ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL SCIENCES

SCHOOL OF GRAGUATE STUDIES

BIOTECHNOLOGY PROGRAM

Micropropagation of two apple (*Malus domestica* Borkh) varieties from shoot tip explants

A Thesis Submitted to the school of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

By

Demsachew Guadie

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Addis Ababa, Ethiopia

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LIST OF ABBREVIATIONS

AC	Activated Charcoal
ANOVA	Analysis Of Variance
BAP	6-Benzyle Amino Purine
CRD	Completely Randomized Design
FAO	Food and Agricultural Organization of the United Nations
GA_3	Gibberlic acid
HARC	Holetta Agricultural Research Center
IAA	Indol Acetic Acid
IBA	Indol-3-Butyric Acid
m.a.s.l	meters above sea level
MS	Murashige and Skoog
PGRs	Plant Growth Regulators
USDA	United States Department of Agriculture
USAID	United States Agency for International Development

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ABSTRACT

Malus x *domestica* Borkh is a perennial pomaceous fruit tree belonging to the family *Rosaceae* and sub-family *Maloideae*. Although the plant is generally found in the northern temperate zones of North America, Europe, Asia Minor and Asia, species within the genus have wide geographical distribution. Apple fruit has been considered to have high health benefits. Most of the species within the genus intercross. Since self-incompatibility is common, apples produced from seed are mostly interspecific hybrids. Therefore, it is difficult to produce true-to-type cultivars from seeds. The objectives of this study, therefore, were to develop micropropagation protocol for two apple (*Malus domestica* Borkh) varieties, MM106 and Anna, which are highly adapted for environmental conditions of Ethiopian highlands, using shoot tip explants and assessing the *in vitro* multiplication potential of this plant up to the fifth sub-culturing stage on MS medium. From the seven combinations of BAP and IBA in full MS medium was used for culture initiation, the servival percentages of the cultured shoots with healthy morphology were 96.7 for MM106 in 1.0 mg/l BAP and 0.1 mg/l IBA medium composition and 93.3 for Anna in 1.5 mg/l BAP and 0.1 mg/l IBA combinations. Among the different treatment combinations used for multiplication, 6.33 ± 0.50 and 4.93 ± 0.42 mean number of shoots were obtained in 1.5 and 2.0 mg/l BAP combined with 0.5 mg/l GA3 and 0.01 mg/l IBA for MM106 and Anna varieties respectively. Sub-culturing stage has also significantly affected multiplication potential of the two varieties. The maximum shoot number was obtained on the 5^{th} (8.70 ± 2.77 mean number of shoots) and 2^{nd} (7.63 ± 0.59 mean number of shoots) sub-culturing stages for MM106 and Anna varieties on the above respective multiplication media compositions. The two auxins, IBA or IAA, with or without activated charcoal (AC) showed significant difference in the number and length of roots of the two varieties. The maximum number of roots was 5.7 ± 0.69 for MM106 and 6.25 ± 0.50 for Anna in media supplemented with 1.0 and 2.0 mg/l IAA combined with AC, respectively. Relatively longer length roots (in cm) were obtained in 1.5 mg/l IBA without AC, 4.55 ± 1.40, for MM106 and 1.0 mg/l IBA with AC, 6.00 ± 0.95, for the Anna varieties. Finally, the survival rate of the acclimatized plantlets in the glasshouse was 91.4% and 65.7% for MM106 and Anna varieties, respectively.

Key words or phrases: Ethiopia, Holetta Agricultural Research Center, Plant growth regulators, plantlet.

1. INTRODUCTION

Malus domestica Borkh (Apple) is a woody plant belonging to the family, *Rosaceae* and sub-family *Maloideae* or formerly *Pomoideae* (Brown, 1992). The fleshy outer cover of apple fruit develops from a compound pistil consisting of two or more carpels and an inferior ovary. The outer part of the pericarp also becomes fleshy, while the endocarp becomes more or less cartilaginous, forming the core of the fruit, containing many seeds. Since the major part of the fruit is not developed from the ovary, apple fruit is an accessory fruit. The center of the apple fruit contains five carpels arranged in a five point star, each carpel containing one to three seeds. It is typically temperate tree fruit and most of the cultivars are diploid with chromosome number of 2n = 34 (Sabir and Shah, 2004).

The exact origin of the plant is not clearly known. However, it is the most ubiquitous of temperate fruits and has been cultivated in Europe and Asia. There are suggestions that it originates from central Asia, where its wild ancestor is still found today (Janick *et al*., 1996). There are 7,500 known cultivars of apple (Dobrzanski *et al*., 2006). Apples can grow from seeds. However, like most of perennial fruits, apples are ordinarily propagated asexually by grafting. This is because apple seedlings are examples of "extreme heterozygous", in this case rather than inheriting DNA from their parents to create a new apple with characteristics, they are instead different from their parents, sometimes radically (Harries *et al*., 2002).

Apples can also form bud sports (mutations on a single branch). Some bud sports turn out to be improved strains of the parent cultivar and others differ sufficiently from the parent tree to be considered new cultivars. Apples are mostly self–incompatible; they must cross-pollinate to develop fruit (Sheffield *et al.*, 2005).

Apple is a very nutritious, aromatic and delicious fruit and very rich in vitamins A, B and C. It contains about 11% sugar besides essential minerals in appreciable amounts. It has color appeal, stimulates appetite and is most refreshing (Anonymous, 2001). Apple fruits are alkaline because they contain pectin with alkaline pH. They are also important to prevent constipation for ease defecation of solid waste, because pectin from the apple takes in excess water in the intestines, making a soft bulk that creates a mild, non - irritating stimulant. This stimulant helps the peristaltic movement and assists in natural bowel elimination. Apple can be used in many

different forms; cooked, made into preserves, jellies, canned, candied, as fresh apple juice and made into cider or vinegar. Moreover, the peal is used for making pectin (Anonymous, 2001). The proverb “an apple a day keeps the doctor away” strengthens the idea that apple fruit is an excellent source of very important food nutrients.

Most temperate tree fruit species are self-sterile and extremely heterozygous and most scion cultivars don‟t come true-to-type if propagated using their own, seldom fertile, seeds. Consequently, when a tree raised from seed showed particularly desirable characteristics, the only way of multiplying it in large number was by asexual (vegetative or clonal) propagation (Webster, 1995).

Clonal propagation is the most important method of commercial production of the majority, if not all, horticultural crops throughout the world (Davies *et al*., 1994). The macropropagation of plants is often more difficult, time taking and less successful. Clonal propagation through tissue culture called micropropagation can be achieved in a short time and space. Thus, it is possible to produce plants in large numbers starting from single individual (Razdan, 1993). Besides, micropropagation of apple to produce self-rooted plants will open up new areas of research and allow changes in conventional fruit tree propagation methods (Bahmani *et al.*, 2009).

Most of Ethiopian‟s rural population lives in a state of chronic food insecurity. Recurrent drought, degradation of natural resources and rapid population growth are among the main causes of declining per capita food production. Average daily energy intake is estimated at 16 to 20 % below the accepted minimum, while diseases due to deficiencies in vitamin A, iron and iodine are widespread. Several times over the past 30 years, Ethiopia's precarious food security has tipped over into full-blown famine (FAO, 2006).

Fruit and vegetables are excellent sources of vitamins. Apples are not particularly abundant in any particular vitamin; however, the fruit contains significant amount of almost all kinds of essential vitamins. Necessarily, an apple is nature‟s perfect multi-vitamin source. Apple fruits are helpful for lowing blood pressure by reducing sodium content in body tissues. Apples also purify the blood by transforming toxic chemicals found in the blood into less harmful compounds and excreted via stools and urine, minimize appetite and thereby lower blood cholesterol and by

removing different free-radicals. This is also beneficial to the lymphatic system (Sabir and Shah, 2004).

Easy accessibility of this fruit can prevent the deficiency diseases and other health related problems. However, this fruit is not easily accessible in Ethiopia due to very little production and very expensive cost of few imported fruits available. At present, one kilogram imported apple fruit costs 60.00 ETB in Addis Ababa, Ethiopia. Moreover, apple seedlings are grown in limited number in few agricultural research centers like Holetta Agricultural Research Center (HARC) and other farm areas including Chencha and Entoto, and it is difficult to obtain these seedlings for cultivation. If available these seedlings are very expensive, single grafted apple seedling costs minimum of 80.00 ETB.

The objective of this study is, therefore, to develop optimal micropropagation protocol for two selected apple varieties, namely the rootstock, MM106 and Anna scion from shoot tip explants. This will have its own contribution to improve the food insecurity and health related problems in Ethiopia, being one of the developing countries suffering from food insecurity and health related problems, by producing the healthy apple seedlings with relatively short period of time and limited space and thereby fruits in large amount.

2. OBJECTIVES

2.1. General objective

The general objective of the present study is to develop micropropagation protocol for two apple varieties, Anna and MM106, from shoot tip explants.

2.2. Specific objectives

A. To optimize different growth regulators concentrations and combinations on MS medium for optimum production of shoots;
B. To determine the sub-culture stage with highest number of multiplied micro-shoots;
C. To investigate rooting response of shoots to different IBA or IAA concentrations in combination with or without activated charcoal;
D. To acclimatize the *in vitro* propagated plantlets in glasshouse;
E. To evaluate the survival rate of plantlets during acclimatization.

3. LITERATURE REVIEW

3.1 Description and taxonomy of *Malus* x *domestica* Borkh

Malus x *domestica* Borkh is a pomaceous fruit tree belonging to the family *Rosaceae* and sub family *Maloideae*. It is a small deciduous perennial tree, 5-12 m tall and age longevity between 60 to 100years. Depending on the rootstock cultivar and the age of the tree, the roots can occupy between 2 to 104 m^2, most frequently ranging between 10-30 m^2. The leaves are alternately arranged, simple oval with an acute tip and serrated margin, 5-12 cm long and 3-6 cm broad which are attached on a 2-5 cm petiole (Dobrzanski *et al*., 2006; Atkinos, 1980; USDA, 2008).

The apple tree is monoecious species. Three to six flowers in cymes (the first being the most advanced) appear in mixed buds (Dennis, 2003). It produces rose epigymous flowers. The development of multicarpellate inferior ovary and the accessory tissue after fecundation produce the pome fruit (Ryugo, 1988).The flowers are produced in spring with the leaves, white to deep pink in color and 2.5 - 3.5 cm in diameter. They are produced in cymes clusters on fairly short pedicels, usually on spur type growth, but in some instances from the terminal or lateral buds of the previous season‟s growth. Typical flower of apple consists of five petals, a calyx of five sepals, about 20 stamens and the pistil which divides into five styles. The ovary (fruit) has five carpals at the center of the fruit which are arranged in a five point star, each usually containing one to three ovules, so that in most cases, the maximum seed content is 10 but some cultivars have more. „Liberty" and „Northern Spy", for example, usually have 12 to 18 seeds and the „Ottawa 3" rootstock often has 20 to 30 seeds (Janick *et al*., 1996). The fruit matures in autumn, and is typically 5-8 cm in diameter (rarely up to 15 cm) (Dobrzanski *et al*., 2006).

