TOWARDS THE FORMATION OF SOMATIC EMBRYOS FROM AVOCADO LEAF EXPLANTS

by

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DECLARATION

I, Rebecca Opeyemi Oyerinde (Student number: 1042860), declare the following:

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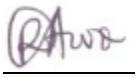
The thesis is being submitted for the degree of Doctor of Philosophy to the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted for any other higher degree or examination in any other institution.

The experimental work described was carried out in the School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, Johannesburg, South Africa, under the supervision of Professor David J. Mycock (School of Animal, Plant and Environmental Sciences, University of the Witwatersrand).

Signature: 

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ABSTRACT

Avocado (*Persea americana* Mill.) is the most important and the only tree that produces edible fruits from the many economically-valuable trees of the Lauraceae. Beyond the fruits, other parts of the plant are highly sought after for further use such as home remedies and beauty products. The increasing demand for avocado outweighs its production and this demand cannot be met by its natural means of propagation, which is characterized by particular difficulties such as flowering pattern, long juvenile period and diseases. The advances in plant biotechnology have facilitated other propagation methods, such as micropropagation, to bypass the difficulties associated with natural propagation. Somatic embryogenesis is a micropropagation method that has been used to generate *in vitro* avocado plantlets in the past. However, this has been achieved mainly by the use of immature zygotic embryos. Considering that zygotic embryos are a result of sexual reproduction, their genotypes are not truly known. Somatic embryogenesis, in some cases, involves the formation of callus as an intermediary phase before plant regeneration. The use of callus, however, is not limited to differentiation towards plant regeneration. Thus, this study focused on callus production from an avocado explant that is true to type and less destructive to the parent plant – the leaf, which was sourced from both greenhouse plantlets and the *in vitro* shoots.

In vitro avocado shoots were generated via three explanted materials: the embryonic axes, the seeds and the axillary buds. Shoots grown from embryonic axes had, significantly, the least number of leaves per shoot, and the smallest leaf size (length and breadth) while those obtained from the seeds had the highest of the observed parameters. However, for the purpose of this study, associated genetic variation with seed-derived shoots needed to be avoided as well as seasonal availability of the seeds. Hence, axillary buds were used for *in vitro* avocado shoot production.

The greenhouse and the *in vitro* growth conditions triggered the expression of phenotypic plasticity in the avocado plants. One of the characteristics observed was the presence or absence of bundle sheath extensions – a criterion with which leaves are accordingly classified as heterobaric or homobaric and thus leaves from the greenhouse- and the *in vitro*-raised materials fall into these classifications, respectively. Another trait was that there were more chloroplasts (3 chloroplasts/100 μm^2) in the greenhouse materials than their *in vitro* counterparts (1 chloroplasts/100 μm^2), which implied that photosynthesis was

more efficient in the former. Also, the *in vitro* leaves were thicker (171.19 ± 21.6 µm) with larger cells than greenhouse leaves (75.98 ± 8.6 µm).

Histomorphological observation showed that *in vitro* materials were not as differentiated as the greenhouse materials. The immature state of differentiation of the *in vitro* plant did not support the development of its nodal explants towards organogenesis but favoured the induction of callus from its leaf explants. It showed that callus originated from all three leaf tissues (i.e. the vascular bundles, the mesophylls and the epidermal layers) in the *in vitro* materials while it was only induced from the vascular bundles and the epidermis of the greenhouse materials. It also showed that in the first two weeks of callus induction, leaf cells had increased in size and cell division had been induced – the processes that eventually led to callus formation. These processes were as a result of the synergistic relationship between 2,4-D and BAP in the induction medium.

Leaf explants from *in vitro* shoots produced more callus (72.7 ± 10.35%) than explants from greenhouse plants (54.2 ± 15.40%). Seventy-nine different media were tested for optimal callus induction. The media were tested using four basal nutrient formulations: Murashige & Skoog (MS), Gamborg's B5, Mango Medium for Somatic Embryos (MMSE) and $B5^{+}$ and three auxin types: 2,4-dichlorophenoxyacetic acid (2,4-D), picloram and naphthaleneacetic acid (NAA). The results showed that Gamborg's B5 basal nutrient medium was more favourable (39.69 ± 14.95%) for callus induction than MS (21.43 ± 9.99%). All the MMSE-based induction media did not support callus formation while less than 10% of explants developed on $B5^{+}$ on some of its associated induction media. The use of auxin as the only plant growth regulator (PGR) did not result in callus formation. However, the addition of the cytokinin, 6-benzylaminopurine (BAP), to the auxin-enriched media resulted in some callus formation. In Gamborg's B5 medium, PGRs within the range of 0.5 - 2.0 mg/L 2,4-D and 0.2 – 1.0 mg/L BAP were optimal for callus induction under dark incubation, of which 1.0 mg/L 2,4-D and 0.5 mg/L BAP was the most effective combination. The order of auxin effectiveness for inducing callus was 2,4-D > NAA > picloram.

