Scientific Research Publishing

American Journal of Plant Sciences, 2017, 8, 2898-2921
http://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Micropropagation of Avocado (*Persea americana* Mill.)

Jayeni Chathurika Amarathunga Hiti-Bandaralage, Alice Hayward, Neena Mitter*

Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane, Australia
Email: *n.mitter@uq.net.au

How to cite this paper: Hiti-Bandaralage, J.C.A., Hayward, A. and Mitter, N. (2017) Micropropagation of Avocado *(Perseaamericana* Mill.). *American Journal of Plant Sciences*, **8**, 2898-2921.
https://doi.org/10.4236/ajps.2017.811197

Received: September 27, 2017
Accepted: October 28, 2017
Published: October 31, 2017

Abstract

Avocado is a high demand, high value tropical fruit recognised for its nutritional value. Being planted as a grafted tree, propagation of avocado refers to propagation of rootstock cultivar, then graft it with bud-wood from a mature scion cultivar. Elite cultivar propagation is critical to maintain the quality of fruit and farm management practices. Avocado propagation through seeds exhibit high genetic variation, hence less appealing for orchard plantings. Rooting of cuttings is only possible through a complex, lengthy and expensive process called "Frolich and Platt method". This creates limitations on rapid industry expansion due to scarcity and high price of plants in many countries. Alternative propagation methods are sought over 5 decades. 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. In this review, we present the developments in micropropagation of recalcitrant species, avocado, over the last 45 years. We summarise the culture media composition, hormones, growth conditions for different stages of avocado micropropagation pipeline, elaborating on cultivar specificity for *in vitro* success, and problems encountered under *in vitro* conditions and during acclimatisation. Overview of the current knowledge is critical to focus on important aspects in protocol optimisation, to develop an efficient and effective micropropagation system for avocado as well as other woody plant species recalcitrant for micropropagation.

Keywords

Avocado, Micropropagation, *In Vitro* Culture, Shoot Regeneration, Root Regeneration, Acclimatisation

1. Introduction

Avocado (*Persea americana* Mill.) is a nutritious and healthy fruit containing all food elements (carbohydrates, proteins and fats), wide spectrum of vitamins (A, B, C, D, E and K) and minerals [1]. 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 [2]. The origin of avocado runs back to 291 B.C. in Mexico but it is one of the first fruits to be domesticated by humans as early as in 6400 B.C. [3]. It is the only economically important species in family Lauraceae and classified in to three land races referring to the place of origin; Mexican race, Guatemalan race and West Indian race [2]. The racial differences exhibit variation in many traits including important agronomical and commercial traits [4]. However, attributes related to vigour under abiotic and biotic stress conditions is a major difference among the cultivars of the three races. For an example, Mexican cultivars are most tolerant to cold and poorly aerated soils [5] [6] and West Indian cultivars are tolerant to high temperature, humidity, soil salinity and soil pH [5] while members of the Guatemalan race behave intermediate to unfavourable environmental conditions [5].

If grown from the seedlings avocados may take up to 10 years to bear fruit. Therefore, as common to many horticultural fruit crops, avocados are grafted with bud-wood from a selected mature tree for precocious production and trueness of type. Beneficial shoot and root characters are integrated by selection of rootstock and scion cultivars to optimum productivity and crop manageability. Considering the consumer preference for fruit quality, which is vital for a commercial crop of avocado, the scion cultivar may draw the most attention during selection for orchard plants. The agronomically important features are related to plant vigour, productivity, quality of fruit, tolerance to pests and diseases and adoptability to different soil conditions [7]. Rootstocks are often selected for dwarf size [8], salt tolerance, adaptation to alkaline soil [8] [9] and pest and disease resistance [9] while scion characters are mostly screened for fruit quality, fruit set, post-harvest fruit quality and consumer preference [10]. Rootstock and scion contribute different attributes to the tree as a whole, and the combination determines the overall productivity of the tree. There are two types of rootstocks used in avocado industry; seedling rootstock (germinated seed of a selected rootstock plant) and clonal rootstock (rooted cutting from a selected mature rootstock plant). The type of rootstock largely influences the field performance and ultimately the harvest of the plant [11] [12].

Avocado bears complete flowers (male and female organs in a single flower), but shows an unusual pollination syndrome, "protogynous dichogamy" of male and female floral activity at different times during the day which favours out crossing [13]. 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 [12]. Moreover, some literature outlines the benefits of clonal rootstocks compared to seedlings

such as early flowering and higher yield [14]. However, this is still a controversial point for the preference for avocado rootstocks. Though this situation prevails, it is understandable that planting complete clonal plants (composed of both clonal scion and rootstock) would result a complete homogenous orchard with predictable offspring with respect to desired on field performance and complying with modern farming practices.

The two avenues, vegetative propagation or seeds obtained from controlled pollination can conserve genetic stability during plant propagation [15]. However, controlled pollination of avocado results very high fruit drop [16] leaving no other option except vegetative propagation to ensure genetic stability. Research into alternative methods, for clonal rootstock production, such as cuttings, air layering and tissue culture have all been attempted for vegetative propagation of avocado [6] [8] [17]. Pretreatment of mother plants (e.g. injecting of giberrellic acid, etiolation, ring barking) prior to obtain the cuttings, or different incubation conditions after rooting treatment have been experimented [18] [19] [20] [21]. However, rooting of cutting has shown limited success with very low percentage of root induction, inconsistent rooting, longer time for root induction and difficulty in managing experiments under field condition occupying a large number of cuttings [21] [22] [23] [24] [25].

Clonal rootstock propagation of avocado is the bottle neck and has become a crucial step in avocado propagation cascade. Commercially, clonal rootstocks are propagated by the Frolich & Platt double grafting method [23]. This requires a seedling nurse plant, to which a rootstock scion is grafted. Upon graft healing the grafted plant is subjected to an etiolation period to etiolate and elongate axillary buds of grafted scion. The base of the etiolated shoot is then treated with commercial rooting hormones containing indole-3-butyric acid and covered with an external plastic pot while attached to the nurse seedling. Once the roots are established, the second grafting takes place on rooted rootstock plant with bud-wood from a selected mature scion cultivar [16] [26] to obtain an orchard ready plant. Though this technique is more than 40 years old, it is the sole technique to obtain clonal rootstocks from mature elite plants despite the disadvantages; being expensive, time consuming and laborious, with a struggle to meet the supply for growers' demand [26] [27], especially in the countries where high labour cost is involved. However, further improvement of Frolich's technique by Earnst [26] using multiple micro cloning with the use of micro containers has been helpful with a limited increase in production, but has not alleviated problems with respect to labour, cost and time.

With the introduction of micropropagation techniques, attention was directed towards establishing an industry applicable tissue culture procedure for avocado clonal propagation. In the modern research sphere, micropropagation is an essential part of clonal propagation, transgenic plant generation, germplasm preservation, plant breeding and novel variety development [28]. Nevertheless, direct application of micropropagation as a mass propagation tool for economically

important plants have gained the most attention due to several reasons. E.g. tissue culture is advantageous being independent from climatic changes, high rate of multiplication, and minimal space requirements under well controlled pest and disease-free plant propagation.

The feasibility of micropropagation of many herbaceous plants has been demonstrated repeatedly [29], however woody plants are well known to be recalcitrant to tissue culture conditions [30] [31] [32]. The regeneration frequencies of asexual tissues of woody species have generally been zero or low, and require cultivar specific procedures [10]. Avocado with no exception behaves highly recalcitrant to *in vitro* conditions [9] [16] [33] [34]. The morphogenetic capacity in tissue culture is lower for adult (after flowering) avocado tissue than juvenile (before flowering) tissues [35] [36]. Direct application of protocols optimised for juvenile material does not ensure success with materials of adult nature [16].

An array of different mature (shoot tips, axillary buds) and juvenile (embryo, shoot tip, axillary buds, leaf, leaf petiole, root pith, stem pith, flower, fruit mesocarp, peduncle, pollen, cotyledon and protoplasts) avocado explants have been cultured *in vitro* for different research goals. However, limited success has been reported with micropropagation attempts, failing to establish comprehensive protocols that can be readily adopted to replace conventional propagation practice [7] [29] [34]. Considerable amount of research requirements exists to develop a successful protocol for *in vitro* regeneration and propagation of avocado.

A comprehensive *in vitro* micropropagation protocol will concentrate on several aspects; a suitable culture system, explants selection, explant and/or mother plant pre-treatment conditions, culture conditions, basal media, and hormone regime at different stages, specific to species and cultivar. This review intends to focus on all the stages of tissue culture system, associated problems and solutions, to better understand the favourable conditions for avocado micropropagation. Further the cultivar specificity of avocado for *in vitro* conditions will be emphasised. This review will concentrate on a summary of *in vitro* systems trialled nearly for five decades, for clonal propagation of avocado from both juvenile and mature material.

