IN VITRO PROPAGATION OF AVOCADO (*PERSEA AMERICANA* MILL.)

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Advances in micropropagation technique have helped to produce true-to-type clones of many important horticulture plants. A procedure for micropropagation of Avocado was developed using stem segments as explant for the *in vitro* establishment and 60 to 100% of explants survived. The highest average shoot length was obtained on Murashing and Skoog (MS) basal medium supplemented with 1 mg/l 6-benzyl adenine (BA) and 0.5 mg/l naphthalene acetic acid (NAA). The maximum number of proliferated shoots (6.2 shoots/ explant) was obtained on MS medium supplemented with 2 mg/l BA, 0.5 mg/l NAA and 10 mg/l arginine. Meanwhile, the highest shoot length (3.7 cm) was obtained on MS medium free from growth regulators (control). Eighty percent of the shoots rooted on half strength MS medium supplemented with 4 mg/l indole butyric acid (IBA), 0.5 mg/l NAA and 160 mg/l phloroglucinol (PG). The complete plants with the maximum average root number / shoot (5) and length (9.3 cm) was obtained on medium containing 4 mg/l IBA, 0.5 mg/l NAA and 160 mg/l PG. The highest survival percentage of 70% was obtained from the plantlets when they were transferred to greenhouse conditions. Avocado can successfully micropropagated beginning with stem segments without significant damage to mother plant.

Keywords: Avocado, micropropagation, acclimatization, stem segment, phloroglucinol

INTRODUCTION

Avocado (*Persea americana* Mill.), a dicotyledonous woody species, is one of the major fruit crops of the tropics and subtropics. Avocado is a high demand, high value tropical fruit recognized for its nutritional value. Due to its high economic importance (Cruz-Hernandez et al., 1998), Avocado is one of the most important fruits in the world with an annual production estimated to be 3.5 million tons in 2008 (FAOSTAT, 2010). México, Chile, the United

States and Indonesia are the leading producers of Avocado. It is a nutritious and healthy fruit containing all food elements (carbohydrates, proteins and fats), wide spectrum of vitamins (A, B, C, D and K) and minerals. It has gained an immense popularity over the last few decades as a luxurious fruit and has become a very important tropical horticultural crop in the modern world (Ayala-Silva and Ledesma, 2014). The racial variation in many traits including important agronomical and commercial traits are related to vigor under a biotic and biotic stress conditions and is a major difference among the cultivars (Wolstenholme, 2003). If grown from the seedlings, Avocado may take up to 10 years to bear fruits. Therefore, as common to many horticulture fruit crops, Avocado is grafted with bud wood from selected mature tree for precocious production and trueness of type. Beneficial shoot and root characters are integrated by-selection rootstock and scion cultivars to optimum productivity and group manageability. Avocado bears complete flowers (male and female organs in a single flower), but shows an unused pollination syndrome (protogynous dichogamy) of male and female floral activity at different times during the day which favors out crossing (Sedgley, 1985). This leads to a high level of heterozygosity creating inconsistency in genetic stability and consequently making Avocado seedlings less preferable as rootstocks for commercial planting compared to clonal rootstocks **(**Ben-Ya'acov and Michelson, 1995). Avocado propagation through seeds exhibits high genetic variation, hence less appealing for orchard plantings (Hiti-Bandaralage et al., 2017). Due to cross-pollination in Avocado, every seedling is genetically different from the mother tree and the other seedlings derived from seeds of the same tree. Consequently, the performance under field condition of seed-propagated rootstocks is unpredictable (Gonzales-Rosas et al., 2003). Rooting of tree cuttings associate long turn-around time and high expense (Hiti-Bandaralage et al., 2020). Avocado is a major fruit of tropics and subtropics, which is highly important from economical point of view with a luxurious nutritional value. Therefore, various attempts have been made to multiply Avocado by tissue culture techniques (Premkumar et al., 2003), however *in vitro* propagation of this species has been proved quite difficult in a number of studies (Nhut et al., 2007). An understanding of the factors controlling *in vitro* developmental process is essential for the establishment of an efficient regeneration system. Shoot proliferation ability of plant tissues and the formation of adventitious roots depend upon the interaction of several endogenous and exogenous factors (Ahmed, 2002). Physiological status of an explant, concentration of plant growth regulators in culture medium and their mutual interaction are one of the major determinants among these factors (Zulfiqar et al., 2007). Ontogeny of explants and their position on mother plant greatly affects the *in vitro* development and according to Chern et al. (1993), different explant sources have different growth potential due to differences in age, endogenous metabolic status and differential genome. Shoot regeneration is considerably difficult to achieve in Avocado from shoot tips and nodal

