IN VITRO REGENERATION OF POMEGRANATE AND GENETIC INTEGRITY ASSESSMENT OF DERIVED MATERIALS

TEBOGO STIMELA

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In Vitro Regeneration of Pomegranate and Genetic Integrity Assessment of Derived Materials

Tebogo Stimela

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DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been submitted to any other college, institution or university for academic credit.

Signed: _____ Date: _____

Tebogo Stimela
MB300-0007/17

This thesis has been submitted with our approval as University supervisors

Signed:_____ Date:_____

Dr. Remmy W. Kasili

Institute of Biotechnology Research (IBR), Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya

Signed: _____ Date: _____

Prof Edward G. Mamati

Department of Horticulture and Food Security, Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya

DEDICATION

I affectionally dedicate this research work to my late sister Keolebogile Stimela.

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ABBREVIATIONS AND ACRONYMS

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
CRD	Completely Randomized Design
CTAB	Cetyl trimethylammonium bromide
GA_3	Gibberellic acid
GRA	Grey Relational Analysis
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KN	Kinetin
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
PGRs	Plant Growth Regulators
SSR	Simple sequence repeats
TDZ	Thiadiazol-5-ylurea
WPM	Wood Plant Medium

ABSTRACT

Pomegranate is an important plant with nutritional, economic and health benefits. In recent years, many people have become aware of health benefits of pomegranate but the existing propagation methods cannot meet the demand for suitable planting materials needed for commercial production. Micropropagation can lead to mass production of plantlets and callus-mediated *in vitro* regeneration can open avenues for application of genetic engineering to improve this crop. The aim of this study was to develop an appropriate *in vitro* regeneration protocol for pomegranate and assess the genetic integrity of derived materials. Cytokinins (BAP, KN, TDZ) were tested for shoot induction from nodal explants while auxins (NAA, IBA and IAA) were tested for root induction from *in vitro* regenerated shoots. NAA in combination with BAP were assessed for their ability to induce callus from cotyledon and leaf explants. For callus induction, there were two experiments; one experiment had five levels of NAA with a fixed level of BAP. The other experiment had five levels of BAP with a fixed level of NAA. Genetic variability between mother plant, derived callus and plantlets was assessed using SSR markers. Highest number of shoots and leaves from regenerated shoots were obtained on MS media supplemented with 6.9µM KN; an average of two shoots and 12.00 ± 1.15 leaves/ explant. The highest number of roots was achieved on half strength MS media supplemented with 4.9µM IBA; an average of 7.00 ± 1.00 roots/shoot. The longest root was obtained on media supplemented with 5.3µM IAA; an average of 15.00 ± 1.00 mm. Both IAA at 5.3µM and IBA at 4.9µM were suitable for root induction. Callus was induced on MS media supplemented with combinations of (2.2-11.0µM BAP) and (2.8-13.2µM NAA).

Hence cotyledons and young leaves are suitable explants for callus induction in pomegranates. Eight SSR markers were used in assessment of variability in tissue culture derived pomegranate. Genetic variation with a similarity coefficient of 0.46-0.92 was found in the derived materials. This variation could be attributed to the seedling materials used in the study. Therefore tissue culture can be used in rapid multiplication of pomegranate propagation materials. However there is a need to optimise conditions for detecting somaclonal variation in derived materials. Combination of BAP (2.2-11.0µM) and NAA (2.8-13.2µM) can be used in pomegranate callus formation.

CHAPTER ONE

1.0 Introduction

1.1 Background information

Pomegranate (*Punica granatum* L.) is a plant from the family *Punicaceae*. It belongs to the genus *Punica* and the species *granatum*. It is an indigenous plant in Iran and the Himalayas in northern India and has been accepted and grown over the Mediterranean and the Caucasus region of Asia. The plant grows well under different kinds of climate, soils, except sandy and saline soils and it is tolerant to drought. Naturally, it grows below 1000m above sea level and it is limited to the tropics and subtropical regions, and does well in both arid and semi-arid areas. Pomegranate trees are grown all over Iran, India, drier parts of South East Asia, Malaysia, the East Indies as well as in the dry and hot areas of United States and Latin America (Glozer and Ferguson, 2011). Countries in the Mediterranean are key areas where pomegranate is cultivated on a commercial scale, followed by Asian countries and former USSR (Union of Soviet Socialist Republics such as Ukraine, Georgia and Russia). Spain has the most efficient cultivation of 18.5 t/ha, followed by USA with 18.3t /ha. The highest export of 60 000 t/year is from Iran, followed by India with an export of 35 176 t/year. Though Spain has a small cultivation area of 2000 ha, it exports 37.8% of 37 000t (total production) followed by Israel with 23.5% and USA with 15.5% of the total production (Da Silva *et al*., 2013).

Pomegranate fruit and juice have gained commercial significance since 2000s with the verification of its health benefits by the scientific works all over the world. Smith

(2014) reported that there was an improved conduct of mice with Alzheimer's disease after administration of pomegranate juice. Standardised pomegranate rind extract showed antibacterial, anti-inflammatory and anti-allergic properties (Panichayupakaranant *et al*., 2010). According to Glozer and Ferguson (2011), pomegranate fruits are high in fiber and are a good source of vitamin A and C. They further reported that three small fresh fruits have more than 50% of the recommended daily intake (RDA) of beta-carotene.

Bioactive phytochemicals which are thought to act against cancer, diabetes and high blood pressure are contained in all plant parts (Kahramanoğlu and Usanmaz, 2016). In ancient times, extracts from pomegranate fruit peels and tree bark were used in the 'Ayurvedic System of medicine (world's oldest holistic healing system that originated in India) in treatment of diarrhoea and dysentery. In India and Middle East, extracts were used in treatment of diabetes through the 'Unani system of medicine (South Asia traditional *system* of healing and health maintenance) (Smith, 2014).

Studies have also provided evidence in support of the possible use of pomegranates in helping to prevent and/or treat cancer, cardiovascular disease, diabetes, dental conditions (juice have antiviral and antibacterial effects against dental plaque), erectile dysfunction, Alzheimer's disease, male infertility, arthritis and obesity (Smith, 2014). Consumption of concentrated pomegranate juice by type II diabetic patients with hyperlipidemia showed juice effect in moderating heart disease risk factors (Esmaillzadeh *et al*., 2006).

Pomegranates are consumed as fresh fruits or used for making fruit juice syrup, meat sauce, spice, molasses or wine spirits. Fruits may be used as a decoration agent and cattle feed due to their richness in fibre and antoxidants (Kahramanoğlu and Usanmaz, 2016). Dhinesh and Ramasamy (2016) reported development of the new pomegranate products such as slightly processed pomegranate seeds, jams, marmalades, jellies, juice concentrates, seeds in syrup, candied arils, arils in vinegar, pomegranate wine and pomegranate syrup.

Currently, demand for pomegranate fruit is increasing worldwide due to high pharmacological and therapeutic properties of the fruit (Jalikop, 2010). However there are challenges in propagation method which include low seed germination due to dormancy. Pomegranate seed coat is hard hence making water penetration and gaseous exchange difficult resulting in a low germination rate (Materechera and Seeiso, 2013). Cuttings have poor root development and the propagation method is season dependent, labour intensive, time consuming and results in slow establishment (Kaji *et al*., 2013a; Kaji *et al*., 2013b; Singh *et al*., 2013). This is a setback for commercial pomegranate production. Therefore an *in vitro* regeneration protocol can be used to produce a suitable pomegranate planting material en mass which will make commercialization achievable. The benefits of tissue culture include; rapid multiplication of genetically uniform plants possessing desirable traits, production of plants in the absence of seeds, regeneration of whole plants from plant cells that have been genetically modified, production of disease free plants and plants from seeds which have low chance of germinating (Idowu *et al*, 2009).

Increased awareness of pomegranate health benefits in Kenya has created an increased demand against very poor supply leading to a net importation of this fruit and therefore making it fetch a high market price. The purpose of this study was to develop an appropriate *in vitro* regeneration protocol of pomegranate which can be used to address the problem of inadequate planting material and meet the increased demand by Kenyan farmers. Appropriate conditions for callus induction were also investigated as callus-mediated regeneration will open avenues for improvement of the fruit tree using modern biotechnology approaches such as genetic engineering. The study was designed to determine the best plant growth regulators (PGRs) and optimum PGRs levels for plant regeneration, callus induction and determine the genetic integrity of derived materials.

1.2 Statement of the problem

According to Oxfarm Organic (2017) there is an increasing demand of pomegranate in Kenya due to increased awareness of its health benefits. However, the supply is low since few farmers produce pomegranate fruits due to insufficient suitable planting materials for commercial production. The insufficiency of planting material is brought about by the challenges experienced with the existing pomegranate propagation methods which include seed dormancy that leads to poor germination rate and poor root development on cuttings. Propagation from cuttings is also slow and time consuming.

1.3 Justification

Increased awareness of pomegranate health benefits in Kenya has created an increased demand against very poor supply leading to a net importation of this fruit and therefore making it fetch a high market price. The purpose of this study was to address the problem of limited pomegranate quality planting material through the development of an appropriate *in vitro* regeneration protocol that could be used for rapid multiplication of planting materials.

1.4 Significance of the study

An appropriate *in vitro* regeneration protocol of pomegranate growing in Kenya can be used for mass production of planting material to meet the increased demand by Kenyan farmers. Regeneration via callus will open avenues for the improvement of the fruit tree by genetic transformation.

1.5 Hypotheses

1. There is no difference between plant growth regulator type and levels on pomegranate shoot and root induction
2. There is no difference in plant growth regulator combination levels for pomegranate callus induction
3. There is no variation in genetic integrity of pomegranate tissue culture derived materials

1.6 Objectives

1.6.1 General objective

To develop an appropriate *in vitro* regeneration protocol for pomegranate and assess the genetic integrity of derived materials.

1.6.2 Specific objectives

1. To determine the best plant growth regulator type and level for pomegranate shoot and root induction.

2. To determine appropriate plant growth regulator combination levels for pomegranate callus induction and organogenesis

3. To evaluate genetic integrity of pomegranate derived materials.

CHAPTER TWO

2.0 Literature Review

2.1 Pomegranate botany and growth conditions

There are only two species in the genus *Punica* which are *P. granatum* and *P. protopunica*. The difference between subspecies is based on the xylem anatomy with *P. protopunica* proposed to be the older subspecies (Holland *et al*., 2009). *P. protopunica* is found on the Socotra Islands in Yemen only whilst *P. granatum* is found in different geographical regions which include the Mediterranean basin, Central Asia, China, India, South Africa and Australia. Nowadays *P. granatum* plant is grown throughout the world both in the tropics and subtropics with different climatic conditions showing its resilience, adaptability and wide range of genetic diversity (Da Silva *et al*., 2013).

2.1.1 The pomegranate fruit tree

P.granatum is a deciduous shrub with a bushy appearance due to the development of multiple trunks. The tree when under cultivation reaches a height of 5m but when growing in the wild it can grow beyond 7m (Smith, 2014). Pomegranate tree can be productive for 15 years after which it loses vigour and its production decreases. A young tree has a brownish-red bark which turns grey as it matures. The tree produces suckers around the base and those suckers can be used for propagation as cuttings or be removed during pruning. When left un-pruned, the suckers take a lot of plant photosynthates reducing fruit yield and quality (Kahramanoğlu and Usanmaz, 2016).

According to Ashton *et al.,* (2006) plants raised from seeds begin fruit bearing at the age of four years, whilst those raised from cuttings begin fruit bearing at the age of three years. Depending on pomegranate variety, geographical region and cultural practices, the tree yield in the third year can be 10-20 kg and in the fifth year can increase up to 60-100 kg (Kahramanoğlu and Usanmaz, 2016).

2.1.2 Leaves

The plant leaves have a length of 1.91-8.89 cm and a width of 1.02- 3.05 cm, with short petioles. They are arranged in groups, with each group containing three leaves. The leaves are reddish in colour when young and turn bright green when they mature, with the top part being more dark green compared to the bottom part but the petiole remains reddish (Ashton *et al*., 2006).

2.1.3 Flowers

Pomegranate flowers occur about one month after bud appearance on the newly developed branches. They occur either as singles or small groups of 2-6 flowers and rarely appear at the branch ends, sometimes found on the auxiliary buds. Flower petals are more than 3cm in diameter and most of the cultivated fruiting types have single flowers with orange-red or intense red petals. For ornamental varieties, flowers are double and the petals have many shades of colours ranging from white to red (Ashton *et al.,* 2006). Most pomegranates have perfect flowers; the flowers have both the stamen and pistil in one flower implying that they can undergo self-

pollination. The availability of another cultivar allows cross pollination that will set an improved crop and increase in fruit set by 38%. Few varieties do not have perfect flowers and therefore require insects such as honey bees as pollinators. Flowering can take place up to 10-12 weeks or more depending on the cultivar being grown and environmental factors (Glozer and Ferguson, 2011).

2.1.4 The fruit

According to Ashton *et al*., (2006), the pomegranate fruit is a fleshy berry with inedible carpellar membrane partitions, separating the cavities (locules) of 4-15 containing the edible parts, and all these are enclosed in a skin which is smooth, thick and leathery. The fruit inner part is full of many fleshy seeds of prismatic shape each surrounded by an edible juicy pulp known as arils. Fruit sizes range from 5.08-15.24cm width with large sized fruits containing 1200-1300 seeds. The fruits are spherical, sometimes pear shaped with a calyx (crown) remaining attached to the fruit. The calyx length differs with varieties and can be 3.18-6.36cm long (Ashton *et al*., 2006). Background fruit colour is greenish yellow or brown with some reddish parts, while the whole fruit skin depending on the cultivar can be pink, red, yellow and dark purple with darks in between. In pomegranate growing regions, fruit maturity in average varieties is achieved six months after bloom with early varieties taking a shorter time to ripen. Pomegranates are non-climacteric fruits, meaning that fruit ripening would not occur once the fruit is off the tree hence harvesting should be done after they have attained complete maturity. During storage, the fruit slightly

dries out causing the sugars to concentrate which makes the fruit sweeter (Ashton *et al*., 2006).

2.1.5 Pomegranate cultivars

There are numerous pomegranate cultivars found in different countries and they are grouped based on the taste, time of harvest and seed hardness. In relation to taste, they are classified as sweet, sweet-sour, sour and in terms of time of harvesting, they are classified as early, average and late. Based on hardiness, they are classified as soft or hard seeded. (Glozer and Ferguson, 2011; Kahramanoğlu and Usanmaz, 2016). Some of the highly productive cultivars include; Wounderful, Mollae de Elche, Hicaznar, Ganesh and Acco.

2.1.5.1 Wounderful

It is an average-late cultivar with high productivity and originates from Florida. The fruit peel is red containing seeds which are not very hard surrounded by dark red tart-sweet arils (Glozer and Ferguson, 2011).

2.1.5.2 Mollar de Elche

It is a highly productive average cultivar from Spain with soft seeds surrounded by red sweet arils enclosed in a red skin (Kahramanoğlu and Usanmaz, 2016).

2.1.5.3 Hicaznar

Hicaznar is a pomegranate cultivar originating from Turkey. It is a very productive late cultivar which yields red fruits containing hard seeds and sweet to sour arils (Smith, 2014).

2.1.5.4 Ganesh

Cultivar Ganesh originates from India. It is an average cultivar which produces soft seeds surrounded by pinkish sweet arils enclosed in a yellowish red rind (Venkatesha and Yogish, 2016).

2.1.5.5 Acco

It is an early cultivar from Israel producing red peel fruits containing soft seeds surrounded by very sweet red arils (Chandra *et al*., 2010).