2. Techniques of Micropropagation for Avocado

2.1. Shoot Tip/Meristem Culture

Culturing extreme apical tissue or small apical tips from woody plant species is extremely challenging, but it is the most effective method for virus elimination in plant tissue culture [37] [38] [39] [40] [41]. Beside virus elimination, regeneration through meristem has the advantage of maximizing regeneration rate [42], which is highly favorable for mass propagation.

Typically, in meristem culture, explant apical sections are 1 mm or less in length with no visible leaf primordia for virus elimination [43]. But with avocado, in order to overcome mortality and lower regeneration capacity, the meriste-

matic dome is excised with one or two leaf primordia, while still minimizing percentage of virus infected plants [43]. To improve regeneration in avocado meristem culture, Schroeder [38] used large shoot tips (3 - 8 mm) with well-developed bracts and leaf primordia. However, shoot regeneration was hindered by massive callus formed at the base of the tissue resulting poor shoot elongation and failure in root induction. To date no meristem culture system has been established to produce intact plantlets, using either juvenile or mature avocado explants, even with the use of large shoot tips.

2.2. Vegetative Axillary Bud Culture/Nodal Culture

Most woody plant species are amenable to nodal culture and shown to be more successful [44] than shoot tip culture, thus has been employed in avocado tissue culture for many years [27] [38]. Avocado shoots activated from axillary buds have been reported to remain alive for longer but associated with poor elongation, which limits the multiplication capacity through nodal segments [38]. Nevertheless, nodal explants are a very reliable source of explants in terms of preserving genetic stability of elite cultivars [16]. 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 and will be discussed in detail at several points in this review.

2.3. Regeneration from Callus

This is an indirect plant regeneration approach in tissue culture using a two-step process where callus is generated through dedifferentiation, then redifferentiated into an intact plant. Callus establishment in avocado has been successful starting from a variety of explants such as stem, leaf, flower, fruit mesocarp, peduncle and cotyledon [43] [45]. Living avocado cells from any tissue shown to be responsive for cellular proliferation to produce amorphous calli masses [46]. According to Schroeder [46] some avocado calli have survived *in vitro* for over 17 years. This trait can be exploited for germplasm preservation if regeneration from callus could be achieved. Under the influence of plant growth regulators, cells can be induced to form pro-embryos or somatic embryos which can then be developed into intact plants [47], which has been possible with immature zygotic embryo tissues of avocado [48].

Ahmed *et al*., [10] have shown that callus induction from avocado embryonic tissue depends on the hormone regime; the minimum level being 2.0 mgL^{-1} 6-benzyleaminopurine (BAP) or 2.0 mgL^{-1} 1-naphthalene acetic acid (NAA). Blumenfeld and Gazit [49] investigated the response of cotyledon tissue to cytokinin to form callus and found that kinetin, BAP, zeatin and 6 (ɣɣ-dimathylallyl-amino) purine at a concentration of 1 mgL^{-1} in conjugation with 5 mgL^{-1} indole-3-acetic acid (IAA), to equally promote callus growth. However, shoot regeneration from avocado callus remains unsolved in spite of the incidental direct root regeneration from callus tissue through continuous

subculture [37] [43] [50].

3. Processes Involved in Avocado Micropropagation

3.1. Explant Selection

Certain attributes of explant material have been reported to determine the success of *in vitro* regeneration of plants. Ontogeny of the mother plant, explant source (apical or axillary), explant's position on the mother plant, metabolic status, and genotype are thought to be influential [33].

Explant harvesting position on the mother plant is accountable for culture response. Zulfiqar *et al*. [33] observed a maximum proliferation rate of 2.5 shoots per explant from axillary buds compared to apical buds (1.58) in cv. 'Fuerte'. This could be due to tissue specific morphogenic potential and the inherent capabilities of different meristematic activity within different plant parts with respect to the position within the plant. Conversely, pruning the mother plant has resulted an opposite pattern, 2.33 shoots per explants with shoot tips compared to 1 shoot per explant with axillary bud nodes in the same cultivar [33].

Several studies support that shoot regeneration is affected by explant harvesting season [51] [52]. However, Castro, Oyanedel, and Cautín [34] have seen no seasonal effects on *in vitro* growth of mature cv. 'Lula' and 'Velvick', explants harvested in summer, fall and spring.

3.2. Preconditioning of Explants and Culture Initiation

The initiation phase of explants in tissue culture is associated with several problems; bacterial and fungal contamination, low sprouting rates, browning due to oxidising exudates [16]. Early attention to these problems will determine the success of the micropropagation procedure as these directly affect subsequent shoot and root regeneration stages.

Measures to reduce microbial contamination and robustly propagate disease free plants start with selection of healthy, stress free, mother plants. Preparation of mother plants by pruning and applying fungicides [53], followed by surface disinfestation/sterilisation procedures, depend on explants type, condition of mother plants and their environment. Surface sterilisation procedures will generally involve washing under running tap water, treatments with ethanol and/or sodium hypochlorite solution with added surfactant (e.g. Tween −20) and several washing steps with sterile distilled water after each step. The ethanol washing step has been shown to be important to reduce infestation levels to a great extent [53], when compared to procedures excluding ethanol. The use of 1% $HgCl_2$ solution in place of 70% ethanol is another standard practice [54].

Explants obtained from field trees have been repeatedly reported to be highly contaminated with bacteria and fungi once initiated *in vitro*. Antibiotic treatments such as rifampicine (25 mgL^{-1}), tetracycline (25 mgL^{-1}) and garamicine (1 mgL^{-1}) have been used as control measures in such instances [7]. Immersion of plant material in a fungicide solution, e.g. Thiobenzole, is effective to control

fungal contaminations [27].

Activation of dormant apical and axillary buds at initiation stage is required for some avocado cultivars to expedite growth. Barceló-Muñoz and Pliego-Alfaro [16] prescribe a roller drum incubation in liquid medium for apical and lateral buds activation within 2 weeks. Etiolation of plants prior to obtaining nodal sections for initiation has been shown to successfully activate bud growth in cvs. 'Hopkins', 'Duke 7' and 'Colin V-33' [8] [29] [53]. However, according to Schroeder [29] etiolated shoots produce large callus at the cut end, though shoot elongation is satisfactory, while light grown shoots remained dormant in culture with a very large callus at base. Nevertheless, several authors have succeeded in braking dormancy using light grown juvenile and mature nodal sections of avocado, with [45] or without hormones [34] in regeneration media showing different responses; an increased shoot proliferation for cv. 'Duke' and only a marginal increase for cv. 'Topa-Topa', 'Hass' and 'Fuerte'.

During explant preparation for initiation, severed plant tissues excrete phenolic compound which can be oxidised by polyphenoloxidases, peroxidises or by air. The oxidised phenolic compounds (quinines) appear brown around cut surfaces of the tissue. Quinones show inhibitory effects on enzymes activity which ultimately results in tissue death [55]. Pretreatment of explants to avoid browning has been considered by many authors to establish quality cultures. Castro, Oyanedel, and Cautín [34] treated explant material with 500 mgL^{-1} ascorbic acid and citric acid mixture containing 50% v/v ice along with dark incubation. In a study using embryonic axis from cvs. 'Catalonia', 'Choquette', 'Dade', 'Maxima' and 'Tower', a period of 2, 7 and 10 days dark incubation has successfully eliminated browning with no inhibited shoot growth compared to explants incubated under illuminated conditions [56]. Nevertheless, 'Duke 7' mature nodal sections grown under 24 hours light regime showed reduced phenolic production in culture [53], possibly due to maturity level of explant material used in the study. Further, explants from trees pruned in the same year, resulted in more browning in culture than explants from trees pruned two years before, suggesting the link between higher phenolic production in young actively growing tissues irrespective of actual ontogenetic age of the plant [33].

3.3. Selection of Basal Media

Basal media is composed of macro nutrients (N, K, P, Ca, Mg, S), micronutrient (Fe, Ni, Cl, Mn, Cu, Zn, B, Mo) and vitamins essential to complete the growth cycle of a plant [57]. Determination of optimum nutritional environment is one of the challenges during tissue culture protocol optimisation [58] as nutrient requirement for optimum growth can be species and cultivar specific. Further, basal nutrient composition plays a vital role in different phases of the micropropagation cycle; initiation, shoot induction, multiplication and root induction. However, it is evident that juvenile avocado explants show no specificity for basal media while mature explants show restricted growth depending on cultivar.

In protocol optimisation for avocado, research into basal media selection has been limited (Table 1) and many of the attempts have been reported on Murashige and Skoog (1962) based media [8] [9] [10] [29] [33] [43] [54] [56] [59] [60] [61] or Modified Murashige and Skoog (MMS) medium [10] [62] for shoot induction and proliferation. This could be one of the reasons for limited success in early stages hindering the shoot regeneration.