explants with limited shoot proliferation, elongation of existing buds and formation of scaly leaves (Barringer et al., 1996). Moreover, *in vitro* root formation is a major problem in Avocado which has been reported to be correlated with the position of buds on a tree and the existence of juvenility gradient in them (Palanisamy and Kumar, 1977). In addition to the explant source, plant growth regulators also have stimulatory effects on shoot regeneration and root induction capacity of plants. In particular, the leaf and root structures developed under *in vitro* conditions largely affect the success of acclimation process (Pospišilová et al., 1999). The hardening or acclimation process is the stage where *in vitro* plants are gradually exposed to normal environmental challenges, thus, are forced to develop complete survival mechanisms for a dynamic septic environment. Being planted as a grafted tree, propagation of Avocado refers to propagation of rootstock cultivar, then grafting it with bud-wood from a mature scion cultivar. Elite cultivar propagation is critical to maintain the quality of fruit and farm management practices. Potential of micropropagation has been well demonstrated for wide variety of economically important plants. Commercial application of micropropagation for Avocado will undoubtedly boost the industry around the globe. Tissue culture has the potential be a very effective and efficient alternative for clonal rootstock production of Avocado. However, Avocado has performed poorly in a tissue culture environment, similarly to other woody plant species (Bairu and Kane, 2011). Improving Avocado rootstock propagation is a major industry/research challenge globally. Clonal propagation is desirable for clonal fidelity of this outcrossing species. Therefore, in the present study an attempt was made to develop a protocol for *in vitro* propagation of Avocado with the aim to identify the best concentration of growth regulators for successful shoot proliferation and rooting.

MATERIALS AND METHODS

1. Plant Material

Activity growing shoots of Avocado were collected as plant material from 10 –years – old trees, an orchard full production located at Alcanater–Alkhayreyah Research Station, Horticultural Research Institute, Agricultural Research Center, Giza, Egypt. Stem nodal segments of about 2 cm in length were isolated from the shoots as explants. The explants were washed in running tap water by adding few drops of tween-20 to remove the superficial dust particles as well as fungal and bacterial spores. The surface sterilization of explants was carried under complete aseptic conditions. The explants were surface sterilized with 30% (v/v) Clorox bleach solution (5.25% sodium hypochlorite) for 30 min providing gentle agitation, followed by three sequential rinses in sterile double distilled water, then immersed in 0.1% (w/v) sterile solution of mercuric chloride for five min and finally rinsed six times with sterile double distilled water (Fig. 1).

2. Nutrient Medium and Culture Conditions

The basal nutrient medium containing macro and micro elements applied throughout this study was Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 100 mg/l myo-inositol, 30 g/l sucrose, 1.8 g/l gelrite, 1.5 g/l activated charcoal and 0.4 mg/l thiamine HCl. The pH value of the nutrient medium was adjusted at 5.7- 5.8 by adding few drops of either 0.1 N NaOH or 0.1 N HCl, prior to the addition of gelrite. The media were dispensed into jars, each contained 50 ml of culture medium. Sterilization of the medium was achieved by autoclaving the jars containing media under pressure of 1.1 kg/cm^2 at 121ºC for 20 min. After culturing, all cultures were incubated in a culture room at 25 ± 2ºC, 60-70% relative humidity and 16-h photoperiod provided by cool white fluorescent lamps.

3. Establishment Stage

Stem nodal segments were inoculated on MS medium supplemented with different concentrations of 6-benzyl adenine (BA) at concentrations of 0, 1, 2 and 3 mg/l either individually or in combination with 0.25 or 0.5 mg/l of isopentenyl adenine (2iP) or naphthalene acetic acid (NAA). Survival percentage, average number of shoots/explant and average shoot length (cm) were evaluated after six weeks from culture.