2.1.6 Growth conditions

Although pomegranate plant can grow well under different climatic conditions, the plant is adapted to a climate which comprise of cool winters and hot summers hence recognized as a mild-temperate to subtropical fruit. The plant can withstand drought but the yield declines with frequent exposure to drought conditions. Severe injury is experienced by the plant at temperatures below -11^{0}C (Kahramanoğlu and Usanmaz 2016). During the ripening period, high temperatures are preferred by the plant as it causes the fruits to become reddish and sweeter. The fruit flavour is greatly affected by day and night temperature difference; the higher the temperature difference, the

sweeter the fruit. Varying soil conditions are preferred by pomegranate trees except sandy, saline, alkaline and calcium carbonate rich (calcareous) soils. Water with salinity of 3.500ppm can be tolerated (Kahramanoğlu and Usanmaz (2016). Alkaline soils of pH 7.5 are slightly tolerated but acidic soils of pH range 5.5-6.5 are preferred (Glozer and Ferguson, 2011).

2.2 Nutritional and health importance

Pomegranates are rich in fiber, vitamin A and C. More than 50% RDA of beta-carotene is contained in three small pomegranate fresh fruits. Beta-carotene is a strong antioxidant important in inhibiting plaque deposits from accumulating in the arteries, shielding eyes from being damaged by the sun and causes deactivation of free radicals which can speed up the aging process and increase the risk of cancer. Beta-carotene is converted into vitamin A which is necessary for good vision and ensures the eyes remains lubricated at all times (Glozer and Ferguson, 2011). Pomegranates contain substantial amounts of iron required for haemoglobin, the pigment found in red blood cells used in transportation of oxygen in the body (Glozer and Ferguson, 2011).

Pomegranates contain many bioactive phytochemicals which have antimicrobial activities, help decrease blood pressure and act against cancer and diabetes. Numerous antique cultures had faith in the health promoting effects of pomegranate tree plant parts. These parts include the roots, bark, fruit, leaves and flowers (Holland *et al*., 2009). Bioactive phytochemical amounts in these different parts changes with

the tree developmental stages and fruit maturity. Type of cultivar and environmental conditions also determine bioactive concentrations in the plant (Smith, 2014). Extracts from pomegranate fruit peels and tree bark were used in the Ayurvedic system of medicine for treatment of infections such as diarrhoea and dysentery. They were also used in the Middle East and India in Unani system of medicine as an anti-parasitic agent and to treat diabetes and ulcers (Smith, 2014). There is verification from recent scientific findings that pomegranates can be used in prevention and/or treatment of diseases such as arthritis, cardiovascular disease, diabetes, cancer, dental conditions (juice have antiviral and antibacterial effects against dental plaque), erectile dysfunction, infant brain ischemia, Alzheimer's disease and male infertility (Smith, 2014).

2.3 Economic importance of pomegranate

Pomegranate fruits can be used to make juice or jelly and syrup can be made by adding sugar in a concentrated juice. The produced syrup is dribbled over desserts dishes such as that of pancakes, piece of plain cake and ice cream. Pomegranate meat sauce is used to moisten meats and chicken in the last culinary steps. Pomegranate wine can also be produced, and in some areas of Asia, pomegranate spices are made by first drying the fruit and pulverizing them into a fine powder (Ashton *et al*., 2006; Glozer and Ferguson, 2011). There are new pomegranate derived products which include processed pomegranate seeds, jams, jellies, marmalades, juice concentrates, frozen seeds, seeds in syrup, candied arils, arils in brandy and in vinegar, pomegranate wine and syrup. Due to nutritious, palatable and good quality dessert,

processed products such as juice, anardana, concentrated syrup and jelly were greatly accepted (Dhinesh and Ramasamy, 2016). There is also granatum plus from Italy which is a line of pomegranate derived products. The Granatum Plus range includes nectars, syrups, cosmetics and capsules which are rich in antioxidants like Punicalagin and Omega 5 fatty acids (Forbon Srl, 2013).

2.4 Pomegranate propagation

2.4.1 Propagation by seed

Sexual propagation of pomegranate is the method used to crossbreed pomegranate varieties. Meaning that the trees produced from seeds will have different genotypes which may show some desirable traits while others bring undesirable traits due to cross pollination (Ashton *et al*., 2006). For commercial production, pomegranates are not propagated through seeds because they do not come as true variety and such seedlings produces fruits which have wide varying characteristics. Examples include fruits from small to large size, woody to juicy, fruit colour ranging from dark red or purple and the taste from sweet to sour (Glozer and Ferguson, 2011). Propagation through seeds involves first drying seeds from a selected fruit for two weeks and once they become very dry, they are sown immediately. If the seeds are to be kept for late planting; after drying they are stored in a sealed container inside a refrigerator. Seed germination is faster for varieties which possess soft seeds than those which produce hard seeds. Soil temperature also determines the time for the seed to germinate and fast germination is promoted by high soil temperatures (Ashton *et al.,* 2006).

2.4.2 Propagation by grafting and layering

Successful pomegranate propagation can achieved through grafting using cleft grafts, wedge grafts, whip and tongue grafts (Ashton *et al*., 2006). Kahramanoğlu and Usanmaz (2016) stated that grafting is not an ideal method because it has a very low chance of success due to a stem which is not strong. The bark can bulge which will cause breaking of the scion during fruit bearing. Grafting as a method of propagating pomegranate does not yield successful results (Glozer and Ferguson, 2011). Layering method can be used whereby in summer a sucker still attached to the mother plant is bent over in such a way that part of the branch is in contact with the ground. The sucker is cut out in early spring after the mother plant has produced leaves, and cutting is done between the mother plant and part of the sucker which is underground (Ashton *et al*., 2006).

2.4.3 Propagation by cuttings

In propagation by cuttings, hardwood cuttings of 15-20cm length from the previous season vegetative shoot growth or suckers are used. They (cuttings) are taken in late season of inactiveness before any bud development and put inside the greenhouse on a sterilized soil in a vertical orientation with exposed top node, or planted directly in nursery beds. Cuttings may be left for a year or two in a nursery however one year is sufficient, then they can be transferred to a permanent orchard (Glozer and Ferguson, 2011). The use of cuttings to propagate pomegranate is labour intensive, time consuming and does not guarantee healthy (disease free) plants though it has been reported as a conventional method (Kaji *et al*., 2013b).

2.5 Plant tissue culture

Plant tissue culture is a term used to refer to collection of techniques used to vegetatively propagate plants under aseptic conditions using small parts of living tissues known as explants on artificial growth media (Yildiz, 2012). The totipotency property of plant cells is the one that makes plant tissue culture possible (Pierik, 1987). The *in-vitro* cell culture concept was developed by Haberlandt through attempts to cultivate various differentiated plant cells in a glucose enriched salt solution and due to utilisation of simple nutrients and lack of sterile conditions, the experiment had little success (Sussex, 2008). Various benefits of plant tissue culture include; rapid multiplication of genetically identical plants of desirable traits, disease free plant production without seeds, complete plant regeneration from genetically modified plant cells and production of plants from seeds with poor germination (Idowu *et al.,*2009). The success of plant tissue culture depends on a number of factors which include; choice of nutrient medium, PGRs, explants source and appropriate environmental conditions. There are generally three major methods used in plant tissue culture which include propagation from pre-existing meristems (shoot tip culture or nodal culture) known as micropropagation, organogenesis and somatic embryogenesis (Sharma *et al*., 2015).

2.5.1 Micropropagation

Micropropagation is a method which involves aseptic excision of any asexual (meristematic) plant part such as shoot tip or bud and culturing it under controlled conditions on sterilized growth media to bring about a plantlet which is the exact

copy of the mother plant (Sharma *et al.,* 2015). Initially, micropropagation technique was developed for the purpose of commercial production of ornamental plants and was later used for production of fruit and vegetable crops as it is considered a rapid method of plant multiplication. It is also used as an important *ex situ* conservation technique of medicinal plants which are endemic, rare and endangered in a particular area (Tasheva and Kosturkova, 2013).

2.5.2 Somatic embryogenesis

Somatic embryogenesis is a process whereby either one cell or a cluster of cells start the development pathway that causes regeneration of non-zygotic embryos which are competent to form a full plant after germination (Sharma *et al*., 2015). Since somatic embryos are formed without fusion of gametes, and so they are referred to as clones because genetically they are similar to the mother plant. During somatic embryogenesis, the cells undergo a sequence of morphological and biochemical alterations which result in non-zygotic embryo formation with the ability to regenerate plants. Somatic embryogenesis show a distinct path of development involving characteristic events which include; cell dedifferentiation, cell division activation and reprogramming of their physiology, metabolism, and gene expression patterns (Yang and Zhang, 2010).

Somatic embryogenesis can be used in examining plant embryogenesis events and is also one of the significant way of multiplying transgenic plants or plants of choice (Simões *et al*., 2010). Somatic embryogenesis can either be direct or indirect. Direct somatic embryogenesis involves somatic embryo formation from the explant without

going through the callus phase whilst the indirect somatic embryogenesis involves formation of somatic embryos through the callus phase (Yang and Zhang, 2010). It has been suggested that PGRs and stresses are the important factors that facilitate the signal transduction cascade that directs gene expression reprogramming. After that, succession of cell division follows which influences muddled callus growth or differentiated growth directing to somatic embryogenesis (Yang and Zhang, 2010).

Sharon *et al*., (2011) induced somatic embryos directly from two root segments of pomegranate *in vitro* raised seedlings cultured on Gamborg's B5 media supplemented with 2mg/L Kinetin (KN). The roots segments which formed embryos were those obtained from the middle and at the root base. Raj Bhansali (1990) obtained somatic embryos from cotyledons explants of immature zygotic embryos. The explants were taken from unripe pomegranate fruits.

2.5.3 Organogenesis

Shoot organogenesis involves formation of adventitious shoots from explants with no pre-existing meristems followed by rooting of formed shoots (Kane *et al*., 1994; Schwarz and Beaty, 2000). The process can be divided into two; direct and indirect shoot organogenesis. Direct involves shoot formation from explants without callus formation whereas indirect involves shoot formation through callus produced from the explant. Normally there are three stages in shoot organogenesis which are dedifferentiation, induction and differentiation (Shen, 2007).

Dedifferentiation is a process whereby explant specialized cells are unspecialized and returns to less dedicated and supple developmental state that can include or not include formation of callus. In a direct shoot organogenesis pathway, some cells will dedifferentiate straight to form a new primordial whereas in indirect pathway, cells dedifferentiate to form callus followed by development of new shoots (Shen, 2007). Some of these unspecialized explants cells have the ability to respond to particular inducing signals such as PGRs leading morphological differentiation and nascent development of organs. Induction stage is the period between when cells become competent and when they become determined fully for primordial production. During this stage, competent cells become devoted to a specific developmental destiny and the stage comes to an end when cells are fully determined and without the inducing signal are able to undergo shoot organogenesis (Schwarz and Beaty, 2000).

2.5.4 Tissue culture media

The following are components of plant tissue culture media: macronutrients, micronutrients, vitamins, amino acids or nitrogen supplements, source (s) of carbon, undefined organic supplements, growth regulators and solidifying agents (Saad and Elshahed, 2012). As stated by the International Association of Plant Physiology, the element whose concentrations is greater than 0.5mmol/L is defined as macro element and those needed in concentrations lower than 0.5mmol/L are referred to as microelements. Several researchers have utilized different media to suit a particular tissue culture requirement and depending on the overall media salt concentrations,

different media have been formulated. Murashige and Skoog (MS) media is the most widely used and is a very effective plant tissue culture medium for almost all types of tissue culture work (Saad and Elshahed, 2012).

2.5.4.1 Plant nutritional requirements (micro and macro nutrients, vitamins)

Plant nutrition is necessary *in vitro* for proper plant growth and development. Without nutrients, growth and development cannot be achieved. Under different culture conditions, different genotypes and explants perform differently, therefore to understand the full genetic potential of cells; it is necessary to provide a medium that will allow cells to grow (Oagile, 2005). Apart from carbon (C), hydrogen (H) and oxygen (O), the fundamental macro-elements required in tissue culture media for growth and satisfactory morphogenesis are nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S). Basic micro elements required as biochemical reactions catalysts include; iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo). Vitamins are required by plants for normal growth and development and they are involved in various plants metabolic processes as catalysts. This means that if plants are grown *in vitro*, they may act as limiting factors for cell growth and differentiation if not provided in the culture medium. The mostly used vitamins in tissue culture media include: thiamine (B1), nicotinic acid and pyridoxine (B6) (Saad and Elshahed, 2012).

2.5.4.2 Solidifying agents

Culture medium firmness significantly affect cultured tissue growth and there are different gelling agents used to solidify culture media which include; agarose, agar and gelrite (Saad and Elshahed, 2012). The mostly used solidifying agent in preparation of semi-solid and solid culture media is agar; a polysaccharide acquired from seaweeds. It is utilised worldwide because of the advantages it has over other agents which are; easy melting at temperature range of 60-100^{0}C when mixed with water, solidifying at approximately 45^{0}C and formation of a gel which is stable at all possible incubation temperatures. Also, agar is not digested by plant enzymes and does not react with media constituents (Saad and Elshahed, 2012). Gelrite was used in the study because it allows formation of clear gel which facilitates visualization of root development, growth and contamination.

2.5.4.3 Plant growth regulators

PGRs are organic compounds other than nutrients which provide energy or mineral elements in small quantities either for the purpose of encouraging, hindering or qualitatively altering any plants growth and development (Decoteau, 2005). According to Saad and Elshahed (2012) PGRs are necessary in plant tissue culture since they play an important role in plant stem elongation, tropism, and apical dominance. Generally there are five groups of PGRs which are auxins, cytokinins, gibberellic acid (GA), abscisic acid (ABA) and ethylene. Auxin to cytokinin proportions is the one that control the type and extent of organogenesis in plant cell cultures. In many plant species, auxins encourage root development while cytokinins

encourage shoot development. GA also induces plant growth and hinders formation of adventitious roots (Oagile, 2005).

Auxins which are commonly used in plant tissue culture media include; 1-Naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA), Indole-3-acetic acid (IAA) and 2,4-Dichlorophenoxy-acetic acid (2,4-D). IAA is the only auxin naturally occurring in plant tissues. Cytokinins commonly used include; Benzylaminopurine (BAP), KN and Thiadiazol-5ylurea (TDZ), 6-Dimethylaminopurine (2-iP) and 6-4-hydroxy-3-methyl-trans-2-butenylaminopurine (Zeatin). Zeatin and 2iP are naturally occurring cytokinins with zeatin considered the most effective cytokinin (Saad and Elshahed, 2012).

2.5.5 Shoot induction and multiplication

Cytokinins are known to cause differentiation of shoot buds from shoot tip and nodal explants into shoots (Sharma, 2017). Though naturally occuring cytokinins such as zeatin and 2-iP are used, artificial cytokinins are preferred due to their power to induce multiple shoots and these are BAP and KN (Benita, 2014). According to Sharma (2017), BAP is the most commonly used hormone due to its effectiveness in promoting axillary shoot development from nodal explants. To carry out shoot multiplication, one is required to repeatedly sub culture the *in-vitro* regenerated shoots until the required quantities of shoots are obtained. Then, the regenerated shoots are induced to develop roots by culturing them on rooting media. Most often, components of shoot multiplication medium remain the same as that of shoot

initiation with only PGRs concentrations modified for shoot proliferation (Benita, 2014).

