In vitro Protocol Development for the production of Virus-Free Plant Materials for selected Elite Citrus cultivars (*Rutaceae*)

HUSSEN KEDIR DEMBI

A Thesis Submitted to the Department of Applied Biology,

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirements for the Degree of Masters of Science in Applied Biology (Specialization in Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

December, 2022

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DECLARATION

I hereby declare that this MSc thesis entitled "***In vitro* Protocol Development for the production of Virus-Free Plant Materials for selected Elite Citrus cultivars *(Rutaceae)***" is my original work. 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.

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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* Protocol Development for the production of Virus-Free Plant Materials for selected Elite Citrus cultivars *(Rutaceae)***" prepared by **Hussen Kedir** under our guidance submitted in partial fulfillment of the requirements for the degree of Mater's of Science in Biotechnology. Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

_____	_____	_____
Major Advisor	Signature	Date
_____	_____	_____
Co-advisor	Signature	Date

APPROVAL OF BOARD OF EXAMINERS

We, the advisors of the thesis entitled "***In vitro* Protocol Development for the production of Virus-Free Plant Materials for selected Elite Citrus cultivars *(Rutaceae)***" and developed by **Hussen Kedir** hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor	Signature	Date
Co-advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by **Hussen Kedir** have read and evaluated the thesis entitled "*In vitro* Protocol Development for the production of Virus-Free Plant Materials for selected Elite Citrus cultivars *(Rutaceae)*" 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).

Chairperson	Signature	Date
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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).

Department Head	Signature	Date
School Dean	Signature	Date
Office of Postgraduate Studies, Dean	Signature	Date

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LIST OF ABBREVIATIONS AND ACRONYMS

2, 4-D	2, 4-Dichlorophenoxyacetic Acid
ANOVA	Analysis of Variance
BAP	6-Benzyl Amino Purine
DDW	Double Distilled Water
FAO	Food and Agricultural Organization
GA3	Gibberellic Acid
MARC	Melkassa agricultural research center
MS	Murashige and Skoog
NAA	α-Naphthalene Acetic Acid
NaOCl	Sodium hypo-chlorite
PGR	Plant Growth Regulator
SE	Somatic Embryogenesis

ABSTRACT

Citrus is one of the most widely grown fruits of the angiosperm subfamily Orantoidia in the family Rutaceae, which plays a key role in the world's economy and commercially grown in the tropical and sub-tropical regions of more than 140 countries. The production of citrus in Ethiopia adversely affected by biotic and abiotic stress. Several diseases, especially graft transmissible diseases such as Citrus tristeza virus (CTV), Citrus exocortis viroid (CEVd), Citrus psorosis virus (CPsV), and huanglongbing (greening), etc., rigorously affect citrus plant growth, fruit quality, and yield. Under such conditions, advanced tissue culture techniques provide the best possible alternative for producing pathogen free planting material from elite citrus cultivars. The aim of this study was to develop an efficient tissue culture protocol that enables producing virus-free elite three citrus cultivars such as Mexican lime (Citrus aurantifolia), Valencia Rhode and Hamelin (Citrus sinensis). For this experiment, style and stigma explants, aseptically cultured on MS (Murashige and Skoog) medium. Explants (unopened flowers) were collected and sterilized using (1.5% and 2%) of NaOCl for (10, 15 and 20 minutes) of time. The MS culture media supplemented with different concentrations and combination of BAP, BAP+2,4-D, and BAP+NAA were used for callus induction and regeneration. The MS medium without PGRs was used as a control. Study results showed that, sterilizing with sodium hypochlorite (NaOCl) at a concentration of 1.5 % for 20 minutes found to be the optimum concentration and exposure of time (100%) for sterilizing of explant. The highest calli initiation (100%) responses were observed on MS medium supplemented with 6-benzylaminopurine (BAP) at 4.0 mg/L followed by MS medium supplemented with the combination of 6-benzylaminopurine (BAP) at 2.0 mg/L and 1-naphthaleneacetic acid (NAA) at 2.0 mg/L (83.33%) in this experiment. Maximum shoot regeneration (66.66%) responses were obtained using MS medium supplemented with 6-benzylaminopurine (BAP) at 4.0 mg/L. MS medium without plant growth regulators showed the best rooting (83.33%) response. Finally, 66.66% of regenerated plantlets on MS medium were in vitro raised by GA3 at 5 mg/l. Among the studied cultivars, Mexican lime showed relatively higher in terms of regenerate ability, indicating that this cultivar could be targeted for callus induction and regeneration to produce virus-free plant. Therefore, the present study had successfully regenerated from style and stigma tissue culture.

Keywords: Callus, Media, Murashige and Skoog, Micropropagation, Regeneration, Rooting, Tissue Culture.

1. INTRODUCTION

1.1. Background

Citrus is a genus of flowering plants and shrubs in the family (Rutaceae). Plants of this genus produce citrus fruits such as oranges, lemons, pommelo, grapefruits and limes. The genus Citrus is native to South Asia, East Asia, Southeast Asia, Melanesia and Australia (Liu *et al*., 2012). Citrus is widely grown in more than 140 countries in six continents in subtropical and tropical regions of 40 degrees north and south. Brazil is the largest producer, followed by the United States (USA), China and Mexico. Spain, the United States and South Africa are the largest exporters, followed by Turkey and Morocco. Citrus fruits are important fruits in international trade and require excellent quality and shelf-life characteristics (Mekbib *et al*., 2006).

Citrus is one of the most important commercial fruit crops in terms of economic value and human nutrition. Fresh fruits and juices are the most important citrus products, but essential oils, pectin, marmalade, candied and dried skins are also of commercial value. This genus has high economic and nutritional value and derives its economic importance from fruits that are consumed fresh or squeezed into juice (Talon and Gmitter., 2008). Citrus peels too have high importance and can be candied, used as livestock feed, in the perfume, bakery and soap industries (Dhanavade *et al*., 2011).

Citrus has been used in many medicinal formulations to treat a variety of diseases, from toothache, diarrhea, constipation, and insomnia to vomiting (Singh and Rajam, 2010). It has a bioactive secondary metabolite that is effective against cancer and degenerative diseases (karimi *et al*., 2012). Health experts often suggest consuming citrus fruits for minerals, vitamins, and other necessary ingredients to restore health by quickly improving appetite (Rakesh *et al.,* 2013). Flavonoids in citrus fruits play an important role in preventing the development of hyperglycemia by increasing glycogen, hepatic glycolysis, and reducing hepatic gluconeogenesis (Shen *et al.,* 2012).

Many citrus genotypes are apomixis and polyembryony; in such genotypes adventitious embryos develop from cells within the nucellus of the mother plant and may develop with zygotic embryos (Carimi *et al*., 2005). Apomixis comes from two Greek word "APO" (away from) and "MIXIS" (act of blending or mingling) or Apomixis is a form of asexual reproduction that happens through seeds, within which embryos develop without fertilization whereas Polyembryony implies that more than one embryo, develops within one seed. It is also referred to as adventitious embryony (Nucellar embryony or Nucellar budding) (Kumar, 2019).

There are many possible causes for Polyembryony. A specific cell within the nucellus, or a particular cell with an integument, has an embryo. Genetically, these embryos have the same genotype as the parent plant and are apomixis. This event produces a variable percentage of nucellus offspring that are genetically the same as to the female parent. Standard citrus rootstocks propagate by growing openly pollinated seeds, whereas many rootstocks are highly polyembryony and propagate species-specific through nucellus embryos (Tzatzani *et al*., 2018).

Among fruit species, citrus fruits are most severely affected by pathogens transmitted through vegetative propagation materials. Diseases caused by viruses and virus-like pathogens cannot be effectively combated by chemical treatment, so prevention is the most important means of combat (Roistacher *et al.,* 2006). Prevention includes the application of certification. This includes a number of legal and technical procedures to ensure the quality of the final breeding material from the selected source plant. These plants need to be individually checked for the presence of viruses and their pomological characteristics (Roistacher *et al.*, 2006).

Ethiopia is a relatively lower citrus production than western country and Ethiopia had been known to export citrus from the 1960's to the Middle East and Western Europe. However, over the past 30 years export volumes have dropped due to poor quality delivered onto the market, which is mainly due to lack of improved production practices and technology transfer has hampered industry growth. (Ayana, 2021). citrus production and productivity in Ethiopia are extremely threatened by many grafts transmissible diseases like, citrus tristeza virus (CTV), citrus exocortis viroid (CEVd), citrus psorosis virus (CPsV), and

huanglongbing, etc. It became a serious citrus production constraint because of its impact on yield, quality and international trade (Ayana, 2021)

Graft-transmissible pathogens are widespread in citrus growing areas, some of which can have serious impacts on the citrus industry, such as citrus tristeza virus (Meziane *et al*., 2017). The phytopathological status of citrus reproductive materials is still unknown in some countries. The use of "healthy" plants is one of the most effective means to prevent the penetration and spread of these infectious agents in new citrus growing areas and to improve the citrus industry. Methods developed for sanitation of citrus plants from graft-transmissible pathogens include the use Nucellar embryos of Citrus, heat-treated buds and *in vitro* shoot-tip-grafting (STG) (Meziane *et al*., 2017). The latter effectively overcome the limitation of previous techniques by producing pathogen-free, true-to-type citrus plants without long juvenile phase (Murashige *et al.* 1972).

However, STG didn't give fitting results in the elimination of some infectious agents (e.g., Citrus psorosis virus) (Carimi *et al*. 2013). Conversely, *in vitro* regeneration by stigma and style (both as a single explant) somatic embryogenesis (SE) is a promising technique for improving citrus plants, free from the main pathogen, true-to-type and with a short juvenile phase (Metoui *et al.*, 2020). Somatic embryogenesis (SE) made using stigma/style explants resulted in 100% "healthy" citrus plant regeneration (Carimi *et al*. 2013). Citrus nucellus seedlings are completely virus-free because the embryo sac and attached tissues are filled at a flowering time with some unknown powerful substances which kills all the viruses (Kumar, 2019). Therefore, to clean graft transmissible of citrus diseases, it is important to optimize the *in vitro* protocol development of the selected cultivar using style and stigma culture (Meziane *et al*., 2017).

Somatic embryo formation from Stigma and style culture is a new technology in citrus sanitation that has been shown to be very effective for completely eliminates 'psorosis, tristeza, infectious variegation, concave gums, impietratura, cristacortis, exocortis and cachexia' (Metoui *et al.,* 2020). Stigma / style somatic embryo formation is one of the most effective methods for plant regeneration in most citrus fruits without inducing somaclonal variation. In addition, style / stigma somatic embryogenesis has been shown to be effective in eliminating major citrus viruses and virus-like diseases (Metoui *et al.,* 2020). This

technique is used in this research activity. Three citrus cultivars (orange, lemon, lime) were selected and tested for the presence of the most important viruses and virus-like pathogens.

1.2. Statement of the Problem

Graft transmissible diseases of citrus are one of the most important limiting factors to citrus (Rutaceae), affect its health, and, thus, contribute to significant losses in yield. Citrus is attacked by several graft-transmissible pathogens that affect its fruit quality. The main graft-transmissible diseases of citrus are Tristeza, greening, stubborn, exocortis, ringspot psorosis, and lots of others (Lee, 2014). In developing countries where the protection and proper handling of fresh fruits are inadequate, losses during transportation and storage can account for more than 50% of harvested crops (Mekbib *et al*., 2006). Pathogen attacks not only reduce plant yields and quality but also result in plant death. To combat this problem the methods for elimination of this pathogen from citrus were needed.

Methods for elimination of graft-transmissible pathogens from citrus plants include the utilization of nucellar embryos citrus, heat-treated buds and *in vitro* shoot-tip-grafting (STG) (Meziane *et al*., 2017). Shoot-tip grafting and thermotherapy have proven to be excellent treatments for removing many pathogens from citrus bud wood, but they do not guarantee 100% success in removing all pathogens. *In vitro* regeneration by somatic embryogenesis (SE) from stigmas and style has been shown to be a promising way to obtain short-lived citrus fruits, free from major infectious diseases, true type and short juvenile phase (Carimi *et al*. 2013).

Somatic embryo (SE) produced using stigma/style explants resulted in 100% "healthy" citrus plant regeneration (Carimi *et al.* 2013). Therefore, we decided to perform somatic embryogenesis from the stigma and style of citrus cultivars. Somatic embryogenesis was used to eliminate many graft-transmissible diseases and also the characteristic polyembryony of most citrus species allows obtaining nucellar progeny identical to mother plants. Ovule culture *in vitro* (in the case of seedless polyembryonic varieties) and nucellus culture *in vitro* (in the case of mono embryonic varieties) were developed for the aim of obtaining nucellar progeny identical to the mother plant. Therefore, production of virus-free citrus cultivar was carried out by an *in vitro* optimization protocol using plant organs (style and stigma) at melkassa Agricultural research center.

1.3. Objectives

1.3.1. General Objective

- ❖ The general aim of this study was to develop an efficient tissue culture protocol that enables producing virus-free elite citrus cultivars.

1.3.2. Specific Objectives

The specific objectives of this study are:

- ✓ To determine the optimum concentration and duration of exposure of elite citrus cultivars to the local bleach (Berekina) for effective surface sterilization.
- ✓ To determine the optimum concentration of plant growth regulators for effective callus initiation, shoot elongation and regeneration.
- ✓ To determine the optimum combinations and concentrations of auxins and cytokinin for effective shoot and root growth and development from callus.

1.4. Significance of the Study

The result of this study will provide information on the production of virus-free, and uniformly high-quality selected citrus (*Rutaceae*) varieties through tissue culture protocol optimization. It also gives illustration for future study to know the concentration of NaOCl for sterilization on citrus regeneration and the effect of genotype on citrus regeneration. In addition to this, it is preliminary study for future study to know the effect of cytokinin and auxins for citrus regeneration protocol. It also contributed significant ideas for other researchers for further studies on the citrus research associated with tissue culture.

2. LITERATURE REVIEW

2.1. The Origin and Distribution of Citrus

Citrus fruits originated in tropical and subtropical regions of Asia, especially Southeast Asia including China, India, and the East Indies where citrus fruits, especially mandarins and pommelo, were considered highly prized tributes and were only available in the courts of the state. Lemons were originally grown in India and sweet oranges and mandarins are native to China (Liu *et al.,* 2012). Citrus fruit is one of the most popular and important fruits in the world. Globally available and common plants contribute to human nutrition. Because of the delicious taste, affordable value, and consumer awareness of the gradually recognized potential health properties, citrus fruits and products are very predominant with widespread nutritional and economic impacts in both developed and developing countries (Kour and Singh, 2012).

