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In vitro Regeneration of Avocado (Persea Americana) West Indian Rootstock

cv.lula Via Tissue Culture

انتاج اصول الافوكادو (West Indian) صنف لولا عن طريق زراعة الانسجة

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Abstract: Avocado (*Persea Americana Mill*) is a subtropical fruit and is highly recommended for its nutritional value. Avocado is known for its sensitivity to biotic and a biotic stress. One way to overcome this problem is to graft on tolerance rootstocks that are mainly obtained via seeds that are subjected to genetic variation. Two different types of explants: axillary bud and shot tip meristem were used in this study. Axillary buds gave the best results in terms of the maximum number of shoots when compared to the apical meristem in all treatments, regardless of the media type. The highest shoot induction in Woody Plant Medium (WP) was better than Murashige and Skooge (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 buds responded better than apical meristem when cultured in BAP containing medium regardless of medium type. The maximum shoot length (4.8 cm) was obtained from axillary bud cultured in WP media supplemented with BAP (1.5mg/l). For rooting, 50% of explant formed root at IBA (1.0 mg/l) when cultured in WP medium. The best number of roots (3.2 roots/explant) was obtained in MS medium supplemented with IBA (1.0 mg/l).

Keywords: Avocado, regeneration, BAP, Acclimatization, Rootstocks. Lula cultivar.

المستخلص: تعتبرفاكهة الأفوكادو (*Persea americana Mill*) من محاصيل الثمار الرئيسية في البلدان المدارية وشبه المدارية. ومن منظور اقتصادي تعتبر هذه الفاكهة مصدر مُدر للدخل نظراً لقيمتها الغذائية العالية. نظراً لحساسية فاكهة الأفوكادو وإمكانية تعرضها للاجهاد 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. لذلك تقدم تقنية زراعة الأنسجة النباتية بديلاً لحل هذه المشكلة. حيث أن الهدف الرئيسي من هذا العمل هو إيجاد بروتوكول فعال وقابل للتطبيق من أجل زراعة أنسجة أصول الافوكادو (West Indian) المُستخدمة بشكل رئيسي من قبل المزارعين الفلسطينيين. تم الحصول على الحد الأقصى من shoots من الترعيم الإبطي وذلك بمعدل 2.5 لكل عملية استنبات تمت في وسط WP مُدعم ب 1.5 ملغم/لتر من الBAP. وعندما تم استخدام القمم النامية، كان الحد الأقصى

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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 ب 1.5 ملغم/لتر من هرمون الBAP. أما بالنسبة لعملية التجذير، 50 % من النباتات انتجت أكبر عدد من الجذور بمعدل 3.2 جذور عند زراعتها على في وسط WP مُدعم ب 1.0 ملغم/لتر من الIBA.

الكلمات المفتاحية: افوكادو. زراعة الانسجة . هرمون BAP. اقلمه . اصول. صنف لولا

INTRODUCTION:

Avocado (*Persea americana* Mill) is a unique fruit. While most fruit consists primarily of carbohydrates, Avocado fruit consists of healthy fats. Numerous studies have shown that it has powerful health benefits. According to Bergh & Eustrand, (1986), 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 carotenoids, lutein and zeaxanthin, (Hutchinson 1984).

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

Besides virus elimination, either avocado micro propagation carried out using shoot tip meristem (Barringer et al. 1996) or by vegetative axillary bud culture (Schroeder 1973) has the advantage of maximizing regeneration rate, which is highly favorable for mass propagation (Rani and Rania 2000). Shoots obtained from axillary buds in avocado are 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 the genetic stability of elite cultivars due to high level of success with many cultivars; most of the avocado research protocols are confined to nodal culture using both juvenile and mature material (Barceló-Mu-oz and Pliego-Alfaro 2003).