Kaliamoorthy *et al*., (2008) observed maximum shoot multiplication of *Harpagophytum procumbens* without callus from nodal explant cultured on MS medium plus Gamborg's (B5) vitamins supplemented with 0.1 mg/L IAA and 5.0 mg/L KN. From micropropagation of *Harpagophytum procumbens* by Grabkowska and Wysokinska (2009), it was observed that the best shoot multiplication was achieved in the presence of 6 µM TDZ. They reported that the shoots which were produced were small and their elongation on Schenk and Hildebrandt (SH) agar medium supplemented with GA_3 was necessary.

2.5.6 Root induction

In vitro grown plant rooting is an important stage because the aim is to have plants growing *in vivo*. Though there are some species whose *in vitro* regenerated shoots can develop roots on a hormone free tissue culture media, some species require particular treatments before root development hence the rooting stage is necessary. PGRs commonly used for the purpose of encouraging root induction are auxins and their ability to induce rooting differs with auxin type, concentration and plant species (Oagile, 2005). Root initiation stage involves the transfer of shoots obtained from shoot initiation and multiplication stage onto a suitable rooting media. Depending on the plant species, rooting media may be changed to some extent with respect to basal medium composition and increased auxin levels for root induction (Benita, 2014).

2.5.7 Callus induction and plant regeneration

Callus is a mass of undifferentiated cells produced by plants in response to stresses caused by pathogen infection or wounding (Ikeuchi *et al*., 2013). The whole plant or many plant parts can have the ability to multiply *in vitro*. However it has been established that in some organs, callus can be induced easily than in others. Young meristematic tissues are the most appropriate, even those (meristemtic tissues) in older plant parts such as the cambium can form callus. Callus which is formed from the original explant is referred to as primary callus but those initiated from primary callus are referred to as secondary callus (George *et al*., 2008).

Auxins such as 2,4-D and NAA have been commonly used for callus formation, with or without cytokinins such as KN. 2,4-D in combination with BAP has been used for callus formation in *Acacia sinuata* (Grabkowska and Wysokinska, 2009). NAA with BAP have been used to obtain callus from leaf explant of *Echinacea purpurea* (Koroch *et al*. 2002). GA_3 was also reported to enhance growth of callus and help in elongation of dwarf plantlets. Sometimes ABA is included in the culture media to inhibit or stimulate callus growth, depending on the plant species (Saad and Elshahed, 2012).

With exception of KN, in all cytokinin combinations with IAA, increase in production of axillary shoots was observed together with callus formation on regenerated shoots (Kaliamoorthy *et al*., 2008). Shushu (2001) reported similar observation in *Harpagophytum procumbens* where callus formation was observed on

explants during direct axillary shoot induction from single nodal segments and shoot tip explants. Successful callus induction was achieved on kola plant explants cultured on media supplemented with varied (0.1-1.0 mg/L) NAA levels combined with constant (2.3 mg/L) BAP level. But no callus was observed in explants cultured on media supplemented with varied BAP levels combined with constant (1.0 mg/L) NAA level (Obembe, 2000).

2.6 *In vitro* culture of pomegranate

Studies have been done on pomegranate tree micropropagation resulting in development of new protocols in which regeneration was through indirect organogenesis using anthers, leaves and cotyledons as explants (Deepika and Kanwar, 2010; Patil *et al*., 2011). Protocols were also established in which plantlets were generated through somatic embryogenesis from explants such as petals (Patil *et al*., 2011).

2.6.1 Tissue culture media for pomegranates

Tissue culture media mostly used for *in vitro* regeneration of pomegranate is MS media. However some authors evaluated effects of different media on *in vitro* regeneration of pomegranate and found other media such as Woody Plant Medium (WPM) gave better results. Nonetheless, MS is still considered the best in almost all pomegranate studies hence it was used in this study. Kaji *et al*., (2013a) investigated two different culture media; MS and WPM on shoot induction and found that culture

media significantly affected the shoots length, number of nodes and leaves on regenerated shoots but there was no significant difference in number of shoots obtained. Plantlets produced on WPM, were more vigorous due to longer shoot length compared to those produced on MS media. Patil *et al*., (2011) also tested two different culture media and obtained the highest shoots number on MS media than WPM. Singh *et al*., (2013) also found that culture media type significantly affected explant establishment. They investigated different types of media and found maximum establishment, highest number of internodes and minimum days to establishment on MS followed by Gamborg (B5) and lastly WPM.

2.6.2 Explant type and source

Different explants from various pomegranate plant parts of different ages have been used in tissue culture studies by different authors. Naik *et al*., (2000) used cotyledonary node explants of 20-days old *in vitro* germinated seedlings whilst Singh *et al*., (2013) used cotyledonary node explants from five weeks old *in vitro* germinated seedlings. Kaji *et al*., (2013a) used shoot tips and nodal explants from two year old pomegranate trees. Deepika and Kanwar, (2010) used cotyledon, hypocotyl, leaf and internode section explants from six year old tree. Patil *et al*., (2011) used nodal segments explants from mature tree and Bonyanpour and Khosh-Khui, (2013) used leaf explants from one year old plant. All of them successfully produced plantlets *in vitro*.

2.6.3 Pomegranate micropropagation

2.6.3.1 Shoot induction

From *in vitro* mass multiplication of pomegranate using cotyledon nodal explant of Ganesh variety, shoot proliferation was obtained on medium supplemented with BAP combined with auxins (Singh *et al*., 2013). Kaji *et al* ., (2013a) observed shoot induction from shoot tips and nodal explants cultured on medium supplemented with and without KN within one to two weeks of culture, but shoot growth failed in the medium without KN. Multiple shoots were obtained when explants were cultured on a medium containing a combination of 0.54µM NAA with varied KN concentrations. Naik *et al*., (2000) reported reduced shoot development and shoot elongation on medium supplemented with higher concentrations of BAP or KN at 23.0µM. This demonstrates that cytokinins with or without auxins, can lead to shoot induction but increased levels of the hormones results in a decrease of shoot development and elongation. Few studies have been reported on the use of TDZ in pomegranate micropropagation, therefore in this study, amongst the cytokins BAP and KN, TDZ effect on shoot induction was also determined.

2.6.3.2 Rooting of shoots

Bonyanpour and Khosh-Khui (2013) obtained 100% root induction with shoots having good root growth and root number. This was observed on WPM containing 0.2 mg/L IBA which was recorded as the best treatment. Kaji *et al*., (2013a) used half-strength WPM supplemented with 5.4µM of NAA and obtained the highest number of roots which was seven roots per shoot. Singh *et al*., (2013) recorded

maximum number of roots and highest root length from half strength MS medium supplemented with 0.5mg/L NAA and 200 mg/L activated charcoal. This shows that auxins without cytokinins can induce root development. Activated charcoal as medium amendment can positively influence rooting of shoots. IAA effect on rooting of pomegranate microshoots has been investigated in this study as there is limited information available on its effect in pomegranate rooting.

2.6.4 Callus induction and differentiation

Callus induction was observed from *Punica granatum* L. leaf explants cultured on MS medium supplemented with a combination of BAP and NAA. Maximum callus was obtained on medium supplemented with a combination of 1 mg/L BAP and 1 mg/L NAA (Bonyanpour and Khosh-Khui, 2013). Deepika and Kanwar (2010) investigated the effect of different types of explants on callus formation and found that cotyledon explants were the best, followed by hypocotyl, internode and leaf explant respectively. Callus differentiation was obtained on MS medium with 9.0 µM BAP and 2.5µM NAA and the highest regeneration was from callus derived from cotyledon explants. Parmar *et al*., (2012) reported optimum callus formation of 93.71% from cotyledon explants cultured on MS media supplemented with 4.0 mg/l BAP and 3.0 mg/l NAA. Shoot induction response was high at 72.34%, obtained on MS medium supplemented with 2.0 mg/l BAP and 2.0 mg/l NAA.

2.7 Somaclonal variation

Somaclonal variation refers to inherited genetic modifications which accrue in callus from somatic explant and get expressed in offsprings of callus *in vitro* regenerants. The variation occurs either during or after *in vitro* regeneration (Purohit, 2011). To plant propagators, somaclonal variation is a problem due to the fact that their main goal is preservation of a particular plant genotype. However to plant geneticists, somaclonal variation is appreciated because it is regarded as a new genetic information source which can result in crop improvement and profound comprehension of molecular and biochemical foundation of inheritance (Purohit, 2011).

Somaclonal variation occurs as a consequence of altered genes or epigenetic marks. It happens mostly in mass of cells which have not differentiated, isolated protoplasts and in morphological characteristics of plants regenerated *in vitro* (Krishna *et al*., 2016). Mutations occurring during tissue culture are activated by various stress issues including; explant wounding, exposure to sterilizing agents during the process of sterilization, lighting conditions and disparity in levels of media constituents (high PGR concentrations, carbon source from the nutrient medium) (Smulders and de Klerk, 2011). According to Bordallo *et al*., (2004) source of explant, genotype, tissue culture conditions and *in vitro* period are the greatest things influencing the occurrence of somaclonal variation. Somaclonal variation is dependent on PGRs, source of explant, genotype, variety difference, age of variety in culture and ploidy level (Salehian *et al*., 2014).

Few approaches including biochemical, cytological, morpho-physiological and DNA based molecular markers have been used to verify the degree of genetic fidelity of plants regenerated *in vitro* (Krishna *et al*., 2016). A common assessment is done using DNA-based molecular markers such as restriction fragment length polymorphism (RFLP), Amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD), microsatellites or simple sequence repeats (SSR) and single nucleotide polymorphism (SNP) (Jiang, 2013).

2.7.1 DNA markers used for genetic variation analysis in plants

DNA marker is a DNA fragment used to show variations or mutations which can be used in the identification of polymorphism between different genotypes (Jiang, 2013). Such DNA fragments are linked to a certain place within the genome and through the use of a particular molecular technology, they can be identified. The two elementary methods for detecting the polymorphism are southern blotting (a nucleic acid hybridization technique) and polymerase chain reaction (PCR) technique. Both methods involve an electrophoresis process such as Polyacrylamide gel electrophoresis (PAGE), Agarose gel electrophoresis (AGE) and capillary electrophoresis (CE). In DNA samples variation can be detected based on the products which include the band mobility and size (Jiang, 2013).

2.7.1.1 RFLP markers

RFLP are markers based on southern blotting or hybridization. Use of these markers involves mixing of restriction enzymes with the extracted genomic DNA. The enzymes are used to cut DNA at recognition sites yielding DNA fragments of different sizes which are used to detect variation among plants. The different fragment sizes produced by restriction enzymes are brought about by base pair insertions, deletions, translocations and inversions which occur at the recognition sites of individual plant genomic DNA (Nadeem *et al*, 2017). Once fragments are produced, they are detected through electrophoresis and DNA probing technique (Jiang, 2013).

2.7.1.2 RAPD markers

RAPD is a marker based on polymerase chain reaction (PCR) system in which genomic DNA is amplified by PCR using a short single random primer of about 10 nucleotides. Genomic DNA amplification, takes place when two hybridization sites in the opposite direction are the same (Jiang, 2013). The generated fragment length depends on the size of both primers for RAPD markers limited to 10 nucleotides and the targeted genome. PCR products are separated by ethidium bromide (EtBr) stained agarose gel electrophoresis and visualized under UV light. The existence of polymorphism from rearrangement or mutations which occurred at or between primer binding sites is confirmed by the appearance or non-appearance of a specific RAPD band in the visualized gel (Nadeem *et al*., 2017).

Ali *et al*., (2017) used RAPD markers to detected somaclonal variation in plants of four tomato genotypes generated *in vitro*. From *in vitro* regenerated plants of *Hibiscus sabdariffa*, somaclonal variation was found through the use of RAPDs markers as polymorphic bands were obtained from PCR products (Govinden-Soulange, *et al*., 2010).

2.7.1.3 AFLP markers

AFLP are markers which combines the technology of RFLP and PCR in which DNA is digested first with restriction enzymes followed by ligation of fragments with synthetic adaptors and amplification using adaptor complementary primers (Nadeem *et al*., 2017). Adapters are used to provide a known sequence for PCR amplification. AFLP primer of 17-21 nucleotides length are used and are made of a synthetic adaptor sequence, restriction endonuclease recognition sequence and an arbitrary, non-degenerate selective sequence of one to three nucleotides (Jiang, 2013).

Shooshtari *et al*., (2013) investigated somalonal variation in *Ducrosia anethifolia* plantlets using AFLP markers. They detected polymorphism among *in vitro* regenerated plants and reported the cause to be genome methylation. Polanco and Ruiz (2002) used AFLP markers for somaclonal variation analysis in regenerated plants of *Arabidopsis thaliana*. Variation was detected among the plantlets and minimum of one variation was displayed by 66.6% plantlets. The range of mutations per plant was 1-18.

2.7.1.4 SNP markers

A SNP is a single nucleotide base difference between two DNA sequences or individuals. They are classified based on the nucleotide substitutions which may either be transition (C substituted with T or G substituted with A) or transversions (C substituted with G or A, A substituted with T or C, and T substituted with G). SNPs can be found in the gene coding sequence, non-coding regions, or intergenic regions at different frequencies in different chromosome regions (Jiang, 2013). SNPs are co-dominant markers, frequently connected to genes therefore making them to be preferred genetic markers for use in genetic studies and breeding (Jiang, 2013).

Miyao *et al*., (2012) observed somalonal variation in rice plantlets obtained from cultured cells of single seed stock. Numerous SNPs, insertions and deletions (InDels) were found in the plantlets genome suggesting that SNPs and InDels play a role in somaclonal variation of regenerated rice plants

2.7.1.5 SSR markers

SSR markers also known as microsatellites or short tandem repeat (STRs) are PCR based markers which involve randomly tandem repeats of short nucleotide of two to six base pairs/nucleotides long. They can be di-, tri- and tetra-nucleotide repeats such as (GT)n, (AAT)n and (GATA)n distributed widely throughout the plant and animal genomes (Jiang, 2013). What differs among individuals is the copy number of repeats and that is what is normally used to detect polymorphism in organisms.

Double stranded DNA recombination, single- strand DNA slippage, mismatches and retrotransposons are the things influencing the occurrence of SSRs. Since SSRs flanking region sequences are conserved, they are usually used in primer development (Nadeem *et al*., 2017)..

Salehian *et al.,* (2014) induced somaclonal variation in two Iranian rice cultivars. Mature embryos used as explants were first cultured on MS callus induction media followed by transfer of callus to shoot induction media. Somaclonal variation was detected from regenerated plants using SSR markers. The similarity coefficient range of 0.52-1.00 was obtained with 36.66% bands being polymorphic. Occurrence of somaclonal variation through callus exposure to UV-C radiation was revealed in *in vitro* regenerated plants of potato (*Solanum tuberosum* Ssp. Tuberosum) using SSR markers. Variation observed among plantlets implies that somaclonal variation can be induced by subjecting callus to UV-C radiation (Karácsonyi *et al*., 2011).

SSRs were markers of choice in the study because they are reproducible and have been characterized in several pomegranate studies.

2.8 Genetic integrity assessment of Pomegranate

Guranna *et al*., (2017) assessed genetic fidelity of pomegranate *in vitro* regenerated plants from callus using RAPDs. The analysis revealed 90.66% monomorphic bands and 9.33% polymorphic bands among pomegranate plantlets. Hence the protocol can be used for large production of pomegranate planting materials and genetically

modified plants. Kanwar *et al* (2010) studied genetic variability of pomegranate plantlets obtained from callus using 24 RAPD markers. Cotyledon explants from *in vitro* seedlings were used as explants for callus induction. They found 74% genetic similarity between the plantlets.