4. Multiplication Stage

Shoot obtained from the establishment stage were transferred to MS medium supplemented with different concentrations of BA (0, 1, 2 and 3 mg/l) in combination with 0.25 or 0.5 mg/l of 2iP or NAA and 10 mg/l arginine (Arg). Shoot proliferation was determined after six weeks of culture. Average number of new shoots formed per explant and average shoot length (cm) were recorded

5. Rooting Stage

For rooting, individual shoots 3-4 cm long were excised from the multiplication stage and cultured on half strength MS medium supplemented with indole butyric acid (IBA) at different concentrations (0, 1, 2 and 4 mg/l), in combination with 0.5 mg/l NAA or phloroglucinol (PG) at concentrations of 0, 80, 120, 160 and 220 mg/l. The percentage of rooted shoots, average number of roots formed per shoot and average root length (cm) were recorded after six weeks of culture on the rooting media.

6. Acclimatization Stage

The rooted plantlets were removed gently from the medium. Washed in running tap water and soaked in 2.0 g/l fungicide solution (Benlate) for three min. Plantlets were then transferred to plastic pots containing peat moss and sand (1: 1) in green house (28±2°C, 70% humidity). The potted plants were irrigated and initially covered with plastic bags, which were gradually eliminated within eight weeks for completing their acclimatization and plantlets were maintained under nursery condition.

7. Statistical Analysis

Experiments were subjected to the completely randomized design. Variance analysis of data was carried out using ANOVA for statistical analysis. The differences among means for all treatments were tasted for significance at 5% level, by using Duncan's multiple range test (Duncan, 1955), as described by Snedecor and Cochran (1990). Means followed by the same letter are not significantly different.

RESULTS AND DISCUSSION

1. Culture Establishment

From data represented in table (1), it could be shown that survival percentage of Avocado cultures ranged from 60 to 100% using different concentrations of BA, 2iP and NAA including the control (without growth regulators). However, the concentration of 2 mg/l BA, was the best concentration concerning the number of shoots (3 shoots/explant) and also the survival percentage attained 100%. The highest average shoot length (2 cm) was obtained on the medium contained 1 mg/l BA and 0.5 mg/l NAA, compared with the medium without NAA. The general trend of responses of shoot growth to different auxin concentrations in the media may be interpreted by the fact that the auxin affects mainly the length of shoots. On the other hand, the effect of some concentrations of auxin on morphogenetic responses of stem nodal segments varied relatively according to concentrations of cytokinin in the media. This might be due to low concentration of endogenous cytokinin that might play a role in the magnitude and effective level of exogenously added cytokinin concentration in the explant tissue. The difference in the interaction between the explants and different growth regulators may be due to the presence or absence of their mechanism of action in the tissue or to the availability of such growth regulator in the explant, the matter which facilitates its function and further concentrations are not needed (Tisserat, 1988). This type of adverse effects of higher concentrations of cytokinin in the initiation stage has been reported in *Caralluma edulis* (Petel et al., 2014), *Passiflora foetida* (Shekhawat et al., 2015) amd *Andrographis echioidus* (Savitikadi et al., 2020).

2. Mutiplication Stage

Effect of various combinations of BA, 2iP and NAA on the multiplication of shoots is presented in table (2). The cytokinin free medium gave the least average number of shoots indicating strong apical dominance. On the control medium, an average of 1.5 shoots per explant was obtained after six weeks of culture with the highest average shoot length (3.7 cm). MS medium containing 10 mg/l Arg plus BA and NAA at concentrations of 2 mg/L and 0.5 mg/l, respectively, induced significantly the maximum shoot number (6.2 shoots per explant) than other treatments (Fig. 2). The increase in shoot proliferation may be due to the effect of cytokinin, especially when

added in appropriate concentration, where it regulators shoot proliferation, cell division and differentiation (Gross and Partiner, 1984). It seems that BA and 2iP could stimulate shoot growth if added to the media at low concentrations, while their high concentrations stimulate shoot multiplication. The results indicate that BA plays a kays role in shoot propagation of Avocado. This cytokinin is an efficient growth regulator for shoot multiplication in other plants, such as Avocado (Araceli et al., 1999), Nemagured and Okinawa peach rootstocks (Edriss et al., 2014) and Volkamer lemon (Ahmed et al., 2017). For Avocado, BA proved to be the most effective cytokinin for shoot multiplication but not for shoot elongation (Table 2). The increase in the number of shoots of Avocado may be due to the physiological role of BA of breaking the apical dominance and stimulating the growth of new shoots (Pruski et al., 2005 and Alvin et al., 2020). Shoot elongation decreased slightly with increasing BA concentration. This result suggests an inverse relationship between the number of shoots and shoot elongation in the propagation protocols (Cueneca et al., 1999 and Abd Alhady, 2018).