As indicated by Broothaerts *et al*. (2004) even though incompatibility is common in most of the cultivars, some cultivars are partially self- compatible. Self-incompatibility in apple is of gametophytic type and is controlled by single multiallelic locus, S-locus. As pollen tubes elongate through the styles, they are attacked by cytotoxic proteins. Expression of specific inhibitors avoids a lethal attack. These style toxic proteins (S- RNases) are expressed by S-genes. Pollen tube growth is inhibited when the pollen shares the same S- allele with the pistil on which the pollen germinates. Eighteen different S-alleles have been identified; only three of them most frequently occur (S_2, S_3 and S_9). Mostly the wild apple cultivars are diploid (2n = 34

chromosomes) and some of them are triploid (3n = 51), even some others are tetraploid (4n = 68). Triploids produce relatively heavier sized fruits. Cabrer *et al*. (2007) have found that 29% of the local cultivars in northern Spain were triploids, producing an average of 15% heavier apple fruits.

Cross-pollination, mainly by insects, is common for better fruit production. Therefore, knowledge of appropriate kinds of cultivars, which are cross-compatible, is prerequisite for best fruit production (Lorenzo *et al*., 2009).

Figure 1. Apple flower and fruit morphology, cross section and longitudinal section.

The genus *Malus* has 25 to 30 species and several sub-species of the so- called crabapples, many of which are cultivated as ornamental trees for their profuse blossom and attractive fruits (Way *et al*., 1991; Langenfeld, 1991 and Li, 1996). Although they are generally found in the northern temperate zones of North America, Europe, Asia Minor and Asia, Species within the genus are widely distributed. Such wide geographic distribution suggests that a wealth of potentially useful traits may exist within the genus that could be utilized by apple breeders in the development of modern apple cultivars and apple rootstocks suited to diverse environmental conditions (Hokanson *et al*., 2001).

There are more than 7,500 cultivars or sub-species of apple available in temperate and sub-tropical climates with different chilling requirements. Each variety has specific commercial requirements for skin color ranging from yellow or green for varieties such as „Granny Smith" and „Golden Delicious" to red for varieties like „Red Delicious." Bi- colored apples such as „Gala" and „Braeburn" are also common (Dobrzanski *et al*., 2006; Watkins *et al*., 2002).

3.2. Botanical origin and distribution of *Malus* x *domestica*

Most of the species of apples intercross. Since self- incompatibility is common, trees produced from seed are mostly interspecific hybrids. Hence, the cultivated apple is likely the result of interspecific hybridization and at present the binomial *Malus x domestica* has been generally accepted as the appropriate scientific name (Korban and Skirvin, 1994). The multiplication sign "X" placed between the genus and species names denotes an interspecific hybridization within the genus. Fertile hybrids are obtained from almost all crosses among *Malus* species (Korban, 1986) and there is ample precedent for significant contributions by species for the improvement of commercial apple cultivars.

The wild ancestor of apple is *Malus sieversii*, which is wild from the Heavenly Mountains (Tien Shan) at the boundary between western China and the former Soviet Union, to the edge of the Caspian Sea (Morgan and Richards, 1993). Investigations made in Central Asia have verified that *M. sieversii* is very diverse and has all the qualities present in *M. x domestica* (Forsline *et al*., 1994; Forsline, 1995). The city where it is thought to originate is Alma Ata or "father of the apples" in Asia. The use of molecular markers has also confirmed that, the wild apple located in Central Asia could be the major maternal contributor to the domesticated one (Harris *et al*., 2002).

The tree is still found wild in the mountains of Central Asia in southern Kazakhstan, Kyrgyzstan, Tajikistan, Xinjiang and China. Other species that were previously thought to have made contributions to the genome of the domestic apples are *M. baccata* and *M. sylvestris*, but there is no hard evidence for this in older apple cultivars. These and other *Malus* species have been used in some recent breeding programmes to develop apples suitable for growing in climates unsuitable for *M .x domestica*, mainly for increased cold tolerance (Dobrzanski *et al*., 2006).

The word apple comes from the old English word „æppel", which has relation with a number of Indo-European language families. The prevailing theory is that "apple" may be one of the most ancient Indo-European words (*„ abl'*) to come down to English in a recognizable form. The scientific name *Malus*, on the other hand, comes from the Latin word for apple, and ultimately from the Greek *melon*. The legendary place name Avalon is thought to come from a Celtic

evolution of the same root as the English "apple", as is the name of the town of Avellino, near Naples in Italy (Dobrzanski *et al*., 2006).

3.2.1 Apple cultivars

Scientific nomenclature for apples has changed since Linnaeus denominated *Pyrus malus*. Other naming in the past have been *M. communis, M. Sylvestris, M. pumila* and *M. domestica* (Harris *et al*., 2002). The number of species in the genus *Malus* is uncertain and still under controversy. Robinson *et al*. (2001) described that the number of species in genus *Malus* depends upon the rank given to several taxa, species being sub- species and putative hybrids and the nomenclature of the taxa is complex. Janick *et al*. (1996) said that *Malus* is a diverse genus with around 25 to 30 species with further sub-species in the crab apple group.

Different scientists have made their own contribution for the identification of different species within the genus *Malus.* Forsline *et al*. (2003) classified 27 primary apple species together with their origin and use. Of these, 22 of them are from Asia (11 mainly located in China), 4 in North America, 2 in Europe and 1 in Japan. Six species are used for fruit production, *M. sieversii, M. sylvestris, M. angustifolia, M. ioensis, M. coronaria*, and *M. hupehensis*. Five out of 27 are recognized as ornamentals and 12 as possible rootstocks.

3.2.2. Root stock varieties

Most of the apple rootstocks used today were derived from collections and selections by East Malling Research Station in England, where most of the initial researches were conducted in the early 1913. Pomologists at East Malling collected and characterized the clonal rootstocks that had been developed by farmers before many centuries in Europe. After selection they form the „M" series, the prefix in early produced apple root stocks. To identify each clone in the series, every clone has number following „M". Subsequent rootstocks were produced in John Innes Institute, at Merton in UK, by crossing some East Malling rootstock types with other apple varieties like "Northern Spy" in the 1920s to introduce resistance to woolly apple aphid for use in New Zealand and Australia. Further collaborative breeding between the John Innes Institute and the East Malling Research Station produced Malling-Merton hybrid, the MM series, rootstocks with aphid resistance. They have also numbers assigned following the symbol and their number series ranges between 100 and 120 (USAID, 2008; Rom and Carlson, 1981; Jackson, 2003).

Depending on their growth behavior, the rootstock clones of apple are divided into three major groups including strong growing (standard), medium-strong growing (semi-dwarf) and weak growing (dwarf) types (USAID, 2008).

The most important apple rootstocks are:

- Weak growing (Dwarf): M27, M9, M26 – Height: Between 1.8-3.0 m
- Medium-strong growing: MM106, M7, M4, MM104, M2 – Height: Around 4.0 m
- Strong growing: MM111, MM109, M10 – Height: Between 4.8-5.5 m.

These rootstocks are important to propagate valued scions that could not be self rooted, by the process of grafting. Grafting rootstocks and scions is also important to increase earlier reproductive development and the amount of dry matter allocated to fruits relative to the vegetative part (fruit production efficiencies per tree), resistance to insects and disease, tolerance to soil and climatic factors and anchorage, ease of propagation, influence the time of leaf senescence, leaf size, photosynthetic rate, flower morphology, flower set time, fruit set, apical dominance, tree shape, cambial differentiation and the duration and rate of tree growth (Webster, 1995).

3.3 Chilling requirement and bud break of apples

Depending on the chilling requirement of different varieties, apples can be found distributed at different geographical locations. It is one of the most widely cultivated temperate fruit crops, produced commercially from Siberia and Northern China, with winter temperatures ranging from –40 ^{0}C to high elevation equatorial locations in Colombia and Indonesia where two crops can be produced in a single year (Janick, 1974).

Apple trees are adapted to different climates. They are cultivated from Northern Europe down to the tropics. Most of the old cultivars require a long rest period, but new selections with less requirements allow them to be cultivated in subtropical areas. Petropoulou (1985) classified apple cultivars according to chilling requirements in six classes and related a shorter rest period with lower growth. Some cultivars are very resistant to low temperatures (- 35^{0}C). Some were selected for very short seasons, three months from blooming, while others require up to six months.

If the chilling requirement is not satisfied, the buds will not open; if it is partially met, the buds will open sporadically and both the bloom and harvest periods will be abnormally extended. Most apple cultivars require between 1200- 1500 chilling hours in the temperature range of 4-7^{0}C (Linden *et al*., 1996).

Table 1. Chilling requirements and days to blooming of some cultivars of apple at varying temperature range (Petropoulou, 1985)

Cultivar	Chilling requirements (in hr)	Days to blooming
Rome Beauty	2700–3100	201
Ingrid Marie	2300–2700	152
Keswick Codlin	2300–2700	136
Antonouka	1900–2300	129
Kidd"s Orange Red	1900–2300	115
Early Victoria	1450–1900	106
Cox	1000–1450	99
Winter Banana	1000–1450	98
Falstaff	300–1000	77
Starkspur Golden Delicious	300–1000	77
Greensleeves	300–1000	74
M16	2300–2700	139
M25	1800–2300	103
M7	1350–1800	65
M27	950–1350	55
M9	950–1350	44

Bud break in apple trees occurs in two distinct stages; the first is completion of an endodormant stage in winter, and the second is the shift from an ecodormant stage to active bud growth in spring (Atwell *et al.,* 1999). Breaking endodormancy in apple trees depends on meeting the chilling requirement (Mehlenbacher and Voordeckers, 1991). The shift from the ecodormant stage to bud break and active growth of apple trees depends on increasing air temperatures in spring (Zhu *et al.*, 1997). Among a chain of effects underlying this process, mobilization of stored carbohydrate and protein reserves to support developing buds and leaf growth often begins before bud break and always precedes the onset of autotrophic growth (Loescher *et al*., 1990). Because apple trees store carbohydrates predominantly in fine and coarse roots (Greer *et al*., 2003), it is likely that root-zone temperatures affect bud break by influencing carbohydrate mobilization.

3.4 Importance of apple

3.4.1 Health benefits of using apple

As indicated by the proverb „an apple a day keeps the doctor away", using apple fruit have been considered to have high health benefit. Different researches suggest that apples can reduce the risk of prostate, colon and lung cancer. Different group of chemicals in apple could protect the brain from neurodegenerative diseases like Alzheimer"s and Parkinsonism (Dobrzanski *et al*., 2006). Fruits and vegetables are high in antioxidants, a diet high in these foods helps to prevent oxidative stress, different chronic diseases and slow aging. Apple fruit is very good source of important phytochemicals like antioxidants, flavonoids, and other free phenolics which are not bound to other compounds (Boyer and Liu, 2004).

Table 2. Apple fruit nutritional value per 100 g (USDA Nutrient Database, 2008)

Element/Compound	Amount
Carbohydrates	13.81 g
Dietary fiber	2.4 g
Fat	0.17 g
Protein	0.26 g
Vitamin A equiv.	3 µg
Thiamin (Vit. B1)	0.017 mg
Riboflavin (Vit. B2)	0.026 mg
Niacin (Vit. B3)	0.091 mg
Pantothenic acid (B5)	0.061 mg
Vitamin B6	0.041 mg
Folate (Vit. B9)	3 µg
Vitamin C	4.6 mg
Calcium	6 mg
Iron	0.12 mg
Magnesium	5 mg
Phosphorus	11 mg
Potassium	107 mg
Zinc	0.04 mg

Apples are produced mainly for the fresh market (Way and Mc Lellan, 1989). Specifically in USA, apples are processed into five basic products, viz., juice, canned puree, canned slices, dried apples, and frozen slices. Apple juice and canned sauce are the dominant products (one-half and one-third, respectively). Apples are also processed into vinegar, jelly, apple butter, mincemeat, and fresh slices. Small quantities are also made into apple wine, apple essence, baked whole apples, apple rings, and apple nectar. Another important product is cider, mainly in France, UK, and Spain.