Citrus sinensis L. is one of the most important fruits known since ancient times. It's an excellent source of vitamin C with high antioxidant potential. The origin of citrus fruits is believed to be in Southeast Asia, including East Arabia to the Philippines, and the Himalaya region to Indonesia or Australia (Talon *et al*., 2020). Although citrus fruits are grown in more than 140 countries around the world, most of the harvests are grown on both sides of the equator, including the tropical and subtropical regions of the world at latitudes of 35 degrees north and latitudes of 35 degrees south with cultivation and production concentrated in major regions within the northern hemisphere (Kaur, 2016). Citrus trees are easy to cross, so new species emerge from time to time (Suryawanshi J, 2011).

Most of the cultivated citrus species are a component of the Citrus genus containing, based on the taxonomists, between 16 (Swingle and Reece 1967) and 156 species (Tanaka1961). Barret and Rhodes (1976) were the first to propose the existence of four basic taxa based on morphological descriptors (*Citrus maxima (Burm.) Merr*. —the pummelos, *C. medica* L.—the citrons, and *C. reticulata* Blanco—the mandarins) from which all cultivated Citrus descended (Badenes & Byrne, 2012). The cultivated species such as (*C. aurantium* L.—the sour orange), *C. sinensis* (L.) Osbeck—the sweet orange, *Citrus micrantha, C. paradisi Macf* —the grapefruit, *C. limon* (L.) *Burmf*. —the lemon and *C. aurantifolia (Christm.*) Swingle—the lime are appeared by recombination of the four taxa (Badenes & Byrne, 2012)

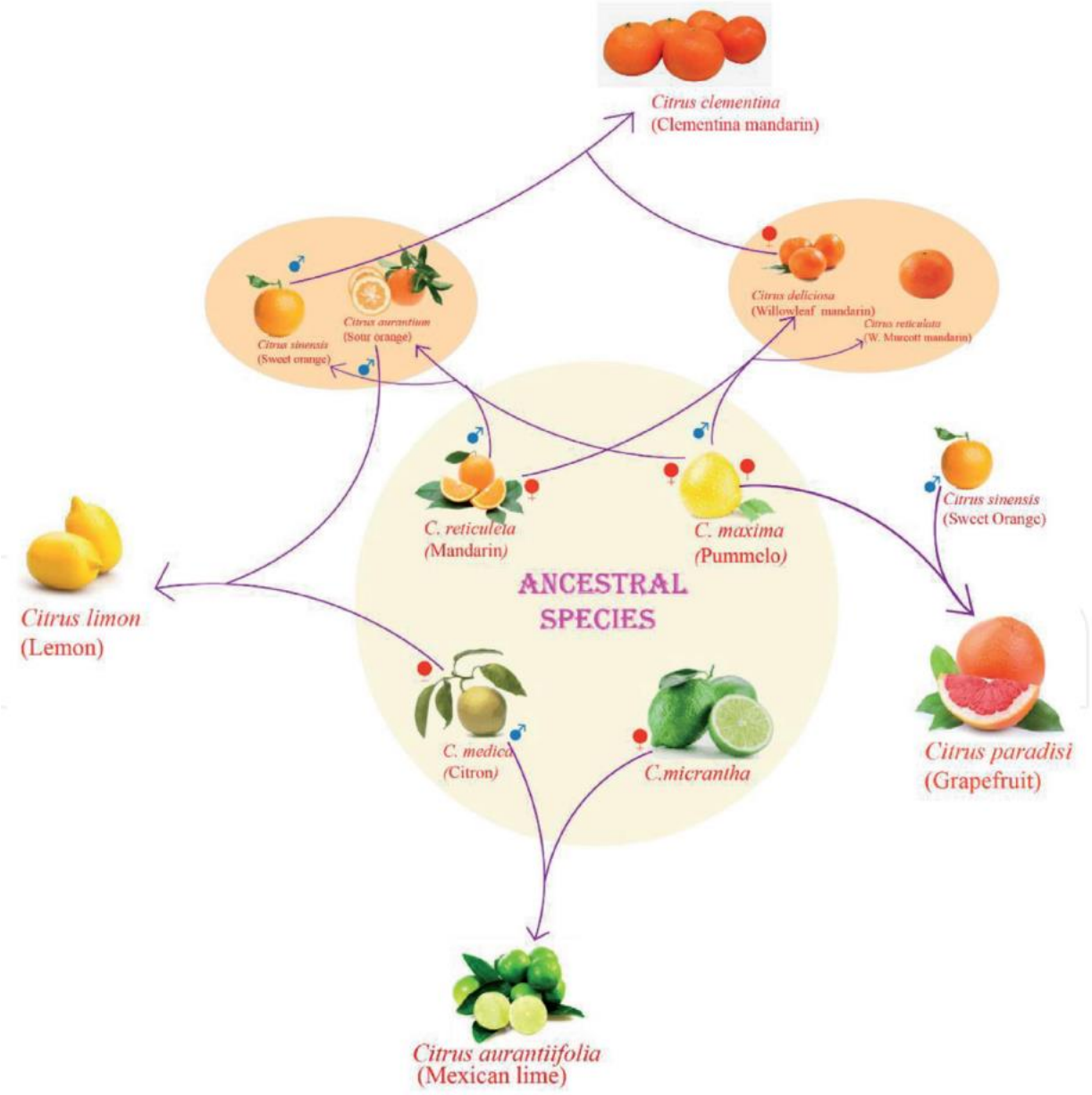


Figure 1. The genealogy and evolution of citrus fruits. Four ancestral species namely; *Citrus reticulata*, *Citrus maxima, Citrus medica,* and *Citrus micrantha*, depicted in the central circle, have contributed to the evolution of cultivated species. Source (Wu *et al.*, 2018).

Citrus fruits form a major fruit group consisting of tangerines, oranges, lemons, pommelo, grapefruits, tangelo, trifoliate orange, and lemons. It is one of the most important fruits in Ethiopia. Cultivation began in the Upper Awash Valley and Melkassa area of central Ethiopia. It is mainly produced in Dire Dawa, central Awash and the Melkassa area in southeastern Ethiopia (Mekonen., *et* al 2015). Ethiopia agricultural climatic conditions are

ideal for the production of high-quality citrus fruits. Five bio-phenological regions (Dire Dawa, lower and central Awash, Melkassa area) have been identified as potential production centers capable of supplying citrus fruits throughout the year, based on flowering time and climate (Mekonen., *et* al 2015).

Citrus production is over 100 million tonnes and the market is saturated, especially when it comes to orange production. Consumers are looking for seedless, easy-to-handle, easy-to-skin, sufficient size and juice content, balanced sugar acid ratios, and attractive, light-colored fruits. Current production trends show that the variety profile is shifting to lighter peels. This is especially true given the fact that the two hemispheres are complementary and that the development of transport technology will enable long-term transport and storage (Abbas, 2009).

Fruits of these varieties should be regarded as health and health-related compounds, and should have good Firmness, juiciness, good taste, good color, good size, seedless, and easy to peel and resistant to post harvest pathogens and to physiological disorders (Liu *et al*., 2012). These are aimed at ensuring the supply of high quality, safe and healthy products to consumers on the market. It includes accounting of all practices and processes performed at the plant at each point in the supply chain, implementing traceability systems and superior production, harvesting, packing, transportation and storage practices, and also implementing quality and management systems that are certified as appropriate and optimal for the citrus sector (Spreen *et al*., 2020)

2.2. Botany of Citrus

The plants are a shrub or tree, usually thorny, with alternating leaves, and coriaceous persistent. The petioles are often winged and the flowers are axillary, individually bundled, white or pink, with a sweet scent. The petals are straight, elongated, oblong, thick, imbricate, and 4-8 in number. Seeds are horizontal or sagging, testa coriaceous or membranous, embryos may be more than one in one seed, cotyledons are plano-convex, often uneven, radicles small and superior (Khalid *et al*., 2010). Citrus trees are evergreens that produce fruits of various shapes and sizes from round to rectangular, full of aroma, flavor and juice. They have a rough, tough, bright color with green to yellow rind or rind known as epicarp or flavedo, which covers the fruit and protects it from damage (Khalid *et al.*, 2010).

The glands contain essential oils that give the fruit a characteristic citrus aroma. It is composed of white, thick, spongy mesocarp or albedo, which together with the outer pericarp form the pericarp or fruit skin. The inner part forms pulp and is divided into individual segments or juice sac (depending on the variety, with or without seeds) by radicals or a thick film of inner pericarp. This part is rich in soluble sugars, ascorbic acid, pectin, fiber, various organic acids and potassium salts, giving the fruit a characteristic citrine taste (Khalid *et al*., 2010).

Citrus fruits aren't only delicious and refreshing; they also provide vitamins, minerals, and lots of other substances. Citrus fruits contain a significant amount of vitamin C. And also, fruits are an excellent source of vitamin B complex (Suryawanshi, 2011). The mild bitterness of juice is because of a glucoside called Naringin which is assumed to own medicinal properties. citrus peels are rich in pectin and essential oils. There are several citrus fruits in the world. Some are ancient and a few are recent (Suryawanshi, 2011).

Before the advent of molecular data, Citrus was classified based on morphology or biochemical techniques such as isozymes. There are currently two commonly used classifications of Citrus: Swingle (Swingle and Reece, 1967) and Tanaka (Tanaka, 1977). There are 16 species in the genus Citrus Swingle and 162 species of Tanaka (De Araújo et al., 2003). Hence classification of the kinds and varieties are complicated. An abundance of locally used names and changing botanical nomenclature also hinders distinct classification. According to Swingle and Reece (1967), important citrus species are:

- ✓ Sweet orange - *Citrus sinensis*
- ✓ Mandarin Orange - *Citrus reticulata*
- ✓ Acid lime - *Citrus aurantifolia*
- ✓ Lemon - *citrus limon*
- ✓ Grape fruit - *Citrus paradise*
- ✓ Pummelo - *Citrus grandis*
- ✓ Citron - *Citrus medica*
- ✓ Sweet lime - *Citrus limettoides*
- ✓ Gaganimma - *Citrus pennivesiculata*
- ✓ Vadlapudi Orange - *Citrus madaraspatana*
- ✓ Sour Orange - *Citrus aurantium*
- ✓ Rough lemon - *Citrus jamberi*

- ✓ Rangapur Lime - *Citrus limonia*

The subtropical climate is ideal for the growth and development of citrus fruits. Dry conditions combined with a well-defined summer (range 75 mm to 250 mm) with little rainfall are best suited for citrus growth. High humidity promotes the spread of many diseases. Frost is very harmful to citrus fruits. Hot summer air leads to dehydration and dripping of flowers and young fruits. Among fruit species, Mandarin (Khasi Orange type) grows up to 2000m in height to adapt to cooler climates. Citrus fruits grow well in many soils. Soil characteristics such as soil reaction, soil fertility, drainage, free lime and salt are several key factors in determining the success of citrus plantations (Yekeen, 2014).

Citrus fruits grow on well-drained, light soil. Deep soils with a pH range of 5.5 to 7.5 are considered good. However, it can grow in the pH range of 4-9. The presence of calcium carbonate levels in the feeding zone can adversely affect growth. Light loam on heavier but well drained sub-soils appears to be ideal for citrus (Yekeen, 2014). Seeds show 45-90% polyembryony. Seedlings are relatively free of rot and other viral diseases that are not transmitted through seeds. However, the yields aren't good compared to budded plants on account of the beneficial responses of the root stock viz., earliness, high yield and adaptability to the environment and soil, resistance to diseases and pests, and good quality. Vegetative propagation methods like budding are practiced (Tzatzani *et al.,* 2018).

Propagating varieties have to be known for their excellent performance and virus-freeness. the root stock should be compatible with the scions and well adapted to the soils of the regions where tress is meant to be grown. The stock and scion should be compatible and able to produce long-lived and productive wood. The influence of the foremost important and widely used root stocks and their characteristics should be known to the grower for selecting the right kind of stock for the locality (Tzatzani *et al*., 2018). Unlike another fruits, citrus fruits do not continue to ripen after being removed from the tree, so it's important to choose them at the right stage of ripening. Ripeness is measured according to various properties like color, juice content, soluble solid (sugar) content, and solid to acid ratio. Citrus fruits are usually harvested by hand (Sood, 2009).

Citrus lemon is the botanical name of lemon and it's a necessary citrus species. In recent years, it has been produced on a large scale all over the world. In juice processing the by-

product obtained is the peel of lemons. The skin is rich in dietary fiber. Lemon contains other useful ingredients that are beneficial to health. It's a vital source of protein, fat and macro minerals (Janati *et al*., 2012). Lemon is used to form juice and utilized in various sprayers and dishwashers (Sandhu and Minhas, 2006). *Citrus Sinensis* is the name of an orange plant and is the fruit of an evergreen tree. It's one amongst the most widely used fruits in the world. They are available in most countries of the world. It's considered one of the best citrus fruits rich in ascorbic acid. Orange has a sweet and bitter taste. Oranges contain a wide variety of carbohydrates, fats, and proteins and are much more nutritious than other fruits. Ascorbic acid is found in a large amount about 45 mg or 75% per 100 g of orange (Buyuktuncel, 2021).

2.3. Uses of Citrus Fruits

Citrus has several uses, including: Waste from oranges can be used as animal feed. It is also used as a fragrance. It is used as a skin bleaching agent. Oranges and their juices are used as energetic drinks. It gives relief when someone is exposed to heat. It is an excellent antioxidant. Ripe orange juice is suitable for all kinds of fever. It contains a lot of potassium and lowers blood pressure. Orange juice is good for arthritis. It is a good medicine for stomach problems (Sandhu and Minhas, 2006). Lime (Citrus aurantiifolia) is a citrus fruit, usually round, green, 3-6 cm in diameter, and contains acidic juice vesicles. There are several types of citrus fruits called lime, such as key lime, persian lime, kaffir lime, and dessert lime. Lime is a rich source of vitamin C, acidic and often used to enhance the flavor of foods and beverages. They are cultivated all year round. Plants with fruits called "limes" have different genetic origins. Lime does not form a monophyletic group (a group of organisms that are descendants of a single ancestor) (Janati *et al*., 2012).

2.4. Nutraceutical Importance

Citrus fruits, different in color and size, are very nutritious. These are beneficial to health due to the presence of bioactive compounds such as carotenoids, flavonoids and ascorbic acid. These compounds have antioxidant, antitumor, anti-inflammatory, and blood clotting inhibiting (antithrombotic) characteristics (Rehman *et al*., 2020). These fruits are also a rich source of vitamins and minerals such as vitamin C, A and B complexes. Vitamin A promotes skin and vision, but vitamin B complexes such as thiamine, folic acid, and pyridoxine are

needed as an external source for replenish. Of the minerals, potassium, magnesium, calcium and sodium are very abundant in citrus fruits. However, trace amounts of zinc, iron and manganese are present (De Moraes Barros *et al*., 2012).

