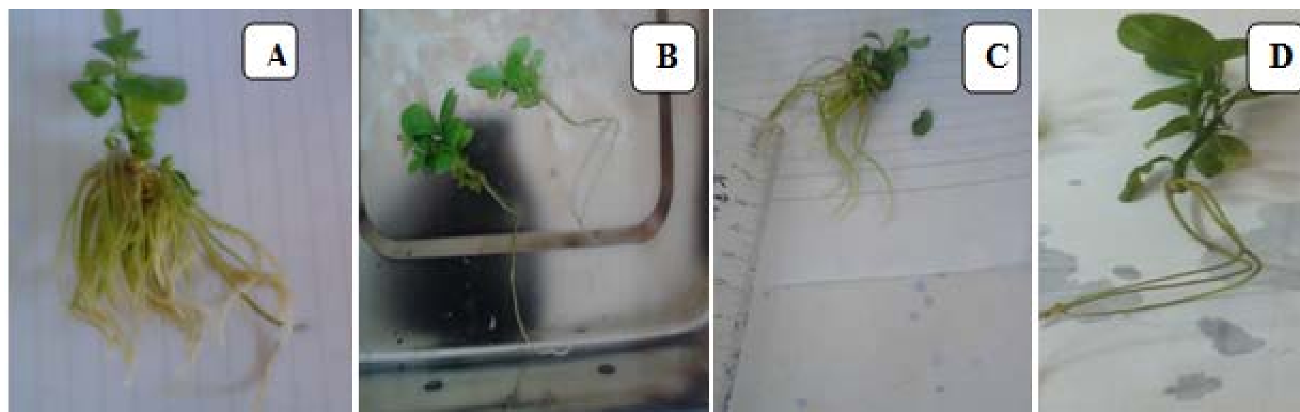

Figure 3: The effect of NAA on *in vitro* rooting A) Lemon rooting at NAA 1 mg/L after 30 days B) Lemon rooting at hormone free media after 30 days C) Lemon rooting at NAA 2 mg/l after 30 days D) Macrophylla rooting at hormone free media after 30 days.

4.4. Acclimatization

Plantlets or *in vitro* grown seedlings were transferred from the jars into pots. Among the acclimatized plantlets, 65% survived which is similar to the results of Singh *et al.,*(1994) who found 60 % success with plants transplanted in potting mixture of garden soil and kept in humid chamber initially for three weeks. It is known that plants suffer from a high rate of water loss immediately after transplanting due to the high size of intercellular spaces, the slowness of stomatal response to water stress and the poor connection between the adventitious roots and the vascular system of stem. Acclimatization of plantlets or the seedlings for three weeks under plastic pots was an essential prerequisite for successful transfer from jars to pots. During this time, the plantlets or seedlings undergo morphological and physiological adaptations enabling them to develop sufficient water control.

5. SUMMARY AND CONCLUION

Citrus is considered as the number one fruit of the world due to its high nutritional value, great production potential and preparation of large number of fruit products from it. It is one of the most economically important fruit crops grown by small holders and commercial farmers in Ethiopia. However, its expansion currently, in Ethiopia is declining (quality and quantity) due to different problems including shortage of planting material and disease transmission.

In vitro shoot multiplication was carried out for lemon and macrophylla genotypes using shoot explants. Seeds were cultured on MS media under light and dark conditions to evaluate percent of shoot initiation and days for shoot initiation. Initiated shoots were cultured on MS medium supplemented with different concentrations of BAP (0.0, 2.5, 5.0 and 7.5 mg/L) and sucrose (0, 15, 30 and 45 g/L) to evaluate their effect on shoot multiplication. The treatments were arranged in 2x4x4 factorial experiment and laid out in Completely Randomized Design (CRD) with three replications. Micro-shoots were then transferred into ½ MS medium supplemented with different concentrations of NAA (0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L) for rooting. In this, experiments the treatments were arranged in a 2x6 factorial experiment and laid out in Completely Randomized Design (CRD) with three replications. The rooted shoots were transferred into greenhouse for acclimatization. Data collected were number of days for shoot initiation and percent of shoot initiation (for shoot initiation experiment), shoot number, shoot length and leaf number (for shoot multiplication) and root number and root length (for rooting).

The analysis of variance revealed that the effect of photoperiod on shoot initiation experiment is highly significantly different ($P<0.01$). In shoot multiplication experiment plant growth regulators, sucrose and genotypes were highly significantly affected at ($P<0.01$) for shoot multiplication parameters, i.e., shoot number, shoot length, leaf number and also the root parameters, i.e., root number and root length highly significant at ($P<0.01$).

Shoot, initiation under light condition was started at the third day for both lemon and macrophylla. However, under dark condition shoot initiation was started on the fourth and fifth

day for both lemon and macrophylla respectively under dark condition. Shoot initiation percentage was higher 100±0.0 for lemon and 90±16.73 for macrophylla under light conditions at the seventh day as compared to 53.33±10.32 for lemon and the same 53.33±10.32 for macrophylla under dark condition at the seventh day.