Indirect regeneration was applied to avocado from different types of explants: stem, leaf, flower, fruit mesocarp, peduncle and cotyledons (Vieitez et al. 1983). Living avocado cells are responsive to cellular proliferation to produce calli masses (Moe and Andersen 1988). According to Schroeder (1980), some avocado calli have survived in vitro for long time. This trait was exploited for germplasm preservation if regeneration from callus tissue 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 and Jonkers 1976) and was possible to obtain with immature zygotic embryo tissues of avocado (Zimmerman 1984).

The objective of this study is to establish a reliable and efficient regeneration protocol for avocado West Indian cv. Lula rootstock.

MATERIAL AND METHODS:

Plant samples:

Certified plants were collected in October in both years of 2016 and 2017 from local nurseries in Jenin and Tulkarim governorate with the help of Ministry of Agriculture

Explant sterilization and inoculation:

Apical meristems and axillary buds (Fig. 1) used in the experiments as an explant were washed under running tap water for 5 minutes and surface sterilized with 2.5% hypochlorite for 20 minutes, washed three times again with sterile distilled water, rinsed again with 70% ethanol for 30 second, and washed again three times with sterile distilled water.

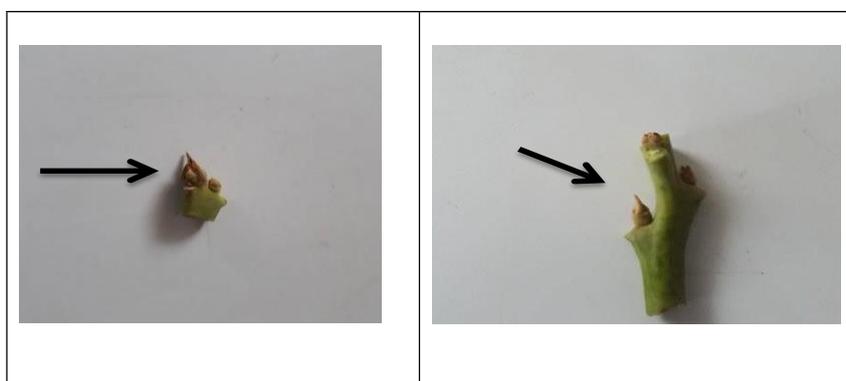


Figure (1) : Explants type (A: apical meristem, B: Axillary bud) used for Micro propagation

Explants were inoculated in petri dishes and magenta boxes containing different medium compositions (either inoculated in Murashige and Skoog (1962) or Woody Plant Medium supplemented with different concentrations of plant growth regulators (PGRs) as shown in (Table 1).

Table (1). WP and MS medium supplemented with different PGRs concentration

PGRs concentration (mg/l)	WP	MS
Thidiazuron (TDZ)	0, 0.5, 1, 1.5 and 2	0, 0.5, 1, 1.5 and 2
kinetin (KN)	0, 0.5, 1, 1.5 and 2	0, 0.5, 1, 1.5 and 2
6-Benzylaminopurine (BAP)	0, 0.5, 1, 1.5 and 2	0, 0.5, 1, 1.5 and 2

Five explants were inoculated in each petridish or magenta boxes for each concentration and repeated three times. The samples were kept in growth chambers at 23 ± 2 °C at 16/8 light dark period for 6 weeks and refreshed every 2 weeks. At the beginning, the samples were kept for 3 days in the dark to minimize phenol production before transferred to light conditions.

Rooting:

Shoots with 4-5cm in length were transferred into WP with different concentrations of Indole-3-butyric acid (IBA) (0, 0.5, 1, 1.5, and 2). Each treatment is composed of three magenta boxes as replicates with three shoots per replicate. Data was reported after 6 weeks for the number of roots in each treatment.