Nutraceutical is a combination of two words. "Nutrition" and "pharmaceuticals"; therefore, the term infers that nutraceutical could be regulated as dietary supplements, medicines, and food ingredients. The term "dietary supplement" is also explained as follows: “Food, or parts of food that provide medical or health benefits, including prevention and treatment of illness" (Kalra EK *et al*., 2003). Nutraceuticals protect against diseases developed due to nutrient deficiencies and also have physiological benefits. These are used as dietary supplements and food ingredients. Because these are dietary supplements, they may contain vitamins, minerals, plant extracts essential amino acids, Poly Unsaturated Fatty Acids (PUFA), and enzymes that may be deficient in most diets (Wu *et al.,* 2018).

There are another category of nutraceuticals or dietary supplements are nutrient-fortified food. For example, iron-enriched fruits protect people from the diseases caused by iron deficiency. Iron deficiency anemia is a good example. These crops can protect disease from humans that caused due to vitamin A deficiency such as night blindness and xerophthalmia (Wu *et al.,* 2018). Various types of citrus fruits are used in Ayurvedic medicines and remedies. *C. medica Linn.* (Bijapur) is used as appetizer, cardio-stimulant, and antiemetic. *C. Jambhiriis* used as antidiarrheal, improves digestion essential oil of *C. Limon* exhibits antioxidant activity in the prevention of lipoperoxidation and exerts antinociceptive activity through a central depressant mechanism by changing the motor coordination of Swiss albino mice treated with naloxone. Citrus fruits are an excellent source of vitamin C, a nutrient that strengthens the immune system and keeps the skin smooth and elastic (Okwu, 2008).

2.5. *In vitro* Micropropagation of Citrus

Citrus trees are usually grafted to nucellar seedlings, which are used as rootstocks, or propagated asexually by cuttings. Most species in this genus are characterized by a high degree of polyembryony consisting in the production, in a single. Citrus seeds are composed of 1-40 adventitious embryos that pass through the nucellar, resulting in the development of two or more embryos. Nuclear embryos resemble (similar) to mother plants, but zygotic

embryos tend to be weak and variable, and very often do not survive because they have to compete for nutrients and space with nucellar embryos (Antonietta *et al*., 2020)

Due to polyembryony, open-pollinated seed germination is a common procedure used in citrus for large-scale propagation of rootstocks in nurseries. Tissue culture is one of the most interesting areas of plant biotechnology, consists of the ability to establish and maintain plant tissues (callus, cells, protoplasts, etc.) and plant organs (embryos, shoots, roots, flower parts) in a nutritive growth medium, in a culture vessel, in aseptic conditions, and under controlled environmental conditions (Antonietta *et al*., 2020).

Micropropagation is a valuable alternative to traditional propagation, especially when shoot regeneration is achieved by direct *in vitro* organogenesis. It provides the nursery with many practical benefits, such as speeding up and improving the vegetative propagation and the production of true-to-type and virus-free plants; the mass propagation of clones; and plant production in a short time in a limited space and throughout the year (Carimi & Pasquale, 2014).

Micropropagation does not appear to be commercially important to citrus, just because most species are characterized by a high degree of polyembryony. In addition, another use for *in vitro* growth is the potential for genetic transformation (Dutt, & Grosser, 2009). In citrus, higher multiplication rates are obtained using juvenile explants, but plants regenerated from these tissues usually exhibit strong undesirable juvenile characteristics and are slow to come into bearing. In order to introduce genetic variation through genetic transformation or mutagenesis, it is especially important to have an efficient protocol available starting with adult tissue. Due to high levels of contamination, few protocols have been developed for micropropagation of mature citrus fruits. *In vitro* regeneration has been obtained from several citrus fruits by various *in vitro* techniques and regeneration pathways such as somatic embryogenesis and organogenesis (Antonietta *et al*., 2020).

2.6. Citrus Production in Ethiopia

World citrus production was 157 million tons. Between 1970 and 2019, global citrus production increased significantly from 39.6 million tons to 157 million tons, with annual rates peaking at 10.47% in 1980 and then declining to 3.59% (FAO, 2019). Citrus production

is 11.1 million hectares, with mass production of oranges (75 million tons), followed by mandarin, tangerine, satsuma (34 million tons), lemon and lime, grapefruit and pomelo (9 million tons) (FAOSTAT, 2018).

Citrus fruits are one of the most economically important fruit crops cultivated by small and commercial farmers in Ethiopia. In 1985 it occupied 7,290 hectares of land in Ethiopia, but its area was reduced to 5,380 hectares (2200 oranges, 1750 mandarins, 1100 hectares of lime and lemon, respectively), producing only 33,500 tonnes (Mekonen, 2015). Currently, the total area coverage and the annual production of citrus in Ethiopia were estimated 5165.28 ha and 38486.5 million tons respectively (Ayana, 2021). The decrease in production area over time is because of many production-restricting biological and abiotic factors (Mekonen., *et* al 2015).

Traditional methods for Citrus propagation are supported bud-wood selection and grafting for scion varieties. Rooted cuttings, or more often nucellar seed propagation, are wont to prepare rootstocks. The selection of rootstock requires too many citrus characteristics, like resistance to biotic and abiotic stress, fruit quality and size, productivity, maturity time, and precociousness (Bowman *et al.,* 2020). Additionally, to stimulating reproductive growth, the best rootstock must be cold-tolerant, drought-tolerant, and heat-resistant and adaptable to a variety of weather conditions (Caruso *et al*., 2020).

2.7. Citrus Disease and Management Strategies

Environmental problems such as physiological disorders, pests, and disease, are the main causes of low citrus yields, but the disease, affects not only the quality of citrus fruits, but also their production. Citrus fruits are in danger of the threat of various diseases caused by various pathogens, and the economic loss of plant diseases is usually serious (Singh *et al*., 2007). Especially graft transmittable citrus diseases are more common to our country. The graft transmissible disease occurs at all stages of development and indiscriminately affects leaves, fruits and young twigs. It destroys citrus production. Citrus fruits are mainly propagated by grafting. Haunglongbing (greening), citrus tristeza virus, gummosis, and psoriasis are citrus diseases transmitted during the grafting period (Ayana, 2021).

Symptoms appear in numerous parts of the tree. Infected trees generally have withered twigs, discolored leaves, and canopy thinning. Symptomatic leaves can be of normal size, yellow, or speckled, or small, upright, and have a variety of whitening patterns (Albrecht *et al.*, 2016). Huanglongbing is related to the presence of the gram-negative bacteria genus *Candidatus* Liberibacter (CL). Three species are known to cause the symptoms of HLB: CL asiaticus (CLas), CL americanus (CLam), and CL africanus (CLaf). The Asian and also the American species can be transmitted via the psyllid Diaphorina citri Kuwayama (Hemiptera: Psyllidae), commonly called Asian citrus psyllid (ACP), and the African species by the insect Trioza erytreae (Hemiptera: Triozidae) (Bové, 2006).

Graft transmissible of citrus diseases can cause 50 to 100% yield loss; and typically renders the fruits don't sell well. It also affects the yield and quality of essential oils extracted from the rind of citrus fruits. In Ethiopia, citrus trees are affected by a variety of diseases caused by many fungi, bacteria, viruses and virus-like organisms (Taye, *et al*., 2018). Viruses and virus-like diseases like psorosis, tristeza, and greening are reported to be of great economic importance and play a crucial role within the decline of citrus orchards in the country. Tristeza and greening are one of the most important and prevalent citrus diseases in many citrus growing areas of Ethiopia. Three aphid species are viz. *Aphis gossypii*, *Toxoptera aurantii*, *Toxoptera citricidus* and the *Citrus psylid* are the sole insects that occur as vectors of citrus diseases in Ethiopia (Taye, *et al*., 2018). Their geographic distribution and population densities among the aphids recorded, A. gossypii was the least common species on citrus. it has been observed in Arba Minch, Dire Dawa, Erer Gota, Gibe and Melka Werer. Previous studies have suggested that at almost all altitudes the bulk is covered by *T. citricidus*. It is referred to as an efficient vector of Tristeza virus and is confined to the middle and upper parts of the Awash Valley (Dagnew *et al*., 2014). Citrus virus, viroid and virus-like infections are widespread in the main citrus growing areas. They spread primarily through the utilization of infected reproductive materials (Roistacher, 2006).

The economic importance of citrus diseases currently present in Ethiopia includes, citrus wither tip, citrus Tristeza virus, citrus gummosis, citrus canker, citrus greening, slow decline (citrus nematode) spreading decline (burrowing nematode), blight, greasy spot, Alternaria brown spot, Phytophthora diseases and postharvest decay of citrus plants (Taye, *et al*., 2018).

2.7.1. Citrus Canker

Citrus canker is a disease that affecting citrus in worldwide and is caused by the bacterium *Xanthomonas campestris* pv. *citri*. This infection causes sores on the leaves, stems and fruits of citrus fruits like lemons, oranges and grapes. The canker isn't harmful to humans, but it's a serious effect on the vitality of citrus trees, causing premature shedding of leaves and fruit. Canker-affected fruit is safe to eat, but unsightly to be marketable (Eshetu, 2007). The disease is believed to possess originated in geographic region, is extremely resistant when it becomes established in a neighborhood, making it necessary for all citrus orchards to be destroyed for successful eradication of the disease (Singh *et al*., 2007). Citrus canker bacteria have a short life in soil and leaves. The short lifespan in natural soils is due to microbial interactions, especially predatory effects of protozoa. If the wound leaves and twigs are moistened on the soil surface or placed at a depth of 3-6 cm, the survival period doesn't exceed three weeks. If plant debris is maintained under dry conditions, survival increases to 2–3 months (Gabriel *et al.*, 2020).

Symptoms:

first raised, watery spots appear on leaves, twigs, thorns, branches and thorns.Later spots become thickened, brown and corky. Infection to petioles, midrib causes premature defoliation. In severe attack symptoms produced on all plant parts (Gabriel *et al*., 2020).

Management:

Crude streptomycin at 100-1000 ppm at 15day intervals. Phytomycin 2500 ppm or streptomycin-100 at 500 ppm Copper Ammonium in reduced 86-90% disease incidence (Gabriel *et al.*, 2020).

2.7.2. Citrus Greening

Citrus greening is known to be one of the most devastating diseases affecting citrus fruits round the world (Halbert and Manjunath, 2004). The disease is related to *Candidatus* Liberibacter, an alpha Proteobacteria which is gram-negative, fastidious, and phloem limited (Damsteegt *et al*. 2010). The *Candidatus* Liberibacter genus contains three species that are known to cause disease in citrus: Candidatus Liberibacter asiaticus (Ca. L. asiaticus), *Candidatus* Liberibacter americanus (Ca. L. americanus), and *Candidatus* Liberibacter africanus (Ca. L. africanus) (Ajene *et al*., 2020). *Candidatus* Liberibacter asiaticus (CLas) is widely believed to be the bacterial agent accountable for the disease in citrus growing

countries. CLas and its related strains, Ca. L. americanus (CLam) and Ca. L. africanus (CLaf), are transmitted by the vector's Asian citrus psyllid Diaphorina citri Kuwayama and African citrus psyllid Trioza erytreae (Ajene *et al*., 2020). The bacterium CLas resides in the plant's phloem. When the psyllid feeds on a leaf of an infected citrus plant, it sucks up the nutrient-dense sap containing CLas bacteria from phloem. Psyllids then fly to the following uninfected plant to repeat the method and spread the pathogen. CLas proliferates within the phloem, thus blocking the transportation system and causing stunted growth, premature and lopsided greening fruit and yellow shoots, and eventual death. Citrus greening disease (HLB) has caused havoc within the citrus industry around the world (Abutineh *et al*., 2021). It penetrates the conductive tissue and causes the citrus to decline, making it unproductive. Citrus greening probably came from China. Today, it's a significant problem in all citrus producing countries (Javed *et al*., 2007).

Symptoms:

zinc deficiency and various other conditions can cause symptoms similar to citrus greening. Symptoms include yellowing of veins and adjacent tissues, yellowing or staining of whole leaves, sometimes corking of veins, subsequent premature defoliation, and dieback of twigs, defoliation, decay of feeder rootlets and lateral roots, decline in vigorous and ultimately, the death of the entire plant. Diseased leaves are hardened, straightened, and bent outwards, but after young leaves and premature defoliation, they're small, thin, and have symptoms of zinc deficiency (Singh, 2000).

Management:

Elimination of Citrus psylla comprises with 0.02% Diazinon or endrin spray with Bavistin and Ledermycin (500 ppm) sixfold at 10-day intervals.

2.7.3. Citrus Wither Tip

The disease is caused by the fungus Colletotrichum gloeosporioides, the branches and leaves of the affected trees dry up and it becomes difficult to require care of the tree. Apply a balanced dose of fungicide 4:5:0 after pruning the branches affected by the trees (Raza *et al*., 2019)

Symptoms:

Die back often progresses slowly and leaves wither, turn yellow and drop. Twigs and branches appear to have been burned by fire. Drying of twigs from top to down ward And When twigs were gasping, minute brown-to-black, slightly raised, clumped pustules are observed which are acervuli of the fungus. Dry twigs are ash colored affected fruit showed tar stain symptoms (Raza *et al*., 2019).

Management:

Spray with Benomyl and Captafol 1-4 applications. Start Bordeaux Mix (1%) or Copper Fungicide (Copper Oxychloride) at an interval of 21 days. Bavistin at 0.1% also inhibits the development of the disease (Raza *et al*., 2019).

2.7.4. Citrus Tristeza Virus

Citrus Tristeza Virus (CTV) is one of the most destructive of the numerous viruses that affects citrus. The virus pathogen has been responsible for the death and debilitation of millions of trees. These strains varied in severity, vector specificity, and host range (Ayana, 2021).

Symptoms:

the symptoms are vein clearing, flecking of veins and Leaves bronze, and golden, yellow. Leaf wilt, drop, reduced fruit size, while hanging on dead tree. Stem pitting- honey combing, pegs Sudden deaths, necrosis of cambium. Trees show bushy appearance. Twigs tend to be brittle and break (Mehrotra, 2006).

Management:

The management of Tristeza involves cultural management and biological control measures including mild strain cross protection. genetic engineering and breeding is being done for virus resistance in commercially acceptable scions and rootstock. There is no direct chemicals treatment against the virus. It’s been suggested that vector control by chemicals over a large area may reduce spread of CTV by reducing aphid populations (Mehrotra, 2006).

2.7.5. Citrus Gummosis

This can be one of several well-known gumming diseases of citrus fruits. The formation of gums on the trunk or branches is a characteristic of symptom. The gums arise from the blisters that contain the gum pockets that are usually found on the trunk. Several factors contribute to the disease, including frost damage, high groundwater levels, and salt buildup. Gummosis is believed to be a weak and damaged tree condition and has been reported to be infectious. it's also known as brown rot gummosis (Javed *et al*., 2007).