Both maximum shoot number and shoot length were recorded at 2.5 mg/L BAP combined with 30 g/L sucrose and the maximum leaf number was recorded at 0 mg/L BAP combined with 45 g/L sucrose for lemon. The effect of BAP was higher than that of sucrose for shoot number and shoot length in both lemon and macrophylla but effect of sucrose on leaf number was higher than BAP in lemon. When comparing genotypes, lemon was better than macrophylla in all parameters. Maximum mean shoot number per explant 10.0±0.0, mean shoot length 8.0±0.1 were recorded in lemon at BAP 2.5 mg/L combined with 30 g/L sucrose and maximum leaf number were recorded at BAP 0 mg/L combined with sucrose 45 g/L 16.75 ±0.25 on lemon.

The maximum results recorded for rooting were, mean root number 20.3±0.57 and mean root length 8.3±0.15 at the concentration of 1 mg/L NAA. Increasing NAA concentration from 0 mg/L to 1 mg/L shows a dramatic change in both root number and root length.

In this study, light condition was found to be optimal for shoot initiation. BAP at a concentration of 2.5 mg/L combined with 30 g/L sucrose was found to be optimal concentration for shoot multiplication. NAA at a concentration of 1 mg/L found to be optimal for rooting.

Hence, shoot initiation under light condition could be recommended for shoot initiation. MS medium supplemented with 2.5 mg/L BAP combined 30 g/L sucrose could be recommended for shoot multiplication and ½ MS medium supplemented with 1 mg/L NAA could be recommended for *in vitro* rooting of lemon and macrophylla.

As the future line of work, further protocol optimization may be required for mass propagation of citrus as this study is limited to two genotypes. Half MS media supplemented with NAA between 0 mg/L to 1 mg/L may be better to be tried for best rooting and also sucrose concentration for leaf number needs further study to get the optimum concentration level.

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7. APPENDIXES

Appendices Table (1): ANOVA summary of the effects of photoperiod on *in vitro* shoot initiation

One-Sample Test(lemon)

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
GPP	8.909	83	.000	39.286	30.52	48.06

One-Sample Test(macrophylla)

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
GPP	8.044	83	.000	33.80952	25.4493	42.1698

Appendices Table (2): ANOVA summary of the effects of BAP sucrose and genotype on shoot multiplication.

		Shoot number		Shoot length		Leaf number	
Source of Variation	**DF**	**Mean Square**	**Pr> F**	**Mean Square**	**Pr> F**	**Mean Square**	**Pr>F**
BAP	**3**	**51.04**	**<.0001*** **	**10.59**	**<.0001*** **	**100.63**	**<.0001*** **
Sucrose	**3**	**2.04**	**<.0001*** **	**8.39**	**<.0001*** **	**3.08**	**<.0001*** **
Genotypes	**1**	**137.76**	**<.0001*** **	**290.16**	**<.0001*** **	**494.5**	**<.0001*** **
BAP*Sucrose	**9**	**12.80**	**<.0001*** **	**2.49**	**<.0001*** **	**23.51**	**<.000*** **
BAP*Genotypes	**3**	**4.06**	**<.0001*** **	**6.82**	**<.0001*** **	**32.67**	**<.0001*** **
Sucrose* Genotypes	**3**	**1.39**	**<.0001*** **	**3.07**	**<.0001*** **	**4.87**	**<.0001*** **
BAP* Sucrose* Genotypes	**9**	**3.18**	**<.0001*** **	**1.65**	**<.0001 *** **	**23.64**	**<.0001*** **
Means		**4.8**		**3.7**		**5.07**	
CV		**2.97**		**2.39**		**5.59**	

DF= Degree of freedom , CV = Coefficient of variance, * = Very highly significant**

Appendices Table (3): ANOVA summary of the effects of NAA and genotype on rooting of micro-shoot.

		Root number		Root length	
Source of Variation	**DF**	**Mean Square**	**Pr> F**	**Mean Square**	**Pr> F**
Genotype	**1**	**452.98**	**<.0001*****	**29.52**	**<.0001*****
NAA	**5**	**116.26**	**<.0001*****	**17.23**	**<.0001*****
NAA * Genotype	**5**	**65.142**	**<.0001 *****	**2.99**	**<.0001*****
CV		**7.85**		**6.58**	
Means		**5.8**		**3.3**	

DF= Degree of freedom , CV = Coefficient of variance, * = Very highly significant**

Appendices Table 4: Components of MS media and its concentration

Category	Chemicals	Conc. in medium (mg/l)
Macronutrients	NH_4NO_3	1650
	KNO_3	1900
	$CaCl_2.2H_2O$	440
	$MgSO_4.7H_2O$	370
	KH_2PO_4	170
Micronutrients	$FeSO_4.7H_2O$	27.8
	$Na_2EDTA2H_2O$	33.6
	KI	0.83
	H_3BO_4	6.2
	$MnSO_4.4H_2O$	22.3
	$ZnSO_4.7H_2O$	8.6
	$Na_2MoO_4.H_2O$	0.25
	$CuSO_4.5\ H_2O$	0.025
	$CoCl_2.6\ H_2O$	0.025
Organic supplements	Myoinositol	100
		0.01
	Nicotinic acid	
	Pyridoxine HCl	0.05
Vitamin	Thiamine HCl	0.05
	Glycine	0.02
Carbon source	Sucrose	30,000
	Agar	8000
Gelling agent		
	BAP	10/100ml
Growth regulators		
	NAA	10/100ml
		10/100ml
		10/100ml1