Plants acclimatization and hardening:

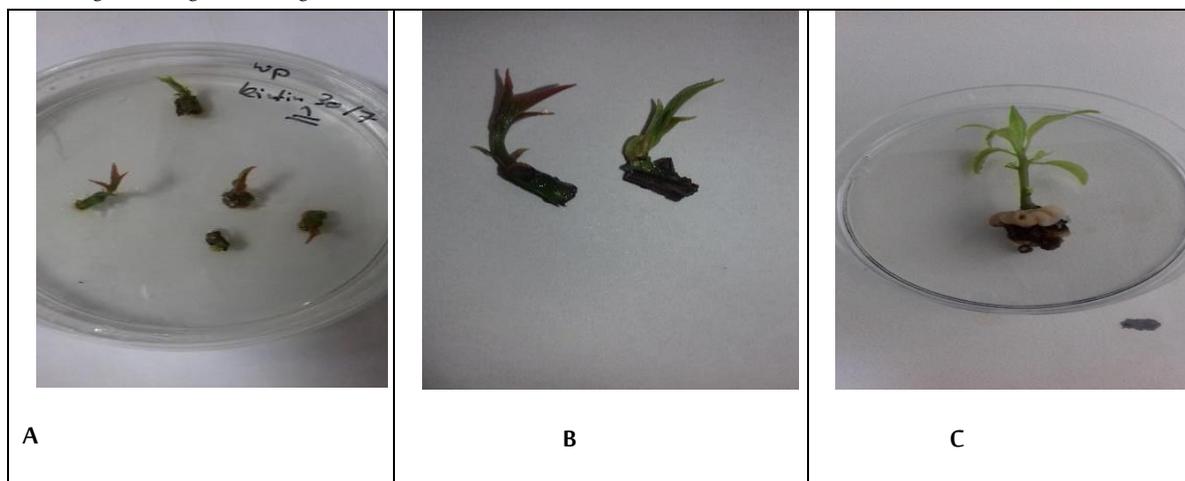
Plants with full root system were transferred to 5 liter capacity hardening medium (Peat moss) and covered with nylon plastic for 1 week under room temperature 25 ± 2 °C for acclimatization.. The plants in pots were transferred later into greenhouse conditions for 1 week before being transferred into open field. .

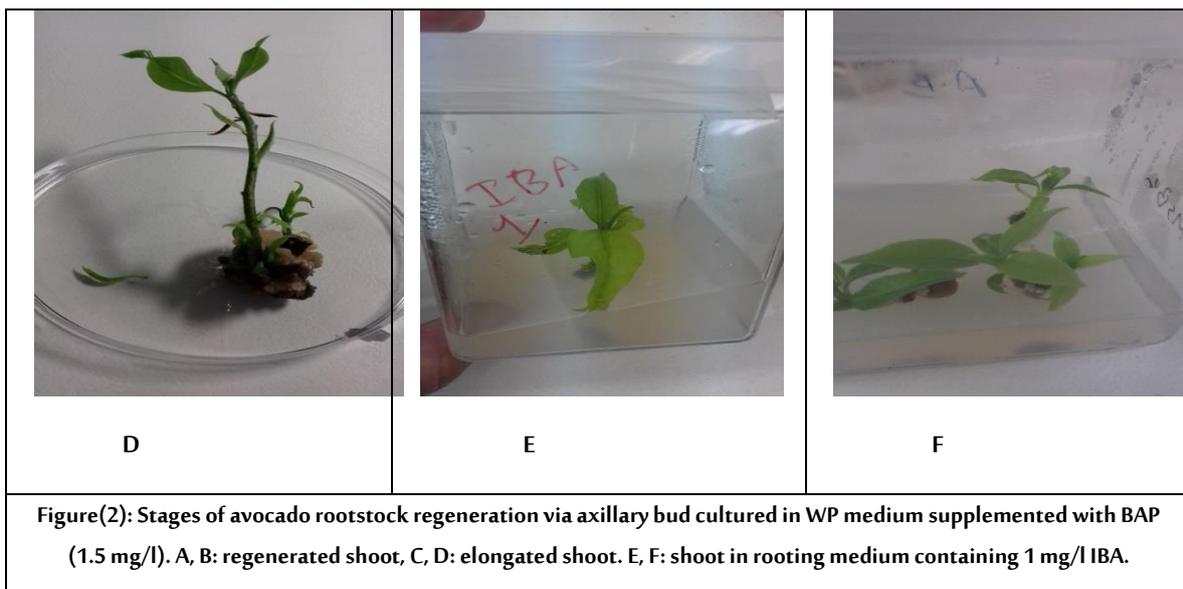
RESULTS AND DISCUSSION:

Culture initiation and shoot regeneration:

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

Explant started responding after 7 days in cultured medium (Fig. 2 A&B). After 5-6 weeks, shoots elongated to 2-4 cm in length (Fig. 2 C). In some explant, multiple shoots (2-4 shoots/ explant) was obtained. Calli were also seen in explants in all treatments, regardless of the type of medium or types of growth regulators (Fig. 2 D).





Figure(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 containing 1 mg/l IBA.

The results of culturing explants (apical meristem and axillary buds) on WP and MS media with different TDZ, BAP and KN concentrations and its effect on shoot number and shoot length are presented in Fig. 3.

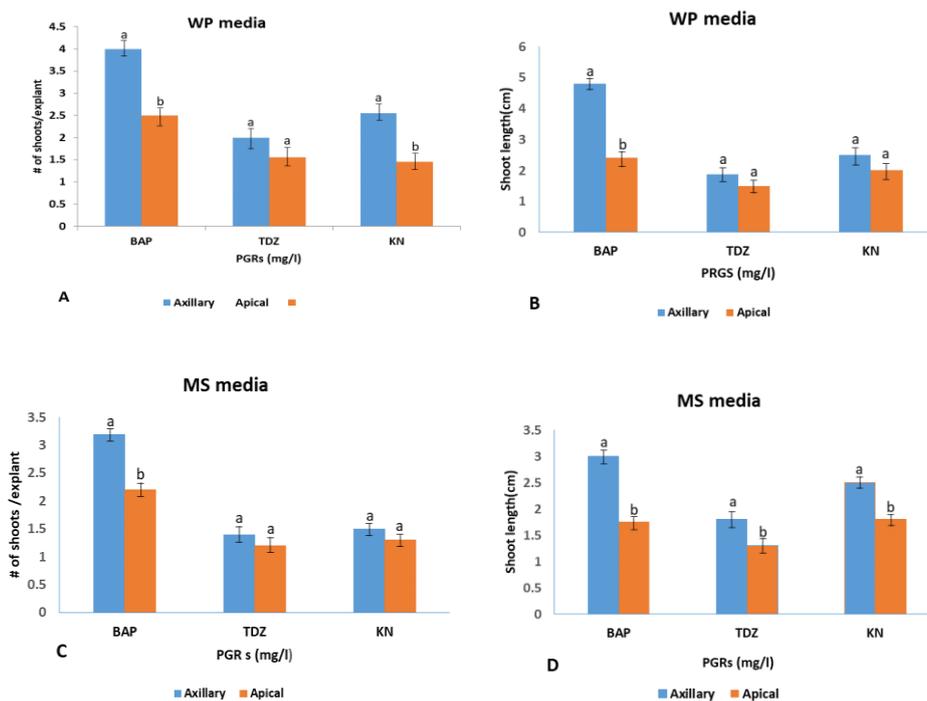


Figure (3): Mean of regenerated shoots and mean shoot length from explants (A, B) and (C, D) cultured on WP and MS medium respectively. BAP (1.5 mg/l), KN (1.5 mg/l) and TDZ (1 mg/l).

columns with same letter are not significantly different after Fisher's test for mean comparison at $P < 0.05$.

Axillary buds had better responses and produced more shoots when compared with the apical meristem in all treatments. WP medium also produced better results when compared with MS medium.