The disease is caused by one or more species of the fungus Phytophthora spp. The disease can affect the root system, underground and above-ground trunks, branches, leaves, flowers and fruits. This is especially troublesome during the long season. Trees with buds bound underground or near them, or in poorly drained areas, are very susceptible. Phytophthora is found in the majority citrus plants (Javed *et al*., 2007).

Symptoms:

Lesions form on the bark at the bottom of the roots of the stem and crown. The infection spreads upward and downward, causing rottening of fibrous root rot. Gum protrusion from the affected trunk and cracks within the bark. During summer, gum remain sticks to the stem. Leaves show signs of nutritional deficiency and drop prematurely (Javed *et al*., 2007).

Management:

The management method for this disease is Bud union 30-45 cm above the bottom and soil level at the time of planting. Paint with Bordeaux paste up to 70 cm and Spray with bordeaux mixture.

2.7.6. Citrus Psorosis Virus

Psorosis is a graft-transmissible viral disease of citrus. The foremost characteristic symptoms of this disease in adult field trees are bark scaling within the trunk and main branches. Gum collects under the scales of the bark and will penetrate the xylem, causing tree stains and vascular occlusions (Roistacher *et al*., 2006). The causative agent of psoriasis is that the Citrus psorosis virus (CPsV). CPsV is classified within the genus Ophiovirus (Greek ‘ophio’ = snake), indicating the elongated twisted and coiled appearance of the virus particles (Achachi *et al*., 2014). The term "citrus psoriasis group" Refers has been used to see several widespread graft-transmissible of citrus diseases. Two forms of psoriasis are

reported. Psoriasis a (PsA) is a more common disease characterized by the presence of bark scales on the trunks and branches of infected cultivable trees and also the discoloration of the wood inside (Achachi *et al*., 2014).

The more aggressive psorosis B (PsB), causes rampant bark scaling even in fine branches in field trees, chlorotic blotching in old leaves with brownish gummy pustules in the leaf underside and sometimes ringspots on fruits (Velazquez *et al*., 2012). The psoriasis virus is transmitted primarily by bud grafting rather than by natural vectors. The disease can have significant economic importance for citrus fruits grown from such transplants, like oranges, grapes and mandarins. serious infections can inhibit growth and fruit yields are reduced by one-third or more. Symptoms vary widely and include the formation of some young leaves of elongated, white to yellow-green spots, rings, or large translucent areas of some young leaves. Certain symptoms tend to diminish as the leaves ripen. Rings bordered by sunken grooves may form on the fruit. On trees 6 to 12 or more years old, the outer bark in localized areas commonly becomes scaly, or small irregular pustules and gum like deposits develop, with the wood being stained underneath. Variously sized cavities or narrow grooves may develop within the massive limbs and trunk (Velazquez *et al*., 2012).

The disease is additionally controlled by eradicating seriously affected trees, planting psorosis-free stock, and using only scions and buds from virus-free trees. Quarantine and certification programs exist in many citrus-growing areas to reduce contaminated stock. The citrus species most severely affected are sweet orange, grapefruit, and mandarin. Certain other species show typical leaf symptoms when infected, but remain freed from bark lesions without obvious cruel effects like sour orange and lemon (Achachi *et al*., 2014)

2.8. Methods for Cleaning of Citrus Diseases

Citrus is one of the most important fruit crops known to man since ancient times. It is a good source of vitamin "C" with high antioxidant potential. Due to suitable agro-climatic conditions, higher planting area, farmers are increasingly interested in the introduction of sweet oranges. However, healthy planting materials to meet planting needs are very limited. A survey of citrus plantations and nurseries based on characteristic symptoms and serological indexes showed that the most common viruses, viroids, and prokaryotic diseases,

citrus Tristeza, citrus variegation, citrus rind, citrus cachexia (wood porosity), citrus greening and Stubbom (Arif *et al*., 2005).

Different methods, such as use nuceller embryo, heat therapy, meristem culture, and shoot tip grafting (STG) to produce virus-free citrus plants. Thermo-therapy is ineffective in eliminating heat-resistant viruses such as rind and wood chips, and cannot be used for heat-sensitive species. In order to overcome these problems, some alternative techniques are used, namely Meristem culture, stem tip embolization and somatic embryogenesis are the preferred methods to eliminate citrus pathogen (Bhat and Rao, 2020).

2.8.1. Nucellar Embryo

As citrus varieties become older, they tend to acquire more and more virus diseases-including scaly bark, quick decline, and stubborn disease. Therefore, it is becoming increasingly important to use virus-free materials for the propagation of new trees. Nucellar embryos in citrus originate directly from cells of a maternal tissue in the ovule-the nucellus. Since sexual reproduction is not involved in their formation, Seedlings that originate from the nucellus, without sexual reproduction, shows a remarkable increase in vigor. The cause of this invigoration is not known but it is a well-established fact that certain virus diseases are eliminated and that this accounts for some of the favorable effects (Bhat and Rao, 2020).

2.8.2. Citrus Shoot-Tip Grafting

Citrus graft-transmissible diseases cause serious economic losses in most citrus growing areas in the world. Many varieties of pathogen-free plants are often not available, so it is necessary to obtain healthy plants from infected plants. In this case, a method is needed to obtain citrus plants that do not contain all graft-transmitted pathogens and have no juvenile characteristics to produce healthy trees for commercial reproduction, and by grafting the tips of diseased plants to young plants grown *in vitro*. Rootstock seedlings came up to obtain some citrus plants (Juárez *et al*., 2015).

Some of these plants do not contain exocortis like viruses and have no juvenile characteristics. This type of shoot-tip grafting (STG) has developed a routine procedure that enables the transplant success rate to reach 30-50%, and the survival rate exceeds 95%, and they have no pathogens transmitted by transplantation. STG has restored hundreds of healthy varieties worldwide and planted hundreds of millions of certified healthy trees. STG is

usually used to regenerate plants from superior genotypes that are extremely difficult to regenerate *in vitro*, such as somatic hybrids, plants from irradiated shoots, haploid and tetraploid plants, and regenerate transgenic plants from transgenic shoots that are difficult to root *in vitro* (Juárez *et al*., 2015).

2.8.3. Micrografting

Micrografting is an *in vitro* grafting technique in which a meristem or shoot tip explant is placed on a decapitated rootstock that has been grown asexually from seed or micro propagated cultures. This technique has been used in woody species, especially fruit trees. Significant studies have been conducted on various citrus fruits to eliminate various viral diseases (Hartmann *et al*., 2002). Since this technology has potential for rapid use *in vitro*, micro grafts are used to improve fruit trees and reproduce by grafting a combination of excellent rootstock and scion combinations with improved productivity. Successful micrograft protocols have been developed for a variety of fruit crops, including citrus fruits, grapes, mulberries, etc (Hussain, *et al*., 2014).

The technology has great potential for the improvement and reproduction of fruit trees on a large scale. It has been used on a commercial scale to produce virus-free plants in fruit crops and viroid-free plants in citrus (Hussain *et al*., 2014). The production of high-quality plants that can be certified genetic and virus free is considered problematic and very challenging. The innovative micro-implantation technique was proposed by Murashige *et al.* (1972) for the production of homogeneous virus-free plants on a commercial scale in a controlled environment. They grafted the short apical shoot of citrus onto the top of a cut seedling grown *in vitro*. Virus indexing of micro grafted plants was performed over a period of 2 years for different viruses using different indicator plants. Out of 33 different varieties of citrus, they were able to grow virus free mother block of 31 varieties through shoot tip grafting. Since then, micro graft technology has been widely used to eliminate viruses, phytoplasma and systemic pathogens in fruit trees, making many fruit trees virus-free (Hussain *et al*., 2014).

3. MATERIALS AND METHODS

3.1. Study Area

The sample collection site was at the Melkassa Agricultural Research Center (MARC) in 2021. The Center is geographically located at a latitude of 8˚24'N, longitude of 39˚21' E and at altitude of 1,550 meters. It is situated about 107 km from Addis Ababa and 17 km from Adama on the way to Assela (Tasfaye., *et al*, 2010).

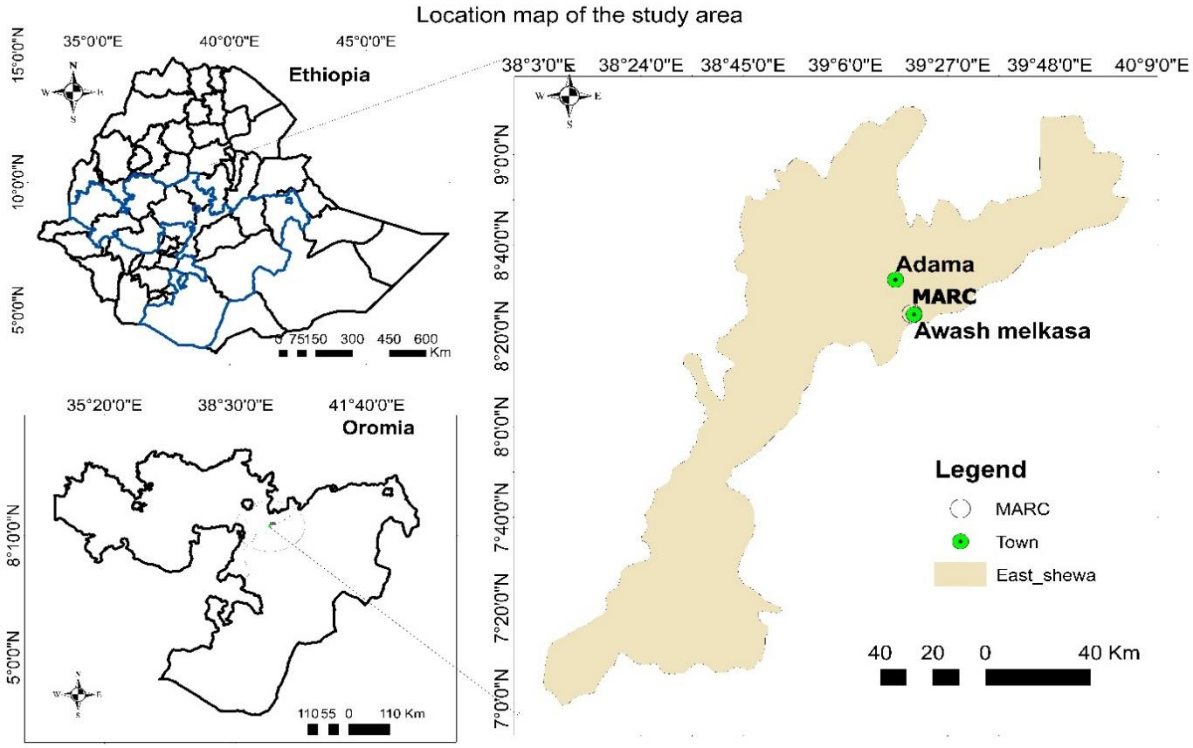


Figure 2. Map of study area.

3.2. Plant Materials

The plants used for this research activity was the popular variety of citrus cultivars (sweet oranges and lime). The internal part of the flower, stigma and style (both as a single explant) from the three citrus cultivars Mexican' lime (*Citrus aurantifolia*), Valencia Rhode **(***Citrus limon* (L.) and Hamelin (*Citrus sinensis*). were used as a source of explant. The unopened flowers of plants were collected from MARC (Melkassa Agricultural Research Center) which was cultivated as one of the cultivars and washed under double distilled water.

3.4. Stock Solution Preparation

Murashige and Skoog (MS) medium was used throughout this research activity. Culture media holds all the nutrients required for the normal growth and development of plants. It's mainly composed of mineral elements such as potassium, calcium, magnesium, nitrogen, phosphorous and Sulfur, which are required in large quantities (macronutrients/ major elements) while others like iron, manganese, copper, zinc, boron and molybdenum are required in small quantities (microelements/minor elements), vitamins, other organic components, plant growth regulators, carbon source and gelling agents (agar) (Ramage *et al*., 2002). Vitamins are supplemented with a medium to achieve the best growth of the tissues. Four vitamins, namely myo-inositol, thiamine, nicotinic acid, and pyridoxine are ingredients in the Murashige and Skoog (1962) medium (Appendix 4). To do so, an appropriate amount of each nutrient was weighed in grams per litter and dissolved in double distilled water sequentially, in such a way that the next nutrient was added after the first one was completely dissolved using a magnetic stirrer. The solution was poured into plastic bottles and stored at 4^0C until used.

3.4.1. Plant Growth Regulators Stock Solution Preparation

This study employed plant growth regulators such as 6-Benzyl Amino-Purine (BAP), 2, 4-Dichlorophenoxy Acetic Acid (2, 4-D), and - Naphthalene Acetic Acid (NAA). All of the plant growth regulator stock solutions were prepared by weighing and dissolving the powder in double distilled water at a ratio of 1:1 (1 mg/ml) and dissolved with 2-3 drops of NaOH based on the requirements of the plant growth regulators. Then, the volume was adjusted by adding double distilled water and stirring using a magnetic stirrer. Upon complete dissolution, the solution of each PGR was poured into labeled 50 ml plastic bottles.

3.4.2. Culture Medium Preparation

The MS salt mixture with its full macro elements, microelements, vitamins and iron source compositions were used to prepare the stock solution. The nutrients in the medium consisted of full-strength MS salts and medium was solidified with 8 g/L agar-agar, supplemented with 40 g/l sugar as carbon source. The pH of the medium was adjusted to 5.7-5.8 with 1 NaOH and 1HCl and then gelled with agar-agar type I. The full-strength MS medium was then dispensed into 30 ml culture bottles (jars) and the culture jars were immediately covered

with caps and then autoclaved at a temperature of 121^0C and pressure of 105 KPa for 20 minutes. Immediately after autoclaving, the medium was taken and kept in culture room until used (Taye, *et al.*, 2018). The medium was taken and stored in the culture room immediately after autoclaving (Taye, *et al*., 2018).

3.5. Explants Preparation and Sterilization

Explants (flowers) were collected from selected elite citrus cultivars. Flowers were collected before opening from three different citrus species (oranges, lime and lemon) belonging to MARC. Then, the explants were transferred to the laminar flow hood cabinet and surface sterilized in 70% ethanol for 2 minutes. Then the treated explants were rinse four times with sterilized distilled water and surface sterilized further using (1.5% and 2%) percent of NaOCl disinfectant solutions plus a few drops of tween-20 (2-3 drops) for 10,15 and 20 minutes.