The other basal salt formulations that have been used for avocado includes, Lloyd and McCown woody plant (WPM) basal media (1981), Anderson basal medium (1980), Dixon and Fuller (DF) (1976), Gamborg (1966), Nitsch (1969), Miller (1956) media [34] [45] [53] [62] [63]. Combination of macro and micro nutrients from one basal formulation, with vitamins from another (MS macro and micro with Gamborg vitamins) has also been reported [27]. It is not possible to closely compare the above studies (Table 1) due to variability in hormone and other supplements used. However, WPM shown to be superior for avocado nodal shoot growth compared to MS, MMS, DF, Andersons, Miller, Nitsch and Gamborg media formulations.

3.4. Shoot Regeneration

Avocado shows limited shoot proliferation and elongation when shoot tip and nodal explants are cultured without exogenous hormones [33]. Juvenile shoots exhibit continuous growth in culture where adult shoots are associated with less vigor, poor stem elongation, heavy callusing, retarded enlargement, shoot tip die back, leaf defoliation and leaf abscission [9]. It can be presumed that correct use of basal media and hormone combination would facilitate axillary bud activation, shoot growth and elongation.

Cytokinins are the primary hormones involved in shoot regeneration process. Optimum concentrations of cytokinin significantly increase RNA, DNA and protein synthesis directing cell division [64], while extreme concentrations (higher or lower) will result in adverse effects, such as stunted shoots, excessive callus production, shoot tip necrosis during shoot regeneration and multiplication irrespective of plant species [65] [66]. Even so, many physiological changes are a collective response to more than one hormone, therefore shoot regeneration *in vitro* can be better manipulated using a combined hormone strategy. For

Table 1. Basal media comparison studies for avocado cultivars at different culture phases.

Basal media	Cultivar	Effects during shoot regeneration	Effects at root regeneration	Reference
MS (half strength macro elements)	Mature Mexican IV-8	3.2 shoots per explant Necrotic leaves	74% rooting	Barceló-Muñoz *et al.* [62]
Gamborg		3.2 shoot per explant No necrotic leaves	90% rooting	
MMS	Juvenile plant material	No callus production	Not tested	Schroeder [29]
Nitsch		Excessive callus produced		

this reason, during shoot regeneration low levels of auxins and gibberellic acid are commonly coupled with cytokinins.

Literature supports that avocado material of juvenile nature, shows 100% shoot regeneration at most instances irrespective of cultivar [54] [56] [67]. Further, some suggests that there is no absolute requirement for hormones to regenerate shoots from juvenile axillary buds [63]. Contrary to that mature material is highly cultivar specific to hormone regime at shoot regeneration (**Table 2**).

Consistency of medium has been considered important for shoot regeneration. Solid, liquid or double phase (liquid layer on solid media) culture media have been tested for avocado to eliminate problems in shoot proliferation and to improve shoot health. Several authors have used double phase medium which includes a lower solid phase (high cytokinin) and a liquid upper phase (low cytokinin) to facilitate shoot proliferation and shoot elongation [36] [62] [68], beside disadvantage of hyperhydric effects on shoot. However, a study by Pliego-Alfaro [68] showed that good quality shoots produced using double phase medium poorly performed during rooting stage compared to shoots generated on solid medium. Further optimizations on a liquid medium using a roller drum has delivered best results for shoot regeneration with 1.3 µM BAP with reduced levels of hyperhydricity symptoms [62]. An early study by Nel, Kotze, and Snyman [69] also stated a liquid medium with a filter bridge was better than the use of solid agar medium for shoot multiplication.

An alternate culture system in which shoots were cultured in liquid medium for 2 weeks in a roller drum followed by six weeks in double phase conditions

Table 2. Summary of findings on effects of different hormones concentrations and combinations for shoot regeneration from nodal explants of mature avocado.

Cultivar	Hormones used	Shoot regeneration results	Reference
'Velvick'	*Meta*-topolin (0.1 mgL^{-1}) + GA3 (0.1 mgL^{-1})	100% shoot regeneration	Bandaralage *et al.* [35]
'Drymifolia'	BAP (0.5 mgL^{-1}) + IBA (0.1 mgL^{-1})	3 to 5 shoots per explants and 71.3% shoot regeneration	Martinez-Pacheco *et al.* [27]
'Hass'	No hormone + 2% (W:V)) peptone	44% shoot regeneration	Nhut *et al.* [54]
'Ness'	BAP (2 mgL^{-1})	0% shoot induction	Barrera-Guerra, Ramirez-Malagon, and Martinez-Jaime [7]
'Mexican IV-8'	Alternate culture in liquid and double phase medium, BAP (1.3 µM) in liquid medium and BAP (0.4 µM) in liquid phase and BAP (2.8 µM) in solid phase	3.2 shoots per explant	Barceló-Muñoz *et al.* [62]
'Lula'	BAP (0.65 mgL^{-1})	70% shoot regeneration	Castro, Oyanedel, and Cautín [34]
'Velvick'	BAP (2 mgL^{-1}) + IBA (0.5 mgL^{-1})	92% shoot regeneration	
'Topa-Topa'	Double phase medium BAP (0.1 mgL^{-1}) in liquid phase and BAP (0.65 mgL^{-1}) in solid phase	3.1 shoots per explant	Zirari and Lionakis [36]
'Hass'		1.6 shoot per explant	
'Fuerte'		1.7 shoot per explant	
'Duke'		1.63 shoots per explant	
'Duke -7'	BAP (1 mgL^{-1})	0% shoot regeneration	Cooper [53]

has been studied by Barceló-Muñoz *et al*. [62] to evaluate shoot proliferation in adult avocado shoots. This system allowed production of longer shoots and increased axillary bud activation. However, shoots continuously grown on double phase medium grow large callus at base with severe hyperhydricity symptoms that was similar to observations made by Pliego-Alfaro and Murashige [9]. Barceló-Muñoz *et al*. [62] also emphasise possible adaptability of this alternate culture system for continuous active proliferation of mature avocado. Nevertheless, Bandaralage *et al*. [35] has presented, the best shoot regeneration from mature material so far achieving 100% axillary bud growth 100% in cv 'Velvick', using 0.1 mgL^{-1} *meta*-topolin and 0.1 mgL^{-1} GA_3 on solid WPM. This suggest better manipulation of hormone along with basal nutrients in a cultivar specific manner would lead to better results with solid media which is practically more suitable in mass propagation. Incorporation of high CO_2 level in culture vessels has been trailed to improve shoot quality, but found to be not helpful in speeding up shoot growth, though it positively affected on shoot elongation with more juvenile appearance than original tissues [7] [70]. This is related to the inhibition of ethylene effect through reducing ethylene production by 5% - 10% (v;v) CO_2 levels within culture environment [7].

3.5. Root Regeneration

In vitro root regeneration is the most onerous and rate limiting step of avocado micropropagation process [9] [33] [43] [71]. Large numbers of factors affect the success of rooting of avocado. Therefore, this stage in micropropagation pipeline has been given prominence by authors specially in mature avocado propagation, and has not been very successful as yet (**Table 3**).

The prerequisite for root induction is high quality shoots [10] [16]. According to Nel, Kotze, and Snyman [69], stronger shoots rooted better at rooting stage and weaker shoots rooted better when an additional culturing period was introduced without any hormones in the basal medium to improve quality of shoots. Furthermore, juvenile shoots with larger leaves have rooted better than that of shoots with smaller leaves [10] [53].

The type of cytokinin, quantity and duration of exposure in shoot regeneration

Table 3. Summary of successful attempts in root induction for nodal explants of mature avocado.

Cultivar	Hormones and special additives used	Root induction (%) results	Reference
'Drymifolia'	IBA—0.5 mgL^{-1} MS macro nutrients with and Gamborg vitamins Activated charcoal—1 gL^{-1} Ascorbic acid—0.4 gL^{-1}	57.5	Martinez-Pacheco *et al*. [27]
'Mexican IV-8'	IBA—1 mgL^{-1}, 3 days MS macro nutrients 0.3 times	90	Barceló-Muñoz *et al*. [62]
'Duke 7'	Shoots grafted to juvenile rootstock IBA—1 mgL^{-1}, 3 days MS macro nutrients 0.3 times Activated charcoal—1 gL^{-1}	50	Pliego-Alfaro and Murashige [9]

phase influence root induction of many woody plants. This is termed as the carry over effect of cytokinins which reduces the rooting ability. In a rooting experiment by Barrera-Guerra, Ramirez-Malagon, and Martinez-Jaime [7], juvenile shoots have shown much higher rooting potential except for the shoots raised in thidiazuron (TDZ) (0.0001 mgL^{-1} - 1 mgL^{-1}) containing medium during the shoot regeneration stage.

