In vitro regeneration of avocado (*Persea americana*): West Indian rootstock cv.lula.

By

Hiba Abed Al-Rahman Qasrawi

Supervisor:
Dr: Naseer sholi

This thesis was submitted in partial Fulfillment of the Requirements For the Master's Degree of Science in: Agricultural Biotechnology.

Deanship of Graduate Studies

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Palestinian Technical University-Kadoorie جامعة فلسطين التقنية –خضوري

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جامعة فلسطين التقنية – خضوري

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This thesis/dissertation "In vitro Micropropagation of avocado (*Persea americana*) West Indian rootstock"

Examination Committee	Signature
- **Dr. NasserSholi, (Supervisor)**	**……**
- **Dr. Hassan Abu Qaood (External Examiner)**	**……**
- **Dr. Mazen Salman (Internal Examiner)**	**……**

DEDICATION

To my mother,

She is the person with a strong and gentle soul, the only one who taught me to believe in myself. I will always be thankful for your continuous support, care and motivation. You have put in me the trust that I put in God as well.

To my father,

Thank you for doing the impossible to make me what I'm today.

Without your encouragement and support I wouldn’t have been able to do this.

To my friends,

Thank you for always being next to me, answering my calls whenever I needed even in midnights. You were very inspiring and motivating.

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Thanks go to National Agriculture Research Center (NARC) and AAUJ for conducting some of the experimental parts in their labs.

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In vitro regeneration of avocado (*Persea americana*): West Indian rootstock cv.lula

By

Hiba Qasrawi

Abstract:

Avocado *(Persea americana Mill)* is subtropical fruit. As it is economically important from the sense that it has a strong cultivation potential especially in Tulkarem. It is also essential to mention that there are many stressors reflect how sensitive avocado. To be more specific occupation is one of the main stressors that plays a huge role in controlling this type of fruit.

Another stressor is important to be mentioned is that present rootstock used by farmers are obtained via seed.

The main objective of this work was to standardize an useful and applicable protocol for micropropagation of West Indian rootstock of avocado (The one used by Palestinians framers).

Axillary bud and apical meristems were used as explants to micropropagate West Indian rootstock. Explants were cultured either in woody plant or Murashing and skoog salt mixture with different combination of cytokines (6-Benzylaminopurin(BAP), TDZ and Kinetin) while rooting was carried out in WP medium supplemented with different concentration of IBA.

Axillary bud gave the best result in term of the maximum number of shoots when compared to apical meristem in all treatments regardless of the medium type. Highest shoot induction in Woody Plant Medium was better than MS medium regardless of the explant type. Explant responded better in BAP containing medium than TDZ and Kn. The maximum number of shoots obtained from axillary bud was 4 shoots/explant when cultured in WP medium supplemented with BAP (1.5mg/l).When apical meristems were used as explant, the best result obtained was 2.5 shoots/explant cultured in WP medium supplemented with BAP (1.5mg/l). For shoot length, axillary bud c

ultured in BAP containing medium was found to be superior to apical meristem regardless of medium type. The maximum shoot length (4.8, 3 cm) was obtained from axillary bud cultured in WP media supplemented with BAP (1.5 and 1mg/l), respectively.

For rooting, the best number of roots (3.2 roots/explants) was obtained in MS medium supplemented with IBA (1.0 mg/l).

ملخص بالعربية

الأفوكادو: تعتبرفاكهة الأفوكادو من محاصيل الثمار الرئيسية في البلدان المدارية وشبه المدارية.
+ومن منظور اقتصادي تعتبر هذه الفاكهة مصدر مُدر للدخل نظراً لقيمتها الغذائية العالية.
في فلسطين،يتم زراعة فاكهة الأفوكادو في محافظتي طولكرم وقلقيلية مع إمكانية زيادة مساحة هذه المناطق المزروعة.
نظراً لحساسية فاكهة الأفوكادو وإمكانية تعرضها للإنهاك الحيوي وغير الحيوي بفعل عوامل الإجهاد البيئي، فإنه من الضروري تركيبها على أصول مقاومه من أجل زيادة مقاومتها والتخفيف من تأثير عوامل الإجهاد البيئي عليها.
عادة ما يتم الحصول على الاصول التي يستخدمها المزارعون عن طريق البذور التي تكون عرضة للتغير الوراثي إضافة إلى تحكم الجانب الإسرائيلي بتوريدها حيث هو المصدر الوحيد لها.لذلك تقدم تقنية زراعة الأنسجة النباتية بعطي بديلاً لحل هذه المشكلة. حيث أن الهدف الرئيسي من هذا العمل هو إيجاد بروتوكول فعّال وقابلا لتطبيق من أجل ازراعة أنسجة أصول الافوكادو (West Indian) المُستخدمة بشكل رئيسي من قبل المزارعين الفلسطينيين.
تم استخدام كل من البرعم الجانبي و القمم النامية في التجربة وزراعتها في أوساط مختلفة (WP, MS) مع تراكيز مختلفة من الهرمونات (BAP, Kin and TDZ)، بينما تمت عملية التجذير في وسط WP وكان مُدعم ب تراكيز مختلفة من ال IBA.
تم الحصول على الحد الأقصى من shoots من البُرعم الإبطي وذلك بمعدل 2.5 لكل عملية استنبات تمت في وسط WP مُدعم ب 1.5ملغم/لترمن الBAP. وعندما تم استخدام القمم النامية ،كان الحد الأقصى ل shoots التي تم الحصول عليها هو 2.5 في وسط WP مُدعم ب 1.5 ملغم/لتر من هرمون الBAP ، بينما تم الحصول على أطول shoot (4.8 سم) عندما تم استخدام البرعم الطرفي في وسط WP مُدعم ب1.0ملغم/لترمن هرمون الBAP. أما بالنسبة لعملية التجذير، تم الحصول على أكبر عدد من الجذور بمعدل 3.2 جذور في وسط WP مُدعم ب1.5ملغم/لترمن ال IBA.

Abbreviations:

Word or sentence	Abbreviation
And others	*et al*.,
6-Benzylaminopurine	BAP/BA
Degree centigrade	C
2,4-Dichlorophenoxyacetic acid	2,4-D
Gamborg *et al*. salt mixture	B5
Indole-3-butyric acid	IBA
6-Furfurylaminopurine	Kinetin
Liter	L
Lloyd & McCown, salt mixture	WP
Milligram	Mg
Milliliter	Ml
Molar	M
Murashige and Skoog salt mixture	MS
Percent	%
Plant growth regulator	PGR
Sterilized Distilled Water	SDW

Chapter One:

Introduction

1. Introduction:

Avocado (*Persea Americana* Mill.) is a major fruit crop of tropics and subtropics, which is highly important from economic point of view with a high nutritional value. Rootstocks type has a great effect on tree development, productivity, and health. It affects tree health, vigor, disease susceptibility and yield (Ben-Ya'acov et al., 2003).

In Palestine, Avocado is mainly cultivated in Tulkarm and Qalqilia area, where the climate is suitable. It is a growing industry. So far only 220 dunums have been cultivated in Tulkarm governorate, where production reaches to 880kg which is more profitable than other crops. In Qalqilia governorate only100 dunums have been cultivated.

Unfortunately, in Palestine, this industry is being based on avocado rootstock namely West Indian type which has been found to be suitable in our soil conditions. These seeds are only brought from Israel as it's the only supplying source, which limits the expansion and will make it more vulnerable in the future.

Most of the rootstock trees are being propagated from seeds; some are very slow to come into production, while some never produce seeds. Seedlings produced from these seeds have been subjected to genetic variation and variable in quality. In addition to that, they usually vary in their degree of tolerance. Unfortunately, the cost of producing clonal rootstocks propagated true to type is high when compared to seedling produced from seeds. The invasion by *Phytophthora cinnamomi*, is the major problems in avocado industry lead to initial investment in clonal rootstocks production.

Many of commercial varieties of avocado are sensitive to either biotic and/ or a biotic stress; to overcome these problems, grafting on rootstocks which is biotic and a biotic stress tolerance was the perfect choice and was first adopted by many farmers. However, These rootstocks were micropropagated through cutting which is laborious, time consuming and in many cases meet with low success. Advancement in biotechnology and mainly in plant tissue culture techniques paved the way for mass scale propagation of many true to type plant including

avocado and avocado rootstocks.

There are few attempts to micropropagate avocado rootstocks. It is complex and is being either carried out mainly by axillary bud (Castro, et al., 1996) or zygotic embryos (Barrera-Guerraetal, 1998, Fuentes et al, 2004) and Somatic hybridization (Richard, 1996).