Table (1). *In vitro* establishment of stem nodal segments of Avocado (*Persea americana*) cultured on MS nutrient medium supplemented with BA, 2iP and NAA. Results were taken after six weeks of culture.

Growth regulators conc. (mg/l)			Survival %	No. of shoots/explant	Length of shoots (cm)
BA	**2iP**	**NAA**			
0	0.00	0.00	60^{d}	1^{c}	1.00^{g}
1	0.00	0.00	80^{c}	1^{c}	1.73^{c}
2	0.00	0.00	100^{a}	3^{a}	1.60^{d}
3	0.00	0.00	90^{b}	1^{c}	1.20^{f}
1	0.25	0.00	90^{b}	1^{c}	1.20^{f}
1	0.50	0.00	100^{a}	2^{b}	1.50^{e}
1	0.00	0.25	90^{b}	1^{c}	1.80^{b}
1	0.00	0.50	90^{b}	1^{c}	2.00^{a}

Table (2). Effect of BA, 2iP, NAA and arginine on the multiplication of Avocado (*Persea americana*) axillary shoots. Results were taken after six weeks of culture.

Growth regulators conc. (mg/l)				No. of shoots/explant	Length of shoots (cm)
BA	**2iP**	**NAA**	**Arg**		
0	0.00	0.00	0	1.5^{g}	3.7^{a}
2	0.25	0.00	10	3.7^{f}	3.2^{b}
2	0.50	0.00	10	4.8^{d}	3.0^{c}
3	0.50	0.00	10	5.2^{c}	2.5^{e}
1	0.25	0.00	10	4.3^{e}	2.6^{d}
2	0.00	0.25	10	5.5^{b}	2.3^{f}
2	0.00	0.50	10	6.2^{a}	1.9^{g}

Concerning the role of arginine, nitrogen is a limiting resource for plant growth in most terrestrial habitats since large amounts of nitrogen are needed to synthesize nucleic acids and proteins. Among the 21 protein organic amino acids is arginine, it has the highest nitrogen to carbon ratio, which makes it especially suitable as a storage form of organic nitrogen. Synthesis in chloroplasts *via* ornithine is apparently the only operational pathway to provide arginine in plants, and the rate of arginine synthesis is tightly regulated by various feedback mechanisms in accordance with the overall nutritional status. A role of arginine as alternative source besides glutamate for proline biosynthesis is still discussed controversially and may be prevented by differential subcellular localization of enzymes. Apparently, arginine is a precursor for nitric oxide (NO), although the molecular mechanism of NO production from arginine remains unclear in higher plants. In contrast, conversion of arginine to polyamines is well documented, and in several plant species also ornithine can serve as a precursor for polyamines (Winter et al., 2015). Amino acids provide plant cells with an immediate source of nitrogen which can generally be taken up by the cells more rapidly than inorganic nitrogen (El-Shiaty et al., 2004). Nitrogen originating from amino acids is assimilated quicker into the carbonic skeletons during the metabolism and synthesis of the proteins, when compared to other inorganic N sources. Montague et al. (1979) reported that arginine serves as a precursor in polyamine synthesis. Fresh mass of explants grown on media containing 5 mM arginine was significantly lower in comparison to 1 mM arginine and 50 µM cysteine (Sotiropoulos et al., 2005).

3. Root Induction and Acclimatization

About 4-5 cm long shoots with two to three leaves were harvested from *in vitro* proliferated shoots, were used for the rooting experiment. The individual shoot was placed in half strength MS medium supplemented with different concentrations of IBA (0, 1, 2 and 4 mg/l) with 0.00, 0.50 and 1.00 mg/l NAA either individually or in combination with PG (80, 120, 160 and 220 mg/l). The shoots showed different responses to rooting after six weeks of culture (Table 3). The highest percentage of rooting (80%) with the highest number (5.1 roots/shoot) and length (9.3 cm) of roots was recorded on half strength MS medium supplemented with 4 mg/l IBA, 0.5 mg/l NAA and 160 mg/l PG (Fig. 3). As shown in table (3), only 10% rooting was obtained on half strength MS medium without IBA. This result is in harmony with that obtained by Abd Ahady (2018), who found that the maximum number of roots / shoot was obtained when IBA and PG were added to half strength MS medium at concentrations of 3 and 160 mg/l, respectively. The use of half strength MS medium for root induction was supported by Beena et al. (2003), who reported that half strength MS medium induced more roots compared to full strength MS medium in *Ceropgia candelaburn*. Moreover, De Klerk et al.