3.5 Apple fruit production

These days, apples are growing all over the world (Ogawa and English, 1991). Apples are the second most important temperate fruit crop next to grapes and fourth among all fruit species following oranges, bananas and grapes regarding world fruit production (Jackson and Palmer, 1999; http://appsl.fao.org, 2004). Globally, China is leading apple production in 2009/2010 cultivation year followed by the 27 member nations of European Union (EU-27), United States, Turkey and India. In the year 2009/2010 total world production of fresh apples exceeded 61.1 million metric tons. Of this, China dominating global apple production, was producing 52.3 % of the world‟s apple fruit and U.S., the third apple producing accounted only for 7.2 % of world wide produced apple fruit (Ballard *et al*., 2011). In the case of Africa, apples are mainly cultivated in some countries including South Africa, Algeria, Tunisia, Egypt and Morocco (http://appsl.fao.org, 2004).

3.5.1 Apple production status in Ethiopia

Temperate fruits including apples, pears, peaches, almonds and others were introduced to Ethiopia before 50 to 60 years ago. Systematic research approaches have been started 30 years back in Agricultural Research Centers such as Holetta Agricultural Research Center (HARC) and the State Farms (Tsedey). Presently, propagation of temperate fruits becomes important component of development programs throughout the highlands of Ethiopia. Production and sales of apple fruits can have great contribution on the economy of farmers (Spohn, 2011).

Apple varieties with low and medium chilling requirement are adapted to Ethiopian highland topography. The most adapted low chilling apple varieties include Anna, Dorset Golden, Princessa and CP 92 which perform best in the Wet and Moist Dega and Woyna Dega, 1900 to 2800 m.a.s.l. and in some cases, even up to 3600 m.a.s.l. The Anna variety has chilling requirement of 250-300 h and it is highly productive. The yield of a fully grown tree can reach 60 to 75 kg in one year (two cycles) and important for commercial production. Anna has 60% self-fertility (Spohn, 2011).

Cultivars with low chilling requirements occur in different locality but most are of poor quality. Oppenheimer and Slor (1968) in Israel crossed some of these low chilling cultivars with good quality ones and introduced „Anna", „Ein Shemer", and „Schlor", cultivars with low chilling requirements and much improved quality. As postulated by Hauagge and Cummins (1991), this low chilling requirement of „Anna" cultivar is controlled by one major dominant and several minor genes.

There is no statistical evidence on the total areas of covered and annual production of temperate fruits in general and apple in particular in Ethiopia. In highland areas with 2000 – 2600 m.a.s.l., low chill requiring apple cultivars are produced. High chilling requiring apple fruits are also produced in areas higher than 2600 m.a.s.l. Most of the apple fruit consumed in Ethiopia is imported from other countries. At present, Ethiopian farmers are producing apple fruits at their home garden. Apple production is expanding in different areas of the country including Gamu Gofa (Chencha and neighboring districts), North Shewa (Degem, Hedabu, Abote, and Alidoro and Dedre Birhan), Gurage, Dabat, etc. (HARC, Horticulture Division Report Paper, 2010).

3.6 Cultural aspects of apple

Dobrzanski *et al*. (2006) indicated that, in many religious traditions, apples are often considered as forbidden and mystical fruit. This tradition is reflected in the book of Genesis. Even though the forbidden fruit in this regard is not clearly identified, popular European Christian tradition has held that it was an apple that Eve incited Adam to share with. The larynx in the human throat has been called Adam's apple because of a notion that it was caused by the forbidden fruit sticking in the throat of Adam. Moreover, the adoption of apple as Christian symbol is that in Latin, the words for „apple" and „evil" are similar (malum). It is often used to symbolize the fall in to sin, or sin itself. When Christ is portrayed holding an apple, he represents the Second Adam who brings life. When held in Adam's hand, the apple symbolizes sin. This also reflects the evolution of the symbol in religion. In the Old Testament, the apple was significant of the fall of man; in the New Testament, it is an emblem of the redemption from that fall, and as such is also represented in pictures of the Madonna and Infant Jesus.

3.7 Mutations and chimeras in apple

In apples, mutation can arise in a cell from which a bud develops. It is called a bud sport. It produces a shoot that differs, usually in only one character, from the plant on which it was produced. These mutations can affect any part of the plant. There are two important types of mutations in apple. These are those that produce single gene differences in some character of the tree or fruit and those that alter ploidy. From the former one, those mutations affecting the appearance of the fruit are easily recognized and most commonly occur. In a mutated fruit, increase in the amount of anthocyanin in the outer cell layers of the fruit skin is common and red sports of many of the popular cultivars have arisen. Some cultivars such as „Cox"s Orange Pippin", „Delicious", „Estar", „Gala", „Jonagold", „Rome Beauty", and „Winesap" are prone to produce mutants of this type, while other cultivars are stable and seldom seem to mutate (Janick *et al*., 1996).

Not all the red sports of one cultivar are necessarily identical; apart from differences in the area of the fruit covered, the intensity of the pigmentation in the outer cell layers can differ (Dayton, 1959). Very often, the mutation is limited to one cell layer in the apical meristem, therefore, the plant is likely to be a periclinal (hand-in-glove) chimera. The mutation is usually not heritable unless the second layer which gives rise to gametes, is involved (Pratt, 1983). Bud sports with increased red color may be extremely valuable and should be propagated and trialed to see if they are superior to the original clone. Reduced russet, russet-free, and total russet occurs and some of these traits may be desirable (Janick *et al*., 1996).

Janick *et al*. (1996) also extended his explanation that, mutations affecting growth habit, particularly the spur or compact types, which produce compact or dwarfish, freely spurring trees, may be extremely valuable and are being sought in all the important commercial cultivars. Mutations inferior to the original clone may also occur and stock trees should be carefully observed so that no inferior mutants are unconsciously propagated. This is the basis for bud selection to maintain the integrity of the cultivar.

The rate at which single gene mutations occur can be increased by irradiation with X-rays, gamma rays, or thermal neutrons. Bishop (1959) produced two dark red sports of „Cortland", two sports of „Sandow" with less color and more russet, and a „Golden Russet" with considerably less

russet than normal. Lapins (1965) and others have also produced compact mutations by means of irradiation. The effective dosage is 3-5 krad for dormant scions and 2-4 krad for summer buds when X-rays are used and 3.9-15.6 $\times$ 10^{12} thermal neutrons/cm^2. Mutants from irradiated material do not readily show themselves and the normal growth, if allowed to develop, will suppress the mutant. When dormant scions have been treated and grafted, the basal ten buds or more of the shoot emerging from each treated bud should be removed and budded onto a dwarfing rootstock.

The mutants are likely to show in the second vegetative generation. Lacey and Campbell (1987) reviewed the production and selection of mutant apples and described the tests and the experimental evidence used to determine the nature of the mutated plants.

As indicated by Einset and Imbofe (1951), the other mutations important in apple breeding are the “large” or “giant” sports which occur due to changes in ploidy level, typically chromosome doubling (diploid to tetraploid or triploid to hexaploid). These sports are usually recognized first by their large fruits, which are sometimes twice as large (in volume) as their diploid counterpart. The fruits, apart from the increase in size, are usually flatter and more irregular in shape and have no commercial value. Giant sports in many apple cultivars have arisen spontaneously and have been discovered by observant growers. Most of these tetraploids are periclinal chimeras and can be grouped according to the arrangement of 2x and 4x tissues. The four layers in the apical meristem (L-1 to L-4) can be classified on the basis of nuclear size as either 2-2-2-2, 2-2-2-4, 2-2-4-4, 2-4-4-4, or very rarely 4-4-4-4 based on whether the cell is diploid or tetraploid. The breeding behavior of these sports differs according to the location of the 4x tissue. Only when L-2 is 4x will the tree breed as a tetraploid. Thus, many giant sports, apart from having tetraploid growth characters, genetically behave as diploids.

The 2-2-4-4 cytochimeras look like a tetraploid, but breed as a diploid (Pratt, 1983). It is possible, however, to produce homogeneous tetraploid plants from 2-2-4-4 sports by inducing shoots to grow from endogenous tissue or from root suckers developed from the scion because roots develop from L-3. One method is to grow one-year-old trees of the giant sports in large containers, cut them down to 30 cm, and remove all the buds and any growth that appears in the region of the removed buds or from the rootstocks. This encourages the formation of

sphaeroblasts in the internodal regions. These will in due course crack and produce adventitious buds which develop into shoots.

Although practically all the 4x sports have arisen spontaneously, it is also possible to encourage the formation of tetraploids by the use of colchicine applied to the growing point. These induced tetraploids are also likely to be cytochimeral (Hunter, 1954).

3.8. Micropropagation and its advantage

Tissue culture has been used by different scholars to propagate different species of plants in the past years. Micropropagation is one form of tissue culture which allows the production of large number of plants from small pieces of the mother plant in relatively short period of time and limited space. It is an aseptic process which requires sophisticated laboratory procedure with unique facilities and special skills (Hartman *et al*., 2004).

Micropropagation confers distinct advantages not possible with conventional propagation method. It is possible to multiply a single explant into several thousands in less than a year. Actively dividing cultures are continuous sources of plantlets without seasonal interruption. It has high commercial potential due to the speed of propagation, clonal propagation, germplasm conservation, genetic transformation and its high quality and ability to produce disease-free plants (Tileye Feyissa *et al*., 2005; Hartman *et al*., 2004).

Most temperate tree fruit species are self-sterile and extremely heterozygous and most scion cultivars don‟t come true-to-type if propagated using their own, seldom fertile, seeds (Webster, 1995). Therefore, this research was designed to develop micrpropagation protocol for two apple varieties, namely MM106 and Anna, to produce true-to-type apple seedlings.

4. MATERIAL AND METHODS

All the laboratory activities and experiments were conducted in the Plant Biotechnology, Plant Tissue Culture Laboratory at Holetta Agricultural Research Center (HARC).

4.1. Stock plant preparation

One year old stock plants of both Anna and MM106 varieties of apple were obtained from temperate fruit cultivation field of Holetta Agricultural Research Center (HARC), Horticulture Division. Seedlings of these varieties were obtained from the field, planted in plastic pots containing red soil, compost and sand in the ratio of 2:1:1, respectively and allowed to grow in glasshouse at HARC at average temperature of 25 ± 2^0 C for about two months under natural sunlight conditions. After two months of growth, mother plants with well developed young shoot tips were used as source of explants.

Figure 2. Anna (A) and MM106 (B) shoot tip explants ready for culture initiation.

4.2. Stock solutions and media preparation

4.2.1. Stock solution preparation

Murashige and Skoog (1962) basal medium was used throughout this research activity. Initially, full strength stock solutions of macronutrients, micronutrients and vitamins and other organic supplements were separately prepared. To do so, appropriate amount of each nutrient was weighted in grams per liter (Appendix 1) and dissolved in double distilled water consecutively in such a way that the next nutrient was added after the first one was completely dissolved. After all the components were completely dissolved using magnetic stirrer, the solution was poured into plastic bottles and stored at +4^0 C until used, for maximum of four weeks.