CHAPTER THREE

3.0 Materials and Methods

3.1 Study site

The study on "*in vitro* regeneration of pomegranate and genetic integrity assessment of derived materials was carried out at Jomo Kenyatta University of Agriculture and Technology (JKUAT) in the Institute for Biotechnology Research (IBR) tissue culture and molecular biology laboratories.

3.2 PGRs stock solutions preparation

Separate PGRs stock solutions were prepared by weighing 10mg of each PGR, then dissolving in a few drops of 0.1M NaOH (for cytokinins) and 70% ethanol (for auxins). The solution volume was topped up to 200ml using distilled water. Stock solutions were then kept in the refrigerator at 4^0C for use when needed.

3.3 Basal media preparation

MS media was prepared by weighing 4g (for full strength) or 2g (for half strength) of MS powder (Duchefa Biochemie) and mixing with 30g sucrose, then dissolving the mixture in 200ml distilled water. The solution was then poured in 1L volumetric flask and topped up to 1L with distilled water. The media pH was adjusted to a range of 5.70 to5.80 using 0.1M NaOH and HCI followed by addition of 2.8g gelrite with constant stirring to ensure uniform distribution of gelrite. Using a measuring

cylinder, 50ml media were dispensed in 350ml culture vessels. The vessels were then sealed and the media sterilized by autoclaving at 121^0C, 120 kPa for 20 minutes.

3.4 Culturing area and sterilization of culturing instruments

All the sterilization of explants, culturing and sub-culturing was done under aseptic conditions in the laminar flow cabinet. The laminar flow cabinet bench was wiped with 70% ethanol and allowed to run for 15 minutes before using it. The forceps and scalpels were washed first, autoclaved and put in a glass bead sterilizer inside the laminar flow cabinet and sterilized up until the sterilizer reached 250^0C. Then they were removed from the sterilizer, allowed to cool and used. After use, they were wiped and returned to a sterilizer, cooled and used again. The vessels which were used for sterilization and dispensing used disinfectants as well as water after rinsing were first washed, autoclaved and kept in the laminar flow cabinet.

3.5 Explant source

Ripe pomegranate fruits were obtained from an orchard in Uasin Gishu county at Eldoret-Kenya. The seeds were grown in the lab from which nodal cuttings for plant regeneration and leaves for callus induction were obtained. Cotyledons from seeds were also used for callus induction.

3.6 Explant preparation

3.6.1 Surface sterilization of fresh seeds

Ripe pomegranate fruit was washed with running tap water for five minutes followed by surface sterilization with 70% ethanol for one minute. It was then rinsed three times with sterilized distilled water. The fruit was cut open using sterilized scalpels and forceps to remove the seeds. Seeds were pressed between filter papers on the bench to remove the juicy pulp and washed in running tap water for ten minutes. Subsequently they were treated with 100mg/L ridomil solution for 45 minutes and rinsed three times with sterilized distilled water.

Seeds were further treated in 100mg/L streptomycin solution for 20 minutes and rinsed three times with sterilized distilled water. After streptomycin treatment, they were immersed in 70% ethanol for ten seconds followed by rinsing thrice with sterilized distilled water. They were then soaked for ten minutes in 20% NaOCI solution and rinsed again thrice with sterilized distilled water under the lamina flow cabinet. In each treatment and rinsing, seeds (explants) were agitated to ensure complete exposure of explants to disinfectants and removal of any traces of disinfectant from explants.

3.6.2 Surface sterilization of dried seeds

Seeds were removed from a ripe fruit, pressed between filter paper to remove the juicy pulp and washed with tap running water. They were then sun dried for two weeks. Sterilization of sun dried seeds was done following the same protocol used for fresh seeds except that the seeds were soaked in 70% ethanol for one minute.

3.7 Seed germination

Surface sterilized seeds were cut open to remove embryos which were then germinated on hormone free full strength MS media. Two weeks old axenic seedlings were used as a source of leaf explants for callus induction whilst four weeks old seedlings served as a source of nodal explants.

3.8 Growth/culture room conditions

Cultures were incubated in a culture room maintained at 25 ± 2^0C temperatures under photoperiodic cycles of 16 hours light and 8 hours dark. Light was emitted by Philips fluorescent tubes of 36W.

3.9 Micropropagation

3.9.1 Shoot induction

The experiment was conducted in a completely randomized design (CRD) with three replications. Full strength MS shoot induction media was supplemented with three cytokinins; BAP, KN and TDZ. The hormones were evaluated to determine the best cytokinin type and cytokinin level for pomegranate shoot induction. These cytokinins were each evaluated at five different levels; BAP at 2.2, 4.4, 6.6, 8.8, 11.0 µM, KN at 2.3, 4.6, 6.9, 9.2, 11.5 µM and TDZ at 1.0, 2.0, 3.0, 4.0, 5.0 µM. Each treatment (cytokinin level) had three culture vessels containing 50ml shoot induction media and one single nodal explant. Culturing of explants involved removal of leaves from seedling stems and cutting them into single nodal explants of 2.0-2.5cm length with

two axillary buds. The explants were then inoculated on shoot induction media in an upright orientation and cultures incubated under light conditions. The number of shoots per explant and the number leaves developed on shoots per explant were recorded after a period of three weeks. The regenerated shoots were then transferred to root induction media.

3.9.2 Rooting of shoots

Root induction experiment was carried out in a CRD with three replications using half strength MS media. The media was supplemented with three auxins; NAA, IBA and IAA. These auxins were assessed to determine the best type of auxin and auxin level for root induction and growth. Each auxin was tested at five different levels; NAA at 0.6, 3.0, 5.4, 7.8, 10.2 µM, IBA at 2.9, 3.9, 4.9, 5.9, 6.9 µM and IAA at 0.5, 1.7, 2.9, 4.1, 5.3 µM. Microshoots which were developed *in vitro* were excised and randomly transferred on root induction media. Each treatment had three culture vessels containing 50ml half strength MS rooting media and one shoot. Cultures were incubated under light conditions for a period of six weeks. Number of roots that developed and length of the longest root in (mm) were recorded.

3.10 Regeneration via callus

3.10.1 Callus induction

The experiment was performed in a CRD with three replications using full strength MS media supplemented with combination of two PGRs; NAA and BAP. The

potential of PGRs combination to induce callus from cotyledon and leaf explants was tested. One experiment had five levels of NAA while BAP was kept constant; 4.4 µM BAP combined with NAA at 2.8, 5.4, 8.0, 10.6, 13.2 µM. In the other experiment; BAP had five levels while NAA was kept constant; 5.4 µM NAA combined with BAP at 2.2, 4.4, 6.6, 8.8, 11.0 µM. Constant concentrations where determined based on the previous pomegranate studies and the ones which were reported to be optimum by most authors were selected as constants. Each hormonal combination had three culture vessels containing 50ml callus induction media and one explant.

Zygotic embryos with cotyledons were removed from sterilized seeds and cut into half then inoculated on callus induction media. The first pair of true leaves from germinated seedlings was excised, leaf edges cut off and the explant placed flat with the adaxial surface in contact with the media.

Table 3.1: Callus induction experiment layout

Explant source	Explant	Number of cultures		Total number of cultures
		Light	Dark	
Fresh seeds	Cotyledons	30	30	60
Dried seeds		30	30	60
2 weeks old *in vitro* germinated seedlings	Leaves	30	30	60

Note. Under each incubation condition, there were ten treatments (hormonal combinations) each done in triplicate.

One culture vessel contained 50 ml callus induction media and one explant

Cultures were kept for a period of four weeks in the culture room. Data taken after four weeks included: percentage explants forming callus, percentage callus forming roots, percentage callus forming shoots and roots as well as colour of formed callus.

3.10.2 Callus differentiation (Indirect organogenesis)

For callus differentiation, different media were tested with media number one, two, three (similar to the treatments used in micropropagation) and hormone free MS being the paramount ones (Table 3.2). The subsequent media used (Table 3.2) were tested after failure to induce shoots from the first three media.

Table 3.2: Media for callus differentiation

Media	Media supplements
1	BAP at 2.2, 4.4, 6.6, 8.8, 11.0 µM
2	KN at 2.3, 4.6, 6.9, 9.2, 11.5 µM
3	TDZ at 1.0, 2.0, 3.0, 4.0, 5.0 µM
4	Constant 0.54 µM NAA combined with BAP: 2.2, 4.4, 6.6, 8.8, 11.0 µM
5	4.4 µM BAP combined with 1.07 µM NAA
6	6.6 µM BAP combined with 1.61 µM NAA
7	8.8 µM BAP combined with 2.15 µM NAA
8	11.0 µM BAP combined with 2.69 µM NAA
9	Constant 2.2 µM BAP combined with 1.6µM (IAA, IBA, 2,4-D)
10	Constant 4.4 µM BAP combined with 2.0 µM (NAA, IAA, IBA, 2,4-D)
11	Constant 4.4 µM BAP combined with 5.4 µM (NAA, IAA, IBA, 2,4-D)
12	Constant 6.6 µM BAP combined with 1.6 µM (IAA, IBA, 2,4-D)
13	Constant 6.6 µM BAP combined with 2.0 µM (NAA, IAA, IBA, 2,4-D)
14	Constant 8.8 µM BAP combined with 1.6 µM (IAA, IBA, 2,4-D)
15	Constant 11.0 µM BAP combined with 2.0 µM (NAA, IAA, IBA, 2,4-D)
16	4.4 µM BAP combined with 4.6 µM KN and 40 mg/L adenine sulphate (ADS)
17	13.3 µM BAP combined with 13.9 µM KN and 40 mg/L ADS
18	22.2 µM BAP combined with 23.2 µM KN and 40 mg/L ADS
19	4.4 µM BAP combined with 2.3 µM KN and 40 mg/L ADS
20	13.3 µM BAP combined with 2.3 µM KN and 40 mg/L ADS
21	22.2 µM BAP combined with 2.3 µM KN and 40 mg/L ADS
22	Constant 2.69 µM NAA combined with 18, 20, 22, 24, 26 µM BAP
23	Constant 0.54µM NAA combined with constant 1.44 µM GA_3 and various BAP concentrations:2.2, 4.4, 6.6, 8.8, 11.0 µM
24	4.4 µM BAP combined with 4.6 µM KN and 1mg/L silver nitrate ($AgNO_3$)
25	13.3 µM BAP combined with 13.9 µM KN and 1mg/L $AgNO_3$
26	22.2 µM BAP combined with 23.2 µM KN and 1mg/L silver nitrate $AgNO_3$
27	4.4 µM BAP combined with 2.3 µM KN and 1mg/L silver nitrate $AgNO_3$
28	13.3 µM BAP combined with 2.3 µM KN and 1mg/L silver nitrate $AgNO_3$
29	22.2 µM BAP combined with 2.3 µM KN and 1mg/L silver nitrate $AgNO_3$
30	Constant 0.57 µM IAA combined with 2.3, 4.6, 6.9, 9.2, 11.5 µM KN
31	Constant 0.57 µM IAA combined with 10%, 15%, 20% coconut milk and various concentrations of KN:2.3, 4.6, 6.9, 9.2, 11.5 µM
32	Hormone free MS with 10%, 15%, 20% coconut milk
33	Constant 0.57 µM IAA combined with 40, 50 and 60 g/L sucrose and various concentrations of KN: 2.3, 4.6, 6.9, 9.2 and 11.5 µM
34	8 µM BAP combined with 6 µM NAA
35	8 µM BAP combined with 6 µM NAA and 10%, 15% and 20% coconut water
36	9 µM BAP combined with 2.5 µM NAA
37	9 µM BAP combined with 2.5 µM NAA and 10%, 15% and 20% coconut water
38	13 µM BAP combined with 13.5 µM NAA
39	13 µM BAP combined with 13.5 µM NAA and 10%, 15% and 20% coconut water
40	11 µM BAP combined with 5 µM NAA

Media	Media supplements
41	11 µM BAP combined with 5 µM NAA and 10%, 15% and 20% coconut water
43	6 µM BAP combined with 6 µM NAA and 10%, 15% and 20% coconut water
44	Hormone free MS media prepared by using 2, 3, 5 and 6 g/L MS powder
45	Hormone free WPM prepared by using 2, 3, 4, 5 and 6 g/L WPM powder
46	Full strength and half strength MS media with 4.1, 5.3 and 6.5 µM IAA.
47	Full strength MS media supplemented with 22 µM BAP alone
48	Full strength MS media containing 22 µM BAP combined with 2.69 µM NAA
49	Half strength MS supplemented with 22 µM BAP alone
50	Half strength MS media containing 22 µM BAP combined with 2.69 µM NAA

3.11 CTAB buffer preparation and DNA extraction

3.11.1 CTAB buffer preparation

Cetyl trimethylammonium bromide (CTAB) buffer (50ml) was prepared by mixing 5ml of 1M Tris (pH 8.0), 2 ml of 0.5M Ethylenediamine tetraacetic acid (EDTA) (pH 8.0), 14 ml of 5M Sodium chloride (NaCI), 0.5g Polyvinylpyrrolidone 40(PVP 40) and 1g CTAB. The volume was then adjusted to 50 ml using sterilized distilled water.

3.11.2 DNA extraction

Total genomic DNA was extracted from young leaves of the mother plant, cotyledon derived callus, leaf derived callus and from leaves of regenerated shoots on nodal explants. Extraction was done using CTAB method by Doyle and Doyle (1987) with modifications. Four of each (callus and plantlets) were randomly selected. The samples were first ground to fine powder in liquid nitrogen using sterilized mortar and pestle. CTAB buffer (250µl) was then added to each sample then mixed until a fine paste was formed. Subsequently, the paste was transferred into labelled 1.5 ml

eppendorf tubes followed by addition of 500 µl CTAB buffer in each tube and vortexed to homogenize the mixture. The samples were then incubated in a hot water bath at 65^0C for one hour. After incubation, they were centrifuged at 10 000 rpm for ten minutes. The supernatant was transferred into new labelled eppendorf tubes, and the pellet discarded. Chloroform: Iso Amyl (24:1) (500µl) was added to the new tubes with samples and mixed by inverting the tube several times followed by centrifugation at 10 000 rpm for ten minutes. The supernatant was transferred to new eppendorf tubes, 500µl of Chloroform: Iso Amyl (24:1) added to the supernatant and centrifuged at 10 000 rpm for ten minutes. Lastly, the supernatant was transferred into new 1.5ml eppendorf tubes followed by addition of 800µl isopropanol and mixed by inverting the tube several times then stored overnight in a freezer at -20^0C. After 24 hours, samples were centrifuged at 12 000 rpm for ten minutes. The supernatant was discarded and DNA pellet washed with 800µl ice cold 70% ethanol to eliminate adhered residues. Samples were then put in the drier and dried at 50^0C for 30 minutes to dry up the DNA. DNA was dissolved in 100 µl of nuclease free water and stored in a refrigerator at 4^0C.

3.12 Determination of DNA quality and concentration

DNA quality was evaluated using 1.8% (w/v) agarose gel prepared by adding 100ml Tris base, acetic acid, EDTA (TAE) buffer in a conical flask containing 1.8g agarose and mixed together by swirling the flask. The mixture was then heated in a microwave until agarose has completely dissolved. The solution was then cooled by swirling the flask under running tap water until a temperature of about 50^0C was

reached. One drop of EtBr was then added to the solution and swirled to homogenize the mixture. Subsequently, molten agarose was dispensed in a casting tray on a flat surface with a gel comb fixed on the tray. The agarose was then allowed to set at room temperature for ten minutes, the comb removed and the gel put in a gel electrophoresis unit filled with TAE buffer. Each DNA sample (3µl) was first mixed with 1x loading dye followed by loading the mixture in the gel wells. The gel was run for one hour at 100V and visualized in a gel documentation system under UV light. DNA concentration was determined using a Nano drop spectrophotometer (Bibby Scientific Ltd, UK).