(2011) observed different effects of PG on rooting depending on the specific auxin applied. A positive rooting response using PG and IBA has been reported for several tree genera, including *Diaspyros*, *Malus* (Dobránszki and Teixeria da Silva, 2010), *Jatropha* (Daud et al., 2013), *Juglans* (Licea-Moreno et al., 2015) and apple rootstocks Ma and M26 (Kim et al., 2020). It has been reported that PG acts synergistically with auxin during the most sensitive phase of root initiation (Dobránszki and Teixeira da siva, 2010). The results show that, in Avocado, PG seems to be essential to induce root formation in the presence of an auxin (IBA and NAA). This confirms the view that PG is an auxin promoter (Perez et al., 2016 and Alvine et al., 2020). PG is a natural precursor of lignin biosynthesis with complex effects on plant tissue culture (Teixeira da silva et al., 2013). Several reports suggest PG can mimic auxin or cytokinin action (Tallon et al., 2012) and PG is effective in attenuating stress damage and removing hyperhydricity caused by poor lignification (Phan and Hegedus, 1985). Licea-Moreno et al. (2015) suggested that *in vitro* plant mortality during acclimatization depends largely on genotype and concentration of PG used. While further research is obviously needed, their results demonstrated that moderate PG concentration during shoot multiplication (0.4 mM), reduced at 0.2 mM, six weeks before root induction, significantly promote walnut *in vitro* performance, without rooting efficiency and plant survival in substantial manner.

Table (3). Effect of MS medium supplemented with different treatments of IBA, NAA and PG on the rooting of Avocado (*Persea americana*). Results were taken after six weeks of culture.

Concentration (mg/l)			No. of roots / shoot	Length of root (cm)	Rooting %
Auxin		PG			
IBA	NAA				
0	0.0	0	0.1^{e}	1.1^{i}	10.0^{h}
1	0.5	0	1.1^{d}	1.5^{h}	30.1^{g}
2	0.5	0	3.1^{c}	4.1^{g}	45.1^{f}
4	0.5	0	3.1^{c}	4.6^{f}	50.1^{e}
0	1.0	0	3.1^{c}	5.1^{e}	45.1^{f}
4	0.5	80	4.1^{b}	8.1^{d}	60.1^{d}
4	0.5	120	4.1^{b}	9.4^{a}	70.1^{c}
4	0.5	160	5.1^{a}	9.3^{b}	80.1^{a}
4	0.5	220	3.1^{c}	8.6^{c}	75.1^{b}

Plantlets regenerated *in vitro* with well developed root system were transferred to a glass house in plastic pots covered with translucent plastic bags to ensured keeping high humidity around the plants. The use of this procedure during the acclimatization phase ensured that most transplanted plantlets to *ex vitro* conditions continued to grow vigorously. After eight weeks the plastic bags were removed, 70% of the plantlets survived in the

green house and showed no sign of water stress and without any morphological abnormalities or variation (Fig. 4).

Fig. (1). Sterilized stem nodal segments of Avocado (*Persea americana* Mill.)

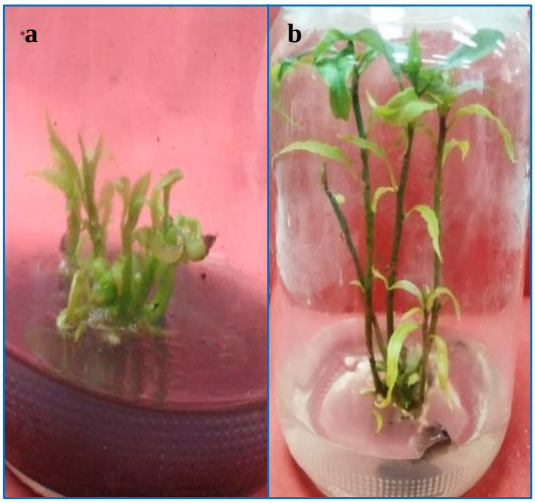

Fig. (2). Multiplication of Avocado (*Persea americana*) on MS medium containing 2 mg/l BA + 0.5 mg/l NAA + 10 mg/l Arg, **a.** after 2 subcultures, **b.** after 3 subcultures.