4.2.2. Plant growth regulators stock solution preparation

Plant growth regulators (PGRs) were prepared in 1mg/ml concentration. The PGRs used for the study were the cytokinin, 6- benzyl amimopurine (BAP), the auxins, indol-3- butyric acid (IBA) and indol acetic acid (IAA), and gibberrellin, gibberrellic acid (GA_3). The powdered crystal of the PGRs was first weighed and dissolved in 3-4 drops of 1N NaOH, 1N HCl and 99% ethanol based on the type of PGR (NaOH for auxins, HCl for cytokinin and ethanol for gibberellin). Upon complete dissolution, the solution of each PGR was poured into labeled 50 ml plastic bottles and filled with double distilled water to the required volume. Then gently stirred and stored at a temperature of $+4^0$ C for short term use (a week) and -5^0 C for long term use (up to a month) until used.

4.2.3 Culture media preparation

In the first category of studies, the culture media for shoot initiation and multiplication contained full strength of MS basal medium (100 ml macronutrient, 10 ml micronutrient, and 10 ml vitamin per liter), 30 g/l sucrose and with or without (for control) PGRs. In the second group of experiments, half strength MS basal medium in the presence or absence of activated charcoal was used with or without different concentrations IBA or IAA for rooting. Seven gram per liter plant tissue culture agar was used as a solidifying agent throughout the experiment. Activated charcoal with 1g/l concentration was also used in the culture initiation and rooting media which was left in the multiplication medium. Finally, the pH of all media was adjusted to 5.7 by using 1N HCl and/or NaOH after addition of agar and activated charcoal because the agar and activated charcoal used have shown slight increase in pH after addition to the media.

After adjusting the pH, the gently mixed medium was boiled on a stove until the agar melted. Then, 40 ml of the prepared medium was dispensed into 10 x 6 cm size magenta GA7 culture vessels. The culture vessels were covered with caps immediately after dispensing the medium and autoclaved by steam sterilization at a temperature of 121^0 C and 105 KPa pressure for 15 minutes. Immediately after autoclaving, the medium was taken and kept in lamina-air-flow-cabinet bench until used (a maximum of a week).

4.3 Establishment of aseptic conditions and culture initiation

Terminal shoot tips of the rootstock, MM106 and the scion, Anna varieties were cut and collected by using sterile blade. The explants were with a size of 2-3 cm and obtained from two-month-old seedlings grown in glasshouse. Also, explants from field grown varieties of Anna, Winter banana, Fuji and Princissa were used. But none of them gave growth response due to high contamination problem and oldness of the shoots used.

The collected shoot tips were washed 4 to 5 times with running tap water in media preparation room. Then in the laminar-air-flow-cabinet bench, the explants were rinsed with sterile double distilled water once followed by sterilization with 70% ethanol for 45 seconds. Then, after rinsing them three times with sterile double distilled water, the explants were further sterilized with 1% sodium hypochlorite (NaOCl) for 8 minutes. Finally, the shoots were washed five times with sterile double distilled water to remove NaOCl off the explants.

The decontaminated shoot tips were kept in fresh sterile double distilled water until final trimming and culturing them in culture medium. Then these shoots were trimmed to 1.5 – 2 cm long in aseptic conditions and cultured on the already prepared culture initiation medium supplemented with 0.1 mg/l IBA in combination with 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 6mg/l BAP (Figure 3). For each of the treatments six magenta GA7 culture vessel (replications) each containing five explants (Figures 4; A and B) were used, that is, a total of 30 explants per treatment for each variety. Each shoot in the culture vessel was observational unit. The culture vessel with cultured explants were properly sealed with parafilm, labeled and placed in dark for three days to reduce the release of phenolic compounds from the explants. After three days, the cultures were transferred and randomly placed on the growth room chamber with 16 hours photoperiod (8 hours dark) and 2700 lux light intensity at 25 ± 2^0 C.

The fluorescent lights were 28 cm away from the top of culture vessels. The stock solution composition, agar concentration and other physical conditions were the same for all the treatments.

Since there was high amount of phenolic compounds released from the explants, they were continuously transferred to new fresh medium twice at three days interval by washing them with sterile double distilled water and removing brown bottom tip of the explants at each transfer.

After a month of growth, to facilitate growth, the micro-shoots with 2 to 3 leaflets were transferred into activated charcoal free medium which has the same composition with the previous one. Every change in growth was carefully observed and recorded. PGR free medium was used as a control. The shoot initiation experiment was repeated twice at different times.

4.4 Shoot multiplication

After 45 days of growth on the culture initiation medium, young and healthy micro-shoots were cultured on shoot multiplication, full strength MS medium, containing different concentrations of BAP alone (Table 3); 0.01 mg/l IBA combined with different concentrations of BAP (Table 4) and, 0.01 mg/l IBA combined with different concentrations of GA_3 and BAP (Table 5). Five shoots per culture vessel and six replications for each treatment were used. The initiated cultures were aseptically cut off and cultured on activated-charcoal-free multiplication medium. The culture vessels were properly sealed, labeled and randomly placed on the growth room chambers with the same culture conditions (temperature, photoperiod and light intensity) as that of the initiation experiment. PGR free medium was used as a control. Sub-culturing was made five times by transferring the newly multiplied micro-shoots to fresh medium of the same composition as the previous one. Sub-culturing was carried out at monthly interval. The growth response of the micro-shoots to different treatments and at different multiplication stages was carefully observed and recorded.

4.5 Rooting

Rooting of the already multiplied shoots of both varieties was conducted on half strength MS basal medium with or without activated charcoal supplemented with different concentrations of IBA or IAA (Tables 9 and 10). PGR free half strength MS basal medium was used as control in all cases. Since the leaf size was not large enough to inhibit growth, for MM106 variety, single shoot with a size of two or more cm was cultured in a test tube containing 20 ml of the rooting medium. Before culturing, the shoots were cut in a „V- shape“ form to increase the surface area of wounding of the cut end of the shoot tip which in turn increases rooting efficiency. Each test tube with cultured shoot was the experimental unit. Twenty replications for each treatment were used (a total of 280 MM106 shoots). But, due to increased leaf size to grow in a test tube, Anna shoots were rooted in Magenta GA7 culture vessels, four shoots per treatment and five

replications for each treatment were used (a total of 280 Anna shoots). The cultures were kept in dark for five days at a temperature of 25 ± 2 ^{0} C. After five days, the cultures were transferred to the growth room chambers with 16 h photoperiod at 25 ± 2 ^{0} C. For those shoots grown in a medium with activated charcoal, the shoots were transferred to this medium after five days of growth in dark in the same medium composition except activated charcoal. Physiological factors (photoperiod, temperature, and light intensity), pH of the medium, agar concentration and other growth conditions were the same as before. The number of roots produced from each shoot and length of the longest roots were measured after four weeks of growth.

4.6 Acclimatization

Agar was removed from roots of the plantlets by gently washing under running tap water and the plantlets were transferred to pots containing sterile soil mix of red soil, compost and sand in their respective ratio of 2:1:1. Then each pot was labeled and covered with polythene plastic bags to ensure high humidity and was transferred to a glasshouse with 70% humidity, 25 ± 2^0 C and 16 h photoperiod in controlled conditions. The plastic bags were totally removed after two weeks. To assess the acclimatization potential, other shoots were also acclimatized on sterile soil without polyethylene coverage and on unsterilized soil mix with or without plastic bag covers. Thirty five plantlets for each condition (140 plantlets for each variety) were acclimatized. After four weeks of growth in the glasshouse, number of survived plants was recorded.

4.7 Experimental design and statistical data analysis

For the present study, Completely Randomized Design (CRD) was used. The number of surviving and dead explants during culture initiation, number of shoots produced per shoot in different multiplication media, the number of shoots produced per shoot at different sub-culturing stages, number of roots per plantlet and lengths of the produced roots were recorded and calculated. Statistical data analysis was done by using excel spread sheet, Sigma Plot Version 8.0 and Statistical Analysis System (SAS) computer software version 9.0 (SAS Institute Inc., 2002). To detect the significance of differences among treatments at or below the probability level of 0.05, analysis of variance (ANOVA) was also made using the software. To detect homogeneity of variance, the means of different treatments were analyzed by using Tukey‟s Studentized Range Test at 5% confidence interval.

5. RESULTS

5.1 Culture initiation

Shoot tips started to develop microleaves after a week of culturing. Though they were cultured on a medium with activated charcoal, the explants had brown tips before producing microleaves (Figure 4; A and B). After 30 days of growth on the initiation medium, 96.7% of MM106 and 93.3% of Anna explants survived and responded better on MS medium containing 1.0 mg/l BAP and 0.1 mg/l IBA or 1.5 mg/l BAP and 0.1 mg/l IBA. None of the explants used as control from both varieties survived. Other media compositions resulted in low number of survived shoots and showed different features like callus formation at the bottom of the explants, production of shoots with folded and brittle leaves and very dwarf shoots (Figure 4; G and H).

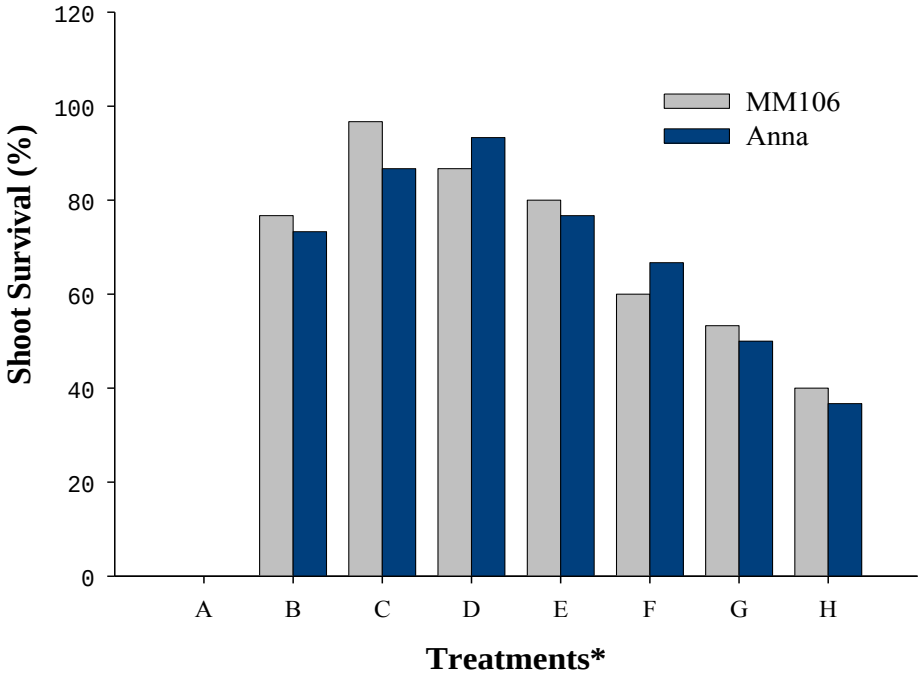

Figure 3. Survival percentage of shoots of MM106 and Anna cultured on MS initiation medium supplemented with different combinations of BAP and IBA.

* Control without PGRs (A), 0.5 mg/l BAP and 0.1 mg/l IBA (B), 1 mg/l BAP and 0.1 mg/l IBA (C), 1.5 mg/l BAP and 0.1 mg/l IBA (D), 2 mg/l BAP and 0.1 mg/l IBA (E), 2.5 BAP mg/l and 0.1 mg/l IBA (F), 3 mg/l BAP and 0.1 mg/l IBA (G), 6 mg/l BAP and0.1 mg/l IBA (H).