Among all plant growth regulators, auxins play an essential role in root induction process of plants. Proving it is no difference with avocado, García-Gómez *et al*. [59] showed that auxin transport inhibitor 2,3,5-triiodobenzoic acid completely inhibited root primordia differentiation of juvenile avocado micro cuttings *in vitro*. Therefore, exogenous supply (specific type and concentration) of auxin is paramount in rooting avocado shoots. Avoiding exogenous application of auxin failed rooting, even shoots of juvenile origin [10] [33]. The optimum concentration for rooting is known to be cultivar specific for avocado. However, sub optimal or supra optimal levels of auxin may adversely affect shoot health and rooting process [33]. Not only root induction but also root elongation is extremely sensitive to the auxin concentration, thus root length tends to reduce with the increase of IBA concentration from optimum level [33]. Auxin conjugates and peroxidase activity is also known to be involved in auxin regulation of rooting [72]. García-Gómez *et al*. [60] studied soluble, ionically and covalently bound peroxidase activity in avocado leaves and stems during *in vitro* rooting of cuttings. They observed two-fold increase in peroxidase activity at stem base after three days exposure to exogenous IBA.

According to literature type of auxin, concentration, exposure time and mode of application has influence on rooting success. IBA is reported to be the most frequently used rooting hormone for avocado while indole-3-acetic acid (IAA) and 1-naphthaleneacetic acid (NAA) usage has been seldom. Another auxin 2-4-dichlorophenoxyacetic acid (2,4-D) has been toxic even at very low concentrations [67], and not frequently used for rooting avocado. IAA is a naturally occurring auxin in plants and has low stability when exposed to light and high temperature [59]. A comparison of IBA vs NAA for rooting (juvenile material) revealed that IBA induces slightly higher rooting percentages while NAA promoting faster rooting [53]. However, Nel *et al*. [69] could achieve only 65% (juvenile shoots) rooting with the media supplemented with 2 mgL^{-1} IBA possibly due to cultivar variation. Nevertheless, rooting percentage increased up to 80% with prolonged incubation in 2 mgL^{-1} IBA. Also, Barringer, Mohamed-Yasseen, and Splittstoesser [56] achieved 30% rooting (juvenile shoots) in medium containing IBA (1 or 2 mgL^{-1}) and activated charcoal (1 gL^{-1}) in media.

Method of exogenous auxin treatment (continuous or pulse exposure) considerably affects rooting success of avocado. A study supports pulse treatment of 1 mgL^{-1} IBA for 1 second being more effective in root induction (100% rooting - juvenile shoots) compared to continuous incubation with 1 mgL^{-1} IBA (60% rooting) with cvs. 'Hopkins' and 'Hass' [53]. Further, this study showed, not only

method of exposure (continuous vs pulse) but also the duration of exposure is crucial, supported by obtaining 100% rooting with 1 sec dipping compared to 60% rooting with 5 sec dipping in 3 gL^{-1} IBA solution. For juvenile shoots, deploying pulse treatment with different exposure times using a range of IBA concentrations, similar successful results were achieved only at 5 second exposure with 4 gL^{-1} IBA, while prolonged exposure to auxin failed to produce roots [7]. This indicates inhibitory effects of supra-optimum auxin concentration for avocado.

The position of bud on the tree and juvenility gradient which exist in explants affect root induction capacity [33] [73]. Shoots from apical buds (29% rooting) proven to be more responsive for rooting treatments compared to those of axillary buds (19% rooting) [33]. On the other hand, higher concentration of IBA has been required for shoots regenerated from axillary buds (1.5 mgL^{-1}) compared to shoots regenerated from apical buds (1 mgL^{-1}). This can be attributed to high endogenous auxin levels present in apical buds and explants' different potential to respond for growth hormones.

Increasing plant maturity or decreasing juvenility diminishes rooting potential of woody perennials [9] [10] [73] [74]. Even *ex vitro*, rooting capacity of cuttings largely relies on age of stock plant for many species, which is known as 'juvenility phenomenon for rooting'. This ontogenetic age dependency for rooting has been observed with avocado. In Kadman's [75] *ex vitro* experiment, rooting percentages decreased from 100% to 30% when cuttings were taken from 12 months old seedlings instead of 2 months old seedling. Similarly, cuttings from young seedlings root at a very high percentage while cuttings from mature trees fail to root or rooting percentages are very low *ex vitro* [24]. Under *in vitro* conditions, juvenile shoots have repeatedly shown to result better rooting percentages while shoots from mature material showed no rooting at most of instances. Juvenile avocado shoots report 100% rooting in agar based media, supplemented with IBA [9] [59] [60] [67]. On the other hand, mature avocado shoots have been highly recalcitrant for any type of rooting treatment either resulting very low percentage of rooting or no root induction [9]. Zirari and Lionakis [36] attempted to root mature avocado shoots (cv. 'Duke', 'Topa-topa', 'Hass', 'Fuerte') using a two-step rooting procedure, but it resulted leaf defoliation leading to 100% mortality within four weeks. In a similar attempt Barrera-Guerra, Ramirez-Malagon, and Martinez-Jaime [7] were not successful in rooting adult avocado shoots using an array of 23 treatments including pulse treatments (5 second to 1 day) ranging from 0.05 gL^{-1} to 6 gL^{-1} IBA, and subculture after rooting treatment period on MS media containing IBA (0.3 mgL^{-1}) and Kinetin (3 mgL^{-1}) for five months.

Researchers have been looking into methods of rejuvenating mature plants to induce juvenile characters considering the high rooting potential of juvenile plants. Evidence for phase reversal has been proven through rooting ability when avocado shoots were subjected to continuous subculturing as well as after grafting

mature material to juvenile rootstock *in vitro*. Grafting mature avocado scion to juvenile rootstock to increase rooting ability has been based on a hypothesis that, phase reversing factors are transmitted to scion from juvenile rootstock. One such experiment by Pliego-Alfaro and Murashige [9], rooting 'Duke 7' shoots (mature, juvenile and grafted mature to juvenile rootstock), resulted 100 % rooting in juvenile shoots, no rooting in adult shoots and 30% rooting in grafted mature shoots to juvenile rootstock. This supports the hypothesis of possible rejuvenation of mature material through grafting. Further experiments by them have confirmed 50% rooting success of adult avocado shoots grafted to juvenile rootstock with 1 mgL^{-1} IBA treatment for three days followed by transfer to hormone free medium. However, repeated grafting (3 times) of adult material to seedling rootstocks has not been effective in improving the rooting percentages, even though the rooted plants retained their rooting competence. The extent to which repeated grafting would sustain rooting ability was not tested in that study. Another rejuvenation method, pruning field mature plants prior obtaining explants for culturing, has also been tested by avocado researchers. Explants obtained from rejuvenated mature plant by pruning has led to the highest ever rooting percentage reported with mature avocado (90%) [62]. At the same time, this result validates the idea of juvenility induction in mature plants through pruning [24].

A dark incubation phase to induce rooting is not confined only to *in vitro* studies of avocado. Etiolated tissues permit more undifferentiated tissues with delayed lignification process creating better stem conditions for rooting [76]. Thus far, there is no evidence on avocado shoots to produce elongated, etiolated shoots under *in vitro* conditions as of *ex vitro*. But most rooting protocols include a dark incubation of 1 - 5 days immediately after auxin application or during continuous exposure followed by transferring to hormone free medium exposed to light [9]. However, a comparison study on effect of dark and light phase for *in vitro* rooting is yet to be demonstrated.

An alternate culture method has been introduced by Barceló-Muñoz *et al*. [62] to induce roots in adult avocado shoots, to compare rooting capacity of shoots obtained through Frolich technique. This occupied a liquid phase (IBA –1 mgL^{-1}) incubation in the roller drum for 3 days at 5 rpm followed by transferring to solid phase (with no auxin) which resulted 90% rooting with cv. 'Mexican IV-8' (mature).

The medium consistency also affects *in vitro* rooting. Liquid media has high water mobility, thus results hyperhydricity while solid media induce growth inhibition due to low water mobility [68]. However, it has been evident that solid-solid composition support poorly for rooting avocado [33] [36]. In contrary, over 50% rooting has been achieved with adult avocado shoots of a Mexican avocado cv. 'Drymifolia' using a solid medium [27]. A double phase medium (upper liquid phase and lower solid phase) will allow maintenance of polarity and higher availability of hormones and nutrients [68]. However, according to

Barceló-Muñoz *et al*. [62], adopting two step procedure; three days incubation in auxin supplemented liquid medium followed by transferring to hormone free condition, induce more roots irrespective of media consistency of the hormone free stage.