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 (Sefasi et al. 2013). Many studies have shown that TDZ stimulates shoot regeneration at low concentrations. In the present study, a lower concentration of TDZ (1 mg/l) was more effective in producing more shoots than higher concentrations. 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 explants with TDZ (1 mg/l) produced multiple shoot (5.8 shoots / explant). Singh et al. (2002) reported a similar result, in his study on *Psidium guajava*, he found that the maximum percentage of shoots regeneration and maximum number of regenerated shoots were obtained by a treatment containing 1.0 μ m TDZ. It was observed that increasing concentrations of growth regulators decreased 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 buds cultured in WP medium supplemented with 1 mg/l TDZ, respectively. (1mg/l) (Fig. 3).

Explants responded differently in formation of multiple shoots in different media compositions, which 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 acts 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.

Axillary buds gave better results than the apical meristem in all treatments. 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. 3). 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 the 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 for the 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) (Zulfaqar et al. 2009). Cytokinins in plant are synthesized by roots and transported upward; therefore, axillary buds are rich in cytokinins due to their presence at a relatively lower position on the mother plant. At a very low concentration of BAP (0.5 mg/l) or at a higher high concentration of BAP (2.0 mg/l), the number of shoots was less than other BAP concentration. Chuenboonngarm et al. (2001) recorded identical symptoms in *Gardenia (Gardenia jasminoides)*. They stated that higher concentration of cytokinins reduced the number of shoots during the 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 started declining with further increase in BAP concentration. Therefore, the selection of the proper concentration of plant growth regulator is critical to shoot regeneration. Our results showed that BAP (1.5 mg/l) produced the maximum number of shoot. Cooper (1987) found that BAP (1 mg/l) produced zero shoots from Duke 7. Zirari and Lionakis (1994) found that BAP (0.65 mg/l) produced 1.7 shoots from nodal culture of the 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 fewer shoots. In a study conducted by Barrera-Guerra et al. (1998), no shoots were obtained from nodal explants when cultured on BAP (2 mg/l) containing medium. In contrary to Barceló-Muñoz et al. (1999) who found that BAP (2.2 mg/l) produced 3.2 shoots from the Mexican IV-8 cultivar. Castro, et al (1995) obtained 3.1 shoots from Topa Topa at BAP (2 mg/l). This clearly shows that the response is genotype dependent.

The maximum number of shoots obtained from axillary buds was higher than that obtained from the 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 KN (1.5mg/l); while in the apical bud, the 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 KN is necessary for shoot development and multiplication. The possible reason of the early response of axillary buds toward KN might be that the axillary buds are rich in endogenous KN so they showed a better response at a relatively higher concentration of 1.5 mg/l. axillary bud and apical meristem, respectively (1.0 mg/l not 1.5 mg/l). The maximum number of shoot length obtained was 2.5 cm from both (Fig. 3).

Rooting:

In vitro root regeneration is the most rate-limiting step of avocado micropropagation process. A large number of factors affect the success of rooting in avocado. Therefore, this stage in micropropagation has been given great attention by researchers especially in mature avocado propagation, which has not been very successful yet.

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

The rooting percentage and root number obtained with inoculation of plantlets to rooting medium supplemented with IBA is giving in Table 2 and 3.

Table(2) : Effect of IBA concentrations on rooting percentage of avocado rootstock. "West Indian "cv Lula" cultured in WP medium.