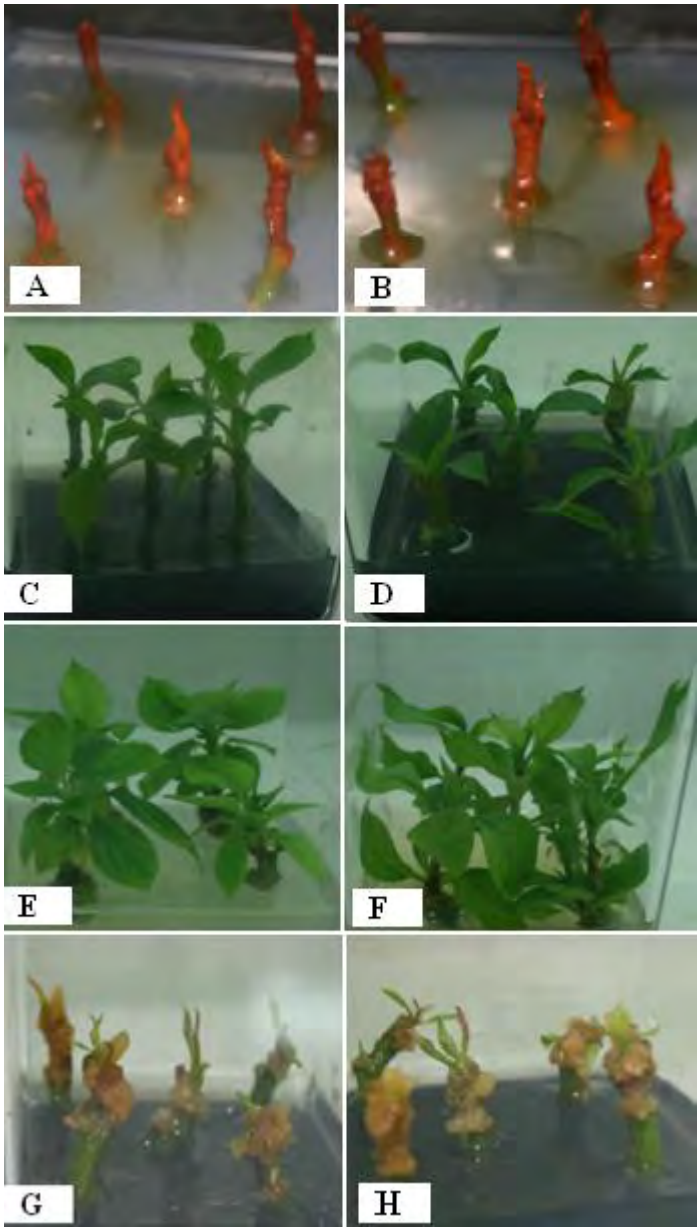

Figure 4. Response of shoot tip explants of MM106 and Anna apple varieties on initiation medium with different combinations of BAP and IBA. MM106 (A) and Anna (B) after 3 days of culturing on initiation medium; MM106 (C) and Anna(D) after a month of growth in initiation medium with activated charcoal and hormonal composition of 1.0 mg/l BAP and 0.1 mg/l IBA, and 1.5 mg/l BAP and 0.1 mg/l IBA, respectively; MM106 (E) and Anna (F) after 15 days of growth in activated-charcoal-free medium after being transferred from the above respective medium of the same hormonal composition; MM106 (G) and Anna (H) after 30 days of growth on initiation medium with 6 mg/l BAP and 0.1 mg/l IBA.

5.2 Shoot multiplication

The shoots on multiplication medium gave different responses based on the hormonal composition differences of the medium (Tables 3, 4 and 5). The best shoot multiplication medium from the used treatments and the better stage of multiplication that provide higher number of shoots was obtained.

The maximum mean number of shoots for MM106 was 6.33 ± 0.50 on the MS multiplication medium containing 1.5 mg/l BAP, 0.5 mg/l GA_3 and 0.01 mg/l IBA (Table 5). The multiplication potential of Anna was relatively less and produced 4.93 ± 0.42 mean number of shoots on the multiplication medium containing 2.0 mg/l BAP, 0.5 mg/l GA_3 and 0.01 mg/l IBA. Other media produced lower number of multiplied shoots for the two varieties. No multiplication was observed on the control unit without PGRs, only the cultured shoots with slow growth were obtained (Table 5). In multiplication medium containing only BAP at different concentrations, as the concentration of BAP increased to 6 mg/l BAP, the shoots of both varieties were found to show very dwarf and bushy appearance (Figure 5). When the sub-culturing stages were considered, the multiplication potential at each stage was fluctuating. However, MM106 gave the highest mean number of multiplied shoots, 8.70 ± 2.77, at the fifth and Anna produced maximum number of multiplied shoots, 7.63 ± 0.59, at the second sub- culturing stages on the above respective media for the two varieties (Tables 7 and 8).

Table 3. Mean number of shoots per explant in MS multiplication medium containing different BAP concentrations, mean values are indicated as ± SD

BAP(mg/l)	Mean No. of shoots/explant	
	MM106	Anna
0	1.0 ± 0.0^{b}	1.0 ± 0.0^{d}
0.5	2.80 ± 0.53^{a}	2.20 ± 0.20^{bc}
1	2.67 ± 0.64^{a}	2.20 ± 0.40^{bc}
1.5	3.13 ± 0.42^{a}	2.67 ± 0.23^{ab}
2	2.27 ± 0.31^{ab}	3.00 ± 0.0^{a}
6	2.33 ± 0.31^{a}	1.67 ± 0.12^{c}

Means connected by the same superscript letters in the same column are not significantly different at 5% probability level.

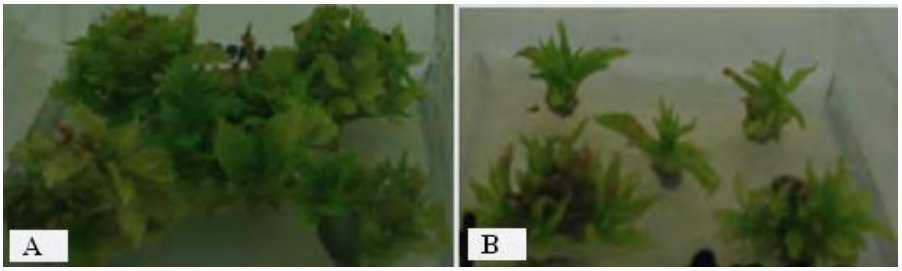

Figure 5. Dwarf and bushy morphology of shoots of MM106 (A) and Anna (B) multiplied on MS medium containing 6 mg/l BAP after a month of growth.

Table 4. Mean number of shoots per explant in MS multiplication medium containing different BAP and IBA concentrations, mean values are indicated as ± SD

		Mean No. of shoots/explant	
BAP(mg/l)	IBA(mg/l)	MM106	Anna
0	0	1.00 ± 0.00^{b}	1.00 ± 0.00^{c}
0.5	0.01	2.27 ± 0.31^{a}	2.67 ± 0.12^{ab}
1	0.01	2.53 ± 0.31^{a}	2.53 ± 0.42^{ab}
1.5	0.01	2.87 ± 0.31^{a}	2.67 ± 0.23^{ab}
2	0.01	2.47 ± 0.42^{a}	2.93 ± 0.31^{a}
6	0.01	2.40 ± 0.20^{a}	2.13 ± 0.31^{b}

Means connected by the same superscript letters in the same column are not significantly different at 5% probability level.

Table 5. Mean number of shoots per explant in MS multiplication medium containing different BAP, IBA and GA_3 concentrations, mean values are indicated as ± SD

			Mean No. of shoots/explant	
BAP(mg/l)	IBA(mg/l)	GA3(mg/l)	MM106	Anna
0	0	0	1.0 ± 0.0^{f}	1.0 ± 0.0^{c}
0.5	0.01	0.5	3.93 ± 0.23^{cde}	3.47 ± 0.42^{ab}
0.5	0.01	1	3.73 ± 0.31^{de}	3.73 ± 0.64^{ab}
1	0.01	0.5	4.93 ± 0.42^{bc}	3.60 ± 0.53^{ab}
1	0.01	1	3.93 ± 0.31^{cde}	3.80 ± 0.35^{ab}
1.5	0.01	0.5	6.33 ± 0.50^{a}	3.80 ± 0.40^{ab}
1.5	0.01	1	5.53 ± 0.46^{ab}	3.73 ± 1.03^{ab}
2	0.01	0.5	4.80 ± 0.40^{bcd}	4.93 ± 0.42^{a}
2	0.01	1	5.40 ± 0.60^{ab}	4.47 ± 0.12^{a}
6	0.01	0.5	3.73 ± 0.50^{de}	2.73 ± 0.58^{b}
6	0.01	1	3.40 ± 0.40^{e}	2.60 ± 0.60^{b}

Means connected by the same superscript letters in the same column are not significantly different at 5% probability level.

Table 6. ANOVA for number of shoots produced per explant in MS multiplication medium supplemented with different PGR combinations and concentrations

Source of Variation	DF	SS	MS	F value	P>F
PGR (BAP only)	5	13.74	2.75	22.98	<0.0001**
Variety	1	0.54	0.54	4.50	0.0455*
PGR x Variety	5	2.13	0.43	3.56	0.0165*
Error	22	2.63	0.12		
Coeff. Var.		15.41			
PGR (BAP + IBA)	5	13.22	2.64	32.72	<0.0001**
Variety	1	0.04	0.04	0.49	0.4891
PGR x Variety	5	0.37	0.08	0.92	0.4842
Error	22	1.78	0.08		
Coeff. Var.		12.42			
PGR (BAP + IBA + GA3)	10	84.00	8.31	39.46	<0.0001**
Variety	1	10.72	10.72	50.91	<0.0001**
PGR x Variety	10	9.90	1.00	4.70	<0.0002**
Error	42	8.84	0.21		
Coeff. Var.		11.93			

* 5% and ** 1% significant levels.

Table 7. Mean number of shoots of MM106 produced at different sub-culturing stage (1-5) in MS medium containing 1.5 BAP, 0.01 IBA and 0.5 GA_3 (in mg/l), numbers in the matrix are ± SD

Stages	Mean No. of shoots/explant
1	6.30 ± 0.50^{ab}
2	4.26 ± 0.72^{b}
3	6.63 ± 0.86^{ab}
4	4.96 ± 0.37^{b}
5	8.70 ± 2.77^{a}

Means connected by the same superscript letters in the same column are not significantly different at 5% probability level.

Table 8. Mean number of shoots of Anna produced at different sub-culturing step (1-5) in MS medium containing 2.0 BAP, 0.01 IBA and 0.5 GA_3 (in mg/l), numbers in the matrix are ± SD

Stage	Mean No. of shoots/explant
1	5.16 ± 0.42^{b}
2	7.63 ± 0.59^{a}
3	4.03 ± 0.54^{b}
4	4.10 ± 0.41^{b}
5	5.20 ± 1.06^{b}

Means connected by the same superscript letters in the same column are not significantly different at 5% probability level.