Finally, stigmas and styles were excised with a scalpel and vertically plated as single explants in Petri dishes (100 X 15 mm) with the cut surface in contact with the medium. Each jar contained three explants, and each of the thirteen treatments required six jars. All steps taken in a laminar flow cabinet are designed to keep the environment aseptic and to protect against viruses and virus-like diseases.

3.6. Callus Induction and Culture Condition

For callus induction, style and stigma explants were excised from an unopened citrus flower and small pieces (0.5 cm^2) were inoculated on MS medium. The experiment has 12 treatments which have 6 replications of each, with different concentrations and combination of plant growth hormones, namely BAP, NAA and 2, 4-D. Visual observations were taken every seven days, and the impact of different treatments was quantified in terms of percent callus induction.

After two weeks, aseptic stigma and style cultures were transferred to MS salt mixture fortified with various concentrations of BAP in combination with NAA to determine the best medium for callus induction. The medium was composed of macro- and micro salts, sucrose and agar according to Murashige and Skoog (1962). In addition, BAP (1, 2, 3, and 4mg/l), 2,4-D (1.5 and 2mg/l) and NAA (0.01, 1.5 and 2mg/l) were added to the MS medium. The sterilized explants were implanted onto the solid Murashige and Skoogs medium with

dissected stigma and style. They were then sealed, labeled, and kept inside the growth room, which had a photoperiod of 16 hours (8 hours of darkness) and a light intensity of 2500 lux from cool white florescent 60-watt bulbs at an average temperature of 25^0C. The subculture was done after four weeks of culture. Weekly data on the percentages of explants that showed callus induction were collected.

3.7. Shoot Regeneration

For regeneration, green healthy friable calli were divided into small pieces and cultured on MS medium (containing 8g/l agar and 40g/l sucrose) supplemented with different concentrations and combinations of plant growth regulators like BAP at (1-4mg/l) alone or combination with NAA at 2mg/l and 2,4-D at 1.5mg/l. Explants in MS medium without plant growth regulators used as a control. The shoot proliferation responses, in terms of number of shoots per explant and average length of shoots (cm) per explant, were evaluated 45 days after the inoculation in shoot multiplication medium. For each 13 treatments, 6 culture tubes were inoculated and cultures were maintained at 26 ± 2 °C with 16 h-day-length provided by fluorescent tubes.

3.8. Rooting

Actively growing shoots (2.53 cm high) were used for *in vitro* rooting. The *in vitro* rooting was done by following the two-step rooting procedure. with some modifications. During this rooting procedure, micro-shoots were initially cultured on MS medium supplemented with NAA (2mg/l) for 24 or 48 h, and cultures were kept in the dark during this step. The NAA-treated microshoots were then transferred to a MS medium containing no plant growth regulators and exposed to normal light conditions (16-hour photoperiod, 42 M m2 s1 illumination) in the second step. Microshoots placed in MS medium (without NAA treatment within the first step) served as a control. Data for rooting percent, the average number of roots per explant, and length of roots per explant were recorded after 45 days of transfer to plant growth regulator-free half-strength MS medium.

3.9. data analysis

Statistical analyses of quantitative data were achieved by means of SPSS software version 26 and Microsoft excel. To detect the significance of variations among treatments below the probability level of 0.05, analysis of variance (ANOVA) was additionally made by using SPSS software. For analyses difference $p < 0.05$ was considered significant. At the $p < 0.05$ probability level, the least significant difference (LSD) is used to indicate separation. Statistical analyses of quantitative data were achieved by means of SPSS software version twenty-six, origin software and Microsoft excel. To detect the significance of variations among treatments below the probability level of 0.05, analysis of variance (ANOVA) changed into additionally made the usage of SPSS software. A difference at opportunity level of $p < 0.05$ was considered significant for analyses. Least significance difference (LSD) become used for imply separation at $p < 0.05$ probability level. the percent of callus induction was obtained by number of explants showing callus induction divide by total number of explants.

$$\% \textbf{ of callus induction} = \frac{\textbf{number of explants showing callus induction}}{\textbf{Total number of explants}}$$

4. RESULTS

4.1. Explant Preparation and Sterilizations

The study was conducted with three PGRs, namely BAP, 2, 4-D and NAA (at different combinations and concentrations) on MS callus induction medium to evaluate their impact on callus initiation and regeneration protocol for three citrus cultivars: Mexican' lime (*Citrus aurantifolia*) and Valencia Rhode **(***Citrus limon* (L.) and Hamelin (*Citrus sinensis*). The plant sample were collected from MARC. In this study, surface sterilization was performed with sodium hypochlorite (1.5%) and 2-3 drops of tween-20 were sufficient to effectively disinfect citrus plants from microbes. The effect of various concentrations of NaOCl at different exposure times on sterilization is presented in Table 1. The percentages of survival rate and contamination in cultures were recorded. *In vitro* cultures that were surface sterilized with 1.5% NaOCl for 20 minutes had the lowest concentration of bleach, showing the minimal contamination and maximum survival responses; it is the optimum NaOCl concentration and exposure time. In cultures, the contamination and survival rates were recorded as 0% and 100%, respectively.

Table 1. Effect of sodium hypochlorite concentration and exposure time on sterilization of citrus explants (Mexican lime cultivar). Data represent mean of 6 repeats.

Sodium hypochlorite concentration %	Time of exposure (minute)	Contamination %	Clean culture %	Tissue survival rate %
1.5	10	83.33	16.67	16.67
1.5	15	16.67	83.33	83.33
1.5	20	0	100	100
2	10	33.33	66.67	37.5
2	15	16.66	83.33	45.83
2	20	0	100	0

Although a high level of sterilization effectiveness was attained when explants were sterilized for 20 minutes with a high concentration of NaOCl (2%), there were adverse impacts on the explants' growth and development as well as their survival rate. Surface sterilization with sodium hypochlorite for 15 minutes at 1.5% was also effective, with an 83.33% survival rate and 16% contamination (Table 1).

The data analysis showed that, for a given quantity of sodium hypochlorite solution, the contamination percentage significantly dropped as exposure time rose. For this observation, the least contamination (0%) and the highest percentage of clean explants (100%) were obtained after disinfection by immersion in alcohol (70%) for 2 minutes and 1.5% of sodium hypochlorite solution for 15 minutes. Comparing the interaction effect posed by the chemical and the duration of disinfection against the aim of sterilization, disinfection of citrus plants with 1.5% of sodium hypochlorite solution for 20 minutes was the most effective sterilization treatment. This resulted in the highest survival percentage (100%) without any contamination. The present finding shows that 1.5% sodium hypochlorite in combination with 2-3 drops of Tween-20 for 20 minutes is the best of all sterilization procedures tested, with the highest survival and lowest contamination response of the variety. The increment in sodium hypochlorite concentration over time had a negative effect on explant, resulting in a yellowish color that led to 0% survival rate.

4.2. Effects of Plant growth regulators (PGR) on Callus Induction

The significant differences of callus induction were observed with different concentrations and combinations of PGR. During the first week, all treatments didn't show callus initiation in all citrus cultivars. During the second week of culturing, significant differences ($P < 0.05$) were observed between BAP and 2,4-D and NAA (Table 2). The highest percentage (100%) of callus induction was observed with BAP at 4 mg/l and also with BAP combined with NAA at 2 mg/l and 2 mg/l, respectively (Table 3 and 4). When BAP combined with 2.4-D at 3 g/l and 2 g/l respectively for all three (Mexican lime, Valencia Rhode and Hamelin) cultivars showed no callus induction.

During the third week, except for MS hormone-free media (the control) and treatment 5 (BAP, 3 mg/l and 2,4-D, 2 mg/l) and treatment 8 (BAP, 2.5 mg/l) all the other media showed response to callus initiation. Using BAP solely at 4 mg/l revealed significant differences as compared to a combination of BAP with NAA or 2,4-D that induced more callus formation than the others. After four weeks of culturing, BAP at 4mg/l was found to be the optimum concentration for callus induction for citrus (Mexican lime, Valencia Rhode and Hamelin) cultivars.

4.3. Characteristics of callus induced with different combinations of PGR

The results of this study revealed that PGRs combination (BAP+2,4-D) gave better compact callus type as compared to BAP alone for Mexican lime, Valencia Rhode and Hamelin citrus cultivars using the style and stigma as explant sources. All three citrus cultivars gave both types of callus morphology the friable and compact features. In terms of friable callus induction with the cultivar Mexican lime, only 41.66% of the PGRs combinations gave friable callus (T3, T4 and T7, T8 and T11, Table 2) and for Valencia Rhode only 33.33% (only T3, T4, T7 and T11, Table 3) and Hamelin 33.33% (T2, T3, T4 and T11, Table 4) of the PGRs combinations gave friable callus and the rest were compact features. The other observation was the variability of the type of callus color observed among the different hormone combination, and cultivar type. The entire friable callus obtained from Mexican lime, Valencia Rhode and Hamelin cultivars showed various color types which is green, yellowish, white/creamy white (Table 2, 3 and 4).

Table 2. Effect of different concentrations and combinations of growth regulator on callus induction for Mexican lime using stigma and style as explant. Data represent mean of 6 repeats.

Plant Growth Regulators concentration (mg/L)				Callus morphology		
Treatment	BAP	NAA	2,4-D	% of induced callus	Callus color	Callus features
T1	1	-	-	66.66	Yellow	Compact
T2	2	-	-	83.33	Whitish	Compact
T3	3	-	-	94.44	Whitish	Friable
T4	4	-	-	100	Green	Friable
T5	3	-	2	0	-	-
T6	0.1	0.01		0	-	-
T7	3	1.5	-	50	White	Friable
T8	2.5	-	-	33.3	Yellow	Friable
T9	1.5	-	1.5	66.66	Brown	Compact
T10	2		2	33.3	Greenish	Compact
T11	2	2	-	100	Green	Friable
T12	0 (control)	-	-	0	-	-

Note: Data presented here represents the final record taken for the last 2 months. Values represent the means of six replicate. T, treatment; BAP, 6-benzayl aminopurine; 2,4-D, 2,4-dichlorophenoxy acetic acid; NAA, naphthaleneacetic acid. control (hormone free).

Table 3. Effect of different concentrations and combinations of growth regulator on callus induction for Valencia Rhode using stigma and style as explant. Data represent mean of 6 repeats.

Plant Growth Regulators concentration (mg/L)				Callus morphology		
Treatment	BAP	NAA	2,4-D	% of induced callus	Callus color	Callus features
T1	1	-	-	44.44	White	Compact
T2	2	-	-	38.8	Yellow	Friable
T3	3	-	-	22.22	Whitish	Friable
T4	4	-	-	100	Green	Friable
T5	3	-	2	0	-	-
T6	0.1	0.01		0	-	-
T7	3	1.5	-	50	White	Compact
T8	2.5	-	-	33.3	Yellow	Compact
T9	1.5	-	1.5	50	Brown	Compact
T10	2		2	33.3	Greenish	Compact
T11	2	2	-	83.33	Green	Friable
T12	0 (control)	-	-	0	-	-

Note: Data presented here represents the final record taken for the last 2 months. Values represent the means of six replicate. T, treatment; BAP, 6-benzayl aminopurine; 2,4-D, 2,4-dichlorophenoxy acetic acid; NAA, naphthaleneacetic acid. control (hormone free).

Table 4. Effect of different concentrations and combinations of growth regulator on callus induction for Hamelin using stigma and style as explant. Data represent mean of 6 repeats.

Plant Growth Regulators concentration (mg/L)				Callus morphology		
Treatment	BAP	NAA	2,4-D	% of induced callus	Callus color	Callus features
T1	1	-	-	72.22	Yellow	Compact
T2	2	-	-	83.33	Yellow	Friable
T3	3	-	-	94.44	Whitish	Friable
T4	4	-	-	100	White	Friable
T5	3	-	2	0	-	-
T6	0.1	0.01		0	-	-
T7	3	1.5	-	50	White	Compact
T8	2.5	-	-	61.1	Yellow	Compact
T9	1.5	-	1.5	66.66	Brown	Compact
T10	2		2	33.3	Greenish	Compact
T11	2	2	-	100	Green	Friable
T12	0 (control)	-	-	0	-	-

Note: Data presented here represents the final record taken for the last 2 months. Values represent the means of six replicate. T, treatment; BAP, 6-benzayl aminopurine; 2,4-D, 2,4-dichlorophenoxy acetic acid; NAA, naphthaleneacetic acid. control (hormone free).

4.4. Differences in callus induction over weeks

The significant differences in calli induction were observed for varying concentrations and combinations of the plant's growth regulatory hormones. The MS medium supplemented with 1 g/l, 2 g/l, 3 g/l and 4 g/l of BAP concentrations revealed significant differences in callus induction within different weeks (Figures 3, 4 and 5). In the first week there is no callus formation in all three citrus cultivars. In the second and third weeks, the highest callus induction of 100% was observed for Hamelin and Mexican lime varieties by BAP alone at 4mg/l and combination with NAA at 2 g/l and 2 g/l, respectively. This is followed by 50 % callus induction for the Valencia Rhode variety at the same concentration.

The highest callus induction (100%) was observed in the fourth weeks at a concentration level of 4 g/l of BAP and BAP combined with NAA at 2 g/l and 2 g/l 100% callus induction

for Mexican lime varieties, and followed by 83.33% Valencia Rhode in the fourth week. When two varieties were compared, MS medium supplemented BAP with NAA for Valencia Rhode varieties revealed a significant difference in callus induction among weeks with the same combination and concentration level, which shows a small difference when compared to Mexican lime varieties (Figure 4). With regard to MS media supplemented with BAP solely or in combination with NAA, significant difference in callus induction was observed. The highest percentage of callus induction (100%) was observed in the fourth week both at 4 g/l of BAP and 2 g/l BAP with 2g/l of NAA hormones, but the callus induction for other varieties is less than this value (Figures 3, 4 and 5).