It is a common practice to reduce concentration of basal nutrients during root induction stage. Avocado seedling shoots have recorded 100% rooting in hormone free 0.3 times strength of salt compared to 10% in full strength medium [67]. However, in this study he has seen no effect on improvement in rooting or general health of the plant by altering vitamins in the medium. Different levels of macro nutrients in basal media, 1/2 [53], 3/4 [33], 3/10 [9], 1/3 [62] have been used to induce rooting. However, it is difficult to draw any conclusions of effectiveness on reducing nutrients due to the lack of comparison studies for the different macro and micro nutrient levels in basal media.

Ex vitro root induction of *in vitro* regenerated shoots has been an alternative strategy to overcome continuous failure in *in vitro* root regeneration for avocado. On the other hand, *ex vitro* root induction will simultaneously reduce time taken for acclimatisation. Pulse treatment of shoots with auxin and planting in a mixture of pumice:peat (50:50 v/v) and incubate with bottom heat of 26˚C has been successful resulting 90% - 100% rooting for *in vitro* raised juvenile shoots [53].

There are other additives that have been used to bring about positive effects on rooting avocado. Peptone is a soluble protein readily used in bacterial culture media. There is no clear understanding of peptone's function at root induction process for any plant species. However, 100% rooting has been achieved with juvenile shoots by incorporating peptone (2% w:v) with 2.7 μM NAA while exclusion of peptone from the medium has not induced roots [54]. Activated charcoal is another common constituent in rooting media. Evidence prevail for its dual function (promoting or inhibitory) of activated charcoal in rooting media [56]. Sucrose in media can also influence rooting. Pliego-Alfaro [67] tested different sucrose levels (1.5%, 6% and 9% w;v) at rooting stage, where 9% sucrose increased large amount of callus production during rooting demonstrating a negative effect. Several organic compounds, including phenolics have shown to facilitate rooting when combined with rooting hormones. Some studies showed diamine putrescine, an aliphatic organic compound as a promoter for rooting when coupled with IBA [10]. Pliego-Alfaro [67] point out aromatic phenolics, catechol and phloroglucinol as positive effectors for root induction and caffeic acid to increase root number per shoot.

Overall, combining different strategies and methodologies, a varying degree of rooting success have been achieved in avocado; maximum possible rooting percentage with juvenile material (100%) in vast range of cultivars, but only 90% with mature material only in cv. 'Mexican IV-8' [62]. This clearly indicates the in depth and refined, cultivar focused research needs for protocol optimisation for rooting mature avocado material.

3.6. Acclimatisation

Anatomical and physiological changes occur in plants under tissue culture conditions due to luxurious growth conditions with plenty of mineral nutrients, moisture, light and devoid of unfavorable external environmental conditions. *In vitro* grown plant lack proper leaf and root structures to withstand dynamic external environment and require a hardening phase prior transferring to external environment.

For avocado, it is recommended to transfer intact plants to greenhouse under 100% humidity followed by daily exposure to gradual increased durations for ambient relative humidity [16]. Different compositions of sterilised potting mixes have been used as potting media; e.g. Peat moss:perlite (1:1, v:v) [27], pumic:peat (50:50, v:v) [53].

Several authors states that micropropagated avocado plants exhibit slow growth during acclimatisation due to absence of vesicular-arbuscular mycorrhizae relationship. Inoculation of *Glomus fasciculatum* on primary root baring plantlets during transition from semi heterotrophic phase to total autotrophic phase has proven to be beneficial with respect to enhanced growth and nutrient content of shoots [71]. But this study emphasises the importance of controlled inoculation at different stages of acclimatisation for better results to reduce mortality due to destructive colonisation of mycorrhizae. Similar observation has been reported with significant improvement of shoot height and number of leaves upon *Glamus sp.* inoculation in non-sterile peat, perlite, sand and sterile soil mix [77]. However, in our opinion there is no enough work done on optimizing acclimatisation practices for micropropagated avocado plantlets, simply due to lesser availability of rooted plants.

4. Culture Incubation Conditions

Illumination and incubation temperature directly influence *in vitro* shoot and root regeneration process. The choice of culture incubation conditions should consider plant species, the type of culture (meristem, nodal or callus) and culture phase (initiation, shoot induction or root induction). It is reported that low temperatures reduce browning but do not facilitate regeneration of shoots of avocado (adult material) [34]. Higher viability of callus induced in leaf sections was observed at 21˚C than at 27˚C, in contrast the study showed shoot elongation of axillary buds was better in 30˚C compared to 20˚C, illustrating effects of different incubation conditions to regenerate callus and shoots from different explant sources.

Most *in vitro* culturing practices have adopted 16 h light period for avocado [8] [9] [33] [34] [35] [69]. However, 18 h photoperiod for the growth of embryonic axis [78] and 24 h for mature nodal sections [53] are also reported.

The irradiance level affect the physiology on multiplication and rooting stages of avocado [68]. De la Vina *et al*. [68] has shown reduction in Chlorophyll and increase in carotenoid levels with the increase irradiance levels. Further, irradiance

level of 35 μmol·m^{-2}·s^{-1} has been able to maintain green shoots while higher irradiance resulting yellowing of shoots.

5. Problems in Avocado Micropropagation

As of with any other woody plant in tissue culture, avocado is also associated with several problems besides being recalcitrant to regeneration of shoots and roots. There have been several research attempts to seek effective solutions to overcome these problems.

Tissue browning is a major obstacle for a successful culture establishment [10] [34]. N-(2-chloro-4-pyridfl)-N-phenylureal (CPPU) [34], sterile Potassium methasulfate [8], Polyvinylepyrolidone (PVP) [10] have been successful chemical control measure for browning. Also, low temperature and dark incubation shown to be effective to prevent browning. Etiolation of mature cvs. 'Duke' and 'Duke 7' have been shown to produce no browning compared to non-etiolated explants [36] [53] demonstrating the effectiveness of dark treatment to overcome tissue browning. Dark treatment of 7 - 10 days after culture initiation has also been used to overcome browning [78]. Use of anti-oxidants such as thiosulfate (1.8 mgL^{-1}) and silver nitrate (6 mgL^{-1}) has been effective to overcome oxidation [7]. Doubling iron (Fe) concentration in basal medium has also been helpful to eliminate browning [63]. Ascorbic acid (25 mgL^{-1}) and charcoal/activated carbon (1 gL^{-1}) supplements also tested successful to reduce lethal tissue browning [27].

Leaf defoliation of regenerated shoots is very difficult to avoid during continuous culture, especially at rooting stage of avocado [34]. During rooting phase, exposure to exogenous auxin (4.9 μM IBA) shown to be causative for rapid defoliation of leaves in mature shoots [9] while juvenile shoots have shown more tolerance to higher auxin concentrations (123 μM IBA). Silver nitrate or silver thiosilphate in culture medium could be helpful to overcome this problem. Further to this, mature avocado shoots in continuous culture tend to reduce vigor and suffer from shoot tip die back or necrosis [10] [16] [34]. Even juvenile shoots reduce nodal multiplication rates after about seven subcultures [61]. Incorporation of PVP at 0.1% (w/v) has shown to greatly address this problem [10]. Moreover, shoot die back has been reported when exposed to exogenous auxin at rooting stage with adult shoots [9] [33]. There is no method of avoidance reported so far to overcome this issue for mature shoots. However, similar problem in juvenile shoots of 'Duke 7' has been overcome by doubling the iron concentration in basal media [63].

Continuous attempts to overcome necrosis, specially associated with mature avocado [33] in culture are much needed as it seems to be a predominant problem in maintaining long term cultures [79]. Boron deficiency is one of the many reasons for gradual destruction of growing points in avocado [80]. Further, Boron deficit conditions gradually disintegrate root system of young avocado seedlings which can be reversible with replacement of Boron in media [80].

Therefore, identifying effective concentration for Boron supplementation could be helpful.

Chlorosis, another common problem in tissue culture, especially associated with fruit crops assumed to be due to high nitrogen content in the medium or due to iron deficiency [63]. According to Harty [63] modification to MS medium varying NH_4^+, NO_3^- and FeEDTA had not been sufficient to overcome this problem. However, this condition can be managed by altering light intensity and cultivar specific micro nutrient adjustments.

Vitrification occurs in avocado causing symptoms of hyperhydric shoots [33]. This is controlled by several factors such as gelling agent type, hormones, organic and inorganic compounds, water potential, incubation conditions (temperature and light) and ecology of container [81]. Hyperhydricity is a physiological disorder normally observed in proliferation stage caused by continuous subculture in media containing high level of cytokinin and/or use of liquid or double phase media [16]. Two types of hyperhydric conditions are associated with avocado; succulent or pathological hyperhydricity in which shoots show symptoms of succulent pale green leaves and shoots with large callus at base with reduced proliferation and rooting capacity, and humid or non-succulent hyperhydricity where shoots and leaves become dark green but do not lose the proliferation or rooting capacity [16]. This condition is effectively managed by timely transfer of *in vitro* shoots to hormone free conditions or solid media depend on the causative factor.