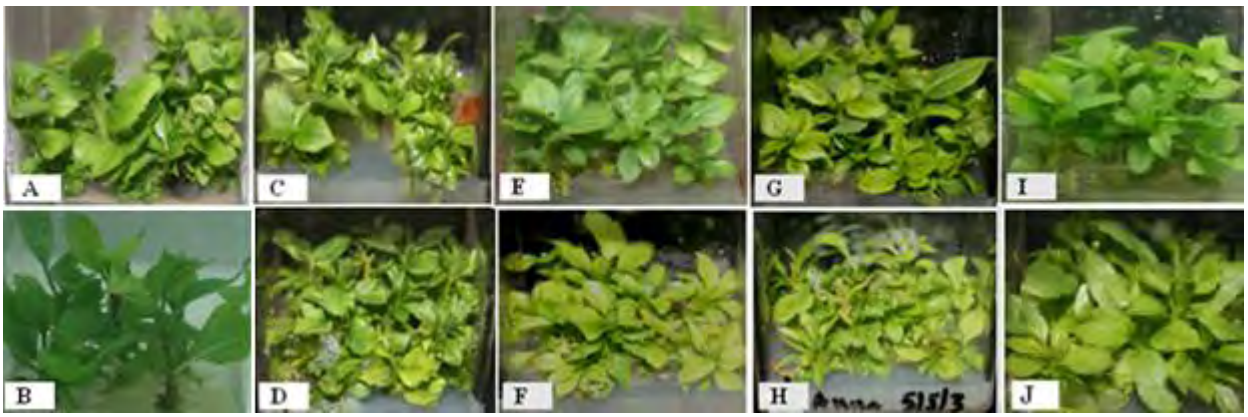

Figure 6. Multiplication of MM106 (upper row) and Anna (lower row) at sub-culturing stages of 1-5 in MS media with hormonal composition of 1.5 BAP, 0.01 IBA and 0.5 GA_3 (in mg/l) for MM106 and 2.0 BAP, 0.01 IBA and 1.0 GA_3 (in mg/l) for the Anna varieties.

5.3 Rooting

The shoots treated with rooting media containing half strength MS basal media supplemented with different concentrations of IBA or IAA and with or without activated charcoal (AC) gave different rooting responses except the control. All the cultured shoots of Anna developed root on the rooting medium containing 1 mg/l IAA and AC (Table 10). MM106 relatively showed reduced rooting percentage *in vitro* and 93.75% of the cultured shoots on the medium containing 2 mg/l IAA and AC (Table 10). Shoots of MM106 cultured on the medium containing 6 mg/l IAA without AC produced callus like swelling at their cultured end in place of root production.

A maximum number of 14 and12 roots were obtained from Anna and MM106 varieties per plantlet treated with 2 and 1 mg/l IAA in the presence of activated charcoal, respectively. These growth regulator compositions produced 6.25 ± 0.50 and 5.70 ± 0.69 mean number of roots for Anna and MM106 varieties, respectively (Table 12).

However, both varieties produced longer roots on the medium containing IBA. Anna produced with of 6.00 ± 0.95 cm on the medium containing 1 mg/l IBA and AC. While MM106 produced 4.55 ± 1.40 root length on the medium containing 1.5 mg/l IBA without AC (Table 11).

Table 9. Percent of rooted shoots of MM106 and Anna on half MS medium containing different IBA concentrations with or without activated charcoal

	Percentage of rooted shoots	
IBA (mg/l)	MM106	Anna
0	0.00	0.00
0.5	31.25	43.75
1	62.50	75.00
1.5	68.75	81.25
2	75.00	87.50
3	50.00	75.00
6	50.00	31.30
0*	0.00	0.00
0.5*	43.75	50.00
1*	87.50	81.25
1.5*	87.50	93.75
2*	81.25	87.50
3*	62.50	75.00
6*	56.25	31.30

* Medium containing 1 g/l activated charcoal.

Table 10. Percent of rooted shoots of MM106 and Anna on half MS rooting medium containing different IAA concentrations with or without activated charcoal

	Percentage of rooted shoots	
IAA (mg/l)	MM106	Anna
0	0.00	0.00
0.5	43.75	62.50
1	75.00	87.50
1.5	81.25	87.50
2	87.50	81.25
3	62.50	81.25
6	50.00	43.75
0*	0.00	0.00
0.5*	50.00	68.75
1*	75.00	100.00
1.5*	87.50	87.50
2*	93.75	81.25
3*	68.75	87.50
6*	62.50	50.00

* Medium containing 1 g/l activated charcoal.

Table 11. Mean number and length of roots obtained on half MS medium containing different IBA concentrations with or without activated charcoal, mean values are indicated as ± SD

	Mean No. of roots/explant		Mean Length of main roots/explant (cm)	
IBA (mg/l)	MM106	Anna	MM106	Anna
0	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}	0.0 ± 0.0^{d}
0.5	1.92 ± 0.35^{ab}	1.0 ± 0.71^{cd}	2.60 ± 0.22^{ab}	1.88 ± 1.25^{cd}
1	1.65 ± 1.18^{ab}	2.35 ± 0.40^{abc}	2.03 ± 1.38^{bc}	3.20 ± 0.57^{bc}
1.5	2.80 ± 0.65^{a}	2.43 ± 1.12^{abc}	4.55 ± 1.40^{a}	3.10 ± 0.70^{bc}
2	2.75 ± 0.68^{a}	2.83 ± 0.99^{abc}	3.33 ± 0.54^{ab}	3.50 ± 0.44^{bc}
3	1.73 ± 1.18^{ab}	2.63 ± 0.48^{abc}	2.90 ± 0.38^{ab}	2.95 ± 0.52^{bc}
6	1.73 ± 1.18^{ab}	1.83 ± 1.29^{abcd}	2.65 ± 1.93^{ab}	2.73 ± 1.87^{bc}
0*	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}	0.0 ± 0.0^{d}
0.5*	2.45 ± 0.39^{a}	1.70 ± 1.15^{bcd}	3.28 ± 0.46^{ab}	2.30 ± 1.61^{bc}
1*	3.30 ± 0.68^{a}	3.60 ± 0.48^{a}	4.50 ± 0.93^{a}	6.00 ± 0.95^{a}
1.5*	3.38 ± 0.82^{a}	3.50 ± 0.96^{ab}	3.98 ± 0.79^{ab}	4.43 ± 0.51^{ab}
2*	1.83 ± 1.22^{ab}	2.23 ± 0.15^{abc}	3.13 ± 2.24^{ab}	4.00 ± 0.73^{abc}
3*	3.18 ± 0.87^{a}	2.53 ± 0.67^{abc}	3.33 ± 0.41^{ab}	3.45 ± 0.44^{bc}
6*	2.25 ± 0.64^{a}	2.00 ± 0.00^{abc}	3.05 ± 0.60^{ab}	3.03 ± 0.95^{bc}

Means not connected by the same superscript in the same column are significantly different at 5% probability level.

* Medium containing 1 g/l activated charcoal.

Table 12. Mean number and length of roots obtained on half MS medium containing different IAA concentrations with or without activated charcoal, mean values are indicated as ± SD

	Mean No. of roots/explant		Mean Length of main roots/explant (cm)	
IAA (mg/l)	MM106	Anna	MM106	Anna
0	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{f}
0.5	3.43 ± 0.82^{b}	2.58 ± 1.90^{b}	2.63 ± 0.15^{ab}	1.93 ± 1.29^{e}
1	3.68 ± 0.84^{ab}	4.03 ± 0.38^{ab}	3.53 ± 0.47^{ab}	3.10 ± 0.48^{cde}
1.5	3.60 ± 0.59^{ab}	3.68 ± 0.47^{b}	3.85 ± 0.69^{ab}	3.63 ± 1.15^{bcd}
2	2.93 ± 2.08^{b}	4.13 ± 0.69^{ab}	2.73 ± 0.62^{ab}	3.28 ± 0.40^{cde}
3	3.88 ± 0.39^{ab}	3.88 ± 0.63^{b}	3.30 ± 0.65^{ab}	3.28 ± 0.61^{cde}
6	2.65 ± 0.70^{b}	2.75 ± 0.65^{b}	2.35 ± 1.60^{b}	2.53 ± 0.54^{de}
0*	0.0 ± 0.0^{c}	0.0 ± 0.0	0.0 ± 0.0^{c}	0.0 ± 0.0^{f}
0.5*	3.45 ± 0.86^{b}	4.33 ± 0.96^{ab}	2.88 ± 0.38^{ab}	2.45 ± 0.33^{e}
1*	5.70 ± 0.69^{a}	4.83 ± 1.33^{ab}	4.25 ± 0.49^{a}	4.93 ± 0.33^{ab}
1.5*	4.00 ± 0.57^{ab}	4.78 ± 0.66^{ab}	4.00 ± 1.09^{ab}	5.58 ± 0.36^{a}
2*	3.63 ± 0.48^{ab}	6.25 ± 0.50^{a}	3.65 ± 0.81^{ab}	4.95 ± 0.87^{ab}
3*	3.30 ± 1.28^{b}	3.83 ± 0.99^{b}	3.70 ± 0.54^{ab}	4.48 ± 0.61^{abc}
6*	3.28 ± 0.75^{b}	3.75 ± 1.31^{b}	3.18 ± 0.92^{ab}	3.83 ± 0.24^{bcd}

Means not connected by the same superscript in the same column are significantly different at 5% probability level.

* Medium containing 1 g/l activated charcoal.

Table 13. ANOVA for number and length of roots in different concentrations of IBA and IAA with or without activated charcoal

Source of variation	DF	SS	MS	F	P>F
Root Number					
Trt. (IBA)	13	117.10	9.01	16.14	<0.0001**
Variety	1	0.29	0.29	0.52	0.4730
Trts.* Var.	13	5.36	0.41	0.74	0.7197
Error	81	45.20	0.56		
Length of Roots					
Trt. (IBA)	13	211.15	16.24	19.74	<0.0001**
Variety	1	0.22	0.22	0.27	0.6039
Trts.* Var.	13	16.24	1.25	1.52	0.1285
Error	81	66.64	0.82		
Number of Roots					
Trt. (IAA)	13	245.12	18.86	24.63	<0.0001**
Variety	1	3.98	3.98	5.19	0.0253*
Trts.* Var.	13	24.17	1.86	2.43	0.0079*
Error	81	62.00	0.77		
Length of Roots					
Trt. (IAA)	13	226.03	17.39	38.68	<0.0001**
Variety	1	2.17	2.17	4.83	0.0308*
Trts.* Var.	13	13.71	1.05	2.35	0.0104*
Error	81	36.41	0.45		

* 5% and ** 1% significant levels.