In our study, the main **goal** was to standardize a reliable and efficient regeneration protocol for Avocado rootstock “West Indian" with the following **objectives:**

1- To optimize a suitable medium and optimal concentration of growth regulators for aseptic culture initiation, shooting, and rooting of avocado rootstock "West Indian"

2- To establish a regeneration protocol for genetic improvement

Chapter Two:

Literature review

2.1 The Avocado

2.1.1 Health Benefits of Avocado

Avocado is a unique fruit. While most fruit consists primarily of carbohydrate; Avocado fruit consists of healthy fats. Numerous studies showed that it has powerful health benefits. According to Bergh, (1991), avocado is a nutrient dense fruit providing four important minerals (iron, magnesium, potassium and copper) and seven essential vitamins (vitamins A, C, E and B6, folacin, niacin and pantothenic acid) in an approximately 2:1 calorie ratio. It is rich in fiber (Gallo-Llobet et al., 1999.), carotenoids lutein and zeaxanthin, (Hutchinson, 1984). Gomez et al. (1994) showed that they're linked to a drastically reduced risk of cataracts and macular degeneration, which are common in older adults (Table1).

Table1: Nutrient composition of edible pulp of' Fuertes' avocado per 100g

Component	Amount
Water	74.0 g
Energy component	
Protein	2.2 g
Lipid	170 g
Carbohydrate	6.0 g
Fiber	1.5g
Vitamins(mg)	14.00 mg
Thiamine.	0.11mg
Riboflavin	0.20 mgNiacin
1.60 mg	
Mineral (mg),	
Calcium	10.0mg
Phosphorus	42.0mg
Iron	0.6 mg
Sodium	4.0mg
Potassium	604.0 mg

Source: Scora and Wolstenholme (1998).

2.1.2 Avocado rootstocks

Rootstocks of avocado are often selected for dwarf size (Barrera-Guerra, et al., 1998) adaptation to alkaline soil and salt tolerance and in addition, pest and disease resistance (Bergh, 1975). The overall productivity of the tree is determined by scion and rootstock including fruit size, quality and fruit set. The types of rootstocks used in avocado industry are either obtained via seed or clonally propagated from selected cuttings with desired characteristics. The type of rootstock being used has great effect on the field performance and ultimately the harvest of the plant (Bergh & strand, 1986).

2.1.3 Origin of various rootstocks

Rootstocks research was started in California where they concentrated on productivity as influenced by Mexican and Guatemalan rootstocks (Halma, 1954). Due to the appearance of *Phytophthora cinnamomi* which is the main causal agent of avocado tree decline in 1942; scientist directed their work at discovering rootstocks resistant or tolerant to this disease. The search for a root rot resistant rootstock was gone well in California when the cultivar Duke was discovered in 1951(Zentmyer, 1972). However it was found that it is not tolerant to *P.cinnamomi.*

Other close related species like *Perseabaronial* (L.) *K. Spreng*and *Persea skutchii* Mez. are species from the Persea subgenus Eriodaphne, but was selected mainly due to its cold tolerance, a trait inherited from its Mexican parentage (Zentmyer etal., 1963) and it is a very significant characteristic in California. The first selection made from Duke Seedlings that were screened *in P. cinnamomi* infested soil was named Duke 6 (Zentmyer & Thorn, 1956). The following year Duke7 was discovered and selected. This selection seemed to perform better than Duke 6 which often appeared chlorotic (Zentmyer, 1977). Barr Duke was a third generation seedling derived from Duke 6 and was found to have an outstanding performance in a severe root rot situation as well as having dwarfing characteristics (Coffey, 1987b).The selection D9 was the result of Duke budwood being subjected to gamma radiation. Although a high level of root rot resistance exists in this selection, it does not propagate as easily as the other Duke rootstocks (Zentmyer&Schieber, 1982, Menge etal., 1992).The G6 clone is the result of bud wood collected in 1971 from a tree in Guatemala. Seed from this tree was also planted and selectionG6 number one was made. However, the parent selection G6 performed better with regard to *P. cinnamomi* resistance. Although G6 originated in Guatemala, it belongs to the Mexican race (Du Plooy, 1991). Another series of rootstocks, consisting of the G755A, G755B and G755C cultivars, originated from fruit collected at a native market in Coban,

Guatemalan, in September 1975. The fruit were said to be coyou that came from a village north-east of Coban (Zentmyer et al., 1988). "Coyou" or "Chucte" are names given by the Guatemalan natives to the fruit of *P. schiedeana*. This rootstock series was found to be more tolerant to *P. cinnamomi* than Duke 7 (Kotze, 1987). G755 have been named Martin Grande after Martin Cumes who died in 1981 (Zentmyer, 1988). Martin Grande establishes easily in severe root rot situations but does not perform well in California due to the cold winter temperatures (Coffey, 1987), and has a history of chlorosis were not expected (Kotze, 1987). Isozyme analysis confirmed in 1986 that the G755 series is a hybrid between *P. Americana* and *P. schiedeana* (Elstrand et al., 1986).No significant differences in tolerance between the three selections with regard to *P. cinnamomi* that could be detected (Coffey & Guillemet, 1987). Thomas was recovered in 1979 from a rootstock of a Fuerte tree growing in Escondido (Du Plooy, 1991). This tree survived in a root rot area where other trees had died, and is thus designated an escape tree. Thomas is a Mexican type and according to (Coffey& Guillemet, 1987) has resistance comparable to Martin Grande. Another Mexican type escape tree is Toro Canyon. Although little is known about this rootstock, Gabor & Coffey (1990) described it as having intermediate tolerance to *P. cinnamomi*, approximately the same as Duke.

Other escape trees include G1008, which belongs to a different Persea

species, *Persea steyermarkii* Allen, and the Parida-series. Little is known about these selections (Du Plooy, 1991).

2.1.4. Common rootstocks:

Guatemalan and Mexican seedlings were used as rootstocks during the first decades. In many cases scions on Guatemalan looked better, but in others they suffered from lime-induced chlorosis; as consequences, the use of Guatemalans was stopped. Then it was replaced by the Mexican cultivar 'Topa-Topa' and became the main source of seeds for rootstocks (Lahav and Lavi, 2002; Newett et al., 2002) It has many advantages mainly in the propagation processes. They were available and cheap, produced a relatively thick shoot and germinated uniformly. Thick shoots is very important when grafting is considered under protected conditions. The main disadvantage was its performance in the field was rarely investigated and did not provide any advantages for the grower. Meanwhile, 'Topa- Topa' is also susceptible to salinity and root rot (Newett et al., 2002)

In California, West Indian avocado rootstocks were very rarely used, although they are resist to salinity much better than the Mexican rootstocks when tested in Israel (Oppenheimer,1947) and Texas (Cooper1951,Cooperetal.1957).

2.2. Rootstocks propagation

Figur1.1: avocado rootstocks and its fruit.

Seedling rootstocks are obtained from seeds onto which the desired scion variety is grafted. Since these trees were obtained from seeds, they are subjected to genetic variation and therefore respond differently to a biotic and biotic stresses. Clonal rootstocks are easier to establish that seedling rootstocks especially if environmental stress occurs during initial phase (Rose,2003). Since seedling rootstock production is cheap, it is still commonly used in most avocado-producing countries (Ernst et al., 2013). Crossing, outcrossing and sexual recombination greatly affect rootstock genetic makeup, so ideally, seedling rootstocks must be derived from isolated (self-pollinated) trees (Crane et al.,2013).

While clonal rootstocks are genetically uniform. Yet, the grafting process is laborious, expensive, and a time for establishment in the field is longer (Whileyetal.,1990). Under standard management practices, clonal

rootstocks have greater consistency of yield (Whiley & Whiley, 2005). Clonal rootstocks selections with high tolerance to Phytophthora root rot are recommended for sites with high disease pressure. (Whiley & Whiley, 2011) recommend that proven seedling rootstocks be planted for new sites, but resistant clonal selections are used for replant sites. In Australia, new plantings of clonal rootstocks have increased from 2% in 1995 (Ben-Ya'acov &Michelson, 1995) to approximately 15% in recent years (Noguera et al., 2011).

2.3 Techniques of Micropropagation for Avocado

2.3.1 Shoot tip/ meristem culture.

Culturing the apical meristem tissue or small apical shoot tips from woody plant species is extremely challenging, but it is the most effective method for virus elimination in plant tissue culture (Barringer et al., 1996, Blickel etal., 1988). Beside virus elimination, regeneration through meristem has the advantage of maximizing regeneration rate which is highly favorable for mass propagation (Fuentes, 2003).

Typically, in meristem culture, the sample size is about 1-2mm or less in length with no visible leaf primordia for virus elimination (Hazarika, 2003). But with avocado, in order to overcome lower regeneration capacity, the meristematic dome is excised with one or two leaf primordia, while still minimizing the percentage of virus- infected plants.