Other factors that were monitored included light and dark incubation, explant size and explant surface in contact with the induction medium. The size of the leaf explant did not have any significant effect on callus formation (p = 0.380794). However, dark incubation significantly favoured more callus formation than light incubation (p = 0.00124 and 0.00182 in B5 enriched with 2,4-D and picloram, respectively). Also, the adaxial surface

making contact with the induction medium resulted in more callus formation (54%) than when the abaxial surface was in contact with the induction medium (22%). The callus formed, in this study, had a range of colour (white, brown cream and green), texture (fluffy, grainy, compact, soft and nodular) and origin (cut edges, end of vein and along the vein and lamina).

There was a switch of preference between induction medium and developmental medium. While the combination of B5 and 2,4-D was optimal for callus induction, B5 was not suitable as a basal medium for further development. Also, phenolic compounds often accumulated in 2,4-D-derived callus which led to deleterious browning and subsequently the callus did not survive manipulation towards differentiation. On the other hand, MS, which was not preferred for callus induction, was more suited for the callus differentiating medium. Similarly, although NAA did not stimulate as much callus as 2,4-D, browning was less pronounced in the NAA-derived callus, the callus cells accumulated callose (one of the markers of embryogenic cells) in their cell walls. The NAA-derived callus subsequently showed some 'greening' responses at the differentiating phase before they died when subcultured. In most cases, subculturing callus to fresh medium led to loss of culture. Some groups of callus cells from the leaves of both the greenhouse- and the *in vitro*-derived materials developed to form proembryo-like structures, which gave an indication of possible further development towards organogenesis.

Considering the paucity of published information on any *in vitro* procedures for the main cultivar 'Edranol' used in this study and for the use of avocado leaves as explants, the work reported here is fundamental. Thus, this study further enhanced our understanding of (i) the selective effectiveness of not fully-differentiated tissues in micropropagation procedures, (ii) the preferential effectiveness of the different basal nutrient media, B5 and MS, at either inducing callus or supporting further development of callus, (iii) the type of auxin used affecting the rate, type and quality of callus formed and (iv) avocado is a recalcitrant species, not only in its post-harvest seed behavior but also in its response to *in vitro* manipulations.

CONTRIBUTIONS FROM THIS WORK

Conferences:

Rebecca Oyerinde and David J. Mycock. Preliminary investigation into somatic embryogenesis from the leaf tissue of avocado. 7th International Workshop on Desiccation Sensitivity and Tolerance across Life Forms (DESWORK 2016), Aquila Private Game Reserve, South Africa, 11 – 15th January, 2016.

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Oyerinde R.O. and Mycock, D.J. Effects of explant size, orientation, incubation type and cultivars on callus formation from the leaves of avocado (under internal review).

DEDICATION

To God Almighty

To my husband and my children

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"In everything give thanks, for this is the will of God in Christ Jesus concerning you (me)"
1Thessalonians 5:18.

I specially want to express my gratitude to my supervisor, Professor David J. Mycock. It is difficult to express my appreciation in a single paragraph, so I will just say a simple THANK YOU as an overarching expression for all you have done and all you have been since the commencement of this journey under your guidance. I cannot but point out your strong belief in my competence and your unfailing ability in identifying my subtle strength, it is mind blowing and I am indeed very grateful.

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LIST OF ABBREVIATIONS

2,4-D	-	2,4-Dichlrophenoxyacetic acid
B5	-	Gamborg's B5 basal nutrient medium
$B5^{+}$	-	Modified Gamborg's B5 basal nutrient medium
BAP	-	6-Benzylaminopurine
BF	-	Bright Field
CIM	-	Callus Induction Medium
FM	-	Fluorescence Microscopy
MMSE	-	Mango Medium for Somatic Embryo
MS	-	Murashige and Skoog basal nutrient medium
NAA	-	1-Naphthaleneacetic acid
PGRs	-	Plant Growth Regulators
SE	-	Somatic embryo
SH	-	Schenk and Hilderbrandt
WPM	-	Woody Plant Medium