Poor leaf expansion of regenerated shoots is also found in avocado. Culture medium supplemented with 40 mgL^{-1} L-arginine and L-glutamine has been supportive for better leaf expansion and shoot growth [63].

6. Conclusion

Over the last 45 years, many of the avocado micropropagation studies have been focused on optimizing protocols for material obtained from juvenile plants with an objective of smooth transfer technique to mature plants. However, so far this has not been possible with avocado. The limited protocols that have been developed for mature avocado propagation are highly cultivar specific and do not meet requirements to adopt it as an industrial practice due to several problems. These include, limited multiplication, low percentage of rooting and inconsistent rooting. Moreover, the smooth transfer of techniques in current protocols is challenging in a mass scale industrial set up. Therefore, cultivar specific optimizations to increase proliferation and rooting success is highly required to achieve an efficient and effective protocols to micropropagate elite avocado cultivars.

Acknowledgements

We would like to thank the Australian Research Council Linkage Programme, 2013 (grant no. LP130100870), the Department of Agriculture and Fisheries, Australia and the University of Queensland, industry partners; Primary Growth

Pty Ltd., Jasper Farms Holdings Pty Ltd., Millwood Holdings Pty Ltd., T/A Delroy Orchards and Anderson Horticulture Pty Ltd., and Australian Postgraduate Award scholarship programme for funding our research in avocado micropropagation.

References

[1] Pierce, H.F. (1959). The Nutritional Value of the Avocado. *California Avocado Society Tear Book*, **43**, 83-85. http://avocadosource.com/CAS_Yearbooks/CAS_43_1959/CAS_1959_PG_83-85.pdf

[2] Silva, T.A. and Ledesma, N. (2014) Avocado History, Biodiversity and Production. In: Nandwani, D., Ed., *Sustainable Horticultural Systems*, *Sustainable Development and Biodiversity*, Springer International Publishing, Switzerland, 157-205. http://link.springer.com/10.1007/978-3-319-06904-3_8

[3] Galindo-Tovar, M., Ogata-Aguilar, N. and Arzate-Fernández, A.M. (2008) Some Aspects of Avocado (*Persea americana* Mill.) Diversity and Domestication in Mesoamerica. *Genetic Resources and Crop Evolution*, **55**, 441-50. https://doi.org/10.1007/s10722-007-9250-5

[4] Bergh, B. and Ellstrand, N. (1986) Taxonomy of the Avocado. *California Avocado Society Year Book*, **70**, 135-146. http://www.avocadosource.com/cas_yearbooks/cas_70_1986/cas_1986_pg_135-145.pdf

[5] Campbell, C.W. and Malo, S.E. (1976) A Survey of Avocado Cultivars. *Proceedings of the* 1*st Sub-Tropical Fruit Short Course*: *The Avocado*, Florida Cooperative Extension Service, 9-15.

[6] Wolstenholme, B. (2003) Avocado Rootstocks: What Do We Know; Are We Doing Enough Research. *South African Avocado Growers' Association Yearbook*, **26**, 106-112.

[7] Barrera-Guerra, J.L., Ramirez-Malagon, R. and Martinez-Jaime, O.A. (1998) *In Vitro* Propagation of Avocado (*Persea drymifolia* Ness.). *Proceedings of the Induced Mutations in Connection with Biotechnology for Crop Improvement of the FAO/IAEA Division of Nuclear Techniques in Food and Agriculture*, Peru, 5-9 October 1998, 63-69. http://www-pub.iaea.org/MTCD/publications/PDF/te_1216_prn.pdf#page=69

[8] Vega, D.E.S. (1989) Propagation *In Vitro* of Rootstocks of Avocado. *California avocado society Year Book*, **73**, 149-151. http://www.avocadosource.com/CAS_Yearbooks/CAS_73_1989/CAS_1989_149.pdf

[9] Pliego-Alfaro, F. and Murashige, T. (1987) Possible Rejuvenation of Adult Avocado by Graftage onto Juvenile Rootstocks *In Vitro*. *HortScience*, **22**, 1321-1324.

[10] Ahmed, M.F., Kantharajah, S.A. and Holford, P. (2001) Factors Affecting Adventitious Shoot and Root Formation from the Avocado Cultivar 'Hass' (*Persea americana* Mill.) *In Vitro*. *Gartenbauwissenschaft*, **66**, 293-297.

[11] Whiley, A.W., Köhne, J.S., Arpaia, M.L. and Bender, G.S. (1990) Future Prospects with New Avocado Cultivars and Elite Rootstocks. *South African Avocado Growers' Association Year Book*, **13**, 16-20. http://www.avocadosource.com/Journals/SAAGA/SAAGA_1990/SAAGA_1990_PG_16-20.pdf

[12] Ben-Ya'acov, A. and Michelson, E. (1995) Avocado Rootstocks. In: Janick, J., Ed., *Horticultural Reviews*, *Volume* 17, John Wikey & Sons, Inc., New York, 381-429. https://doi.org/10.1002/9780470650585.ch11

[13] Sedgley, M. (1985) Some Effects of Daylength and Flower Manipulation on the Floral Cycle of Two Cultivars of Avocado (*Persea americana* Mill., Lauraceae), A Species Showing Protogynous Dichogamy. *Journal of Experimental Botany*, **36**, 823-832. https://doi.org/10.1093/jxb/36.5.823

[14] De Villiers, A.I. and Ernst, A.A. (2015) Avocado Rootstock Research: Principals and Practices. *Proceedings of the World Avocado Congress VIII*, Lima, 13-18 September 2015, 40-45.

[15] Jain, S.M. and Ishii, K. (2003) Micro Propagation of Woody Trees and Fruits. Springer Science & Business Media, Netherlands. https://link-springer-com.ezproxy.library.uq.edu.au/content/pdf/10.1007%2F978-94-010-0125-0.pdf https://doi.org/10.1007/978-94-010-0125-0

[16] Barceló-Mu-oz, A. and Pliego-Alfaro, F. (2003) Micropropagation of Avocado (*Persea americana* Mill.). In: Jain, S.M. and Ishii, K., Eds., *Micropropagation of Woody Trees and Fruits*, Springer, Berlin, 519-542. http://link.springer.com/chapter/10.1007/978-94-010-0125-0_17 https://doi.org/10.1007/978-94-010-0125-0_17

[17] Ben-Yaacov, A. and Zilberstaine, M. (1999) Clonal Avocado (*Persea americana* Mill.) Rootstocks in Israel. *Revista Chapingo Serie Horticultura*, **5**, 39-42.

[18] Barrientos-Priego, A., Borys, M.W. and Barrientos-Perez, F. (1986) Rooting of Avocado Cuttings (*Persea americana* Mill.) Cvs. "Fuerte" and "Colin V-33". *California Avocado Society Year Book*, **70**, 157-164. http://avocadosource.com/CAS_Yearbooks/CAS_70_1986/CAS_1986_PG_157-163.pdf

[19] Cutting, J.G.M. and Vuuren, S.P.V. (1988) Rooting Leafy Non-Etiolated Avocado Cuttings from Gibberellin-Injected Trees. *Scientia Horticulturae*, **37**, 171-176. https://doi.org/10.1016/0304-4238(88)90160-4

[20] Frolich, E.F. (1951) Rooting Guatemalan Avocado Cuttings. *California Avocado Society Year Book*, **36**, 136-138. http://www.avocadosource.com/CAS_Yearbooks/CAS_36_1951/CAS_1951_PG_136-138.pdf

[21] Reuveni, O. and Raviv, M. (1980) Importance of Leaf Retention to Rooting of Avocado Cuttings. *Journal of American Society for Horticultural Science*, **106**, 127-130.

[22] Eggers, E.R. and Halma, F.F. (1937) Rooting Avocado Cuttings. *California Avocado Society Year Book*, **21**, 121-125.

[23] Frolich, E.F. and Platt, R.G. (1972) Use of the Etiolation Technique in Rooting Avocado Cuttings. *California Avocado Society Year Book*, **55**, 97-109. http://www.avocadosource.com/CAS_Yearbooks/CAS_55_1971/CAS_1971-72_PG_097-109.pdf

[24] Kadman, A. and Ben-Ya'acov, A. (1965) A Review of Experiments on Some Factors Influencing the Rooting of Avocado Cuttings. *California Avocado Society Year Book*, **49**, 67-72. http://www.avocadosource.com/CAS_Yearbooks/CAS_49_1965/CAS_1965_PG_067-072.pdf

[25] Leal, F.J. and Krezdorn, A.H. (1964) Rooting Avocado Cuttings. *Proceedings of Florida State Horticulture Society*, **77**, 358-362. http://www.avocadosource.com/Journals/FSHSP/FSHSP_VOL_77_PG_358-362_1964.pdf

[26] Ernst, A.A. (1999) Micro Cloning: A Multiple Cloning Technique for Avocados Using Micro Containers. *Revista Chapingo Serie Horticultura*, **5**, 217-220.