To improve regeneration in avocado meristem culture, (Gomez, et al., 1994) used large shoot tips (3-8mm) with well-developed bracts and leaf primordia. However, shoot regeneration has been hindered by massive callus formed at the base of the tissue resulting poor shoot elongation and failure in root induction.

2.3.2 Vegetative axillary bud culture

Bud culture and shown to be more successful than shoot tip culture in most woody plant species. For this reason, they have been employed in avocado tissue culture for many years (Burtin, etal., 1990). Shoots obtained from axillary buds in Avocado have been reported to be associated with poor elongation but remain alive for longer, which limits the multiplication capacity through nodal segments. Nevertheless, nodal explants are a very reliable source of explants in terms of preserving genetic stability of elite cultivars due to high level of success with many cultivars; most of the avocado research protocols that have been reported are confined to nodal culture using both juvenile and mature material.

2.3.3 Regeneration from callus

This is an indirect plant regeneration approach in tissue culture usinga two- step process where callus is generated through dedifferentiation, then differentiated into an intact plant. Callus establishment in avocado have been successfully initiated from different types of explants: stem, leaf, flower, fruit mesocarp, peduncle and cotyledons (Vieitez et al, 1983). Living Avocado cells from any tissue are found to be responsive for cellular proliferation to produce amorphous calli masses (Moe & Andersen 1988). According to Schroeder,(1980) some avocado calli have survived in vitro for long time. This trait can be exploited for germplasm preservation if regeneration from callus could be achieved. Under the influence of plant growth regulators, cells from callus tissue can be induced to form pro-embryos or somatic embryos which can then be developed into intact plants (Fortanier& Jonkers, 1976) which has been possible with immature zygotic embryo tissues of Avocado (Zimmerman, 1984). Ahmed et al.(2003) have shown that hormone concentration greatly affect callus induction from avocado embryonic tissue depending on the minimum level being 2.0 mg/l of 6-benzyleaminopurine (BAP) or 2.0 mg/l naphthalene acetic acid (NAA). Meyer et al., (2004) has investigated the response of cotyledon tissue to cytokines to form callus and found that kinetin, BAP, zeatin and 6(ɤɤ-dimethylallyl-amino) purine at a concentration of 1mg/l in conjugation with 5mg/l indole-3-acetic acid

(IAA), to equally promote callus growth.

2.3.4 Regeneration of Avocado from cell and tissue culture

Genetic engineering of plant species can only be achieved if there is an efficient regeneration procedure from cell cultures with Avocado and other perennial tree species. This involves the reduction of the whole plant to a single cell that itself has the potential for regenerating the complete tree. Woody plants have traditionally been characterized as recalcitrant in this respect, and have been difficult to regenerate. However, a few important subtropical tree crop species have been regenerated, e.g., citrus (Rangaswamy, 1958) and mango from the nucellus (Litz et al, 1982), and the longan from leaves (Litz, 1988). Witjaksono (1997) reported that it is also possible to regenerate Avocado from the nucellus, a maternal tissue in the young developing seed.

2.3.5 Somatic embryogenesis:

Regeneration from embryogenic cultures is found to be very problematic in most woody species (Márquez-Martín et al., 2012).

Many researchers have used different source of explant to induce somatic embryogesis such as: immature zygotic embryos and nucellus (Mooney & van Staden, 1987, Pliego Alfaro & Murashige, 1988, Witjaksono & Litz, et al., 1999). Avocado propagation from somatic embryogesis is possible and success was achieved by Witjaksono et al., (1999a) when the

shoots that emerge from germinating somatic embryos are removed and micropropagated using standard procedures.

Somatic embryo loses their regeneration capacity very fast (Witjaksono & Litz, 1999b), due to incomplete maturation (Ammirato, 1987). Perán-Mineral salts, sucrose concentration, gellan gum, coconut water and abscisic acid affected the maturation of avocado SEs (Quesada et al.,(2004). Although the production of avocado SEs occurs with high efficiency, the percentage capcity is genotype-dependent and is low (Pliego-Alfaro & Murashige, 1988, Witjaksono & Litz, 1999b, Raharjo and Litz, 2003, Avenido et al., 2009). This low rate of SE conversion is currently the main problem in avocado regeneration via somatic embryogenesis (Litz et al., 2005). Avenido et al. (2009) showed that to obtain plants ready to transplant remained, time- consuming. Efendi, (2003) and Raharjo et al., (2008) were able to obtain genetically modified Avocado plantlets from SE cultures after transformation procedures but at extremely low percentages. Recently, (Palomo-Ríosetal. 2012) reported a culturing in liquid medium significant improvement in the germination rate of genetically transformed Avocado SEs; however, the conversion rate to mature plantlets remained low at 0.5–2%**.**

2.3.6 Protoplast culture.

Protoplasts were successfully isolated from Avocado mesophyll layers (Witjaksono et al., 1998, Witjaksono, 1997).

Chapter Three:

Materials and Methods

3.1 Chemicals and reagents for *in vitro* culture

Basal salts for plant growth, PGRs, and other reagents were purchased from Duchefa Biochemical and Sigma-Aldrich chemical companies. Woody Plant medium (Sigma Cat. No: 047k2329) (Lloyd and McCown, 1980 Murahige and Skoog (MS) medium (Murahige and Skoog, 1962) including all vitamins (Duchefa Prod. No: M0222.0050), were used in this study. The chemical composition of these media is listed in Table4.1.

Table 2:Basic composition of basal growth media used in this study; Lloyd & McCown (1980), Murashige and Skoog (1962).

All components expressed in mg/L	Lloyd & McCown Woody Medium(WP)	Murashige and Skoog (MS)
Ammonium Nitrate (NH_4NO_3)	644	5.424
Boric Acid(H_2BO_3)	4.5	4.5
Calcium Chloride ($CaCl_2$.2H2O)	14	664
Calcium Nitrate ($Ca(NO3)_2$.4H2O)	225	-
Cobalt Chloride ($CoCl2_6$.H2O)	-	4.452
Cupric Sulfate ($CuSO_4$.5H2O)	4.452	4.452
Magnesium Sulfate (MgSO4)	274	274
Manganese Sulfate ($MnSO_4$H2O)	55.2	54.1
Potassium Nitrate (KNO_3)	-	5.1444
Potassium Phosphate (KH_2PO_4)	574	574
Sodium Molybdate ($Na_2MoO_4.2H_2O$)	4.452	4.52
Zinc Sulfate ($ZnSO_4$7H2O)	1.4	1.4
Ferrous Sulfate ($FeSO_4$·7H2O)	57.1	57.1
Na_2-EDTA	27.2	27.2
Inositol	544	544
Nicotinic Acid	4.2	4.2
Pyridoxine-HCl	4.2	4.2
Thiamine-HCl	5.4	4.6
Agar	8	8

3.2 Media preparation and sterilization

Woody plant (WP) salts was used as a basal media for *in vitro* plant growth. The assigned weight was dissolved together with sucrose at 30g/l in deionized water. Growth regulators (in mg bases) were added according to the required amount. Finally, pH was adjusted to 5.8 with 1.0M NaOH or1.0 M HCl, and media was solidified with agar at 8.0 g/l. The media was poured into clean 250 ml magenta boxes (50 ml each) or in 1.5×15 cm test tubes were closed with caps. Media was sterilized by autoclaving at 121◦C and 115 psi for 20 min and then transferred to the media storage room where they kept till their further use.

3.3 Collection of plant materials

Explants of avocado (***Persea Americana***) West Indian rootstocks were collected from Al-Carmel greenhouse located near Jenin.

3.4 Surface sterilization

Explants were surface sterilized by washing thoroughly under running tap waterfor10min,then soaking with sterile distilled water (SDW) and few drops of tween 20 for 30 min. Aseptic solution of sodium hypochlorite (Clorox®) was prepared at final concentration of 1.5-2 %. Under the laminar air-flow cabinet, explants were rinsed with SDW for three times (2 min. each). Finally, a 70% ethanol solution was added to the explants

for 30 seconds then washed with SDW three times (2 min. each)

3.5Micropropagation

3.5.1Culture initiation and shoot regeneration

Apical meristem (0.4cm) and axillary bud explants (Fig 2, A and B) were cultured either on to WP or MS media with different hormonal concentration as shown in Table (3.2).