3.13 Evaluation of somaclonal variation

Assessment of genetic integrity was accomplished using eight SSR markers shown in Table 1. Markers were selected based on the previous *P. granatum* SSR analysis studies (Hasnaoui *et al*., 2010; Sinjare, 2015). PCR amplification was carried out in a final reactions volume of 10µl comprised of; 2 µl of 5x My Taq reaction buffer containing 5mM dNTPs and 15mM $MgCI_2$,, 0.5µl of 10 µM forward primer, 0.5µl of 10 µM reverse primer, 0.2µl of 1 U My Taq DNA polymerase (Bioline), 0.5µl of diluted DNA template equivalent to 30 ng and 6.3µl of PCR water. Amplification was performed using the following temperature profile; 5min at 95^0C followed by 35 cycles of 30 s at 94^0C, 45s at 45^0C and 45s at 72^0C ending with a final extension of 5 min at 72^0C. PCR products (for allele size identification) were separated on 2% (w/v) agarose gels run for one hour 30 minutes and visualized under UV light in a gel

documentation system. The photographs of resolved DNA bands and 1kb ladder were captured for analysis.

Table 3.3: SSR markers for DNA amplification

Locus name (marker)	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
POM 10	CCTCATTGCTGATGAATCTT	ACTCGAGAAGCTCTGTGAAG
POM 13	CACACCCTTCATCAAAAGAT	GGACTAACAACCAGCCATAG
POM 46	CTTCCTCCTACCGAACTATG	CCCACTTTGACACTTCTACC
POM 55	GAGACAATTGGGATCAGAAA	AGTCGACGAACTGTGAAATC
PGCT033b/ FN677553	TAATAAGCTGCCCCGAAGTC	CGGTGATGTCCCTATTGGAG
PGCT038b/ FN677558	CGTGCCAAATGGGTAAATAA	AGAACTCCACGACCCATAAA
PGCT091b/ FN677611	ATCAGAATTGGAATCGGAAC	ACCGAGGTCATCGAACTAAA
PGCT101b/ FN677621	GAACGCCAAATTCAAGAACC	GACGATTCTTTCCTGCCTTG

Source: Hasnaoui *et al*., (2010); Sinjare (2015)

3.14 Data analysis

Comparison of the effect of three cytokinins and their levels on shoot induction was done by analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS) software version 19. The means ± S.E significance between treatments was determined using the multiple comparison test performed by least significant difference (LSD) at $P \leq 0.05$. Data on the effect of different auxins and their levels on root induction were subjected to ANOVA using SPSS version 19. LSD multiple

comparison test at P≤0.05 was also computed to determine significance between treatments.

Based on gel electrophoresis results, number of alleles per marker was determined by counting maximum number of bands in the PCR product of each sample. PCR amplification profiles of samples were then compared with that of the mother plant for polymorphism. Amplified DNA fragments were scored as 1 denoting allele presence or 0 denoting allele absence. Using this binary data, Jaccard's similarity coefficient was computed by subjecting the data to SPSS software version 19.

3.15 Determining the best hormone and hormonal level using Grey Relational Analysis (GRA)

Grey relational analysis (GRA) method (Ertugrul *et al*., 2016) was used to make a decision on which cytokinin and cytokinin level is suitable for shoot induction. Regardless of the difference between treatments in number of shoot and number of leaves, grey relational analysis computation aggregates the two variable parameters (number of shoots and number of leaves) into a single value of each treatment. Using this method, the experimental data was initially normalized to a scale varying between 0 and 1.0 followed by grey relational coefficients and grades computation. The grades were then ranked with the highest grade being the best and given the lowest rank number. The same method was used to determine the best auxin and auxin level on root induction and growth.

CHAPTER FOUR

4.0 Results

4.1 Micropropagation

4.1.1 Effect of cytokinins on shoot induction

Shoots were induced from nodal explants cultured on MS media supplemented with cytokinins after three weeks of culture. No shoot induction was observed on hormone free MS cultures. The maximum mean number of shoots formed per explant was two, achieved on MS supplemented with KN in all treatments (Figure 4.1). The least mean number of shoots formed per explant was 0.67±0.33 observed at 3.0 µM TDZ. Total number of leaves formed on shoots per explant increased with increasing concentrations of both BAP and KN. Further increase in concentration above 6.9µM KN and 8.8µM BAP led to a decrease in number of leaves (Figure 4.2). There was no significant difference in number of leaves per explant among TDZ treatments (Figure 4.2) at $P \leq 0.05$. The highest mean number of leaves on shoots per explant was 12.00±1.15 obtained on the media supplemented with 6.9µM KN. The lowest mean number of leaves on shoots per explant was on media with 2.0 and 3.0µM TDZ (Figure 4.2).

TDZ was inferior in terms of number of shoots and leaves formed compared to BAP and KN since it had the highest rank number (Table 4.1). Shoot induction appeared to be favoured on MS media supplemented with 6.9µM KN followed by 9.2µM KN as these treatments showed the highest number of shoots and leaves. This is depicted in Figure 4.1, Figure 4.2 and by the lowest rank in Table 4.2. Shoots which were

produced on media supplemented with 6.9µM KN were more dark green than those which were produced on media with 8.8µM BAP (Plate 4.1).

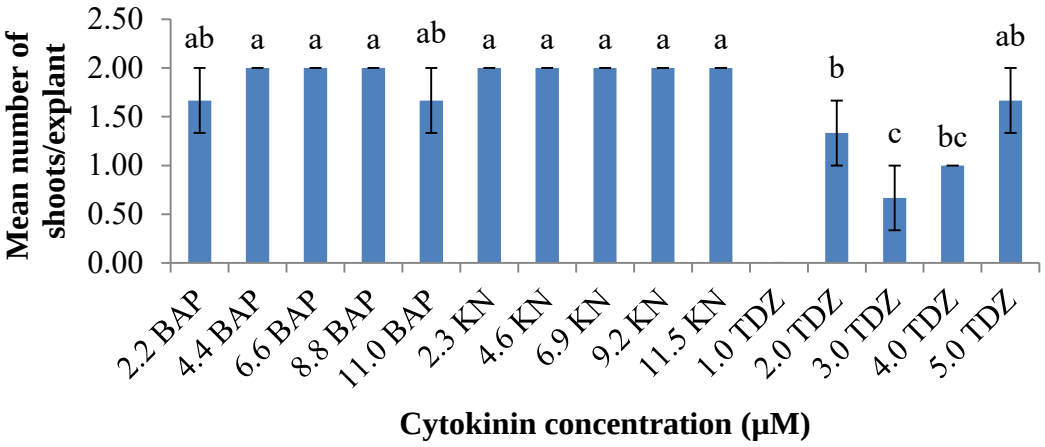


Figure 4.1: The effect of cytokinins on number of induced shoots per explant

Note. Bars with different letters are significantly different at P ≤ 0.05 (LSD multiple comparisons test)

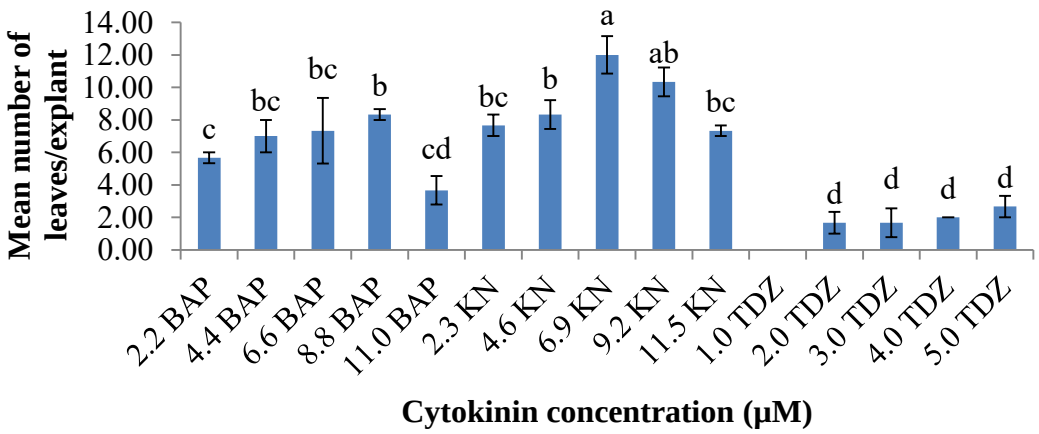


Figure 4.2: The effect of cytokinins on number of leaves on shoots per explant

Note. Bars with different letters are significantly different at P ≤ 0.05 (LSD multiple comparisons test)

Table 4.1: Correlation between cytokinin, number of shoots and number of leaves

Cytokinin	Normalized scores		GRA coefficient		R_i	Rank
	Shoots	Leaves	Shoots	Leaves		
BAP	0.88	0.64	0.80	0.58	0.69	2
KN	1.00	1.00	1.00	1.00	1.00	1
TDZ	0.00	0.00	0.33	0.33	0.33	3

Note. R_i (Grey relational analysis grade)

Table 4.2: Correlation between KN level, number of shoots and number of leaves

KN concentration (µM)	Normalized scores	GRA coefficient	R_i	Rank
	Leaves	Leaves		
2.3	0.07	0.35	0.35	4
4.6	0.21	0.39	0.39	3
6.9	1.00	1.00	1.00	1
9.2	0.64	0.58	0.58	2
11.5	0.00	0.33	0.33	5

Note. R_i (Grey relational analysis grade)

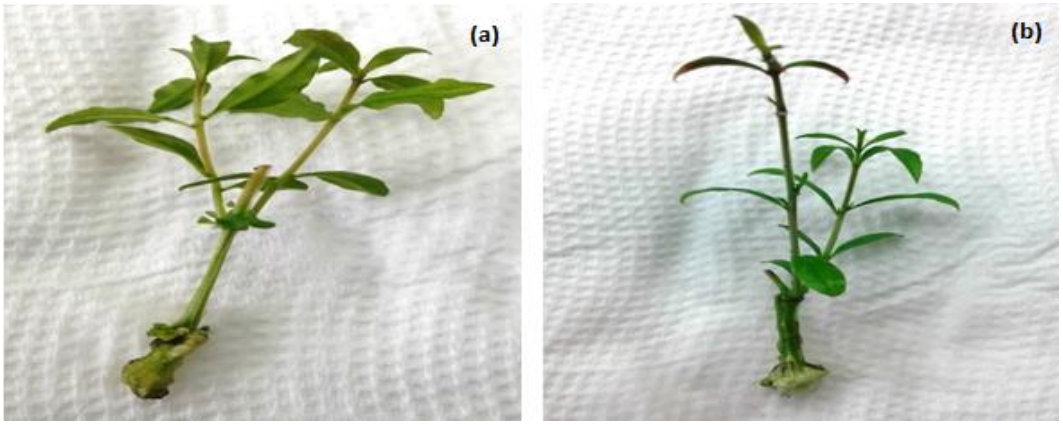


Plate 4.1: Shoots developed from nodal explants

Note: Shoots on nodal explants cultured on MS media supplemented with 8.8µM BAP (a) and 6.9µM KN (b)

4.1.2 Effect of auxins on rooting of shoots

Cultures on half strength MS medium devoid of hormones and all those supplemented with NAA failed to induce roots after six weeks of culture. Supplementation of half strength MS media with IBA and IAA appeared vital for both root induction and root growth. The highest mean number of roots was 7.00±1.00 roots/ shoot found on half strength MS media supplemented with 4.9 µM IBA (Figure 4.3). There was no significant difference in number of roots on media with 5.3µM IAA and 4.9µM IBA at P≤ 0.05. Significant difference in number of roots was observed with increasing IBA level up to 4.9µM, concentrations above this resulted in low number of roots (Figure 4.3). There was no significant difference in number of roots between IAA treatments except at 5.3µM IAA where the highest number of roots was recorded (Figure 4.3).

The highest mean length of the longest root was 15.00±1.00 mm, observed on media supplemented with 5.3 µM IAA (Figure 4.4). Both IBA and IAA treatments showed no significant difference in mean length of the longest root with exception at 5.3µM IAA and 6.9µM IBA (Figure 4.4). Using grey relational analysis method, both auxins appeared to have the same influence on root induction and root growth (Table 4.3). The correlation between auxin levels, root induction and root growth was the same for both auxins. Despite this, the difference was found at hormonal levels of each auxin (Table 4.4). On rankings (Table 4.4), the optimum IAA and IBA levels for rooting of shoots were 5.3 µM and 4.9 µM respectively.

Short and thick roots were observed from shoots cultured on MS media supplemented with 4.9µM IBA (Plate 4.2(a)) whilst long and thin roots were found from microshoots cultured on MS media supplemented with 5.3µM IAA (Plate 4.2(b)). Shoots which were cultured on MS media supplemented with 4.1 and 5.3µM IAA, formed callus at the base where the explant was in contact with the media. Multiple shoots were formed from that callus (Plate 4.3(b)).

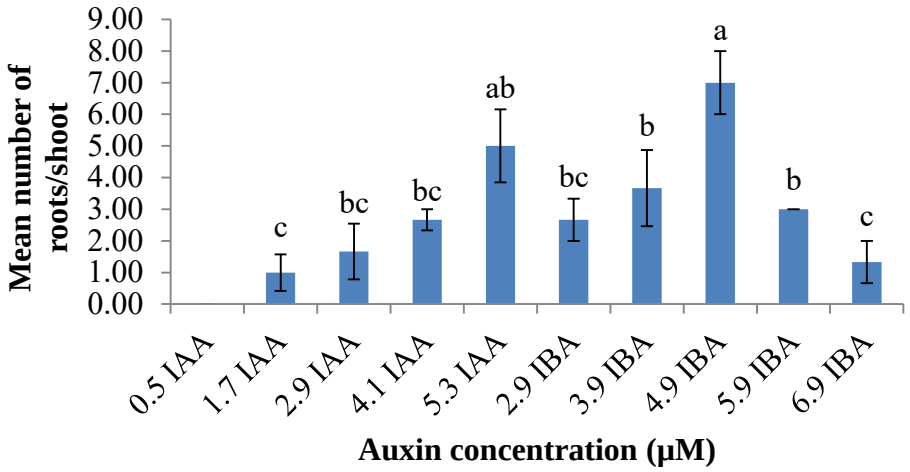


Figure 4.3: The effect of auxins on number of induced roots per shoot.

Note. Bars with different letters are significantly different at $P \leq 0.05$ (LSD multiple comparisons test)

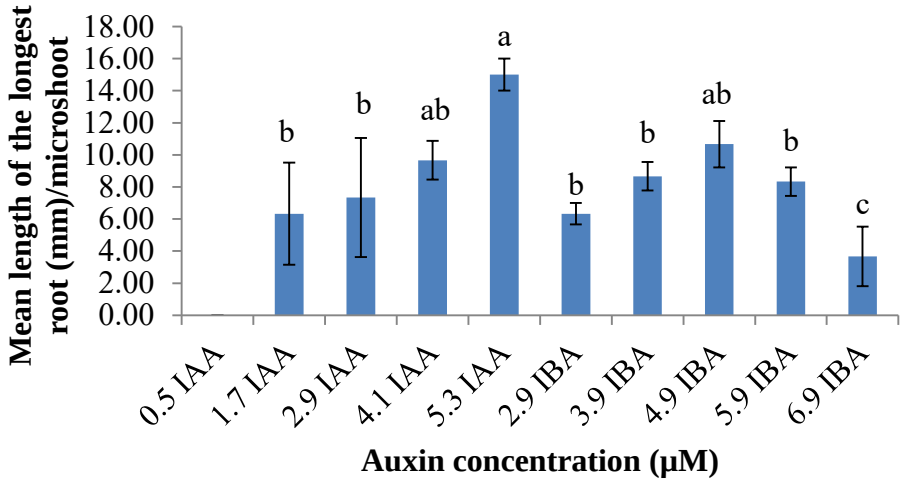


Figure 4.4: The effect of auxins on length of the longest root per shoot.