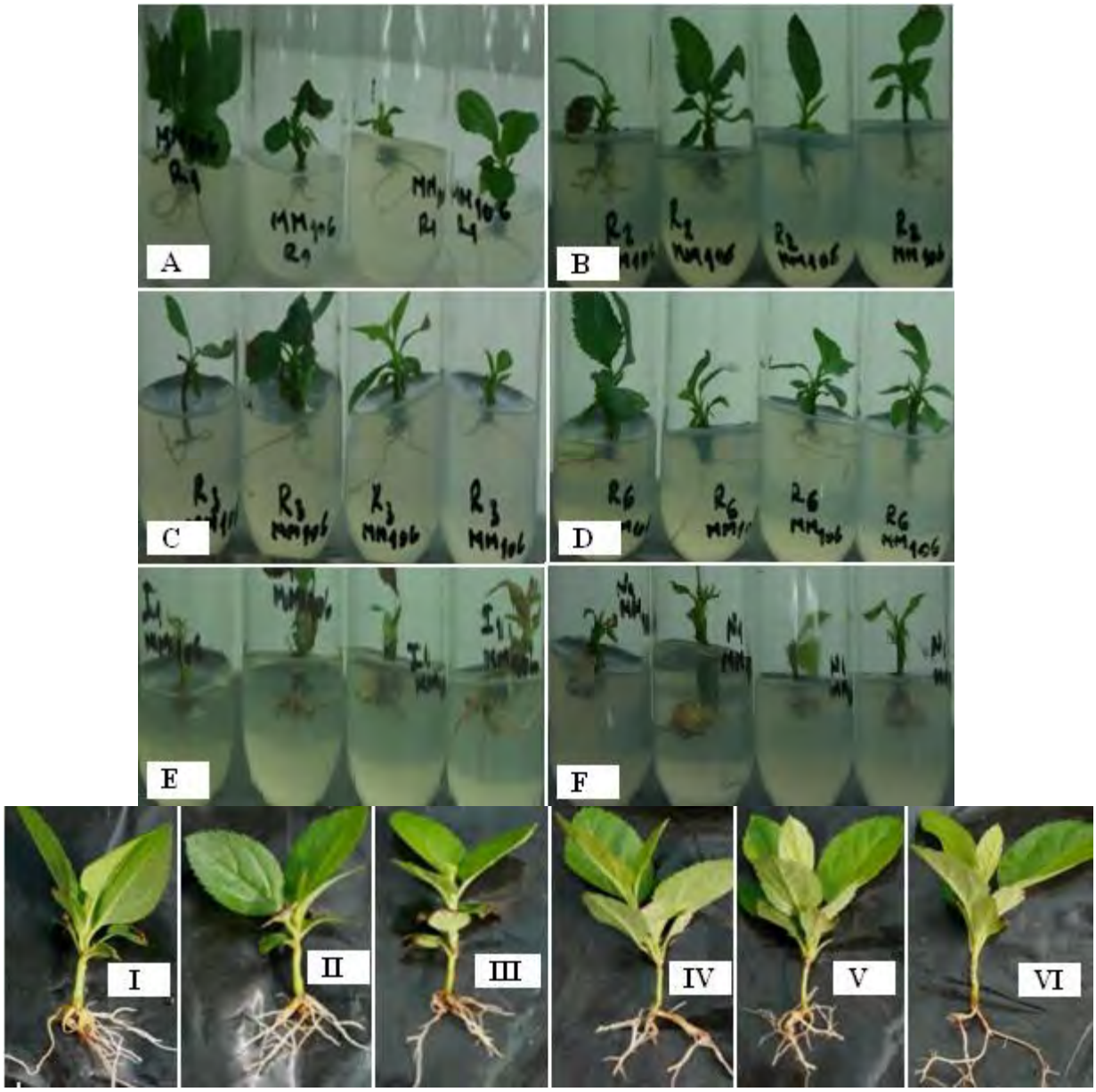

Figure 7. Rooting response of MM106 rootstock on half MS medium containing different concentrations of IBA and IAA. 1.5 mg/l IBA (A), 2 mg/l IBA (B), 3 mg/l IBA (C), 6 mg/l IBA (D), 1 mg/l IAA (E), 6 mg/l IAA (E) without activated charcoal and 1 mg/l IAA (I, II and III), 1 mg/l IBA (IV, V and VI) with activated charcoal.

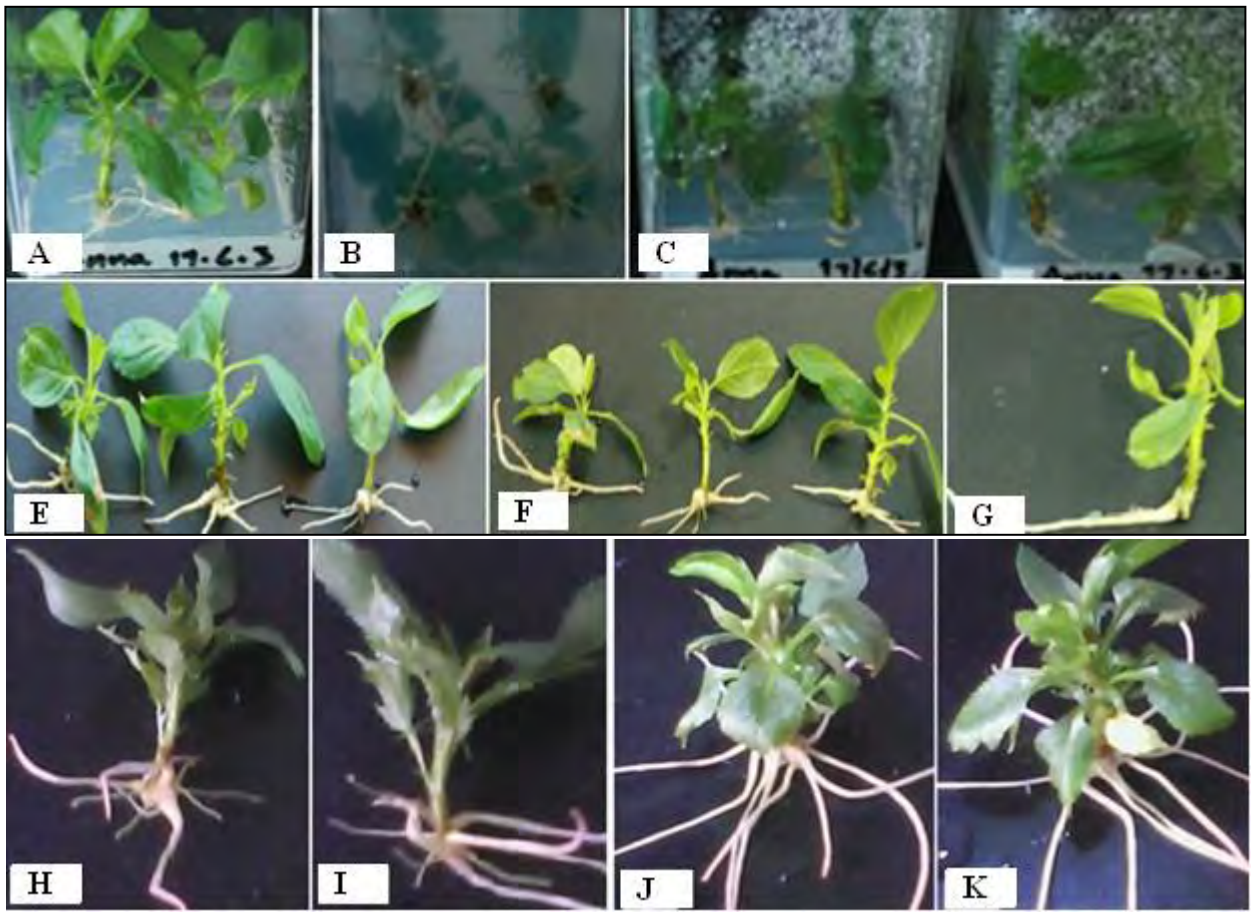

Figure 8. Rooting response of Anna scion on half MS medium containing different concentrations of IBA or IAA.2 mg/l IAA (A and B), 2 mg/l IBA (C) without activated charcoal and 1 mg/l IBA (E and F), 1.5 mg/l IBA (G), 1 mg/l IAA (H and I), 2 mg/l IAA (J and K) with activated charcoal.

5.4 Acclimatization

The plantlets which were planted on soil mix of red soil, compost and sand in the ratio of 2:1:1 were transferred to the glasshouse for hardening. After a month of growth in the glasshouse, the survival percentage was found to be 65.7% and 91.4% for Anna and MM106 varieties, respectively. Most of the dead Anna plantlets were due to fungal contamination and rotting. None of the shoots acclimatized on sterile soil mix without plastic bag covers and on unsterilized soil mix were survived. Further observations on the already acclimatized plantlets in the glasshouse show no aberrant phenotype.

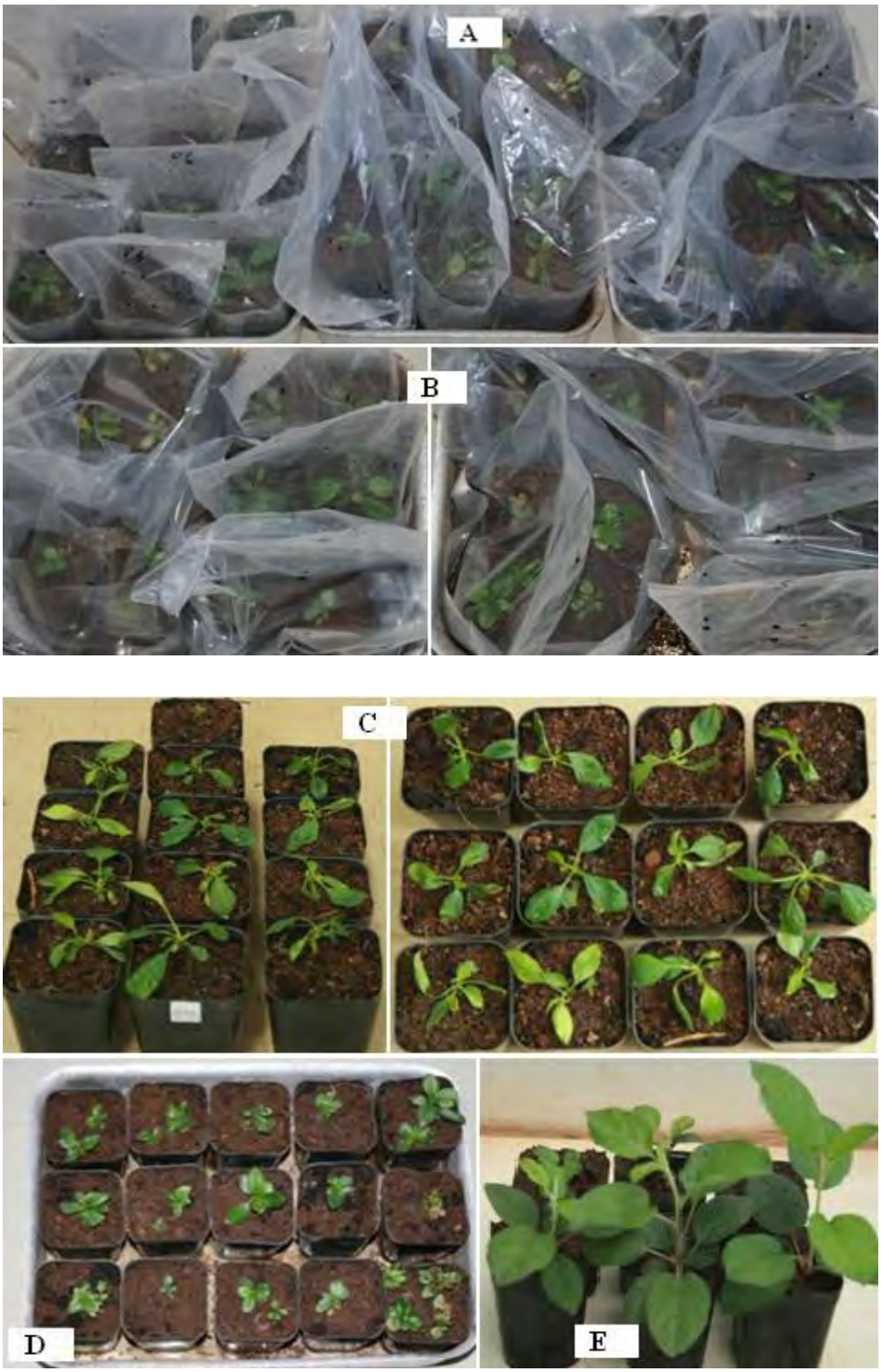

Figure 9. Acclimatized plantlets in the glasshouse after a week of acclimatization MM106 (A) and Anna (B), after two weeks of development in the glasshouse Anna (C) and MM106 (D) and after 45 days of growth in the greenhouse (E).