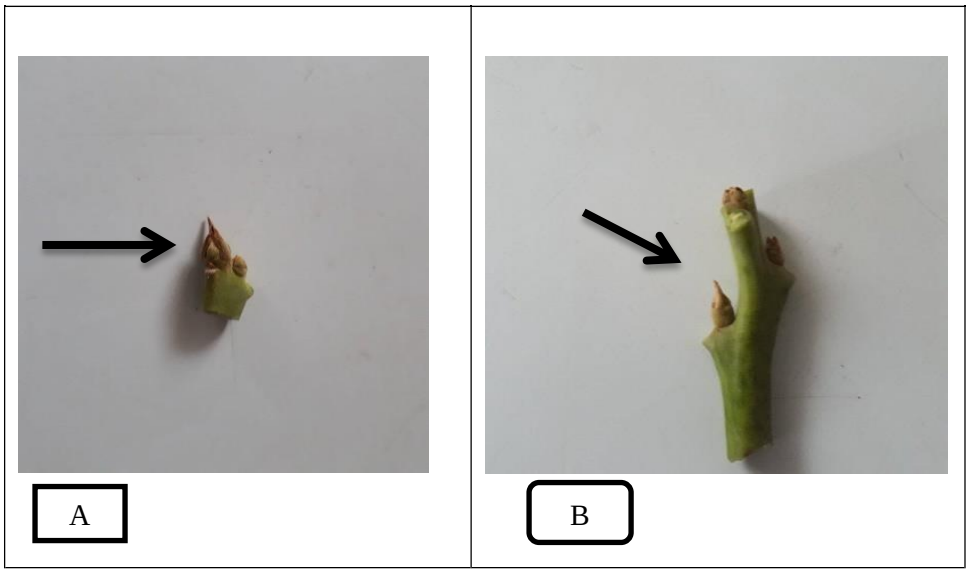


Figure 2: Explants type (A: apical meristerm, B: Axillary bud) used for micropropagation

treatments were arranged in complete randomized design (CRD). In each Petridish, 5 explants were inoculated and repeated three times. Data were reported after 4 weeks of subculture for the number of proliferated shoots and shoot length.

Table 3.: Woody plant medium supplemented with different hormonal concentration used in our study.

PGRs (mg/l)	WP	MS
TDZ	0, 0.5, 1, 1.5 and 2	0, 0.5, 1 ,1.5 and 2
Kn	0, 0.5, 1, 1.5 and 2	0, 0.5 ,1 ,1.5 and 2
BAP	0, 0.5 ,1 ,1.5 and 2	0, 0.5, 1, 1.5 and 2

Inoculated explants were cultured in growth room at 23-25 °C under cool fluorescence light with 2000 lox with 16/8 Light/dark photoperiod. The cultures were kept under these conditions throughout the experiment.

3.5.2. Rooting

Shoots with 4-5cm were transferred into WP with different concentration of IBA (0, 0.5, 1, 1.5, 2). Each treatment composed of three magenta boxes as replicates with three shoots per replicate. Data were reported after 6 weeks for the number of roots for each treatment.

3.5.3. Hardening

Plant with full root system were transferred into hardening medium (Petmoss) covered with nylon plastic for 1 week and kept under the same conditions as stated before. The plants were transferred later into plastic pot under open field conditions.

3.5.4. Statistical analysis

Each experiment were set up at completely randomized design (CRD). Data in each experiment was analyzed with the analysis of variance (ANOVA) using Sigma Plot version 11.0 (Inc. Sigma Plot for Windows).

Means were separated according to Fisher's least significant difference (LSD) taste at the p =0.05 level of probability. Sample numbers for each measurement are provided in the caption of related illustration.

Chapter Four:

Results and Discussion

4. Results and Discussions

The objectives of this study were to: (1) Establish a reliable and efficient protocol. (2) Optimize a suitable medium and optimal concentration of growth regulators for initiation, multiplication, and rooting,

(3) Establish a regeneration protocol for genetic improvement.

4. 1.Micropropagation

4.1.1 Culture initiation and shoot regeneration

For obtaining desired responses in tissue culture, the level of growth regulators and their concentration have to be carefully chosen. In shoot proliferation, high levels of cytokines are used to overcome the apical dominance of shoot and to enhance the branching of axillary bud. The proliferation of shoot culture was carried out by culturing either apical shoot meristem as explants. Explants were cultured on WP media with kinetin, TDZ, BAP and Kin at 0, 0.5, 1.0, 1.5, or 2mg/l.

In our study, in all treatments, Avocado showed a very limited shoot proliferation and elongation wither apical meristems or axillary buds are cultured without exogenous hormones. Castro, et al., (1995) stated that Avocado produce poor result when cultured without growth regulators regardless of the explants types used as a starting material.

The results of culturing explants (apical meristem and axillary buds) on

WP and MS media with different TDZ, BAP and Kin concentrations are shown in table 4.1. 4.2 and 4.3.

In general, the responses varied depending on the type of explant, media type and concentration of growth regulators used in the experiments. The effect of TDZ and explant type on shoot number and shoot length are presented in table 4.1.1 and 4.1.2

Table 4.1.1 and 4.1.2: Effect of TDZ concentrations and medium type on the average shoots number and average shoot length (cm) of (*Persea americana*) rootstock West Indian cv.lula

TDZ (mg/l)	WP			
	Axillary	Apical	Axillary	Apical
	Shoot #	Shoot #	Shoot length	Shoot length
T(0.0)	0d	0 c	0 c	0 c
T(0.5)	0.7 c	0.89 b	1.05 b	0.98 b
T(1.0)	2 a	1.55 a	1.88 a	1.5 a
T(1.5)	1.67 b	1.54 a	1.5 ab	1.44 a
T(2.0)	1.33 b	1 b	1.3 ab	1 b
Mean	1.14 a	0.996 a	1.146 a	0.984 a

TDZ (mg/l)	MS			
	Axillary	apical	Axillary	Apical
	Shoot #	Shoot #	Shoot length	Shoot length
T(0.0)	0 c	0 c	0 d	0 c
T(0.5)	0.7 b	0.6 b	0.2 c	0.6 b
T(1.0)	1.4 a	1.2 a	1.8 a	1.3 a
T(1.5)	1.1 ab	1.0a	1.6 a	1.2 a
T(2.0)	0.75 b	1.0a	1.3 ab	1.0
Mean	0.79 a	0.76 a	0.98 a	0.82 a

Any two means not sharing a letter significantly different at P<0.05

Thidiazuron (TDZ) is widely used for promoting shoot initiation for the micropropagation of some woody plants including species from the Ericaceae family such as lingonberry and blueberry, (Van Staden et al., 2008 and Sefasi et al. 2013).

Axillary bud gave better responses and produced more shoots when compared with apical meristem in all treatments. WP medium also produced better results when compared with MS medium (table 4.1).

Many studies showed that TDZ stimulates shoot regeneration at low concentrations. In the present study, lower concentration of TDZ (1 mg/l) was more effective in producing more shoot than higher concentration. The maximum number of shoots obtained was 2.0 shoots per explant (1 mg/l), followed by 1.67 shoots per explant (1.5mg /l) in WP. Meanwhile, the maximum number of shoots obtained from apical meristem was 1.55 (1 mg/l). Similar result was obtained by Yaseen et al., (1992) who found that culturing nodal explant with TDZ (1 mg/l) produced multiple shoot (5.8 shoots / explant). Similar results have been also reported by other studies, but the optimum level of TDZ varies among different species. Singh, in his study on *Psidium guajava*, found that the maximum percentage of shoot regeneration and maximum number of regenerated shoots were obtained by a treatment consisting of 1.0 µm TDZ (Singh *et al*. 2002). Also, in a study of factors affecting direct shoot regeneration of

pear, Yousef et al. (2014) found that the highest percent of shoot regeneration was achieved at 7.5 μm of TDZ. Furthermore, in contrary, found that direct shoot regeneration of *Primula heterochroma* was stimulated by a high TDZ concentration (2.0mg/l) more efficiently than lower concentrations (0.2-1.0 mg/l). It was observed that increasing concentration of growth regulators decrease the number of shoots in all treatments. On the other hand, the maximum shoot length was 1.88 cm and 1.8 cm obtained from axillary bud cultured in WP medium supplemented with 1 mg/l TDZ, respectively. (1mg/l).

Explant responded differently may be due to the activation of shoot multiplication signal (SMS) in different sites. SMS was identified as a branching factor in highly branched mutants of petunia (Snowden et al., (2005)). Conversely, the presence of higher levels of auxins inhibits the action of SMS and act as a shoot branching inhibitor. Hence, it may be possible that in apical buds, SMS act as a shoot branching inhibitor due to the presence of high endogenous levels of auxins. On the other hand, SMS associated with a lower concentration of auxin in axillary buds may act as a shoot branching factor.