Note. Bars with different letters are significantly different at P ≤ 0.05 (LSD multiple comparisons test)

Table 4.3: Correlation between auxin, number of roots and length of the longest root

Auxin	Normalized scores		GRA coefficient		R_i	Rank
	RN	RL	RN	RL		
IAA	0	1	0.33	1.00	0.67	1
IBA	1	0	1.00	0.33	0.67	1

Note. RN (roots number); RL (length of the longest root); R_i (Grey relational analysis grade)

Table 4.4: Correlation between auxin level, number of roots and length of the longest root

Auxin	Concentration (µM)	Normalized scores		GRA coefficient		R_i	Rank
		RN	RL	RN	RL		
IAA	0.5	0.00	0.00	0.33	0.33	0.33	5
	1.7	0.20	0.42	0.38	0.46	0.42	4
	2.9	0.33	0.49	0.43	0.49	0.46	3
	4.1	0.53	0.64	0.52	0.58	0.55	2
	5.3	1.00	1.00	1.00	1.00	1.00	1
IBA	2.9	0.24	0.38	0.40	0.45	0.43	4
	3.9	0.41	0.71	0.46	0.64	0.55	2
	4.9	1.00	1.00	1.00	1.00	1.00	1
	5.9	0.29	0.67	0.41	0.60	0.51	3
	6.9	0.00	0.00	0.33	0.33	0.33	5

Note. RN (roots number); RL (length of the longest root); R_i (Grey relational analysis grade)

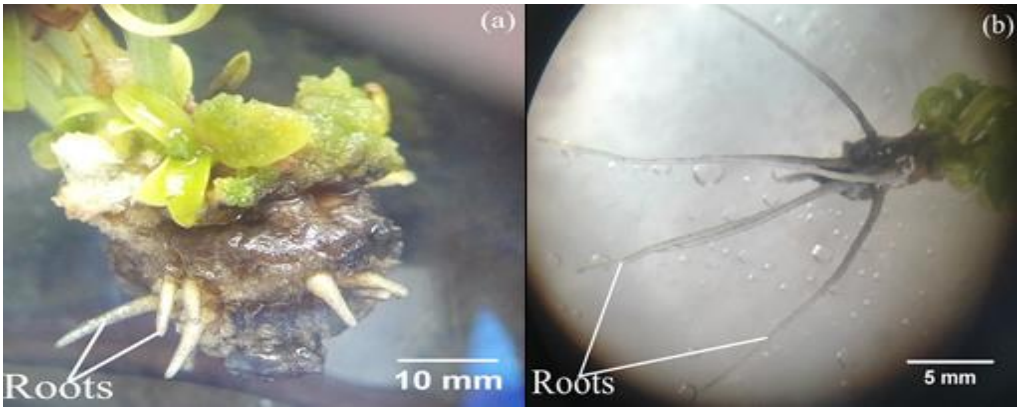


Plate 4.2: Roots developed from shoots

Note: Roots developed from shoots cultured on half strength MS media supplemented with 4.9µM IBA (a); 5.3µM IAA (b)

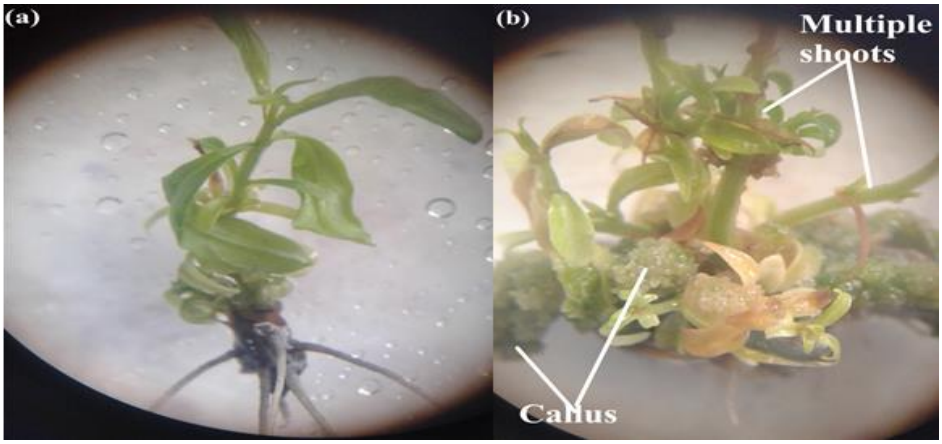


Plate 4.3: Plantlet (a), callus and multiple shoots developing at the base of a shoot(b)

Note: Plantlet growing on half strength MS media supplemented with 5.3 µM IAA (a); callus development at the base of a shoot with multiple shoots (b)

4.2 Regeneration via callus

4.2.1 Effect of hormonal combination on callus induction

None of the explants cultured on MS media devoid of hormones (control) formed callus as well as those which were incubated under dark conditions. All the cultures which contained cotyledon explants from fresh fruit and leaf explants, formed callus when incubated under light conditions. Both explants formed callus in all the treatments (Table 4.5).

4.2.2 Effect of different media supplements on callus differentiation

Few callus from cotyledon explants formed roots and shoots, some formed roots only (Table 4.5 and Plate 4.4(b)) during callus induction but when other pieces of callus

where transferred to the same media, no differentiation was observed. All supplements in MS media tested for callus differentiation did not show organogenesis. Only callus proliferation was observed except in media supplemented with 2,4-D where the callus was turning brown and eventually died.

Table 4.5: The effect of BAP-NAA combination on callus induction from leaf and cotyledon explants of *Punica granatum* after four weeks of culture

TREATMENT/ EXPLANT		COTYLEDON				LEAF			
BAP (µM)	NAA (µM)	% EFC	% CFR	% CFSR	CC	% EFC	% CFR	% CFSR	CC
0	0	0	0	0		0	0	0	
2.2	5.4	100	66	0	GC	100	0	0	G
4.4	5.4	100	0	33	GC	100	0	0	G
6.6	5.4	100	0	0	GC	100	0	0	G
8.8	5.4	100	0	0	GC	100	0	0	G
11.0	5.4	100	0	0	GC	100	0	0	G
4.4	2.8	100	0	0	GC	100	0	0	G
4.4	5.4	100	66	0	GC	100	0	0	G
4.4	8.0	100	33	0	GC	100	0	0	G
4.4	10.6	100	0	0	GC	100	0	0	G
4.4	13.2	100	0	0	GC	66	0	0	G

Note. EFC= explant forming callus; CFR = callus forming roots; CFSR= callus forming shoot and roots; CC= Callus colour

Callus colour: GC (Green and Cream), G (Green)

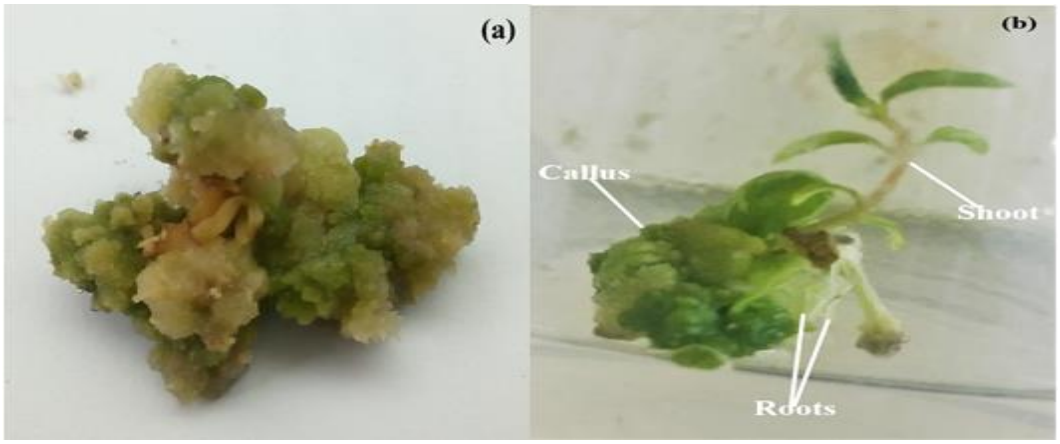


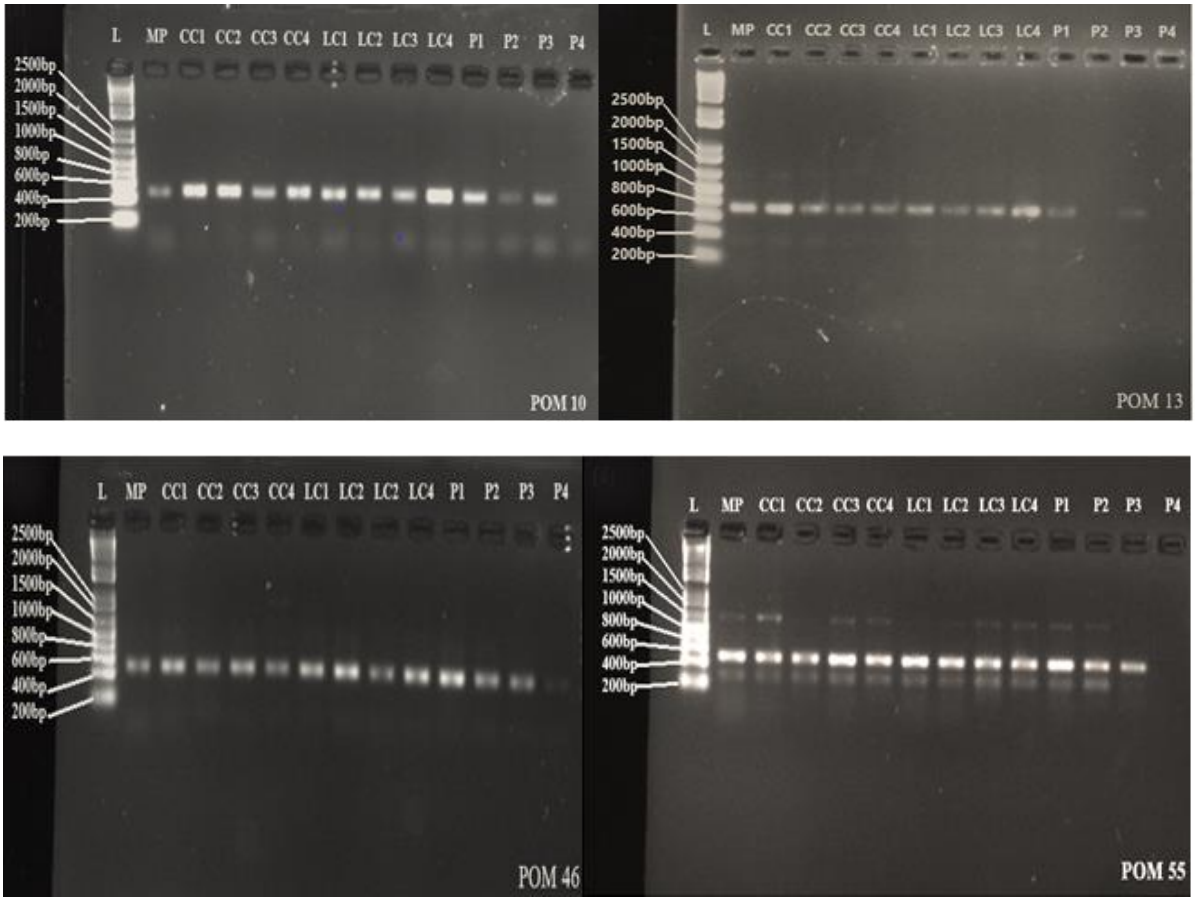
Plate 4.4. Callus generated from cotyledon explant (a); shoot and roots formed from callus (b)

4.3 Genetic integrity of derived callus and plantlets

Eight SSR markers generated a total of 13 alleles with an average of 1.63 alleles per marker (Table 4.6). The allele sizes ranged between 200 and 2000bp. Marker POM 10, POM13, POM 46, PGCT033b/FN677553 and GCT038b/FN677558 gave one allele whilst marker PGCT101b/ FN677621 gave two alleles. POM 55 and PGCT091b/FN677611 gave 3 alleles each (Figure 4.5 and Table 4.6). Out of 13 alleles, 8 were polymorphic and 5 were monomorphic (Table 4.6).

Jaccard's similarity coefficient was computed using SPSS version 19 to evaluate genetic similarity between the mother plant, calli and *in vitro* regenerated shoots leaves. The similarity coefficients ranged from 0.46 to 1.00 (Table 4.7). The lowest genetic similarity 0.46 (46%) was found when plantlet 4 (P4) was compared with the mother plant (MP). The highest genetic similarity of 1.00 (100%) was observed when cotyledon callus 1 (CC1), cotyledon callus 3 (CC3), cotyledon callus 4 (CC4),

Leaf callus 2 (LC2), Leaf callus 3 (LC3) and plantlet 1 (P1) were compared to the MP (Table 4.7). Less genetic similarity was obtained after comparison between MP and plantlet 2 (P2), plantlet 3 (P3), P4. P2, P3, P4 had genetic similarity coefficients 0.77, 0.69 and 0.46 to the MP (Table 4.7).