[27] Martinez-Pacheco, M.M., Suarez-Rodriguez, L.M., Hernández-García, A., Salgado-Garciglia, R., Fernández, I.V., Palomares, M.E.A., Cortés-Rodríguez, M.A. and Lopez-Gomez, R. (2010) *In Vitro* Propagation of Mexican Race Avocado (*Persea americana* Mill. Var Drymifolia). *Acta Horticulturae*, **923**, 47-52. http://www.actahort.org/books/923/923/_5.htm

[28] Thorpe, T.A. (2007) History of Plant Tissue Culture. *Molecular Biotechnology*, **37**, 169-180. http://link.springer.com/article/10.1007/s12033-007-0031-3 https://doi.org/10.1007/s12033-007-0031-3

[29] Schroeder, C.A. (1980) Avocado Tissue *In Vitro*. *California Avocado Society Year Book*, **64**, 139-141. http://www.avocadosource.com/CAS_Yearbooks/CAS_64_1980/CAS_1980_PG_139-142.pdf

[30] Benmahioul, B., Dorion, N., Kaid-Harche, M. and Daguin, F. (2012) Micropropagation and *Ex Vitro* Rooting of Pistachio (*Pistacia vera* L.). *Plant Cell, Tissue and Organ Culture*, **108**, 353-358. https://doi.org/10.1007/s11240-011-0040-6

[31] Amiri, E.M. and Elahinia, A. (2013) Optimization of Medium Composition for Apple Rootstocks. *African Journal of Biotechnology*, **10**, 3594-3601.

[32] Duhoky, M.M.S.S., Omar, M.S. and Yaseen, S.A.H. (2012) *In Vitro* Rooting of AppleMM106 (*Malus domestica* Borkh.) and Pear (*Pyrus calleryana*) Rootstocks. ATINER'S Conference Paper Series, No: AGR2012-0346. https://www.atiner.gr/papers/AGR2012-0346.pdf

[33] Zulfiqar, B., Abbasi, N.A., Ahmad, T. and Hafiz, I.A. (2009) Effect of Explant Sources and Different Concentrations of Plant Growth Regulators on *In Vitro* Shoot Proliferation and Rooting of Avocado (*Persea americana* Mill.) Cv. 'Fuerte'. *Pakistan Journal of Botany*, **41**, 2333-2346.

[34] Castro, M., Oyanedel, E. and Cautín, R. (1995) *In Vitro* Shoot Proliferation in Avocado (*Persea americana* Mill.) Induced by CPPU. *Proceedings of the World Avocado Congress III, Israel*, **3**, 223-226. http://209.143.153.251/WAC3/wac3_p223.pdf

[35] Bandaralage, J.C.A.H., Hayward, A., O'Brien, C. and Mitter, N. (2015) Gibberellin and Cytokinin in Synergy for a Rapid Nodal Multiplication System of Avocado. *Proceedings of the World Avocado Congress VIII*, Lima, 13-18 September 2015, 95-98. http://www.avocadosource.com/WAC8/Section_02/HitiBandaralageJCA2015.pdf

[36] Zirari, A. and Lionakis, S.M. (1994) Effect of Cultivar, Explant Type, Etiolation Pretreatment and the Age of Plant Material on the *In Vitro* Regeneration Ability of Avocado (*Persea americana*). *Acta Horticulturae*, **365**, 69-76. https://doi.org/10.17660/ActaHortic.1994.365.6

[37] Schroeder, C.A. (1976) Responses of Avocado Stem Pieces in Tissue Culture. *California Avocado Society Year Book*, **60**, 160-163. http://avocadosource.com/CAS_Yearbooks/CAS_60_1976/CAS_1976_PG_160-163.pdf

[38] Schroeder, C.A. (1973) Apical and Other Responses of Tissues of Avocado in Aseptic Culture. *California Avocado Society Year Book*, **57**, 138-141. http://www.avocadosource.com/cas_yearbooks/cas_56_1972/cas_1972_pg_138-141.pdf

[39] Romberger, J.A. (1963) Meristems, Growth, and Development in Woody Plants: An Analytical Review of Anatomical, Physiological, and Morphogenic Aspects. Government Printing Office, United States.

[40] Grout, B.W.W. (1999) Meristem-Tip Culture for Propagation and Virus Elimination. In: Hall, R.D., Ed., *Methods in Molecular Biology*, *Plant Cell Culture Protocols*, Humana Press Inc., Totowa, New Jersey, 115-125. http://link.springer.com/10.1385/1-59259-583-9:115 https://doi.org/10.1385/1-59259-583-9:115

[41] Yakuwa, H. and Oka, S. (1988) Plant Regeneration through Meristem Culture from Vegetative Buds of Mulberry (*Morus bombycis* Koidz.) Stored in Liquid Nitrogen. *Annals of Botany*, **62**, 79-82. https://doi.org/10.1093/oxfordjournals.aob.a087638

[42] Rani, V. and Raina, S.A. (2000) Genetic Fidelity of Organized Meristem-Derived Micropropagated Plants: A Critical Reappraisal. *In Vitro Cellular & Developmental Biology—Plant*, No. 36, 319-330. https://doi.org/10.1007/s11627-000-0059-6

[43] Nel, D.D. and Kotzé, J.M. (1984) The Role of Tissue Culture in the Avocado Plant Improvement Scheme. *South African Avocado Growers' Association Year Book*, **7**, 25-26. http://www.avocadosource.com/Journals/SAAGA/SAAGA_1984/SAAGA_1984_PG_25-26.pdf

[44] Schroeder, C.A. (1979) Etiolation and Avocado Bud Elongation *In Vitro*. *California Avocado Society Year Book*, **63**, 86-89. http://www.avocadosource.com/CAS_Yearbooks/CAS_63_1979/CAS_1979_PG_086-089.pdf

[45] Young, M.J. (1983) Avocado Callus and Bud Culture. *Proceedings of Florida State Horticulture Society*, **96**, 181-182. http://www.avocadosource.com/Journals/FSHSP/FSHSP_VOL_96_PG_181-182_1983.pdf

[46] Schroeder, C.A. (1977) Longevity of Plant Tissue Cultures. *California Avocado Society Year Book*, **61**, 72-74. http://avocadosource.com/CAS_Yearbooks/CAS_61_1977/CAS_1977_PG_72-75.pdf

[47] Welander, M. (1988) Plant Regeneration from Leaf and Stem Segments of Shoots Raised *In Vitro* from Mature Apple Trees. *Journal of Plant Physiology*, **132**, 738-744. https://doi.org/10.1016/S0176-1617(88)80238-4

[48] Encina, C.L., Parisi, A., O'Brien, C. and Mitter, N. (2014) Enhancing Somatic Embryogenesis in Avocado (*Persea americana* Mill.) Using a Two-Step Culture System and Including Glutamine in the Culture Medium. *Scientia Horticulturae*, **165**, 44-50. https://doi.org/10.1016/j.scienta.2013.10.019

[49] Blumenfeld, A. and Gazit, S. (1971) Growth of Avocado Fruit Callus and Its Relation to Exogenous and Endogenous Cytokinins. *Physiologia Plantarum*, **25**, 369-371. https://doi.org/10.1111/j.1399-3054.1971.tb01457.x

[50] Skene, K.G.M. and Barlass, M. (1989) Avocado (*Persea* spp.). In: Bajaj, Y.P.S., Ed., *Trees II. Biotechnology in Agriculture and Forestry*, Vol. 5, Springer, Berlin, Heidelberg, 3-12. http://link.springer.com/chapter/10.1007/978-3-642-61535-1_1

[51] Dhar, U. and Upreti, J. (1999) *In Vitro* Regeneration of a Mature Leguminous Liana (*Bauhinia vahlii* Wight & Arnott). *Plant Cell Reports*, **18**, 664-669. https://doi.org/10.1007/s002990050639

[52] Shekhawat, N.S., Rathore, T.S., Singh, R.P., Deora, N.S. and Rao, S.R. (1993) Factors Affecting *In Vitro* Clonal Propagation of *Prosopis cineraria*. *Plant Growth Regulation*, **12**, 273-280. https://doi.org/10.1007/BF00027208

[53] Cooper, P.A. (1987) Advances in the Micropropagation of Avocado (*Persea americana* Mill.). *Acta Horticulturae*, **212**, 571-576. http://www.actahort.org/members/showpdf?session=10239 https://doi.org/10.17660/ActaHortic.1987.212.92

[54] Nhut, D.T., Thi, N.N., Khiet, B.L.T. and Luan, V.Q. (2008) Peptone Stimulates *In Vitro* Shoot and Root Regeneration of Avocado (*Persea americana* Mill.). *Scientia Horticulturae*, **115**, 124-128. https://doi.org/10.1016/j.scienta.2007.08.011

[55] Bhat, S.R. and Chandel, K.P.S. (1991) A Novel Technique to Overcome Browning in Tissue Culture. *Plant Cell Reports*, **10**, 358-361. https://doi.org/10.1007/BF00193159

[56] Barringer, S.A., Mohamed-Yasseen, Y. and Splittstoesser, W.E. (1996) *In Vitro* Multiplication and Plantlet Establishment of Avocado. *In Vitro Cell and Developmental Biology of Plant*, **32**, 119-121. https://doi.org/10.1007/BF02823142

[57] George, E.F., Hall, M.A. and De Klerk, G. (2007) Plant Propagation by Tissue Culture: Volume 1. The Background. 3rd Edition, Springer Science & Business Media, Netherlands.