Table 4.2: Effect of BAP concentrations and medium type on the average shoots number and average shoot length (cm) of (*Persea americana*) rootstock West Indian cv.lula

BAP (mg/l)	WP			
	Axillary Apical		Axillary	Apical
	#of shoot	Shoot #	Shoot length	Shoot length
T(0.0)	0 d	0	0 e	0 d
T(0.5)	1.8 c	1.5 b	1.2 d	1.5 b
T(1.0)	3 b	1.8 ab	3.8 b	1.75 ab
T(1.5)	4 a	2.5 a	4.8 a	2.42 a
T(2.0)	2.9 b	2 ab	2.73 c	1.1 c
Mean	2.34 a	1.56 b	2.506 a	1.354 b

Any two means not sharing a letter significantly different at P<0.05

BAP (mg/l)	MS			
	Axillary Apical		Axillary	Apical
	#of shoot	#of shoot	Shoot length(	Shoot length
T(0.0)	0 d	0 d	0 d	0 d
T(0.5)	1.7 c	1.3 c	1.43 c	1.3 c
T(1.0)	2.5 b	1.66 b	2.5 ab	2.43 a
T(1.5)	3.2 a	2.2 a	3:00 AM	1.76 b
T(2.0)	1.96 c	1.83 ab	2.73 a	1.63 bc
Mean	1.872 a	1.398 ab	1.932 a	1.424 ab

Any two means not sharing a letter significantly different at P<0.05

Axillary buds gave the better results than apical meristem in all treatments (table 4.2). The maximum number of shoots was 4.0 shoots/ explant at 1.5mg/l of BAP cultured in WP (1.5mg/l); while when cultured in MS medium, the maximum number of shoots was 3.2 at 1.5 mg /l of

BAP (1.0mg/l)**(** Fig. 2a & b). No shoots were obtained in control treatment (0.0mg/l) regardless of the type of the explants (0.0mg/l). The maximum shoots number obtained from apical meristem was 2.5 and 2.2 in WP and MS media, respectively (1.0mg/l). This shows that, BAP is necessary for shoot development and multiplication. The possible reasons of early response of axillary buds toward BAP might be that the axillary buds are rich in endogenous BAP so they show a better response at a relatively lower concentration of BAP (1mg /l). Cytokines is being synthesized by roots and trans located upward; therefore, axillary buds are rich in cytokines due to its presence at a relatively lower position on the mother plant. Treatments differed significantly with regards to their effects on shoot number. At very low concentration of BAP (0.5 mg/l) or at higher high concentration of BAP (2.0 mg/l), the shoot number was less than other concentration. Identical symptoms were recorded in *Gerenia jasmonoides* by (Chuenboonngarm et al., 2001 and Hu & Wang 1983). They stated that higher concentration of cytokines reduced the number of shoots during micropropagation process. Our results showed that, by increasing BAP concentration, the shoots number obtained from both explants (apical meristem and axillary buds) increased up to 1.5 mg/l, showing a positive relation between BAP and shoot number after which it starts declining with further increase in BAP concentration. Therefore, selection of proper concentration of plant growth regulator is

critical to shoot regeneration. The optimal concentration of cytokinin resulted in a marked increase not only in RNA but also in DNA and protein synthesis leading to initiation of shoot primordia (Mok & Mok, 2001). Our results showed that BAP (1.5 mg/l) produced the maximum number of shoot. They are in contrary with Ahmed et al., (1998) who found that BAP at 1 mg/l was optimum to produce the maximum number of shoot. Cooper, (1987) found that BAP (1 mg/l) produced zero shoot from Duke 7. Zirari and Lionaki, (1994) found that BAP (0.65 mg/l) produced 1.7 shoots from nodal culture of Fuerte cultivar. Martinez-Pacheco et al., (2010) found BAP (0.5 mg/l) produced 3-5 shoots from 'Drymifolia cultivar. In our study, BAP at 2 mg /l produced less number of shoots. In study conducted by Barrera-Guerra et al., (1998), no shoot were obtained from nodal explant when cultured on BAP (2 mg/l) containing medium. Contrary to Barceló-Muñoz et al. (1999) who found that BAP (2.2 mg/l) produced 3.2 shoots from Mexican IV-8 cultivar. Castro, et al (1995) obtained 3.1 shoots from Topa Topa at BAP (2 mg/l). This clearly showed that the response is genotype dependent.

The effect of Kin and explants type on shoot number and shoot length are presented in table 4.3

Table 4.3.1 and 4.3.2 Effect of Kin concentrations on the average shoots number and average shoot length (cm) of (*Persea americana*) rootstock West Indian.cv.lula

Kin (mg/l)	WP			
	Axillary Apical		Axillary Apical	
	Shoot #	Shoot #	Shoot length	Shoot length
T(0.0)	0 d	0 d	0.5 cd	0.3 bc
T(0.5)	0.95 c	1.05 c	0.85 c	0.7 b
T(1.0)	1.5 b	1.4 b	2.5 a	2 a
T(1.5)	2.55 a	1.45 b	1.44 b	1.8 a
T(2.0)	1.3b	1.8 a	1.2 b	1.77 a

Any two means not sharing a letter significantly different at P<0.05

Kin (mg/l)	MS			
	Axillary Apical		Axillary Apical	
	Shoot #	Shoot #	Shoot length	Shoot length
T(0.0)	0 c	0 c	0 d	0 c
T(0.5)	0.85 b	0.7 b	0.5 c	1 ab
T(1.0)	1.5 a	1.2 a	2.5 a	1.8 a
T(1.5)	1.5 a	1.3 a	2 ab	1.4 a
T(2.0)	0.85 b	1.3 a	1.2 b	1.2 ab

Any two means not sharing a letter significantly different at P<0.05

The maximum number of shoots obtained from axillary bud was higher than one obtained from apical meristem in both WP and MS medium. The maximum number of shoots was 2.55 shoots/ explant was recorded at 1.5 mg/l kinetin (1.5mg/l); while in apical bud maximum number of shoots (1.5) per proliferated explants was recorded at 1.0 and 1.5 mg/l (1.5mg/l). No shoot regeneration was obtained in control. This shows that Kinetin is necessary for shoot development and multiplication

as any other cytokines. The possible reason of early response of axillary buds toward Kin might be that the axillary buds are rich in endogenous Kin so they showed a better response at relatively concentration of 1.5 mg/l.

The maximum number of shoot length obtained was 2.5 cm from both axillary bud and apical meristem, respectively (1.0mg/l not 1.5mg/l)

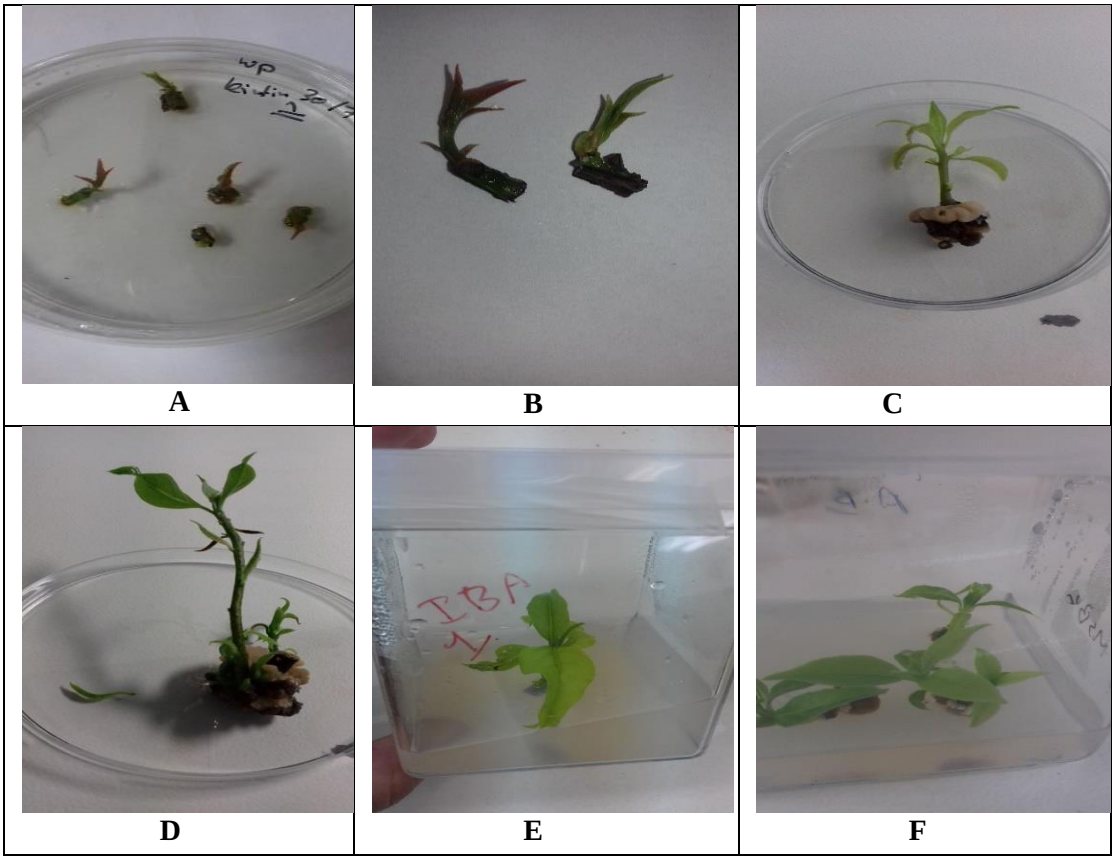


Fig 4.2: Stages of avocado rootstock regeneration via axillary bud cultured in WP medium supplemented with BAP (1.5 mg/l). A, B: regenerated shoot, C, D: elongated shoot. E, F: shoot in rooting medium with 1 mg/l IBA.