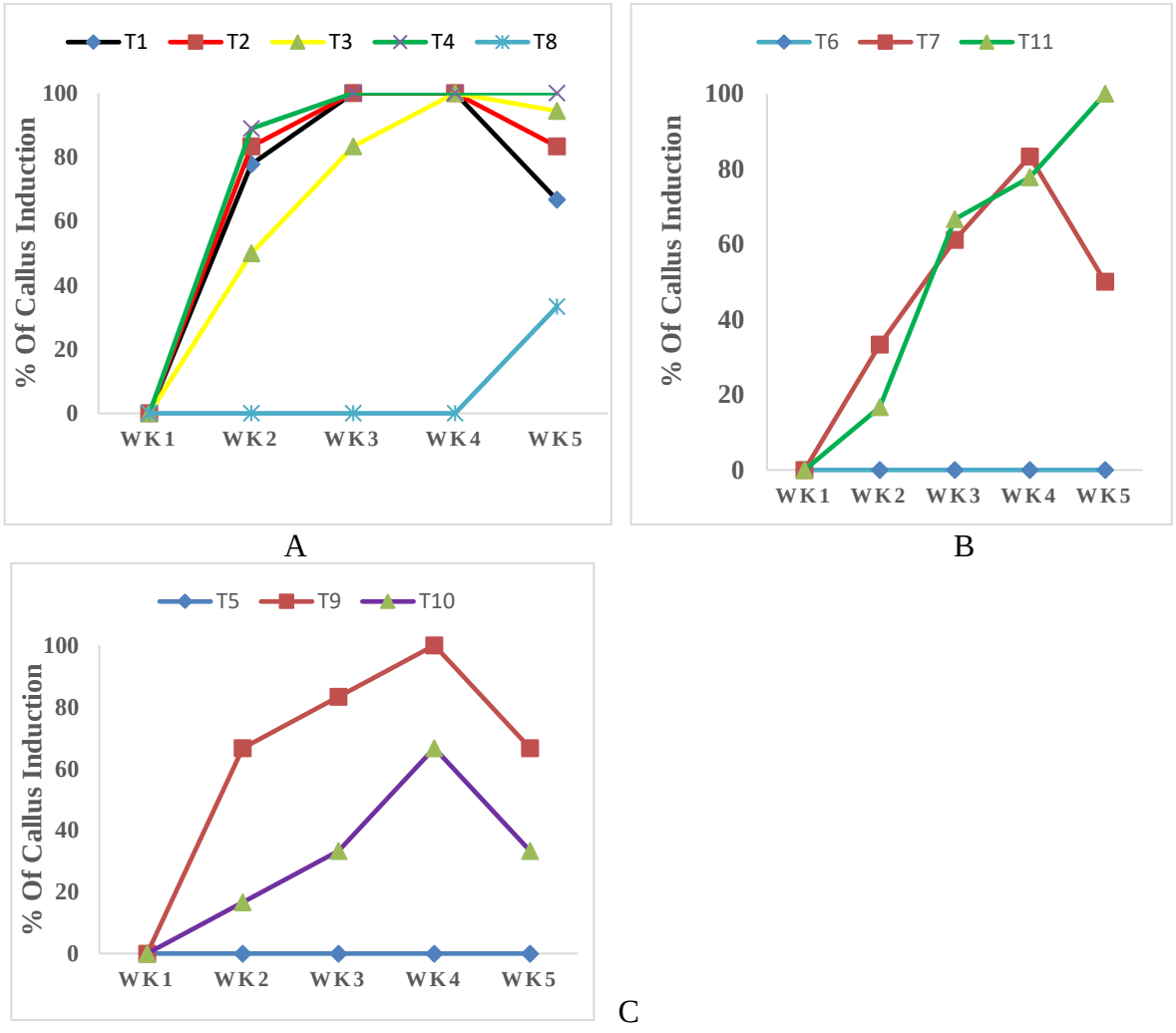
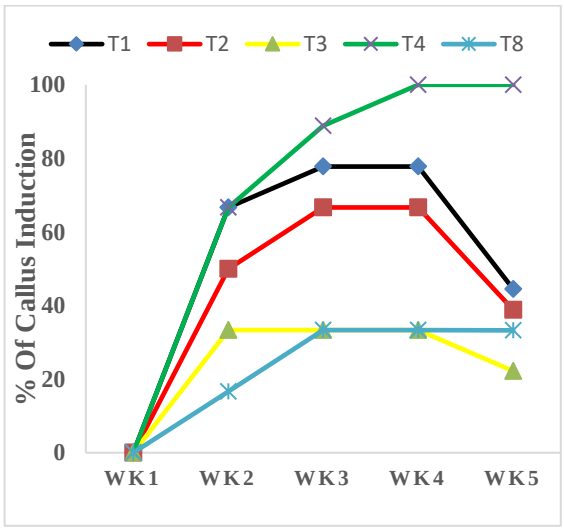


Figure 3. Effects of Plant growth regulators on callus induction over five weeks for Mexican lime cultivar.

Note: A) represents BAP, B) represents BAP+NAA and C) represents BAP+ 2,4-D

WK, represents week

Where : T, treatment; T1 = 1 mg/l BAP; T2 = 2 mg/l BAP; T3 = 3 mg/l BAP; T4 = 4 mg/l BAP, T5 = 3 mg/l BAP + 2 mg/l 2,4-D, T6 = 0.1 mg/l BAP + 0.01mg/l NAA, T7 = 3 gm/l BAP + 1.5 mg/l NAA, T8 = 2.5 mg/l BAP, T9 = 1.5 mg/l BAP + 1.5 mg/l 2,4-D, T10 = 2 mg/l BAP + 2 mg/l 2,4-D, T11 = 2 mg/l BAP + 2 mg/l NAA, T12 = control (hormone free).



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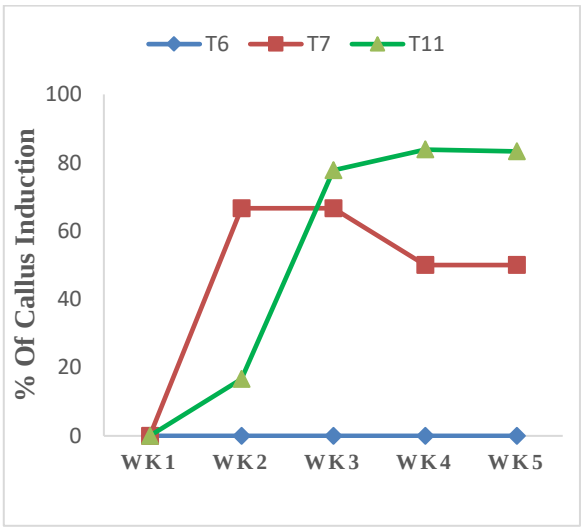

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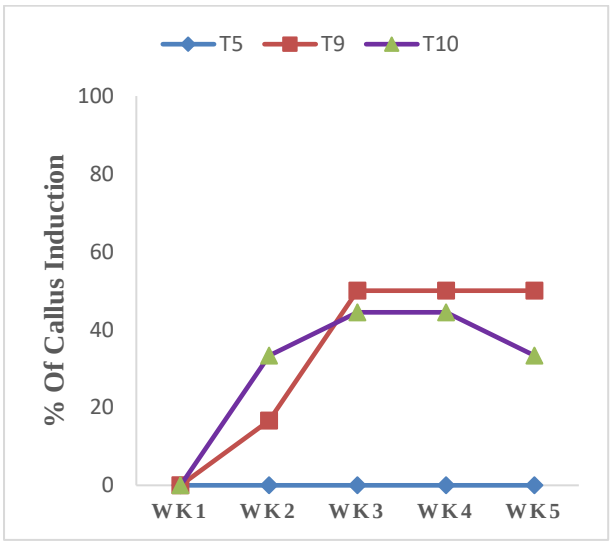

C

Figure 4. Effects of Plant growth regulators on callus induction over five weeks for Valencia Rhode cultivar.

Note: A) represents BAP, B) represents BAP+NAA and C) represents BAP+ 2,4-D.

WK, represents week

Where : T, treatment; T1 = 1 mg/l BAP; T2 = 2 mg/l BAP; T3 = 3 mg/l BAP; T4 = 4 mg/l BAP, T5 = 3 mg/l BAP + 2 mg/l 2,4-D, T6 = 0.1 mg/l BAP + 0.01mg/l NAA, T7 = 3 gm/l BAP + 1.5 mg/l NAA, T8 = 2.5 mg/l

BAP, T9 = 1.5 mg/l BAP + 1.5 mg/l 2,4-D, T10 = 2 mg/l BAP + 2 mg/l 2,4-D, T11 = 2 mg/l BAP + 2 mg/l NAA, T12 = control (hormone free).

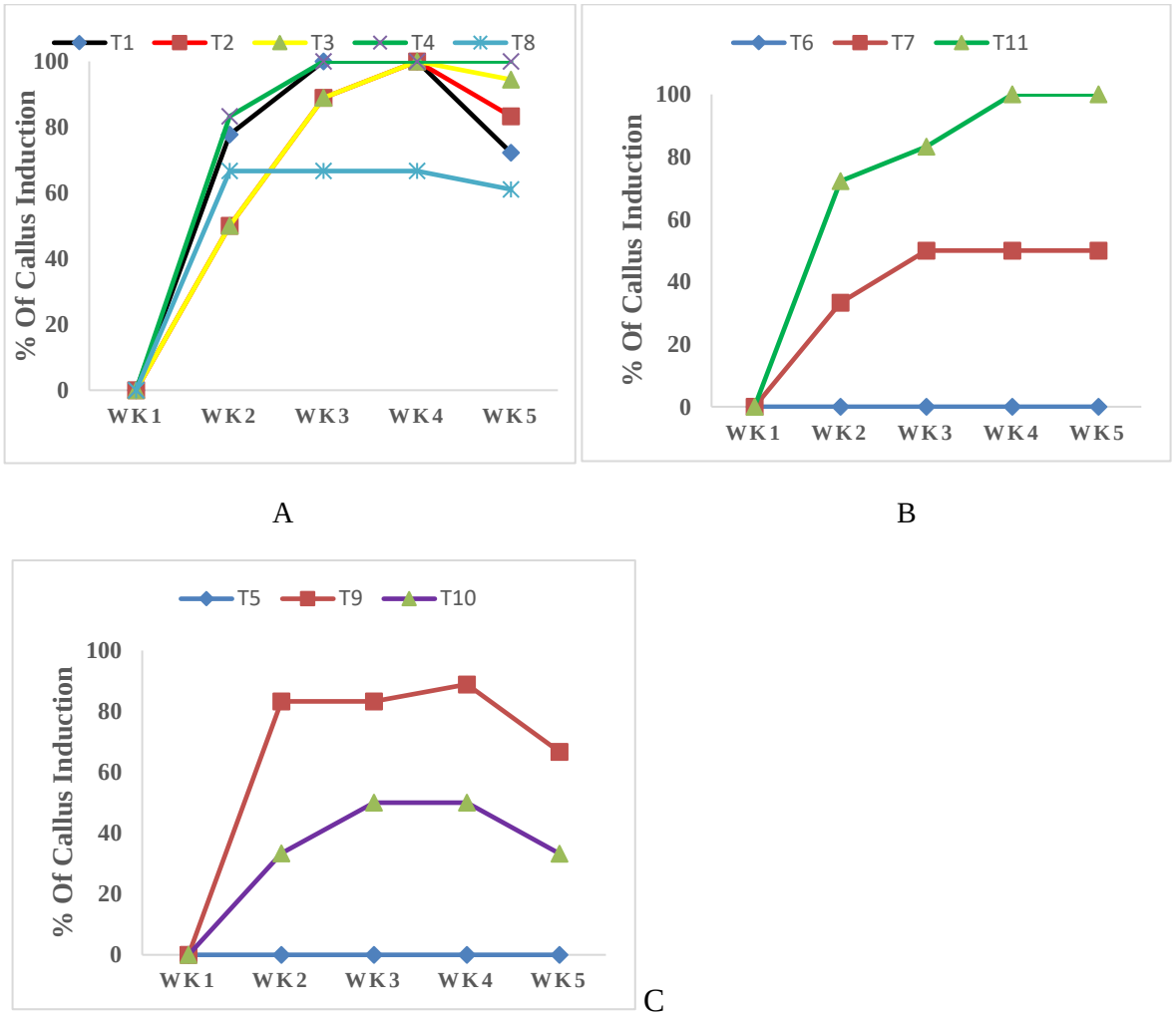

Figure 5. Effects of Plant growth regulators on callus induction over five weeks for Hamelin cultivar

Note: A) represents BAP, B) represents BAP+NAA and C) represents BAP+ 2,4-D.

WK, represents week**.**

Where : T, treatment; T1 = 1 mg/l BAP; T2 = 2 mg/l BAP; T3 = 3 mg/l BAP; T4 = 4 mg/l BAP, T5 = 3 mg/l BAP + 2 mg/l 2,4-D, T6 = 0.1 mg/l BAP + 0.01mg/l NAA, T7 = 3 gm/l BAP + 1.5 mg/l NAA, T8 = 2.5 mg/l BAP, T9 = 1.5 mg/l BAP + 1.5 mg/l 2,4-D, T10 = 2 mg/l BAP + 2 mg/l 2,4-D, T11 = 2 mg/l BAP + 2 mg/l NAA, T12 = control (hormone free).

4.5. Somatic Embryo Development

Indirect SE (somatic embryogenesis) was observed and somatic embryos were developed 90 - 150 days after culture initiation according to the genotype. Somatic embryos were green and easy to detach (Figure 6). Embryos at different stages of development were observed at the same time. The percentage of responsive styles varied from 0% ('Valencia Rhode') to 16% ('Mexican lime), depending on species and genotype (Figures 6 and 8), respectively. Embryos were developed for all cultivars that were supplemented with BAP at 4 mg/L. Indeed, in our experiment, explants cultured on MS medium supplemented with BAP showed a greater potential response in terms of the percentage of embryos produced. Mexican lime showed a higher embryogenic potential at 4 mg/L (Figure 8). Nevertheless, the highest somatic embryogenesis rate was obtained with Hamelin cultivar, Somatic embryos were formed more rapidly in 'Mexican lime explants (90 days after culture initiation) than in explants of other species.

The embryogenic process occurred indirectly on a friable callus initiated at the basal part of the style. The different sequential phases leading to the formation of somatic embryos took approximately 3-7 months. Normally, germinating embryos (with culinary and radicular apices) evolve into plantlets starts to develop when transferred to hormonal-free medium. Embryogenesis in Mexican lime occurred first at 90 days after culture initiation, but with the lowest percentage of somatic embryos formation (16%). The potential embryogenic rate of other Citrus cultivar, the Hamelin (10%) which was lower but consistent and very important.