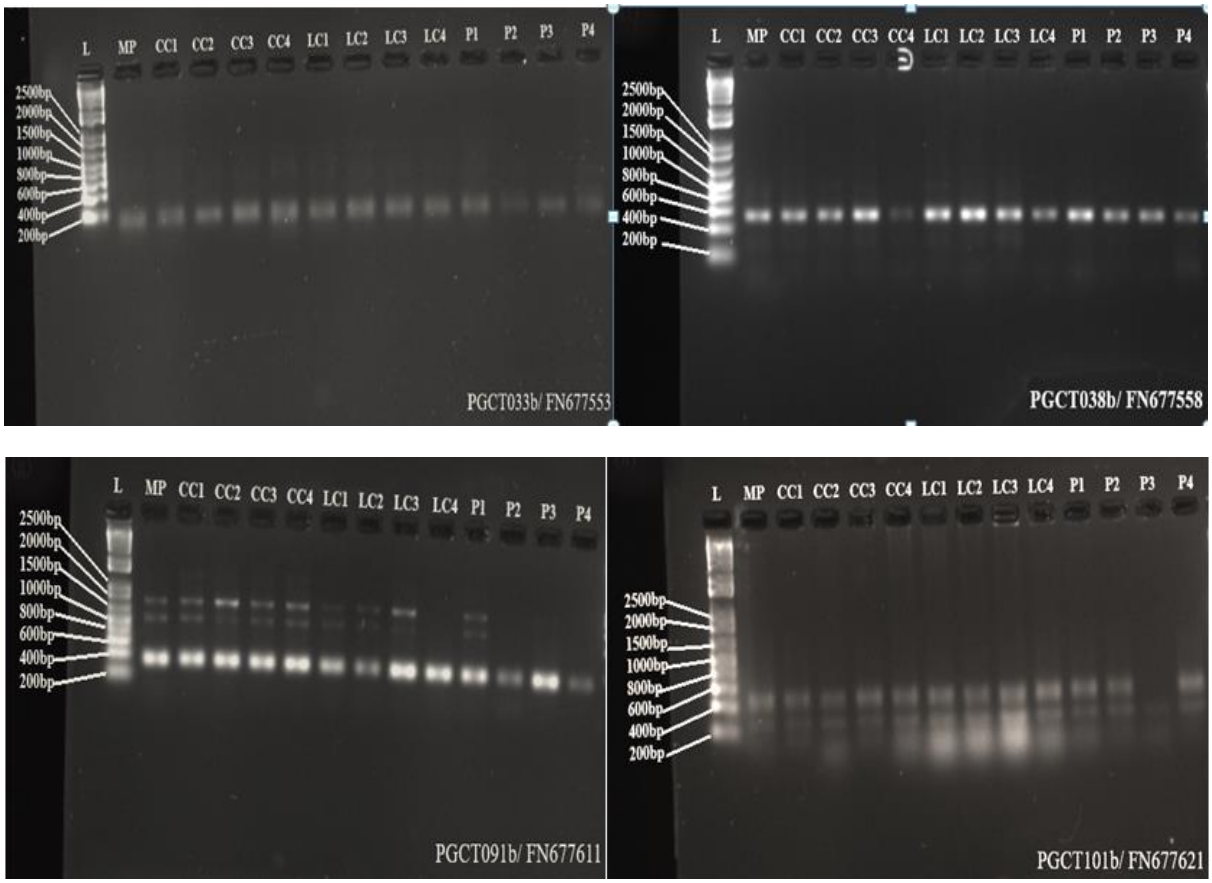


Plate 4.5: PCR products from mother plant, callus (cotyledons and leaves) and from regenerated shoots using SSR markers

Note. MP-mother plant, CC1-cotyledonary callus 1; CC2-cotyledonary callus 2, CC3-cotyledonary callus 3; CC4-cotyledonary callus 4; LC1-leaf callus 1; LC2-leaf callus 2; LC3-leaf callus 3; LC4-leaf callus 4; P1- Plantlet 1 ; P2- Plantlet 2; P3- Plantlet 3; P4- Plantlet 4

Table 4.6: Polymorphism between the mother plant and somaclones as revealed by SSR markers

Marker	Amplified alleles	Monomorphic allele	Polymorphic allele	Allele size (bp)
POM 10	1	0	1	600
POM 13	1	0	1	800
POM 46	1	1	0	600
POM 55	3	0	3	400-1500
PGCT033b/ FN677553	1	1	0	200
PGCT038b/ FN677558	1	1	0	600
PGCT091b/ FN677611	3	1	2	400-2000
PGCT101b/ FN677621	2	1	1	200-400
TOTAL	13	5	8	

Table 4.7: Genetic similarity between *P. granatum* mother plant, derived callus (cotyledons and leaves) and regenerated shoots based on SSR markers

	MP	CC1	CC2	CC3	CC4	LC1	LC2	LC3	LC4	P1	P2	P3	P4
MP	1.00												
CC1	1.00	1.00											
CC2	0.92	0.92	1.00										
CC3	1.00	1.00	0.92	1.00									
CC4	1.00	1.00	0.92	1.00	1.00								
LC1	0.92	0.92	1.00	0.92	0.92	1.00							
LC2	1.00	1.00	0.92	1.00	1.00	0.92	1.00						
LC3	1.00	1.00	0.92	1.00	1.00	0.92	1.00	1.00					
LC4	0.85	0.85	0.77	0.85	0.85	0.77	0.85	0.85	1.00				
P1	1.00	1.00	0.92	1.00	1.00	0.92	1.00	1.00	0.85	1.00			
P2	0.77	0.77	0.69	0.77	0.77	0.69	0.77	0.77	0.91	0.77	1.00		
P3	0.69	0.69	0.75	0.69	0.69	0.75	0.69	0.69	0.82	0.69	0.73	1.00	
P4	0.46	0.46	0.50	0.46	0.46	0.50	0.46	0.46	0.55	0.46	0.60	0.50	1.00

CHAPTER FIVE

5.0 Discussion

Protocols on pomegranate regeneration have been developed with the use of different PGRs limited to cytokinins (BAP and KN) and auxins (NAA and IBA). The use of TDZ for shoot induction and IAA for root induction was not explored in pomegranate studies as compared to other hormones which resulted in limited information available concerning their effects in *in vitro* regeneration of pomegranate. Therefore this study was carried out with the objective to develop an appropriate *in vitro* regeneration protocol for pomegranate with the use of TDZ and IAA among other frequently used hormones in micropropagation of pomegranate. The results of this research work have been discussed in this chapter.

5.1 Micropropagation

5.1.1 Shoot induction

PGRs are not only important in tissue culture of pomegranate but also in other plant species. Studies have been done to identify the best hormone and hormone levels for shoot and root induction as well as for callus formation in pomegranate and other plant species (Patil *et al*., 2011; Bonyanpour and Khosh-khui, 2013; Kaji *et al*., 2013a; Kaji *et al*., 2013b; Parmar *et al*., 2013; Geetha *et al*., 2016; Guranna *et al*., 2017). The reason for TDZ being inferior in shoot induction compared to BAP and KN according to the study results might be that it is not stable in the media. Hence broken down and remain available to plant tissues in low quantities compared to

other hormones resulting in less effect on shoot induction. It was found that the presence of cytokinins (BAP and KN) in the culture media significantly induced shoots and leaves on nodal explants. This shows that the hormones have more effect in stimulating the process of mitosis in the explant, activating the enzymes for cell division and promoting synthesis of RNA hence resulting in significant development of shoots and leaves.

The response varied between treatments of each cytokinin with KN giving an elite hormonal level for optimum shoot induction and number of leaves on shoots. The difference in response between cytokinin type might be due to the difference in physiological activity of each hormone and the degree at which they are metabolized and translocated through the explant. Buah *et al*., (2010) reported that the difference in the aptitude of different PGRs in promoting *in vitro* shoot formation is due to the difference in the hormone mobility through the explant, stability as well as the way they are conjugated and oxidized.

Naik *et al*., (2000) reported contrary results to the study findings where KN was less effective compared to BAP in shoot development from pomegranate cotyledon nodal explants. They reported the best shoot development of 94% on MS media supplemented with 9.0µM BAP but from KN the highest percentage shoot development was 83% obtained on MS media with 9.0 µM KN. The contrasting results could be due to the difference in explant type and cultivar. Other contrasting results from Golozan and Shekafandeh (2010); Kaji *et al*., (2013b); Thombare *et al*., (2017) could have been due to the difference in cultivar, explant age, explant type

and type of culture media. Kaji et al., (2013b) observed variation in shoot induction response between two pomegranate cultivars and reported the cause to be different levels of internal PGRs in each cultivar.

Bonyanpour and Khosh-khui, (2013) also reported that the contrary results obtained in shoot induction response were due to the difference in cultivar and explant type. Different results were also obtained from other plant species as well (Khateeb *et al*., 2012; Baskaran *et al*., 2013). Idowu *et al*., (2009) reported that in general explants obtained from different organs vary in their regeneration capabilities and young tissues have higher micropropagation potential than mature tissues.

5.1.2 Rooting of shoots

Though there was no response in rooting of shoots on half strength MS media supplemented with NAA and hormone free media, other researchers have successfully used NAA in half strength media for root induction in pomegranate (Naik et al., 2000; Patil *el al.,* 2011; Kaji *et al*., 2013a; Kaji *et al*., 2013b; Guranna *et al.,* 2017). Patil *el al*., (2011); Thombare *et al*.,(2017); Desai *et al.,* (2018) obtained roots from pomegranate nodal explants cultured on media devoid hormones which is contrary to the study results. The reason might be due to explant age, type of media used and cultivar.

Different media differs with quantities of micro and macro nutrients in them. For example sulphur is present in WPM at high levels than in MS media, but there are cultivars which can tolerate the high levels and perform better (form roots) in WPM

as compared to MS. The high levels of sulphur can be tolerated and promote gene expression in one cultivar resulting in protein synthesis which will function as enzymes, catalyzing metabolic reactions and also act as transport molecules which could help in translocation of hormones. Hence promoting root development. But with other cultivars, this process can be inhibited in WPM and promoted in MS media due to sulphur levels present in the media. Explants of different ages have different levels of endogenous hormones, making the explant age a critical factor in successful rooting.

It was shown in this study that IBA and IAA are essential for rooting pomegranate shoots. Roots were obtained on media supplemented with either IBA or IAA. There was a significant difference between auxins and auxins levels in terms of number of roots and length of the longest root. The highest root number was obtained on media supplemented with IBA and the longest root was from media supplemented with IAA. The significant difference between treatments could be due to the correlation between auxin level and nodal segment position on the shoot where it came from. This could be the cause of roots being induced on shoots cultured on media with low hormonal level. Determining the effect of cutting position (node) on rooting ability, Nor Aini, *et al*., (2010) observed that *Gonystylus bancanus* nodes obtained at the bottom (from the seedling base), produced many short and thick roots whilst nodes from the top (towards seedling tip) yielded single thin and long roots.

5.2 Regeneration via callus

No callus was formed on explants cultured on MS medium devoid hormones as well as cultures incubated under dark conditions. Similar findings were reported by Harsha (2009) who found that there was no callus formed on leaf explants incubated in the dark for three weeks. Bonyanpour and Khosh-Khui (2013) reported contrasting results where they observed callus induction from pomegranate leaf explants incubated under dark conditions for four weeks. This could be due to the difference in pomegranate cultivar and age of explant source.

From this study, both leaf and cotyledon explants formed callus when cultured on callus induction media supplemented with a combination of NAA and BAP under light conditions. This implies that both hormones are essential for callus formation in both explants. Deepika and Kanwar (2010); Kanwar *et al*., (2010); Parmar *et al*., (2012); Guranna *et al.,* (2017); observed similar results; they induced callus from different pomegranate explants cultured on media supplemented with BAP and NAA. Guranna *et al*., (2017) reported poor quality callus when pomegranate nodal, shoot tip and leaf explants were cultured on MS media supplemented with 2,4-D alone. Most studies suggest that a combination of auxins and cytokinins is necessary for callus induction in pomegranates.

Although Deepika and Kanwar (2010); Kanwar *et al*., (2010); Parmar *et al*., (2012); Guranna *et al*., (2017) under the same Temperature (25 ± 2^0C), photoperiod cycle (16 hours light/ 8 hours dark) and culture media type (MS**)** induced shoots from callus, no callus differentiation was observed in this study after transferring callus to

different shoot induction media. Callus proliferated instead of differentiating into shoots. Similar results were reported by Ramdas (2002) who observed no callus differentiation but callus multiplication both under dark and light conditions. They used the same culture media type and their cultures were kept under the same temperature and photoperiod as to those used in this study and by the mentioned authors.

5.3 Genetic integrity of derived callus and plantlets

Five out of eight SSR markers detected polymorphism in the cultured pomegranate. The results revealed variation between the mother plant, callus derived from leaves and cotyledons and regenerated shoots from nodal cuttings. The genetic similarity range obtained signified a wider genetic variation among the tested samples. This could be attributed to variability among the seeds used in obtaining different explants. The variation could be due to segregation or recombination resulting in the observed variation.

From previous studies on pomegranate and other plant species, different authors used *in vitro* germinated seedlings to carry out their studies and assessed genetic fidelity of regenerated plantlets. Some did not find genetic variation whilst others observed somaclonal variation. However the variation observed in the study could be attributed to genetic variability in the seeds used to produce explants. Kanwar *et al*., (2010) assessed the genetic integrity of *in vitro* raised plants of pomegranate using

RAPDs. They used cotyledon explants from germinated seeds for callus induction and induced shoots from the callus. When genetic integrity of plantlets was assessed, they obtained 74% similarity and concluded that the variation is due to somaclonal variation.

Khateeb *et al*., (2012) detected no genetic variation from *in vitro* propagated plants of *Moringa peregrina* (Forsk.) using ISSR markers. Mallaya and Ravishankar, (2012) also found no genetic variation between micropropagated plants of eggplant (*Solanum melongena* L.) cv. Arka Shirish using RAPD markers. The explants which were used for *in vitro* regeneration were hypocotyls from 15 days old *in vitro* germinated seedlings. Phenotypic and molecular characterization of *Phaseolus vulgaris* plants from non-cryopreserved and cryopreserved seeds revealed no variation between control and cryopreserved samples using SSR markers. This implies that exposure of seeds to liquid nitrogen does not always cause changes in integrity of the genome (Cejas *et al*., 2013).

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Supplementation of MS media with 6.9µM KN was the best for shoot induction from pomegranate nodal cuttings. Half strength MS media containing either 5.3µM IAA or 4.9µM IBA was the best for rooting pomegranate microshoots.

Combination of BAP (2.2-11.0µM) and NAA (2.8-13.2µM) were suitable for callus induction from leaf and cotyledons explants.

There was variability in the *in vitro* derived pomegranate materials which was attributed to segregation and recombination in the seeds that were used.

6.2 Recommendations

Established plant growth regulators and their levels could be used for rapid multiplication of pomegranate planting materials from nodal cuttings.

Protocol for callus induction can be used in modern bio-techniques on improvement of pomegranate.

Studies should also be done on nodal explants for callus induction as they have shown potential to form callus that resulted in multiple shoots.

Further studies recommended establishing conditions for indirect organogenesis.

Studies should be done with appropriate design and more regeneration under tissue culture conditions to assess somaclonal variation in pomegranate derived materials.

REFERENCES

Ali, A.A., El –Denary, M. E., El-Gendy, A. Galal, O. A., Ahmad, M. E and El-Sayed, T. R. 2017. Detection of variations in Tomato using RAPD markers. *Egypt, J.Genet. Cytol*, 46:89-99

Ashton, R., Baer, B. and Silverstein, D. 2006. The incredible pomegranate plant and fruit. Third Millennium Publishing, United States of America.

Baskaran, P., Moyo, M. and Van Staden, J. 2013. *In vitro* plant regeneration, phenolic compound production and pharmacological activities of *Coleonema pulchellum*. *South African Journal of Botany*. 90:74-79

Benita, K. 2014. *Micromorphology and phytochemistry of the foliar secretory structures of Stachys natalensis Hochst. and development of an in vitro propagation protocol* (Masters dissertation, University of KwaZulu-Natal, Durban, South Africa).

Bonyanpour, A. and Khosh-Khui, M. 2013. Callus Induction and Plant Regeneration in *Punica granatum* L. '*Nana'* from Leaf Explants. *Journal of Central European Agriculture* 14(3):928-936

Bordallo, P. N., Silva, D. H., Maria, J., Cruz, C. D. and Fontes, E.P. 2004. Somaclonal variation on *in vitro* callus culture of potato cultivars. *Horticultura Brasileira*, 22(2):300-304

Buah, J. N., Danso, E., Taah, K. J., Abole, E. A., Bediako, E. A., Asiedu, J. and Baidoo, R. 2010. The Effects of Different Concentrations Cytokinins on the *in vitro* Multiplication of Plantain (Musa sp.)