The results showing the best PGR concentration in producing the maximum number of shoots and maximum shoot length is presented in Fig 4.3

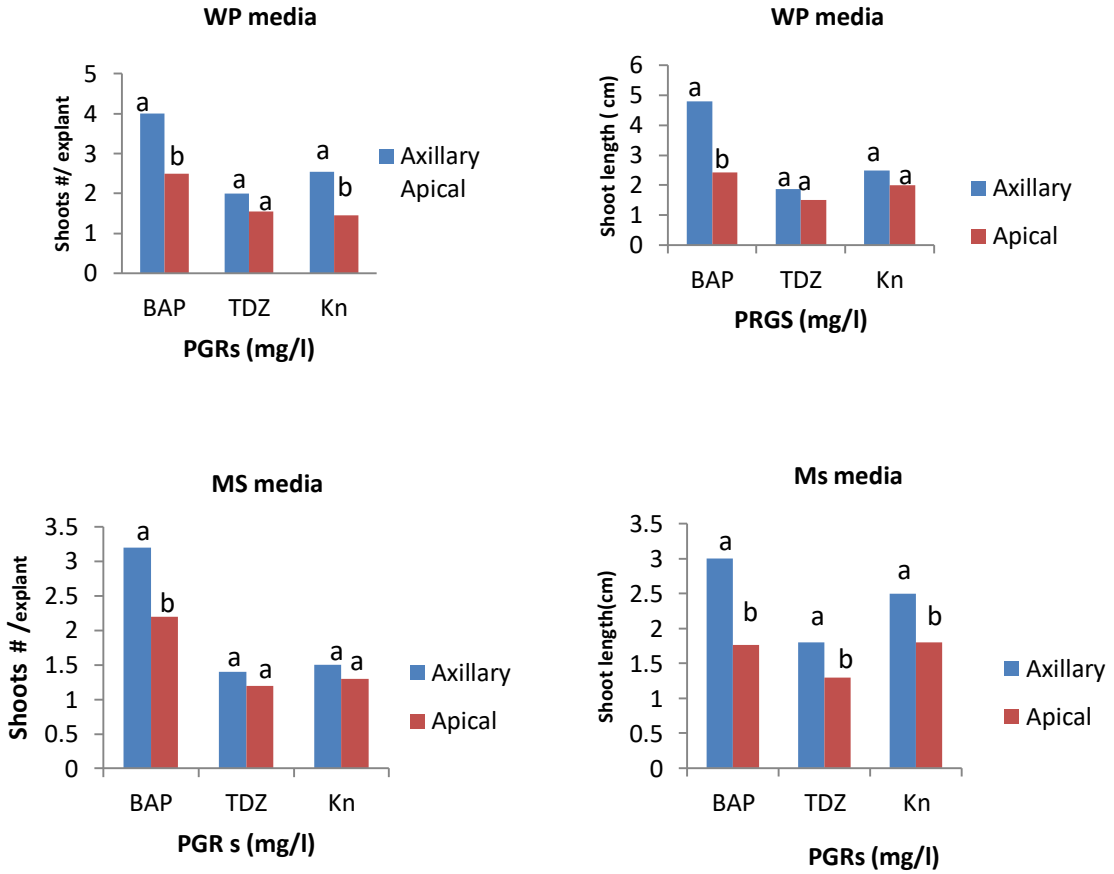
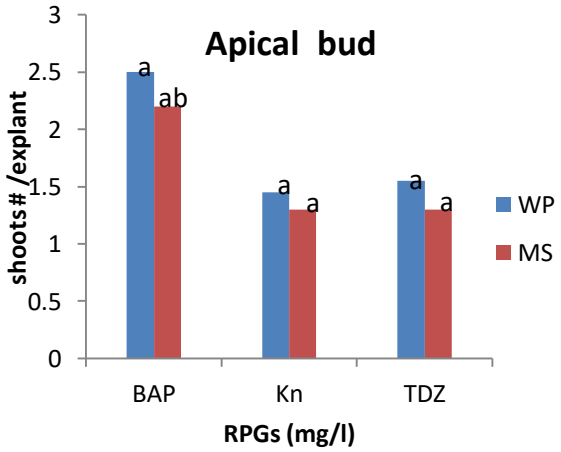


Figure 4.3: Mean of regenerated shoots and mean shoot length from explants (A, B) and (C, D) cultured on WP and MS medium respectively. BAP (1.5 mg/l), Kn (1.5 mg/l) and

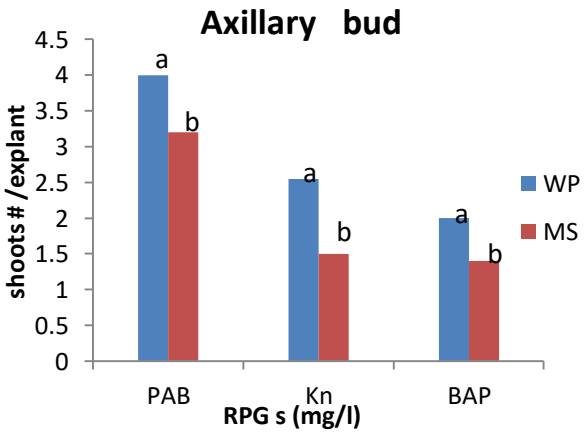
TDZ (1 mg/l). Bars with the similar letters are statistically similar at Fisher's test for mean comparison at P<0.05.

The results clearly showed that Axillary bud gave better response than apical meristem regardless of the medium type. BAP was the best choice for producing the maximum number of shoots and shoot length, followed by Kin. TDZ was the poorest one to produce shot and shoot length .and kinetin produce more shoot and shoot length. In fact, zeatin, benzyladenine (BA), kinetin, and N6-(2-isopentenyl) adenine (2-iP) are the most common cytokinins used in micropropagation of plants (Chawla, 2009).

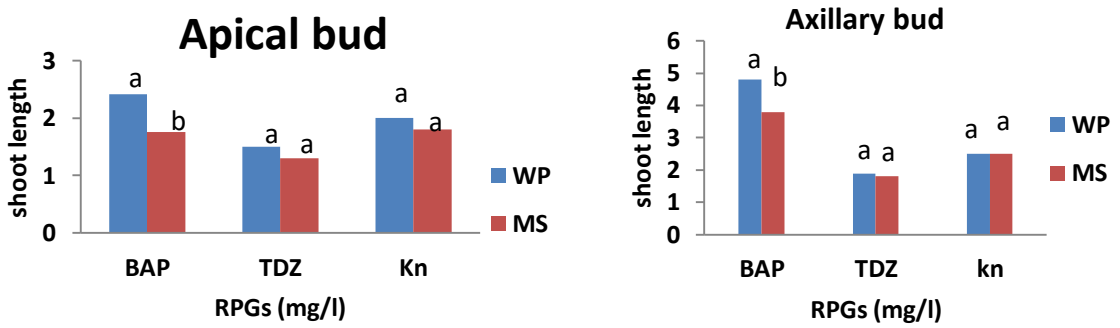
Finally, the WP medium gave better response than MS medium in all treatment (Fig4.5)



A



B



C D

Figure 4.4 Effect of explant type on mean number of regenerated shoots and shoot length (A, C apical meristem .B, D: Axillar bud. cultured on WP and MS medium respectively.
BAP (1.5 mg/l), Kn (1.5 mg/l) and TDZ (1 mg/l). Bars with the similar letters are statistically similar at Fisher‘s test for mean comparison at P<0.05.

4.2 In vitro rooting

*In vitr*o root regeneration is the most rate-limiting step of Avocado micropropagation process. Large numbers of factors affect the success of rooting of Avocado. Therefore, this stage in micropropagation has been given great attention by researcher's especially in mature Avocado propagation and has not been very successful as yet.

Shoots **(Fig. 2 C&D)** of 3-4 cm were transferred into WP medium supplemented with different concentration of IBA **(Fig 2 E &F)** for root induction.