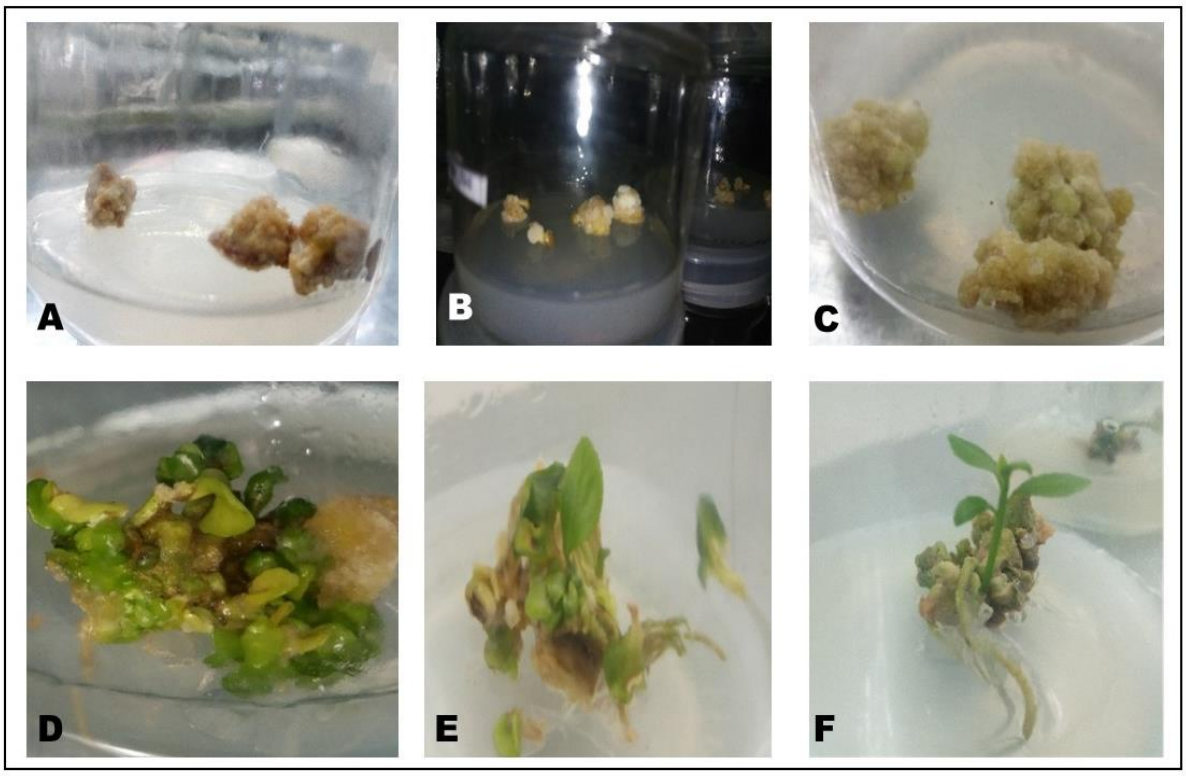

Figure 6. Induced callus and regeneration from stigma and style explants on Mexican lime with different concentration and combination of PGRs after 12 weeks of growth.

NB: Difference between hormone concentration for treatment 1-4, where A= 1 mg/l BAP, B= 2 mg/l BAP, C= 3 mg/l BAP, D,E and F= 4 mg/l BAP

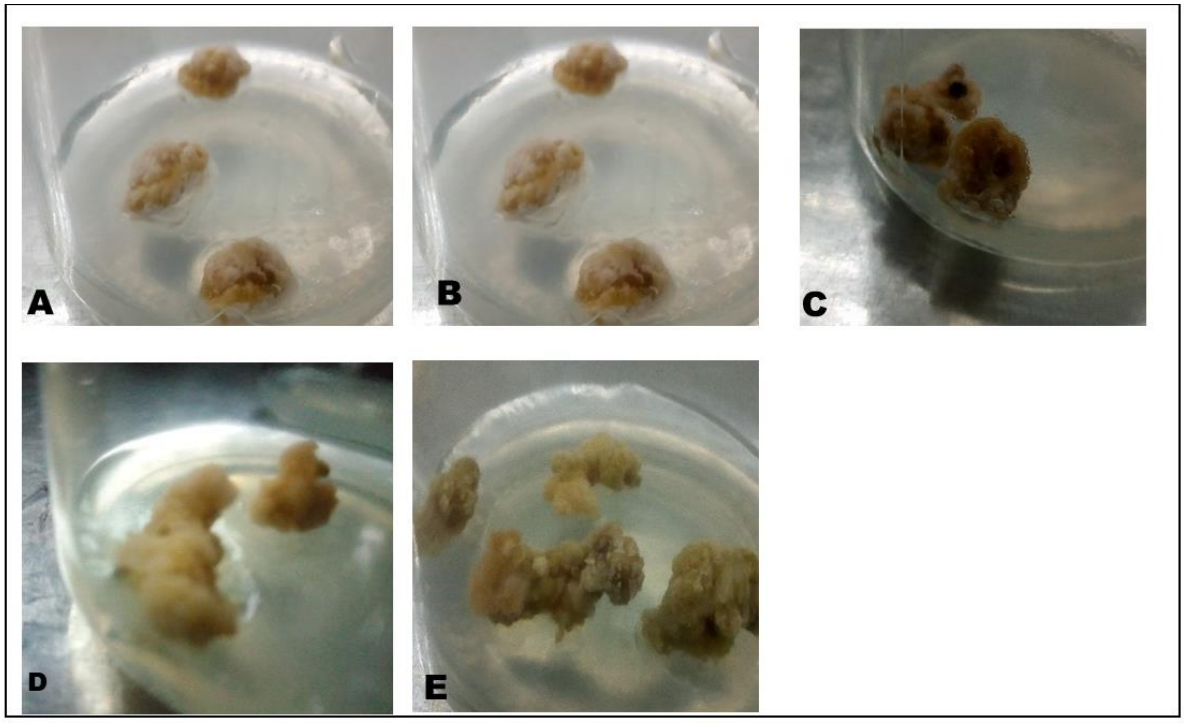

Figure 7: Induced callus from stigma and style explants on Valencia Rhode with different concentration and combination of PGRs after 12 weeks of growth

NB: Difference between hormone concentration for treatment 1-4, where

A= 1 mg/l BAP, B= 2 mg/l BAP, C= 3 mg/l BAP, D and E= 4 mg/l BAP

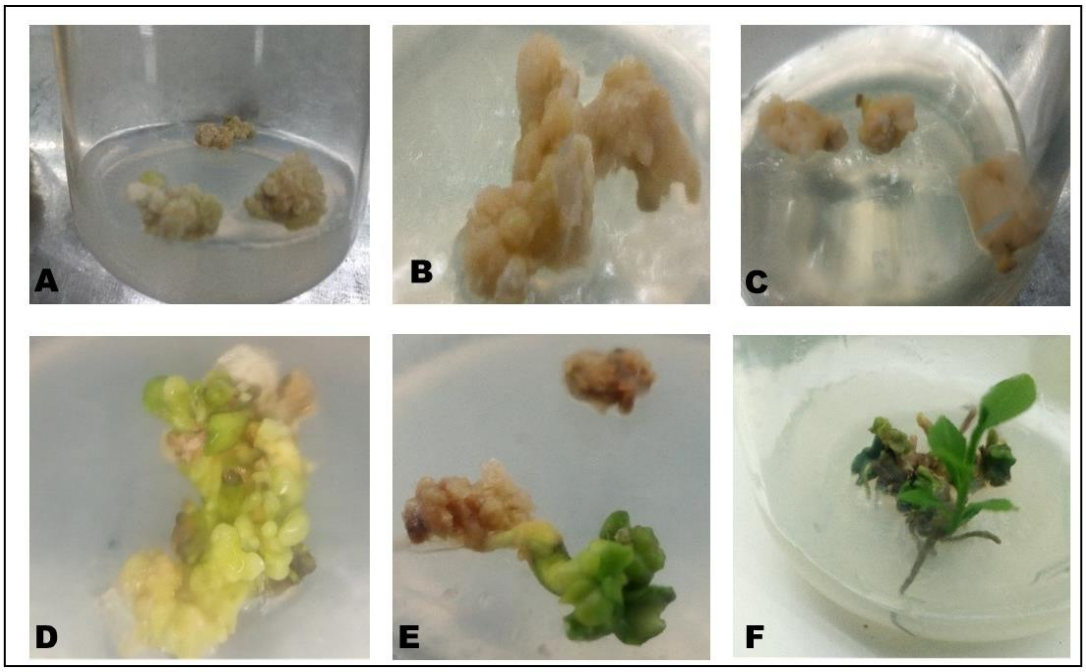

Figure 8: Induced callus and regeneration from stigma and style explants on Hamelin with different concentration and combination of PGRs after 12 weeks of growth

NB:-Difference between hormone concentration for treatment 1-4, where A= 1 mg/l BAP, B= 2 mg/l BAP, C= 3 mg/l BAP, D, E and F= 4 mg/l BAP

4.6 Shoot regeneration

For regeneration, 90-day old green healthy friable calli were divided into small pieces which were cultured on MS medium supplemented with different concentrations and combinations of BA, 2,4-D and NAA. Maximum shoot regeneration response (83.33 %) was observed at Mexican lime with BAP (4 mg/L) (Fig. 6F) and followed by Hamelin (66.66%) at the same concentration. However, when BAP (2mg/L) was used in combination with NAA (2 mg/L) regeneration response was observed but less than that of BAP at 4mg/L, which was (50 %) for Mexican lime and Hamelin (33.33%) cultivars respectively. Whereas Valencia Rhode cultivar did not regenerate at all concentration and combination of PGRs. The addition of GA3 in the MS media at 5mg/L enhanced (66.66 %) shoot elongation. The regenerated shoots readily formed roots (83.33%) when transferred to MS medium supplemented with 40 g/ L

sucrose and 7 g/ L agar without any growth regulator and kept at 25 ± 1 °C with a 16-h photoperiod under 2,000 lux light intensity.

5. DISCUSSION

5.1. Explant Preparation and Sterilizations

This study was the first report to use style and stigma as an explant source to assess *in vitro* protocol development and sanitation of citrus cultivars from viruses and virus-like pathogens. Thus, the present study was undertaken to evaluate sanitation protocol by using NaOCl at different concentration levels with different exposure time and assess the influence of three different PGRs, namely BAP, NAA and 2, 4-D. After 7 days of culture, 0% tissue death was recorded with unaffected growth rate. The result shows that, NaOCl is believed to be a very effective sterilizing agent and is frequently used for surface sterilization of plants for *in vitro* culture at 1.5% for 20 minutes.

The present result is in line with earlier work of Iqbal *et al*., (2019),that reported sodium hypochlorite as good sterilizing agents for many other plants. And also agreed with (Niedz and Bausher, 2002), who state that, The occurrence of a high contamination rate in culture at a relatively lower concentration and shorter exposure time in treatment combinations was possibly due to the insufficiency of sterility concentration and duration of exposure to remove or kill the contaminant agents, mainly bacteria and fungi. For this finding, the increase in sterilant concentration and exposure time above a certain optimum limit (2% and 20 minute) causes the loss of explants because of the oxidant chemical ingredient also kills the plant tissue.

The explants collected from field grown citrus plants harbor many microbial pathogens, like fungi and bacteria, in addition to adhered soil particles; it is therefore necessary to perform a thorough and effective surface sterilization of the explants before culturing. Explants transferred to the laminar flow hood cabinet and rinse repeatedly four times with sterilized distilled water and surface sterilized further using 1.5% of NaOCl (sodium hypo-chlorite) disinfectant solutions plus a few drops of tween-20 (2-3 drops) for 20 minutes gave 100% free from virus and bacteria diseases. This result agreed with Upadhyay *et al., (*2010) who reported that many sterilization procedures based on sodium hypochlorite solution and 70% ethanol rinse are usually considered as good sterilizing procedures for *in vitro* culture

cleaning up. An increase in sterilant concentration and exposure time above a certain optimum limit (2% and 20 minute) causes the loss of explants because of the oxidant chemical ingredient also kills the plant tissue.

This result was in line with Niedz & Bausher, (2002). who reported that, the high concentration of NaOCl and long exposure duration of explants in the sterilizing solution resulted in better removal of microbes due to the concentration, which is negatively correlated with bactericidal activity.

5.2. Effects of Plant Growth Regulators on Callus Induction

About 7 to 14 days after the incubation, most of the explants of different genotypes produced a friable, creamy-white callus at the cut end of the styles. The variable size of callus production and proliferation rates were observed during the callus induction period. Statistical analysis revealed that the species and genotype were significantly affecting the percent of callus induction. Indeed, the percentage of calli production varied from 40 ('Valencia Rhode') to 100 % ('Mexican lime') according to genotypes and species. Among three citrus cultivars, explants of Mexican lime showed the highest callus induction rate which was also of large size.

Explants of Valencia Rhode on the other hand, had smaller calli (less than 1 cm in diameter). Explants of Mexican lime cultivars showed a high rate of browning 1 week after culture initiation, whereas explants of Valencia Rhode and Hamelin cultivars showed callus induction 2 weeks later, and variation in the time to form callus induction was most likely due to genotypic effect. The present results are in line with the findings of Hasbullah *et al*. (2011), who stated that the combinations of three growth regulators (2,4-D, NAA and BAP) had a positive effect on callus induction. Moreover, this study suggests that BAP alone or in combination with the lower concentrations of NAA at 2 mg/L constitutes the foremost favorable combination of growth regulators for the assembly of callus in citrus cultivars by using the floral part. These findings are supported by Can *et al*. (2008), who found that higher concentrations of auxins and cytokinin inhibited meristematic cell division and callus induction and decreased chemical reactions, resulting in stunted growth and finally the death of explants

5.3. Effect of PGR on Callus Morphology

Citrus sinensis from stigma/ styles were highly responsive for the induction of callus. Callus produced have friable and creamy-white color. A strong effect of hormone concentration/ combination, and genotype on somatic embryogenesis induction was observed. The embryogenic response of style and stigma observed in this study is lower compared with that observed by (Meziane, 2012)

The current study demonstrates the existence of significant inter-varieties differences in callusing responses. Mexican lime responds compactly and loosely with light yellow, green, greenish yellow, brown, and creamy yellow. Valencia Rhode responds to more friable callus with yellow, brown, whitish, greenish and greenish yellow colors, although gave compact and loose compact callus with white, yellow and brownish colors. And Hamelin responds with light yellow, green, greenish yellow, brown, creamy yellow, and yellow in both compact and loosely compact forms. From the result, it is possible to recognize that Mexican lime cultivars are more responsible for compact callus. This difference may be due to the cultivars' different reactions to the culture media component. So, Mexican lime was established to be more responsive for citrus regeneration and was supposed to be more appropriate for transformation experiments. This finding is consistent with previous research (Carimi and De Pasquale 2003; Carimi 2005; Meziane et al. 2012), which found that the percentage of explants producing embryos varied by genotype and species.

5.4. Hormonal Effect on callus induction

This study investigated about 12 different combination and concentration of PGRs (BAP, GA3, NAA and 2, 4-D) in callus induction of ‘Mexican lime, Valencia Rhode and Hamelin’ varieties using style and stigma as explants. The regeneration was observed in the presence of BAP alone or in combination with other phytohormones. This finding is in line with (Rattanpal et al. 2011) who reported that, the important role of BAP in shoot induction in citrus species is very high. The higher concentration of BAP (4.0 mg/L) induced the highest number of calluses (100 %), followed by the mid-level of growth regulators (2 mg/L BAP), which induced (83%) calluses. Moreover, the lower level of BAP (1 mg/L) resulted in lower callus induction frequency (66 %).