Treatments	Rooting percentage (%) ± SE	
IBA (mg/l)	Axillary bud	Apical meristem
T1(0.0)	0.1 ^c ±0.001	0.01 ^c ±.001
T2(0.5)	22 ^d ± 2.01	17 ^d ± 2.4
T3(1.0)	50 ^a ± 3.5	39 ^a ± 3.4
T4(1.5)	38 ^b ±2.1	30 ^b ± 3.23
T5(2.0)	35 ^{bc} ± 3.1	25 ^c ± 4.25

Mean	29.02 ^a ± 1.54	22.20 ^b ± 1.45
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Data with different letters are significantly different after fisher test at $p < 0.05$

Axillary buds gave 50% rooting while the apical meristem exhibited a comparatively low rooting percentage (39%) when cultured in WP medium supplemented with 1 mg/l IBA (0.5 mg/l) (Table 2). Variation in the response of apical and axillary buds may be due to the slight change in the endogenous phytochemicals between the explants. It may be possible that apical buds are rich in phytochemicals that enhance rooting percentage. Gomez et al., (1995) found peroxidase activity in the apical buds of avocado and its involvement in the regulation of auxin during the rooting process. They also found that peroxidase activity is associated with 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, a higher rooting rate of 39% was observed in apical meristem at same IBA concentration (1.5 mg/l) (Table 2). Similar to our results, Barceló-Muñoz et al. (1999), 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). Nonetheless, incubation in 2 mg/l IBA for an extended period increased rooting percentage by up to 80%. In addition, Barringer, et al., (1996) achieved 30% rooting (juvenile shoots) in medium containing IBA (1 or 2 mg/l) and activated charcoal (1 g/l).

The possible reasons behind these different responses were that different explants have different potential responses to growth hormones. The optimum concentration of auxins is known to be involved in cell enlargement and is thought to be the controlling factor in the rooting process (Tsipouridis et al. 2006). However, either its suboptimal or supra-optimal concentration may negatively 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 the root meristem.

The maximum roots number obtained from a single axillary bud was 3.2, while the single apical meristem gave only 2.83 roots per explant (Table 3). Our results are in contrary to those of Palanisamy and Kumar (1997) who compared the apical and axillary bud performance for root formation and achieved a higher number of roots in apical buds compared to axillary ones. They attributed this better response of root formation in apical buds to a higher concentration of endogenous auxins. Observations regarding the interaction between various IBA concentrations and explant types revealed that apical buds gave maximum number of roots per rooted explant (3.2) when IBA was used at a concentration of 1.0 mg /l. While in the apical meristem, the maximum root number per rooted explants (2.8) was attained at 1.0 mg /l of IBA. However, rooting was not observed in the absence of IBA, 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 axillary buds at a relatively low concentration of IBA (1 mg/l) might be

that apical meristem is relatively rich in endogenous auxins as compared to the axillary buds. Explants cultured in the medium without IBA (0.5 mg/l) did not show any response to root development. The above results provide evidence that the optimum concentration of auxins is very critical for better rooting response. Preece and Read (2003) 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.

Table(3): 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)	Root number± S.E	
	Axillary bud	Shoot meristem
T1(0.0)	0 ^d ± 0	0 ^d ± 0
T2(0.5)	1.8 ^c ± 0.1	1.4 ^c ± 0.12
T3(1.0)	3.2 ^a ± 0.11	2.8 ^a ± 0.12
T4(1.5)	2.83 ^{ab} ± 0.32	2.2 ^{ab} ± 0.24
T5(2.0)	2.6 ^b ± 0.22	2.4 ^{ab} ± 0.23
Mean	2.086 ^a ± 1.01	1.76 ^a ± 1.01

Data with different letters are significantly different after fisher test at $p < 0.05$

Plant acclimatization and hardening:

Plants with full root system were successfully hardened in 5 liter capacity Pot having Petmos under greenhouse conditions located in Palestine Technical Kadoorie with temperature about $25 \pm 2^\circ\text{C}$ where survival rate was 60 %. The survived plants were transferred to open field where 100 % success was obtained

CONCLUSION:

An efficient and well-standardized regeneration protocol was successfully obtained from both the apical meristem and the axillary bud. WP medium was better than MS medium for in vitro regeneration. Results clearly show that the cytokinins type and concentrations used for regeneration of woody plants are genotype-dependent.

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