4.2.1 Rooting percentage:

Statistical analysis revealed that plantlet obtained via apical meristem proved to be more responsive toward rooting treatments than axillary

buds (Table4.4).

Table 4.4. Effect of IBA concentration on rooting percentage of avocado rootstock. "West Indian"cv lula cultured in WP medium.

Treatments	Rooting percentage (%)	
IBA (mg/l)	Axillary bud	Apical meristem
T1(0.0)	0.1	0.01
T2(0.5)	22 d	17 d
T3(1.0)	50 a	39a
T4(1.5)	38 b	30b
T5(2.0)	35 bc	25 c
Mean	29.02 a	22.202 b

Any two means not sharing a letter significantly different at P<0.05

Axillary buds gave 50 % rooting while apical meristem exhibited comparatively low rooting percentage (39%) when cultured in WP medium supplemented with 1 mg/l IBA (0.5 mg/l). In contrary to our results, Cheng et al., (1992) also compared the activity of apical and axillary buds for rooting capacity and found higher rooting percentage in apical meristem as compared to axillary ones. Variation in the response of apical and axillary buds may be due to the slight change in the endogenous phytogradients between the explants. It may be possible that apical buds are rich in phytogradients that enhance rooting percentage. Gomez et al., (1995) found peroxides activity in apical buds of Avocado and its involvement in the regulation of auxin during rooting process were highlighted by (Berthon et al.,1989). They also found that peroxidase activity is associated with the cambial cell division and differentiation which are primary events in the process of root formation. The maximum

rooting percentage (50%) was recorded in axillary buds when low concentration of 1.0 mg/l IBA (1.5 mg/l) was used; however, higher rooting rate of 39% was observed in apical meristem at same IBA concentration (1.5 mg/l). Similar to our results, Barceló-Muñoz et al. (1999) and Pliego-Alfaro and Murashige (1987) obtained 90 % & 50 % rooting at IBA (1 mg/l) respectively. Meanwhile, Martinez-Pacheco et al (2010) obtained 57.7 % rooting at IBA (0.5mg/l).

Nel et al. (1983) could achieve only 65% (juvenile shoots) rooting with the media supplemented with IBA (2 mg/l). Nevertheless, rooting percentage increased up to 80% with prolonged incubation in 2 mg/l IBA. Also, Barringer, et al., (1996) achieved 30% rooting (juvenile shoots) in medium containing IBA (1 or 2 mg/l) and activated charcoal (1 g/l) in media

The possible reasons behind this different response were that different explants have different potential response to growth hormones. (Kulkarni et al., 2000) stated that the threshold concentrations of growth regulators required for organogenesis induction and optimal response among the different treatments. However, control condition (0.5 mg/l) resulted in all the shoots to be rootless. These results revealed that auxins play a major stimulatory role in induction of roots. The optimum concentration of auxins are known to be involved in cell enlargement, thought to be the controlling factor in rooting process (Tsipouridis et al., 2006); however,

either its suboptimal or supra-optimal concentration may negativetly affect the rooting percentage. According to Hartmann et al., (1997), suboptimal concentration of IBA results in an inhibition of free endogenous IAA activity by IAA oxidase with a subsequent decrease in outgrowth of root meristem.

4.2.2 Number of roots per explants:

Apical buds showed significantly ($p<0.05$) better root development, manifesting superiority over axillary buds with respect to root number (Table 4.5).

Table 4.5. Effect of explant types and IBA concentration on the number of roots of avocado rootstock "West Indian" cv. Lula cultured in WP medium.

Treatments IBA (mg/l)	Roots #	
	Axillary bud	Shoot meristem
T1(0.0)	0 d	0 d
T2(0.5)	1.8 c	1.4 c
T3(1.0)	**3.2 a**	**2.8a**
T4(1.5)	2.83 ab	2.2 ab
T5(2.0)	2.6 b	2.4 ab
Mean	2.086 a	1.76 a

Any two means not sharing a letter significantly different at $P<0.05$

The Maximum root number obtained axillary bud was 3.2, **(Fig 4.4 A)**, while apical meristem gave only 2.83 roots per explants. Different

responses of apical and axillary buds may be linked to some physiological differences between them; moreover, variations in the biochemical status of explants may also influence their morphogenetic potential (Han et al., 1997). Our results in contrary to Palanisamy and Kumar (1997) compared the apical and axillary bud performance for root formation and achieved higher root number in apical buds compared to axillary ones. They attributed this better response of root formation in apical buds to higher concentration of endogenous auxins. Observations regarding the interaction between various IBA concentrations and explants types revealed that apical buds gave maximum number of roots per rooted explants (3.2) when IBA was used at concentration of 1.0 mg /l. While in apical mersitem maximum root number per rooted explants (2.8) was attained at IBA concentration (1.0 mg /l) of IBA. However, rooting was not observed in the absence of IBA application, either in apical or axillary buds. It is inferred from the results that in both explants (apical and axillary buds), root number per rooted explants showed the tendency to increase with increasing concentration of IBA up to 1.5 mg/l and 1.0 mg/l in axillary and apical buds respectively. The possible reason of this better response of axiallry bud at relatively low concentration of IBA (1 mg/l) may be due to the fact that apical meristem is already rich in endogenous auxins as compared to axillary buds. Explants cultured in the medium without IBA (0.5 mg/l) did not show any response to root

development. Above results provide an evidence that optimum concentration of auxins is very critical for better rooting response. Preece and Read (2005) reported that optimum concentration of IBA plays an efficacious role in early dedifferentiation of xylem with subsequent development of root initials. Moreover, it stimulates the individual quiescent cells to differentiate and proliferate to form roots primodium. An inhibitory effect of auxins was also observed when explants were exposed to a too high concentration of IBA. Baker and Wetzstein (1994) reported that higher concentration of auxin induces the higher level of derivative metabolites in tissue thus blocking the regeneration process.

4.2.3 Root length:

Data regarding root length of avocado is given in Table 5.6.

Table 4.6. Effect of different explant sources and IBA concentration on roots length of avocado rootstocks "West Indian".cv.lula

Treatments IBA(mg/l)	Root length (cm)	
	Apical meristem	Axillary bud
T1(0.0)	0 c	0 d
T2(0.5)	3.0 a	2.8 ab
T3(1.0)	3 .2a	3.1 a
T4(1.5)	2.3 b	2.5 b
T5(2.0)	1.7 bc	1.2 c
Mean	2.04 a	1.914 a

Any two means not sharing a letter significantly different at $P<0.05$

The results shows no significant difference at $p<0.05$ between explants

sources. Increased root length (3.2 cm) was noticed in apical meristem; however, in the axillary buds (3.1 cm). Results agree with those found in Azadirachta et al., (1997) who revealed appositive response of apical meristem towards root length as compared to the axillary buds; however, results conflict with observations made by Volkaertetal.,(1989) who stated that rooting ability is not influenced by explants source. It is probable that varying biochemical status or physiological state may lead to the variation between apical and axillary buds interms of root primordial elongation. Cheng et al., (1992) stated that root formation is influenced by the physiological and biochemical status of individual plants. Furthermore, Han et al., (1997) stated that morphogenetic potential is dependent on biochemical status of source explants. Higher levels of polyamines in apical buds compared to axillary ones during rhizogenesis were detected by Rey et al., (1994). Moreover, Burtin et al., (1990) reported that root formation involves intensive mitotic activity and metabolic changes accompanied by changes in polyamine level. Polyamines are involved in a wide range of important processes including cell division, protein synthesis and DNA replication and play important role in various morphogenic responses Bais & Ravishankar, (2002). Furthermore, Friedman et al., (1985) found that polyamines in combination with auxins have a regulatory role in root development in mung bean cuttings. The trend observed in the previous parameters of

rooting percentage and roots number was maintained here for the interaction between explants (apical meristem and axillary buds) and IBA concentrations (Table 4.6). However, IBA free medium (0.0 mg/l) failed to support rooting in both the explants. Differential response of apical and axillary bud may be due to the change in differential uptake of IBA.

Sujatha and Reddy (1998) also demonstrated the same reason that different response of growth regulators in apical and axillary buds is due to difference in uptake, recognition by the cells, or in the mechanism of action of IBA.