[58] Schroeder, C.A. (1968) The Longevity of Avocado Tissue *In Vitro*. *California avocado society Year Book*, **52**, 128-130. http://www.avocadosource.com/CAS_Yearbooks/CAS_52_1968/CAS_1968_PG_128-130.pdf

[59] García-Gómez, M.L., Sánchez-Romero, C., Barceló-Munoz, A., Heredia, A. and Pliego-Alfaro, F. (1994) Levels of Endogenous Indole-3-Acetic Acid and Indole-3-Acetyl-Aspartic Acid during Adventitious Rooting in Avocado Microcuttings. *Journal of Experimental Botany*, **45**, 865-870. https://doi.org/10.1093/jxb/45.6.865

[60] García-Gómez, M.L., Sánchez-Romero, C., Heredia, A., Pliego-Alfaro, F. and Barceló-Munoz, A. (1995) Peroxidase Activity during Adventitious Root Formation in Avocado Microcuttings. *Canadian Journal of Botany*, **73**, 1522-1526. https://doi.org/10.1139/b95-164

[61] Nel, D.D., Kotze, J.M. and Snyman, C.P. (1982) *In Vitro* Propagation of Persea Indica. *South African Avocado Growers' Association Year Book*, **66**, 167-168. http://www.avocadosource.com/Journals/SAAGA/SAAGA_1983/SAAGA_1983_PG_92.pdf

[62] Barceló-Mu-oz, A., Encina, C.L., Simón-Pérez, E. and Pliego-Alfaro, F. (1999) Micropropagation of Adult Avocado. *Plant Cell, Tissue and Organ Culture*, **58**, 11-17. https://doi.org/10.1023/A:1006305716426

[63] Harty, P.A. (1985) Propagation of Avocado by Tissue Culture: Development of a Culture Medium for Multiplication of Shoots. *South African Avocado Growers' Association Year Book*, **8**, 70-71. http://www.avocadosource.com/Journals/SAAGA/SAAGA_1985/SAAGA_1985_PG_70-71.pdf

[64] Dan, Y. and Reichert, N.A. (2001) Cytokinin Metabloism and Action. *Annual Review of Plant Physiology and Plant Molecular Biology*, **52**, 89-118. https://doi.org/10.1146/annurev.arplant.52.1.89

[65] Cautín, R., and Vardja, T. (2001) The Effect of Cytokinin Type and Concentration and the Number of Subcultures on the Multiplication Rate of Some Decorative Plants. *Proceedings of the Estonian Academy of Sciences. Biology, Ecology*, **50**, 22-32.

[66] Muhammad, A., Rashid, H., Hussain, I. and Naqvi, S.M.S. (2007) Proliferation-Rate Effects of BAP and Kinetin on Banana (*Musa* spp. AAA Group) 'Basrai'. *HortScience*, **42**, 1253-1255.

[67] Pliego-Alfaro, F. (1988) Development of an *In Vitro* Rooting Bioassy Using Juvenile-Phase Stem Cuttings of *Persea americana* Mill. *Horticultural Science*, **63**, 295-301. https://doi.org/10.1080/14620316.1988.11515862

[68] De la Vina, G., Pliego-Alfaro, F., Driscoll, S.P., Mitchell, V.J., Parry, M.A. and Lawlor, D.W. (1999) Effects of CO_2 and Sugars on Photosynthesis and Composition of Avocado Leaves Grown *In Vitro*. *Plant Physiology and Biochemistry*, **37**, 587-595. https://doi.org/10.1016/S0981-9428(00)80111-4

[69] Nel, D.D. (1983) Progress in Tissue Culture of Avocado. *South African Avocado Growers' Association Year Book*, **6**, 90-91. http://www.avocadosource.com/Journals/SAAGA/SAAGA_1983/SAAGA_1983_PG_90-91.pdf

[70] Prusky, D., Hamdan, H., Ardi, R. and Keen, N.T. (1996) Induction of Biosynthesis of Epicatechin in Avocado Suspension Cells Treated with an Enriched CO_2 Atmosphere. *Physiological and Molecular Plant Pathology*, **48**, 171-178. https://doi.org/10.1006/pmpp.1996.0015

[71] Vidal, M.T., Azcón-Aguilar, C., Barea, J.M. and Pliego-Alfaro, F. (1992) Mycorrhizal Inoculation Enhances Growth and Development of Micropropagated Plants of Avocado. *HortScience*, **27**, 785-787.

[72] Tonon, G., Kevers, K. and Gaspar, T. (2001) Changes in Polyamines, Auxins and Peroxidase Activity during *In Vitro* Rooting of *Fraxinus angustifolia* Shoots: An Auxin-Independent Rooting Model. *Tree Physiology*, **21**, 655-663. https://doi.org/10.1093/treephys/21.10.655

[73] Rasmussen, A., Hosseini, S.A., Hajirezaei, M., Druege, U. and Geelen, D. (2015) Adventitious Rooting Declines with the Vegetative to Reproductive Switch and Involves a Changed Auxin Homeostasis. *Journal of Experimental Botany*, **66**, 1437-1452. https://doi.org/10.1093/jxb/eru499

[74] Ahuja, M.R. (2013) Micropropagation of Woody Plants. Forestry Sciences. Springer Science & Business Media, Berlin.

[75] Kadman, A. (1976) Effect of the Age of Juvenile Stage Avocado Seedlings on the Rooting Capacity of Their Cuttings. *California Avocado Society Year Book*, **59**, 58-60. http://www.avocadosource.com/CAS_Yearbooks/CAS_59_1975/CAS_1975-76_PG_058-060.pdf

[76] Escobedo, V. and Escobedo, J.A. (2010) Adventitious Root Formation without Rooting Medium in Etiolated Shoots of 'Duke' Avocado (*Persea americana*) Growing on Nurse Plants. *Acta Horticulturae*, **923**, 227-232. http://www.actahort.org/members/showpdf?booknrarnr=923_34&frames=N&justloggedin=3&ipbasedconfirmatie

[77] Azcón-Aguilar, C., Barcelo, A., Vidal, M.T. and De La Vina, G. (1992) Further Studies on the Influence of Mycorrhizae on Growth and Development of Micropropagated Avocado Plants. *Agronomie*, **12**, 837-840. https://doi.org/10.1051/agro:19921017

[78] Mohamed-Yasseen, Y. (1993) Morphogenesis of Avocado *In Vitro*. A Review. *California Avocado Society Year Book*, **77**, 107-111. http://avocadosource.com/CAS_Yearbooks/CAS_77_1993/CAS_1993_PG_101-105.pdf

[79] Bairu, M.W. and Kane, M.E. (2011) Physiological and Developmental Problems Encountered by *In Vitro* Cultured Plants. *Plant Growth Regulation*, **63**, 101-103. https://doi.org/10.1007/s10725-011-9565-2

[80] Haas, A.R.C. (1943) Boron Content of Avocado Trees and Soils. *California Avocado Society Year Book*, **28**, 41-52. http://www.avocadosource.com/CAS_Yearbooks/CAS_28_1943/CAS_1943_PG_41-52.pdf

[81] Turner, S.R. and Singha, S. (1990) Vitrification of Crabapple, Pear, and Geum on Gellan Gum-Solidified Culture Medium. *HortScience*, **25**, 1648-1650.

Avocado (Persea americana Mill) is a species from Mexico and Central America and the only one of commercial importance, from an economic point of view, of the Lauraceae family, which includes about 2 200 species. This family includes woody plants, producers of essences producing that grow in warm regions and where the laurel (Laurus nobilis L.) are also included, Camphor (Cinnamomum.Â There are approximately 400 varieties, so we can find fruits of different shapes and weights, which may through 150 to 350 gr (19). Some aspects of the species related to systematic Persea americana Mill are (8): Kingdom: Plantae Division: Magnoliophyta Class: Magnoliopsida Order: Laurales Family: Lauraceae Genre: Persea Species: Persea americana Mill. GENETIC CHARACTERISTICS.