Cejas, I., Méndez, R., Villalobos, A., Palau, F., Aragón, C. Engelmann, F., Carputo, D. Aversano, R., Martínez, M. E. and Carlos Lorenzo, J. C. 2013. Phenotypic and Molecular Characterization of *Phaseolus vulgaris* Plants from Non-Cryopreserved and Cryopreserved Seeds. *American Journal of Plant Sciences*, 4:844-849

Chandra, R., Jadhav, V. T., Sharma, J. 2010. Global Scenario of Pomegranate (*Punica granatum* L.) Culture with Special Reference to India. *Fruit, Vegetable and Cereal Science and Biotechnology* 4(2):7-18

Da Silva, J. A.T., Rana, T. S., Narzary, D., Verma, N., Meshram, D. T. and Ranade, S. A. 2013. Pomegranate biology and biotechnology: A Review. *Scientia Horticulturae* 160:85-107

Decoteau, D. R. 2005. Instructor's manual: Principles of Plant Science Environmental Factors and Technology in Growing Plants. Pearson Education, Inc.New Jersey

Deepika, R. and Kanwar, K. 2010. In vitro regeneration of Punica granatum L. plants from different juvenile explants. *Journal of Fruit and Ornamental Plant Research*, 18(1):5-22

Desai, P. Patil, G., Dholiya, B., Desai, S., Patel, F. and Narayanan, S. 2018. Development of an efficient micropropagation protocol through axillary shoot proliferation for pomegranate variety 'Bhagwa'. *Annals of Agrarian Science*.

Dhinesh, K.V. and Ramasamy, D. 2016. Pomegranate Processing and Value Addition: Review. *J Food Process* Technol, 7: 565.

Doyle, J.J. and Doyle, J.L. 1987. A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue. *Phytochemical Bulletin*, 19:11-15

Ertugrul, I., Oztas, T., Ozcil, A. and Oztas,G. Z. 2016. Grey Relational Analysis Approach In Academic Performance Comparison Of University: A Case Study Of Turkish Universities. *European Scientific Journal*, 128-139

Esmaillzadeh, A., Tahbaz, F., Gaieni, L., Alavi-Maid, H. and Azadbakht, L. 2006. Cholesterol-lowering effect of concentrated pomegranate juice consumption in type II diabetic patients with hyperlipidemia. *Int J Vitam Nutr Res*.,76(3):147-51.

Forbon Srl. 2013. Italy: Granatum Plus-a line of products derived from the best pomegranates. Retrieved November 10, 2017, from http://www.freshplaza.com/article/114278/

Geetha, G., Harathi, K. and Naidu, C. V. 2016. Influence of silver nitrate on in vitro callus induction and indirect shoot organogenesis of *Solanum nigrum* (l.) -an important antiulcer medicinal plant. *International Journal of Pharmacy and Biological Sciences,*6(2):89-99

George, E.F., Hall, M.A. and De Klerk, G.J. 2008. Plant Propagation by Tissue Culture. 3rdedition Volume 1. Springer.The Nertherlands.

Glozer, K. and Ferguson, L. 2011. Pomegranate production in Afghanistan. Mark Bell: Editing and manual layout.

Golozan, A. B. and Shekafandeh, A. 2010. Effects of Plant Growth Regulators on Pomegranate (*Punica granatum* L. cv. Rabbab) Shoot Proliferation and Rooting. *Advances in Horticultural Science*, 24(3):207-211

Govinden-Soulange, J., Somanah, D., Ranghoo-Sanmukhiya, M., Boodia, N. and Rajkomar, B. 2010. Detection of somaclonal variation in micropropagated *Hibiscus sabdariffa* L. using RAPD markers. *University of Mauritius Research Journal,* 16:435-447

Grabkowska, R. and Wysokinska, H. 2009. Micropropagation of *Harpagophytum procumbens* (Burch.) DC. ex Meisn.;the effect of cytokinins on shoot multiplication. *Herba polonica*. 55(3):244-249

Guranna, P. Hosamani, I., Sathyanarayana, R., Hegde, R. and Hipparagi, K. 2017. Micropropagation in Pomegranate (*Punica granatum* L.) cv. 'Bhagwa' through Indirect Organogenesis and Assessment of Genetic Fidelity by RAPD Marker. *Biotechnology Journal International*. 20(3):1-8

Harsha, A. S. 2009. *Induction of somatic embryogenic activity in pomegranate (Punica granatum L.) cultures of mature origin*. (Masters thesis, Department of Biotechnology University of Agricultural Sciences GKVK campus, Bangalore).

Hasnaoui, N., Buonamici, A., Sebastiani, F., Mars, M., Trifi, M. and Vendramin, G. G. 2010. Development and characterization of SSR markers for pomegranate (*Punica granatum* L.) using an enriched library. *Conservation Genetics Resources*. 2:283-285

Holland, D. Hatib, K., Bar-Ya'akov, I. 2009. Pomegranate: botany, horticulture, breeding. In: Janick, J. (Ed) *Horticultural Reviews*, 35:127-191

Idowu, P. E., Ibitoye, D. O. and Ademoyegun, O. T. 2009. Tissue culture as a plant production technique for horticultural crops. *African Journal of Biotechnology*, 8(16):3782-3788.

Ikeuchi, M., Sugimoto, K. and Iwase, A. 2013. Plant Callus: Mechanisms of Induction and Respression. RIKEN Center for Sustainable Resource Science, Yokohama 230-0045, Japan.

Jalikop, S. H. 2010. Pomegranate Breeding. *Fruit, Vegetable and Cereal Science and Biotechnology* 4(2):26-34

Jiang, G. L. 2013. Molecular Markers and Marker-Assisted Breeding in Plants, Plant Breeding from Laboratories to Fields, Prof. Sven Bode Andersen (Ed.), InTech.

Kahramanoğlu, I. and Usanmaz, S. 2016. Pomegranate Production and Marketing. CRC Press, London, New York.

Kaji , B. V., Ershadi, A. and Tohidfar, M. 2013a. *In vitro* propagation of pomegranate (*Punica granatum* L. Cv. 'Males Yazdi'. *Albanian j. agric. Sci*. 12(1):1-5

Kaji, B. V., Ershadi, A. and Tohidfar, M. 2013b. *In vitro* propagation of two Iranian commercial pomegranates (*Punica granatum* L.) cvs. 'Malas Saveh' and 'Yusef Khani'. *Physiol Mol Biol Plants*, 19(4):597–603

Kaliamoorthy, S., Naidoo, G. and Achar, P. 2008. Micropropagation of *Harpagophytum procumbens*. *Biologia Plantarum* 52(2):191-194

Kane, M. E., Philman, N. J. and Jenks, M.A. 1994. A laboratory exercise to demonstrate direct and indirect shoot organogenesis using internodes of *Myriophyllum aquaticum*. *HortTech*, 4:317-320

Kanwar, K., Thakur, K., Verma, V. and Sharma, R. K. 2010. Genetic Variability of *in Vitro* Raised Plants of *Punica granatum* L. by RAPDs. *Fruit, Vegetable and Cereal Science and Biotechnology*, 4 (2):144-147

Karácsonyi, D. E., Chiru, N. and Nistor, A. 2011. Microsatellite analysis of somaclonal variation in potato (*Solanum tuberosum Ssp. Tuberosum*) plantlets regenerated from callus. *Romanian Biotechnological Letters*, 16(1):81-83

Khateeb, W. A., Bahar, E., Lahham, J., Schroeder, D. and Hussein, H. 2012. Regeneration and assessment of genetic fidelity of the endangered tree *Moringa peregrina* (Forsk.) Fiori using Inter Simple Sequence Repeat (ISSR). *Physiol Mol Biol Plants*, 19(1):157–164

Koroch, A., Juliani, H. R., Kapteyn, J. and Simon, J. E. 2002. *In vitro* regeneration of *Echinacea purpurea* from leaf explants. *Plant Cell, Tissue and Organ Culture* 69:79–83

Krishna, H., Alizadeh, M., Singh, D., Singh, U., Chauhan, N., Eftekhari, M. and Sadh, R. K. 2016. Somaclonal variations and their applications in horticultural crops improvement, 3*Biotech* 6: 54-72

Mallaya, N. P. and Ravishanka, G. A. 2012. *In vitro* propagation and genetic fidelity study of plant regenerated from inverted hypocotyl explants of eggplant (*Solanum melongena* L.) cv. Arka Shirish. 3*Biotech* 3:45–52

Materechera, S. A. and Seeiso, T. M. 2013. Seed Treatment to Improve Water Imbibition and Germination of Pomegranate (*Punica granatum*). *Acta Horticulturae*, 979:713-722

Miyao, A., Nakagome, M., Ohnuma, T., Yamagata, H., Kanamori, H., Katayose, Y., Takahashi, A., Matsumoto, T. and Hirochika. H. 2012. Molecular spectrum of somaclonal variation in regenerated rice revealed by whole-genome sequencing. *Plant Cell Physiol*, 53(1):256-64

Nadeem, M. A., Nawaz, M. A., Shahid, M. Q., Doğan, Y., Comertpay, G., Yıldız, M., Hatipoğlu, R., Ahmad, F., Alsaleh, A., Labhane, N., Özkan, H., Chung, G. and Baloch, F. S. 2017. DNA molecular markers in plant breeding: current status and recent advancements in genomic selection and genome editing. *Biotechnology & Biotechnological Equipment*, 32 (2):261–285

Naik, S. K. Pattnaik, S. and Chand, P. K. 2000. High frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate (*Punica granatum* L.). *Scientia Horticulturae*. 85:261-270.

Nor Aini, A. S., Guanih, V. S. and Ismail, P. 2010. Effect of cutting positions and growth regulators on rooting ability of *Gonystylus bancanus*. *African Journal of Plant Science*, 4(8):290-295

Oagile, O. 2005. *African yam bean: morphology, clonal propagation and nitrogen fixation*. (Doctoral dissertation, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire. LE12 5RD, UK).

Obembe, O. O. 2000. Studies on Kola Tissue Culture II: Effect of plant growth regulators on callus induction. *Nigerian Journal of Science*, 34(4):277-281

Oxfarm Organic. 2017. Why pomegranate fruit demand is on the rise in Kenya-Oxfarm Organic Ltd. Retrieved January 25, 2018, from http://www.oxfarmorganic.com/our-products/pomegranate-fruits/why-pomegranate-is-the-most-expensive-fruit-in-kenya/

Panichayupakaranant, P., Tewtrakul, S. and Yuenyongsawad, S. 2010. Antibacterial, anti-inflammatory and anti-allergic activities of standardised pomegranate rind extract. *Food Chemistry*,123(2):400-403

Parmar, N., Kanwar, K. and Thakur, A. K. 2012. *In Vitro* Organogenesis from Cotyledon Derived Callus Cultures of *Punica granatum* L. cv. Kandhari Kabuli. *Natl. Acad. Sci. Lett*. 35(3):215–220.

Parmar, N., Kanwar, K. and Thakur, A. K. 2013. Direct Organogenesis in *Punica granatum* L. cv. Kandhari Kabuli from Hypocotyl Explants. *Proc. Natl. Acad. Sci. India, Sect. B Biol*. Sci. 83(4):569-574

Patil, V. M., Dhande, G. A., Thigale, D. M. and Rajput, J. C. 2011. Micropropagation of pomegranate (*Punica granatum* L.) 'Bhagava' cultivar from nodal explant. *African Journal of Biotechnology*, 10(79):18130-18136

Pierik, R. L. M. 1987. *In vitro* Culture of Higher Plants, Martinus Nijhoff, Dordrecht.

Polanco, C. and Ruiz, M. L.2002. AFLP analysis of somaclonal variation in *Arabidopsis thaliana* regenerated plants. *Plant Science,*162(5):817-824

Purohit, S. S. 2011. Practical Plant Biotechnology. Student Edition Publishers, Jodhpur.

Raj Bhansali, R. (1990). Somatic Embryogenesis and Regeneration of Plantlets in Pomegranate. *Annals of Botany* 66:249-253

Ramdas, C. R. 2002. S*tudies on micropropagation and callus induction in pomegranate (Punica granatum l.) cv. mridula* (Masters thesis, Department of horticulture, Post graduate institute, Mahatma Phule Krishi Vidyapeeth, India).

Saad, A. I.M. and Elshahed, A. M. 2012. Plant Tissue Culture Media, Recent Advances in Plant in vitro Culture, Dr. Annarita Leva (Ed.), InTech.

Salehian, H., Babaeian, N., Bagheri, N., Sedaghati, B., Banaeiasl, F. and Kabirnataj, S. 2014. Study of somaclonal variation in two rice cultivars regenerated from embryo culture, using SSR markers. *International Journal of Agronomy and Agricultural Research* (IJAAR), 5(1):23-28

Schwarz, O. J. and Beaty R. M. 2000. Organogenesis. In: Trigiano R.N and Gray D. J. (eds) Plant Tissue Culture Concepts and Laboratory Exercises. 2nd edition. CRC Press, Boca Raton, London,125-138

Sharma, H. 2017. Role of growth regulators in micropropagation of woody plants-a review. *Int. J. Adv. Res*. 5(2):2378-2385.

Sharma, G.K, Jagetiya, S. and Dashora, R. 2015. General Techniques of Plant Tissue Culture. Lulu Press Inc.Raleigh, North Carolina, United States.

Sharon, M., Sinha, S. and Sharan, M. 2011. Somatic embryogenesis in different root segments of *Punica granatum* L. *Annals of Biological Research*, 2 (5) :104-112

Shen, X. 2007. *Indirect shoot organogenesis and selection of somaclonal variation in Dieffenbachia.* (Doctoral dissertation, Graduate school of the University of Florida).

Shooshtari, L., Omidi, M., Majidi, E., Naghavi, M., Ghorbanpour, M. and Etminan, A. 2013. Assessment of somaclonal variation of regenerated *Ducrosia anethifolia* plants using AFLP markers. *Journal of Horticulture, Forestry and Biotechnology*. 17(4):99-106

Shushu, D. D. 2001. *In vitro* regeneration of the Kalahari devil's claw, *Harpagophytum procumbens*. *South Africa. Journal. Botany*.67:378-380

Simões, C., Albarello, N., Callado, C. H., Castro, T. C. and Mansur, E. 2010. Somatic Embryogenesis and Plant Regeneration from Callus Cultures of *Cleome rosea* Vahl. Braz. *Arch. Biol. Technol.*, 53(3):679-686

Singh, P., Patel, R. M. and Kadam, S. 2013. *In vitro* mass multiplication of pomegranate from cotyledonary nodal explants cv. *Ganesh*. *African Journal of Biotechnology*. 12(20):2863-2868

Sinjare, D. Y. 2015. Application of Microsatellite SSR Markers in a Number of Pomegranate (*Punica granatum* L.) Cultivars in Kurdistan Region/ Duhok Province. *International Journal of Chemical and Biomolecular Science*, 1(3);117-122

Smith, R. E. 2014. Pomegranate botany, postharvest treatment, biochemical composition and health effects. Nova publishers, New york.

Smulders, M. and de Klerk, G. 2011. Epigenetics in plant tissue culture. *Plant Growth Regul.,* 63:137–146

Sussex, I. M. 2008. The Scientific Roots of Modern Plant Biotechnology. *The plant cell*. 20:1189-1198

Tasheva, K. and Kosturkova, G. 2013. Role of biotechnology for protection of endangered medicinal plants. Chapter 11: Environmental Biotechnology-New approaches and prospective application. 235-285

Thombare, D., Tiwari, S., Sapre, S. and Dattgonde, N. 2017. An efficient regeneration protocol for pomegranate (*Punica granatum*) cv bhagwa from nodal and meristem explants. *The bioscan* 11(0):1-4

Venkatesha, H. and Yogish, S. N. 2016. High-yielding varieties of pomegranate. *International Journal of Applied Research*, 2(2): 73-75

Yang, X. and Zhang, X. 2010. Regulation of Somatic Embryogenesis in Higher Plants. *Critical Reviews in Plant Science*, 29:36–57

Yildiz, M. 2012. The pre-requisite of the success in plant tissue culture: high frequency shoot regeneration. Chapter 4 in Recent advances in plant in vitro culture, 63-90