Fig. (3). Rooting of Avocado (*Persea americana*) on MS medium supplemented with 4 mg/l IBA + 0.5 mg/l NAA + 160 mg/l PG after 60 days of incubation.

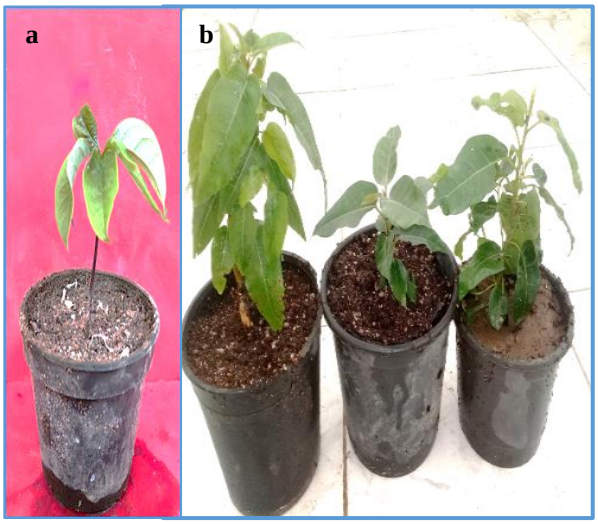

Fig. (4). Hardened *in vitro* derived plantlets of Avocado (*Persea americana*), **a.** after 30 days of hardening, **b.** after 60 days of hardening.

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الإكثار المعملي لنبات الأفوكادو

رضا السيد السيد أبوالفضل، منال الصلاة على النبي أحمد* و محمد رضا عبد المجيد عبد الهادي
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ساعدت التطورات في تقنية الإكثار المعملي في إنتاج نباتات مطابقة للنبات الأم وذلك للعديد من النباتات البستانية المهمة. تم تطوير طريقة الإكثار الدقيق للأفوكادو باستخدام الأجزاء الساقية البرعمية كمنفصل نباتي في المعمل وقد أدى ذلك إلي الحصول علي نسبة حياة للأجزاء النباتية من ٦٠ إلى ١٠٠٪ مع حدوث تضاعف للأفرع بنسبة عالية وكذلك تم الحصول على أعلى متوسط طول للمجاميع الخضرية للمنفصل النباتي على بيئة موراشيجي وسكوج والمحتوية على ١ ملجم / لتر بنزيل أدينين و٠.٥ ملجم/لتر نفثالين حامض الخليك. كان أعلي معدل لتضاعف المجاميع الخضرية (متوسط ٦.٢ فرع/منفصل نباتي) علي بيئة موراشيجي وسكوج المضاف إليها كلًا من ٢ ملليجرام / لتر بنزيل أدينين و ٠.٥ ملليجرام /لتر نفثالين حامض الخليك و١٠ ملليجرام /لتر أرجنين. في الوقت نفسه فقد تم الحصول على أعلى معدل لطول النموات الخضرية (٣.٧ سم) على بيئة موراشيجي وسكوج الخالية من منظمات النمو. ثمانون بالمائة من النموات الخضرية تم تجذيرها عند إستخدام نصف قوة تركيز بيئة موراشيجي وسكوج كوسط غذائي المحتوية علي ٤ ملجم /لتر أندول حامض البيوتريك و٠.٥ ملجم/لتر نفثالين حامض الخليك وذلك في وجود ١٦٠ ملليجرام / لتر فلوروجلوسينول بأعلى متوسط لعدد الجذور (٥ جذور/نبات وأعلى معدل لطول الجذور (٩,٣ سم) كان على بيئة موراشيجي وسكوج والتي تحتوى على٤ ملجم /لتر أندول حامض البيوتريك و٠.٥ ملجم/لتر نفثالين حامض الخليك و ١٢٠ ملليجرام / لتر فلوروجلوسينول. تم الحصول على نسبة عالية من الحيوية (٧٠٪) عندما تم نقل النباتات إلى ظروف الصوبة الزراعية. تم بنجاح إكثار نبات الأفوكادو في المعمل باستخدام الاجزاء الساقية البرعمية دون إحداث ضرر لنبات الأم.