Similar to the previous parameter, treatments showed variable responses with regards to their effects on root length. The root length progressively decreased on further increasing the IBA concentration from the optimal concentration (0.5mg/l). Present results highlighted the crucial role of IBA in optimum concentration in root elongation. Optimum concentration of IBA is imperative for activation of enzymes, for the explants cell wall loosening and extensibility leading to increase in root length Hasnat et al., (2007). Furthermore, IBA is involved in the regulation of many aspects of cambial development and its presence is essential for procambial initiation, cambial cell division and primary cell wall expansion Taiz & Zieger (2002). A little variation in response of explants at low and high concentrations of IBA revealed that growth and elongation of roots is

extremely sensitive to the auxin concentration and root length tend to reduce with higher concentration than optimum level (0.5 mg l-1). Inhibition of root elongation at supra optimal concentration may be linked with enhancement of ethylene biosynthesis. (Hopkin 1995) stated that root length is particularly sensitive to excessive auxins because it inhibits root length due to ethylene production, a root growth inhibitor. Results corroborate with that of (Ahmad et al., 2003) who indicated that root elongation phase was very sensitive to auxin concentrations and was inhibited by higher concentration.

Regeneration rate of the juvenile explants was much higher than mature explants. Although mature explants formed, shoots but they developed slowly. It has been widely reported for many plants that juvenile materials usually give higher *in vitro* responses while mature explants, in some cases such as sugarcane, passion fruit and neem, did not form even callus (Virupakshi et al., 2002, Becerra et al., 2004, Quraishi et al., 2004). In addition, shoot regenerated from mature plants was not of satisfactory quality and after some proliferation, shoots exhibit the symptoms of necrosis; leading to death afterward Harty, 1985; Cooper, 1987; Capote deSol et al., (2000).Necrosis is a major problem in multiplication of mature explants. Pliego-Alfaro et al. (1987) prevented the necrosis in IV-8 rootstock by using double-phase medium (solid and liquid medium) and

observed that mature explants retained some regeneration capacity and development of shoot. In addition, the explants produced callus at the base with vitrification symptoms (Pliego-Alfaro et al., 1987; Zirari and Lionakis (1994) used half-strength MS medium supplemented with BA (2.9 and 0.4mM in solid and liquid phases, respectively) to multiply some mature plants (Fuerte, Hass, Topa-Topa and Duke cultivars). However, they did not report properties of subculture tissues. In our experiment, mature explants of avocado were able to regenerate on MS medium containing peptone, albeit it at a lower efficiency than the juvenile ones. We further characterized the ability to form roots of these shoots since this may be indicative for the future success of avocado tissue propagation.

4.3 Plant hardening:

Plants with full root system were successfully hardened in Petmos (Fig 4.4) with about 100 % success.

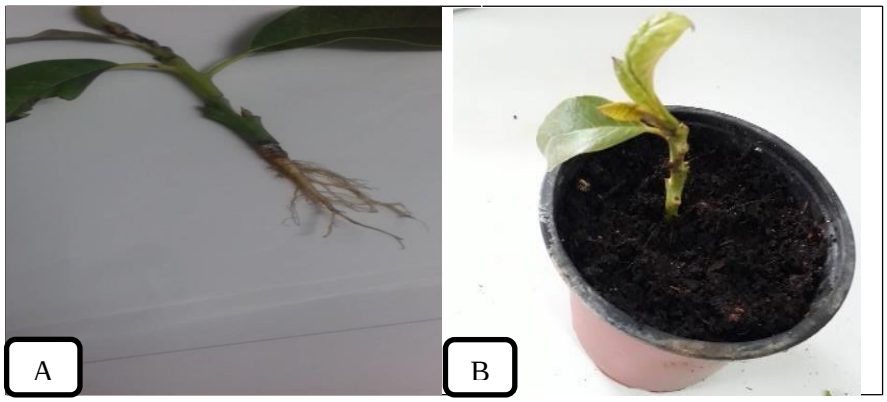


Fig. 4.5: (a) Rooting of shoot obtained from apical buds at 1.0mg/l IBA
(b)Plant hardening

Chapter Five:

Conclusions and Recommendations

5.1. Conclusions:

Efficient and well-standard regeneration protocol was successfully obtained from both apical meristem and axillary bud. WPM medium was better than MS medium for in vitro microropagation. Results clearly showed that the cytokinins type and concentration used for micropropagation of woody plants are genotype-dependent. Thus, analysis of the effect of all cytokinins on regeneration phase of avocado rootstock showed that BAP gives the best results. In this study, TDZ, and Kn did stimulate shoot proliferation but less when compared to BAP. Axillary bud was found to be more efficient in regeneration capacity when compared to apical meristem culture. Addition of peptone enhanced root formation.

5.2 Recommendations:

1- Other research should be conducted to standardize another rootstock.
2- Collaboration with private sector to get involved in any upcoming Future plans\work
3- Involvement and coordination with Ministry of Agriculture for further studied regarding economically importantcrops
4- Manipulation of this rootstock for biotic stress and a biotic stress

through genetic engineering

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Appendix

Table 4.1.1

Analysis of variance (ANOVA) for type on the average shoots number and average shoot length (cm) rootstock at different levels of (TDZ) after six weeks of *in vitro* microshoots of avocado (*Persea Americana*) West Indian cv.lula.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	4	5	1.25	0.769267
Column 2	5	4.98	0.996	0.40153
Column 3	5	5.73	1.146	0.50258
Column 4	5	4.92	0.984	0.36068

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.218103	3	0.072701	0.148028	0.929346	3.287382
Within Groups	7.36696	15	0.491131			

Total	7.585063	18				

4.1.2

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	3.95	0.79	0.2755
Column 2	5	3.8	0.76	0.228
Column 3	5	4.9	0.98	0.682
Column 4	5	3.141667	0.628333	0.379431

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.315677	3	0.105226	0.268959	0.846817	3.238872
Within Groups	6.259722	16	0.391233			
Total	6.575399	19				

Appendix table 4.2.1

Analysis of variance (ANOVA) for type on the average shoots number and average shoot length (cm) rootstock at different levels of(BAP) after six weeks of *in vitro* microshoots of avocado (*Persea Americana*) West Indian cv.lula.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	11.7	2.34	2.318
Column 2	5	7.8	1.56	0.893
Column 3	5	12.53	2.506	3.74318
Column 4	5	6.77	1.354	0.80308

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.84076	3	1.613587	0.832039	0.495623	3.238872
Within Groups	31.02904	16	1.939315			

Total	35.8698	19				

Table 4.2.2

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	9.36	1.872	1.42492
Column 2	5	6.99	1.398	0.71562
Column 3	5	9.66	1.932	1.52117

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.85572	2	0.42786	0.350541	0.711284	3.885294
Within Groups	14.64684	12	1.22057			
Total	15.50256	14				

Appendix table 4.3

Analysis of variance (ANOVA) for type on the average shoots number and average shoot length (cm) rootstock at different levels of(Kinetin) after six weeks of *in vitro* microshoots of avocado (*Persea Americana*) West Indian cv.lula

Anova: Single Factor

Table4.3.1

SUMMARY				
Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	6.3	1.26	0.85175
Column 2	5	5.7	1.14	0.47675
Column 3	5	6.49	1.298	0.57802
Column 4	5	6.57	1.314	0.57998

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.09282	3	0.03094	0.049773	0.984773	3.238872

Within Groups	9.946	16	0.621625			
Total	10.03882	19				

Table 4.3.2

Anova: Single Factor

SUMMARY				
Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	4.7	0.94	0.38175
Column 2	5	4.5	0.9	0.315
Column 3	5	6.2	1.24	1.063
Column 4	5	5.4	1.08	0.452

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.356	3	0.118667	0.214611	0.884808	3.238872
Within Groups	8.847	16	0.552938			
Total	9.203	19				

Appendix table 4.4

Analysis of variance (ANOVA) for rooting percentage (%) of apical and axillary bud on different concentration of IBA of avocado (*Persea Americana*). CV.lula

Groups	*Count*	*Sum*	*Average*	*Variance*
Column1	4	156	39	131.3333
Column2	4	110	27.5	95

Sourceof Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*

Between Groups	264.5	1	264.5	2.337261	0.177178	5.987378
Within Groups	679	6	113.1667			
Total	943.5	7				

Appendix table 4.5

Analysis of variance (ANOVA) for explant sources (apical and axillary buds) and IBA concentration on number of roots per rooted explant of avocado (*Persea Americana*) . cv.lula

Groups	*Count*	*Sum*	*Average*	*Variance*
Column1	5	10.43	2.086	1.62298
Column2	5	8.8	1.76	1.228

Sourceof Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.26569	1	0.26569	0.186385	0.677342	5.317655
Within Groups	11.40392	8	1.42549			
Total	11.66961	9				

Appendix table 4.6

Analysis of variance (ANOVA) for explant sources (apical and axillary bud) and IBA concentration on root length of avocado (*Persea Americana*).

Groups	*Count*	*Sum*	*Average*	*Variance*
Column1	5	10.71	2.142	1.94212
Column2	5	10.49	2.098	1.57977

Sourceof Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00484	1	0.00484	0.002749	0.959474	5.317655
Within Groups	14.08756	8	1.760945			
Total	14.0924	9				