It was supported a selected combination of the organ nature and medium composition. These results are agreed with previous reports on seedless sweet orange (Citrus sinensis L. Osbeck)

(Cardoso *et al.,* 2017), sweet orange and lemon (D'Onghia *et al.,* 2000), and Citrus aurantium (orage) and Poncirus trifoliata, and swingle citrumelo (Tzatzani *et al.*, 2018). The results of this study revealed that PGRs (plants growth regulators) are directly corelated with growth of plants. This finding is agreed with finding of (Khan *et al*., 2009), and (Moreira-Dias *et al*., 2001; Costa *et al*., 2004) which reported that the success of citrus organogenesis is closely associated with the hormonal composition of the medium.

The control treatment (with no growth regulator) didn't show callus induction (Table 2), probably due to the lack of optimum auxin concentration required for the induction. Thus, in the present study, the higher concentration of BAP at 4 mg/L induced more calluses from style and stigma when compared to the other PGRs. The higher concentration of 2,4-D combined with the lower concentrations of BAP and NAA affect callus formation, as 2,4-D was herbicide.

Herbicides can act by inhibiting cellular division, photosynthesis or organic compound production or by mimicking natural plant growth hormones, causing deformities (Ross and Childs 1996). As per this study, the provision of 4 mg/L BAP within the medium appeared necessary for the callus formation and regeneration protocol. All three cultivars gave maximum percentage of callus induction on MS media supplemented with 4 mg/L BAP for 'Mexican lime' (100 %) with whitish green friable feature, Valencia Rhode (100%) with dark yellow loose compact feature and Hamelin (100%) with white, yellow compact feature as showed in (figures 1,2 and 3).

These results are in agreement with the findings of Tavano *et al.* (2009) who reported that, shoot bud emergence on the Calli surface by suppling BAP hormone. Conversely with finding of (Skoog , 2012), who reported that lower amount of BAP (0.2 mg/L) had a positive effect on callus induction and BAP promotes RNA and protein synthesis which activates enzyme activity for cell division and cell wall loosening.

5.5. Effect of Genotypes on callus induction

Genotype is one of the most prominent factors that influence the respond of tissue culture and it is the most determinant of callus induction on citrus since variance were observed between the three varieties for callus induction percentage as well as callus features. The result of the present study indicated that, 'Mexican lime' variety respond highest percentage

of callus when compare to ‘Valencia’ and ‘Hamelin’ variety and respond compact callus formation. On the other side ‘Valencia Rhode’ relatively minimum percentage to induced callus with MS callus culture medium but it gave friable callus especially after three weeks. This variation is associated with differential sensitivity of tissue to the callus culture medium.

According to the findings of this study, SE derived from stigma and style culture has been successfully used to regenerate different genotypes of important Citrus species. Percentages of explants producing embryos varied according to genotypes and, within species, a different regeneration potential was observed as reported in previous papers (De Pasquale et al., 1994; Carimi *et al*. 1999; Carimi and De Pasquale 2003; Carimi 2005; Meziane *et al.* 2012).

These results may also be used for the establishment of the regeneration process using somatic embryogenesis by the floral organ culture and sanitation of citrus genotypes. present result was matched with the finding of Meziane *et al*. (2012) Who reported that, Style/stigma somatic embryogenesis is considered one of the most effective methods for eradicating major citrus virus and virus-like diseases. This genotypic difference with respect to callus initiation was also observed in many other plants, such as flax (*Linum usitatissimum L.*) (Burbulis *et al*., 2007), *Coffea arabica* L. F1 hybrid (Mwaniki *et al.,* 2019), sugarcane (*Saccharum* sp.) (Gandonou *et al*.,2005). Therefore, one has to focus on citrus genotype in order to improve citrus through tissue culture techniques, as it is more suitable for *in vitro* regeneration.

5.6. Development of Somatic Embryo

This result show that it’s possible to induce somatic embryogenesis in all explants for the removal of viruses and virus-like diseases that affect citrus plants. Regeneration in citrus species is a slow process (Singh and Rajam, 2009). Processes of callus induction and organogenesis in C. limon are long-lasting and much more difficult than in herbaceous plants (Benabdesselam *et al*., 2011). In our studies, indirect SE was observed and somatic embryos developed 90 - 150 days after culture initiation according to the genotype. Somatic embryos were green and easy to detach (Figures 1,2 and 3). These results are in line with the findings of Meziane (2017), who reported that somatic embryos appeared 38–150 days after culture initiation, according to the genotype. Embryos at different stages of development were observed at the same time. The percentage of responsive were varied from 0% (‘Valencia Rhode’) to 20% (‘Mexican lime), depending on species and genotype (Figures 3d and 1d), respectively.

Previous reports indicated that the addition of BAP to the medium greatly increased the embryogenic response of styles (Carimi *et al.*, 1994). Indeed, in our experiment, explants cultured on MS medium supplemented with BAP showed a greater potential response in terms of the percentage of embryos produced. Mexican lime showed a higher embryogenic potential (Figure 1d). After a similar experiment, Carimi *et al*., (1994) and De Pasquale *et al*., (1994) reported that lemon genotypes were successfully regenerated from stigma and style. Somatic embryogenesis rates were also obtained with the Hamelin (orange) genotype for our study. Somatic embryos were formed more rapidly in 'Mexican lime explants (90 days after culture initiation) than in explants of other species.

In accordance with previous reports (Carimi and De Pasquale, 2003; Carimi, 2005), the success of somatic embryonic regeneration in citrus depends on the genotype, thus a great deal of variability is shown within the same species. This paper described attempts to induce somatic embryogenesis in stigma and style explants for the removal of viruses and virus-like diseases that affect citrus plants. The explants were cultured on BAP, 2,4-D and NAA enriched medium. It is well reported that *in vitro* organogenesis in citrus is enhanced by cytokinin supplementation (Moreira-Dias *et al*., 2000, Schinor *et al.*, 2011). In this experiment, style/stigma excised from unopened flowers were used as explants to induce SE on MS culture medium containing BAP alone and combined with 2,4-D and NAA as it has previously been recommended for many citrus genotypes (De Pasquale *et al*., 1994, Carimi *et al*., 1998).

The result of our study shows that the embryogenic process occurred indirectly on a friable callus initiated at the basal part of the style. The different sequential phases leading to the formation of somatic embryos took approximately 3-7 months. Normally germinating embryos were easily detached and greenish in color; they evolved into plantlets when transferred to a hormonal-free medium. To the best of our knowledge, this is the first report on somatic embryogenesis from stigma and style explants of citrus in our country. This finding is agreed with D'Onghia *et al*. (2000) reported that mandarin and grapefruit showed a lower percentage of embryo regeneration.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

In general, preliminary findings indicated that somatic embryogenesis using stigma and style culture was successfully used to regenerate different genotypes of the main citrus species grown at the Melkassa Agriculture Research Center. Our ultimate objective was to develop a protocol for virus-free citrus cultivars and to produce somatic embryos for a citrus virus sanitation program. Style and stigma explants have been used successfully in regenerating SE (Somatic embryogenesis) on BAP, BAP+2,4-D, and BAP+NAA enriched MS medium for this purpose. Since the tested genotypes were cultivars grown in the country for a long time, the successful results obtained by our experiment are the very first report. Even though callus formation rates varied widely among the three citrus cultivars (Mexican lime, Valencia Rhode, and Hamelin), they were successfully regenerated by SE from stigma and style cultures.

From the three varieties, relatively, 'Mexican lime' is best for compact callus indication. Among the different tested hormone combinations, the maximum callus percentage was found at 4 mg/l BAP for both varieties with compact features. This implies that it is favorable for further citrus *in vitro* culture. Very promising results were obtained with Mexican lime and Hamelin cultivars

It is also recommendable that 4 mg/l BAP for citrus callus investigation to get the best result. Moreover, the successful and easy application of this technique showed that SE could be largely applied not only for *in vitro* conservation but also for the production of healthy citrus plants. Therefore, the present study generated valuable information that is important for the protocol development for production of virus- free from selected elite citrus cultivars and provides baseline information for future studies.

6.2. Recommendations

Due to the long duration of this type of experiments various activities should be performed in the future. As this protocol was tested on only three citrus cultivars, further studies using other genotypes is required so as to have a reliable protocol for *in vitro* protocol development of citrus cultivars. Further studies on the performance of plant growth regulator toward best shoot induction and best plant growth regulator for rooting. And we recommend to Evaluate the survival rate of plantlet in the field condition. And also, we recommend that, further studies need to be carried out in order to investigate the embryogenic potential of other citrus genotypes of economic importance of citrus species.

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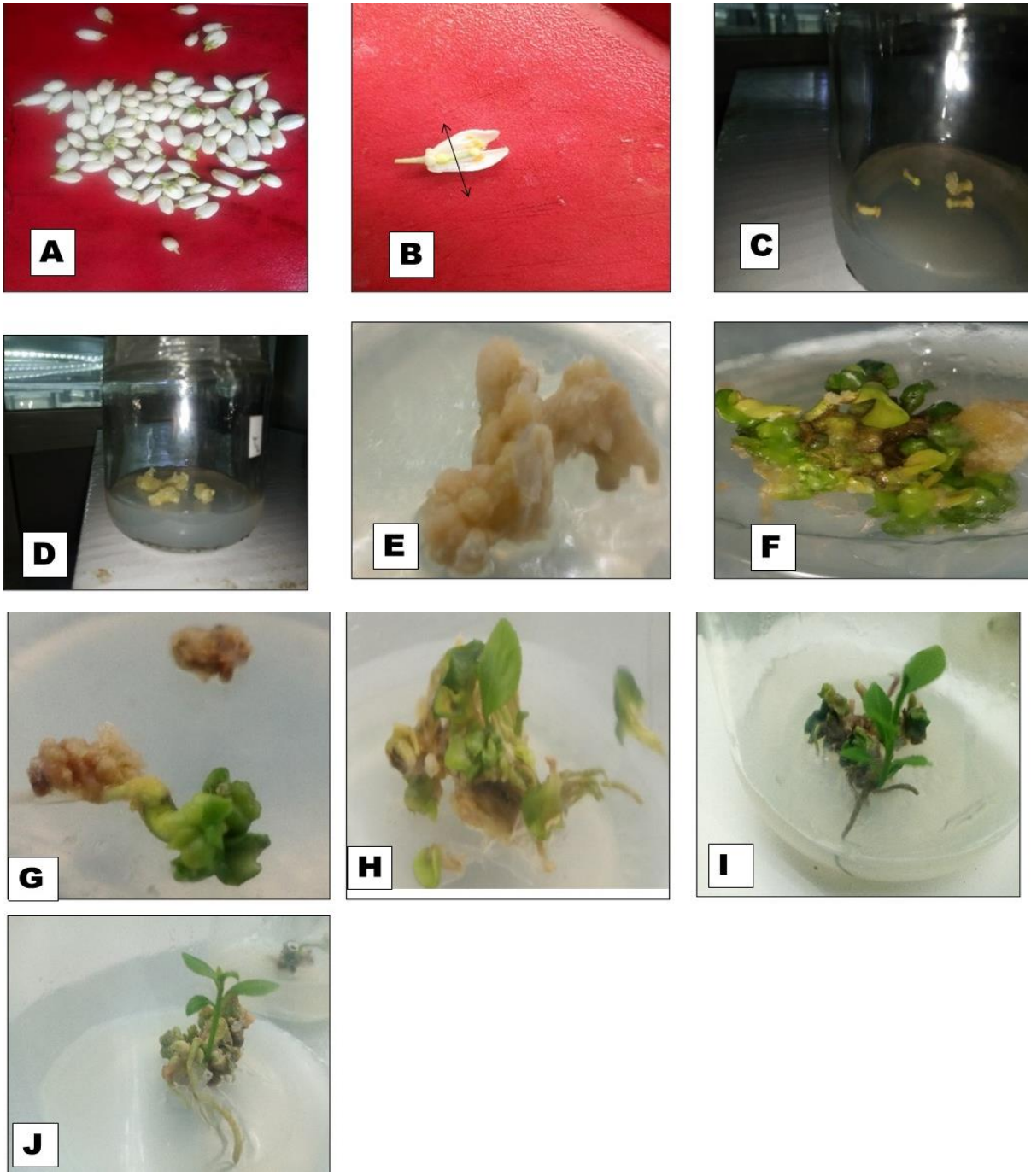
APPENDICES.

Appendix 1. Some of components of the preparation room and equipment used in this study;

Refrigerator (1); Electronic beam balance, PH meter, Magnetic stirrer hot plate, glassware and shelf for keeping chemicals (2); Autoclave (3), Laminar air flow hood cabinet (4); Water bath (5); and Growth room (6).

Appendix 2. Representative pictures of practical works in sample collection and laboratory experiments.

Appendix 3. Sequential stages of indirect somatic embryogenesis from style/stigma explants of "Mexican lime'' sweet orange cultured on BAP, 2,4-D and NAA-enriched MS medium.



Explants collected from the mother tree in open field (A); excised explants which is going to be planted in MS media (B); Swelling of style/stigma explants after a week of culture (C). Friable callus appeared at the basal part of the style after two weeks (D). Proliferation of callus along the style axis (E). Green globular somatic embryos produced on callus after four months (F and G) shoot regeneration (H, I and J).

Appendix 4: preparation of MS stock solution

Nutrients and vitamins	Stock solution preparation		Amount of stocks solution (g/L)
	Reagents	Chemicals formula	
Macronutrients (40x)	Ammonium nitrate	NH_4NO_3	66
	Potassium nitrate	KNO_3	76
	Magnesium sulphate	$MgsO4.7H_2O$	14.8
	Potassium dibasic phosphate	KH_2PO4	6.8
	Calcium chloride	CaCl2	2.9
Micronutrients (1000x)	Boric acid	H_3BO_3	6.2
	Manganese sulphate	$MnSO_4.H_2O$	22.3
	Zinc sulphate	$ZnSO_{4.7}H_2O$	8.6
	Sodium molybdate	$Na_2MO_4.H_2O$	0.25
	copper sulphate	$CuSO_{4.5}H_2O$	0.025
	Cobalt chloride	$Cocl._6H_2O$	0.025
	KI	KI	0.83
	Fe-EDTA	C10H12FeN2O8-	3.73
vitamins	Thiamine- HCL	C12H18Cl2N4OS	0.1
	Myo-inositol	C6H12O6	0.1
	Pyridoxine- HCL	C8H12ClNO3	0.05
	Nicotinic amide	C6H6N2O	0.05
	Glycine	$C_2H_5NO_2$	0.2
	Malt extract	C6H12O6	0.05
	Sucrose	C12H22O11	40
	Agar	C14H24O9	8