6. DISCUSSION

6.1 Culture initiation

From this study, it was observed that, cultured shoot tips gave varying responses to all of the culture media compositions except the control (without plant growth regulators). From the observed results, 96.7% MM106 and 93.3% Anna cultured shoots survived on the initiation medium supplemented with 1.0 and 1.5 mg/l BAP combined with 0.1 mg/l IBA, respectively. These initiated shoots were with healthy morphology and have sufficient number of leaves and shoot height which facilitates their growth. This result is obtained due to combined effect of different factors. The first one is the hormonal composition of the medium. Dalal *et al*. (2006) reported that, 1.0 mg/l BAP combined with 0.1 mg/l IBA is best for *in vitro* culture initiation of the other apple root stock, M9.

Moreover, the addition of 1 g/l activated charcoal and dark treatment for few days is highly important for the adsorption and reduction of phenolic compounds released from the cultured explants which otherwise inhibit growth of them. As indicated by Hartman *et al*. (2004), release of these compounds is common in tree plants. Dark treatment of initiated shoots for few days reduces the release of phenolic compounds from the explants. Moreover, transfer of the cultured shoots to new fresh medium is important to reduce the risk of death of explants. This result is also in consistent with the work of Dalal *et al*. (2006).

Cultured shoots were found to grow fast after being transferred to activated charcoal free medium of the same composition. This can be due to the absence of the negative effect of activated charcoal by adsorbing PGRs and lowering pH of the medium. This observation is also in agreement with the work of Tileye Feyissa *et al*. (2005) while he is initiating the multipurpose woody plant *Hagenia abyssinica*.

However, at relatively high concentration of BAP (6mg/l) combined with 0.1mg/l IBA the explants of both varieties were producing callus at their shoot tips. In line with this result, Caboni *et al.* (2000) reported the induction of callus from shoot apices of other apple varieties on media containing high concentration of BAP and NAA (17.8 µM BA and 2.7 µM NAA).

6.2 Effect of different growth regulators on shoot multiplication

In the present study, full strength MS medium containing different types and concentrations of PGRs have been used to assess the multiplication potential of the already initiated shoots of the two apple varieties. From all the combinations, the maximum numbers of shoots for the two varieties were counted in hormonal composition of BAP, IBA and GA_3. Dalal *et al*. (2006), for *in vitro* multiplication of *Malus domestica* Borkh rootstock variety M9 using shoot tip explants, they obtained 6.8 ± 0.80 mean number of shoots in MS medium supplemented with 2.22 µM BAP, 0.49 µM IBA and 2.32 µM Kn (kinetin). However, in this study, 6.33 ± 0.50 for MM106 and 4.93 ± 0.49 for Anna mean number of shoots were obtained on MS medium containing 1.5 mg/l BAP, 0.01 mg/l IBA and 0.5 mg/l GA_3, and 2.0 mg/l BAP, 0.01 mg/l IBA and 0.5 mg/l GA_3, respectively.

When the multiplication potential of the two apple varieties was compared on other media compositions, relatively higher number of shoots was obtained when BAP was used alone rather than combining it with IBA. The reduced number of shoots in the combination of BAP and IBA may attribute to the inhibitory effect of IBA for shoot multiplication. In line with this, Hartman *et al*. (2004) suggested that, auxins should be either completely absent, or used in small concentration in shoot multiplication medium.

High concentration of BAP (6 mg/l) in the multiplication medium had suppressing effect on the growth of shoots. As a result, on one hand, shoots of MM106 were found to be very dwarf and bushy and, that of Anna were found to be very dwarf and with folded, scaly like and easily fragile leaves on the other hand (Figure 5). This result is in agreement with other literature reports (Banilas and Korkas, 2005). Dunsta *et al*. (1985) during *in vitro* propagation of the apple root stock M4 also reported that, increased concentration of BAP in multiplication medium produced deteriorated and vitrified shoots.

Significant difference in the multiplication rate of the two varieties was observed in all media compositions at 0.05 probability level. In the case of the variety MM106, there was no significant difference observed within and among the multiplication media supplemented with BAP alone and, that of BAP and IBA together (Tables 3 and 4). However, the multiplication rate was significantly affected at different concentrations of the combination of the three hormones

BAP, IBA and GA_3 (Table 5). To the contrary, the Anna variety showed significant difference on the multiplication medium supplemented with different concentrations of BAP alone (Table 3). But it showed least significance difference when BAP was combined with IBA, and both IBA and GA3 (Tables 4 and 5). This may be due to the difference in the internal hormone content of the two varieties. Dalal *et al*. (2006) also reported similar result while *in vitro* multiplying different apple rootstock varieties.

6.2.1 Effect of sub-culturing stage on multiplication potential

The multiplication potential of the two apple varieties was assessed up to the 5^{th} sub-culturing stage of multiplication. The media compositions 1.5 mg/l BAP in combination with 0.01 mg/l IBA and 0.5 mg/l GA_3 for MM106, and 2.0 mg/l BAP, 0.01 mg/l IBA and 0.5 mg/l GA_3 for the Anna variety were used for this purpose. These media compositions were selected based on the higher multiplication potential revealed during the first multiplication step.

The results of this experiment showed that sub-culturing significantly affected the multiplication potential of the two apple varieties. The multiplication potential of the shoots do not show regular pattern. However, the fifth sub-culturing stage of MM106 produced 8.70 ± 2.77 mean number of shoots which was the highest from the other sub-culturing stages (Table 7). Whereas, in the case of, Anna variety the second sub-culturing stage produced 7.63 ± 0.59 mean number of shoots (Table 8) which was the highest as compared to other sub-culturing stages. This variation in multiplication potential at different stages of sub-culturing is in harmony with other reports (Tileye Feyissa *et al*., 2005; Dalal *et al*., 2006).

This repeated sub-culturing and determination of the stage where relatively higher number of multiplied shoots is obtained is very crucial for mass propagation of woody plants like apple in which *in vitro* culture initiation from shoot tip explants is highly difficult (Webster and Jones, 1989).

6.3 Rooting

To assess the rooting response of the two apple varieties, half MS medium containing IBA or IAA at different concentrations in the presence or absence of activated charcoal was used. Half MS medium is recommended by different authors for rooting *in vitro* (Dalal *et al*., 2006; Hartman *et al*., 2004). George (1996) also reported that use of activated charcoal in rooting medium enhances rooting. Transferring the cultured shoots on rooting medium to dark was better for root initiation. Related to this result, there are many reports that, different apple cultivars readily show root initiation when the cultured shoots are placed in dark for few days in a medium supplemented with IBA (Welander, 1985; Marin and Marin, 1998 and Zimmerman and Fordham, 1985; Sharma *et al*., 2007).

The two varieties responded differently to different concentrations of IBA or IAA. IBA is reported to be most effective for root induction *in vitro* (Dalal *et al*., 2006; Sharma *et al*., 2007). In the present study, however, from the comparison made between the rooting hormones IAA and IBA, IAA was more effective to increase root number in both varieties. IBA relatively increased the root length. From the ANOVA (Table 13) it was observed that, IAA significantly affected length and number of roots in both varieties, but not IBA, at 5% probability level.

6.4 Acclimatization

The plantlets produced *in vitro* are highly susceptible to the *ex vitro* condition until they develop adaptation mechanisms to best cope the stress up. The observation made for the two varieties after a month of growth in the green house revealed higher survival percentage for the root stock variety, MM106 than the scion variety, Anna. This may be due to the higher rooting potential and adaptability character of root stock varieties than scions. Supporting this result, Webster (2002) reported that, apple rootstocks have better rooting efficiency and different traits which make them adaptable to *ex vitro* condition than scions.

7. CONCLUSION

Based on the results of the present study, the following conclusions are made:

- Use of activated charcoal and placing the cultures for few days in dark together with transferring them to new fresh medium after two or three days of culture, until production of phenolic compounds cease or reduced to the minimum possible, is important to reduce the risk of death of cultured shoot tip explants of the two apple varieties.
- Shoot initiation was optimal on MS medium containing 1.0 and 1.5 mg/l BAP combined with 0.1 mg/l IBA for MM106 and Anna varieties, respectively. Slight increase or decrease in hormonal concentration can also initiate shoots of both varieties to a reduced level, supra-optimal PGR concentrations produced callus, however.
- Relatively higher number of multiplied shoots with best morphology was obtained in a medium containing the combination of BAP, IBA and GA_3.
- Though the multiplication potential of the two apple varieties do not follow regular pattern with sub-culturing stage, the fifth and the second sub-culturing stages produced higher number of multiplied shoots for MM106 and Anna varieties respectively.
- Both varieties have better number of roots when the shoots were supplemented with IAA combined with activated charcoal (AC). But root length was observed relatively to be longer in IBA without AC for the variety MM106 and when IBA is combined with AC for Anna.
- The root stock variety, MM106, has better adaptation potential than the scion, Anna, to external environmental conditions during acclimatization.

8. RECOMMENDATIONS

Future perspectives, based on the present study, should focus on the following areas:

A. The effect of other phenolic compound adsorbing chemicals like polyvinylpyrrolidone (PVP) on initiation potential of the explants should be investigated with respect to activated charcoal.
B. Determining the genetic stability using molecular markers whether repeated sub-culturing stage affected stability of cultures or not should be assessed.
C. Rooting response of multiplied shoots at each sub-culturing stage should be investigated.
D. Developing *in vitro* grafting protocol by using already micropropagated rootstock and scion varieties should be carried out.
E. Use of the already developed micropropagation protocol for large scale production of the two apple varieties and distributing them to farmers to increase apple fruit production in Ethiopia should be given due attention.
F. Research on further optimization of acclimatization should be done particularly for Anna variety to increase the survival percentage during acclimatization.

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APPENDIX

Appendix 1: Full MS basal medium stock solution composition

Components	Concentration(g/l)
Inorganic nutrients	
Macronutrients	
NH_4NO_3	16.5
KNO_3	19.0
$CaCl_2.2H_2O$	4.4
$MgSO_4.7H_2O$	3.7
KH_2PO_4	1.7
Micronutrients	
Fe-Na-EDTA	4.0
$ZnSO_4.7H_2O$	0.86
H_3BO_3	0.62
$MnSO_4.4H_2O$	2.23
$CuSO_4.5H_2O$	0.0025
KI	0.083
$NaMoO_4.2H_2O$	0.025
$CoCl_2.6H_2O$	0.0025
Vitamins and other organic supplements	
Myo-inositol	1.0
Nicotinic acid (NaOH)	0.05
Pyridoxine.HCl (B_6)	0.05
Thiamin.HCl(B_1)	0.01
Glycin (glycocoll)	0.2
Sucrose	30

Declaration

I, the undersigned, declare that this thesis is my original work and has not been presented for any degree in any other University. All sources of materials used for the thesis have been accordingly acknowledged.

Name: Demsachew Guadie

Signature _____

Date _____