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Avocado: Family Lauraceae

The Lauraceae family is listed among the 'basal angiosperms' i.e. world's oldest flowering plants (Bost *et al*., 2013). The family comprises about 45 genera, a few of which are *Persea, Cinnamomum, Cassytha* and *Camphora* (Bergh, 1969; Chanderbali, *et al*., 2008; Bijzet, 2011; Bender, 2013a) and approximately 2850 species (Christenhusz, and Byng, 2016). Most of the species in this family are trees and shrubs that are usually evergreen while some are deciduous. However, the *Cassytha* is a genus of herbaceous, rootless and parasitic vines. The family includes some species that are of cultural and economic importance and others that are common household names. For example, leaves of *Laurus nobilis* were used in ancient Greek culture to signify and reward major achievements (Chanderbali *et al*., 2008). *Cinnamomum pauciflorum* produces essential oil (Kong *et al*., 2009) while *Persea americana* is not only valuable for its oil but for its edible fruit as well. Other species such as *Chlorocardium, Beilschmiedia, Eusideroxylon zwageri, Ocotea spp* and *Cinnamomum kanehirae* are valuable for commercial timber production (Moura-Costa *et al*., 1993; Glimn-Lacy and Kaufman, 2006; Pelegrini *et al*., 2013; Schaffer *et al*., 2013; Chang *et al*., 2015; Gibson and Rebicca, 2016). Furthermore, *C. kanehirae* is the only natural host of the medicinal fungus *Antrodia cinnamomea* (Chang *et al*., 2015); while the leaves of *Cinnamomum verum* tree, commonly known as bay leaves, are used in cooking (Sharif *et al*., 2018).

1.1.1 Origin and distribution of avocado (*Persea americana* Miller)

Avocado (*Persea americana* Miller) is considered the most valuable species of economic importance in the Lauraceae. The trees are a fruit crop of tropical and subtropical origin as for most genera of the Lauraceae (Ben-Ya'acov and Michelson, 1995; Bender, 2013a). They are native to Central and South America and have been cultivated there since around 8,000 BC (Storey *et al*., 1986). Avocados were introduced to Jamaica in the mid-17th century, Asia in the mid 1800s, South Africa in the late 19th century and cultivation in the United States of America (Florida and California in particular) started in the early 20th century (Ben-Ya'acov and Michelson, 1995). There are three main subspecies or horticultural races of avocado grown at different elevations: (i) the west Indian or Antillean race (*P. americana* var. *americana*) which grows best in a lowland tropical climate at altitudes of 0 to 1000 m (ii) the Guatemalan race (*P. americana* var. *guatemalensis*) is adapted to medium elevations of about 1000 to 2000 m above sea level in semitropical climates and (iii) the Mexican race (*P. americana* var. *drymifolia*) is grown at high

elevations between 500 and 3000 m in subtropical climates (Ben-Ya'acov and Michelson, 1995; Anguiano *et al*., 2007; Chanderbali *et al*., 2008; Crane *et al.,* 2013). There are hundreds of cultivars or varieties of avocado, but according to the report of the South African Department of Agriculture, Forestry and Fisheries (DAFF) (2012), seven are grown for commercial purposes in South Africa. These are 'Fuerte', 'Hass', 'Bacon', 'Pinkerton', 'Edranol', 'Ryan', and 'Reed', with 'Fuerte' being the most popular. However, the 'Hass' cultivar is the leading variety in the United States of America (USA) and globally (Dreher and Davenport, 2013). Many varieties of avocado are crossbreeds of two or more races, e.g. the 'Fuerte' cultivar is a hybrid of Guatemalan x Mexican races (Popenoe, 1941; Gómez-Lim and Litz, 2004).

1.1.2 Importance of avocado

Commercially, avocado fruit is an important crop that is gaining popularity worldwide because of its exceptional nutritional value (Guzmán-García *et al*., 2013a). Within a decade, world production of avocado increased from 2,935,316 metric tonnes in 2002 to 4,311,049 metric tonnes in 2012 (FAO, 2002; 2012) and the value was ca. 6 million metric tonnes in 2017. For more than 10 years, countries such as Mexico, Dominican Republic, Peru, Indonesia, US, Kenya, Brazil, among others, have been the top countries producing avocado (Table 1.1). According to FAOSTAT (FAO, 2017), Mexico is consistently the world-leading exporter of avocado while USA is the leading importer even though it is one of the top producer countries (Table 1.1). In South Africa, avocado is cultivated in the subtropical/tropical climate of Limpopo, Mpumalanga and KwaZulu-Natal provinces; and its production increased from 81,900 to 98,200 metric tonnes within five years (2010/11 – 2014/15) (Sippel, 2011; DAFF, 2015; 2019). Domestic sale of the crop increased from 15,038 to 22,196 metric tonnes in 2017 and 2018, respectively (SAFTF, 2018).

Avocado fruit fits into the group of foods referred to as functional human foods (Pieterse *et al*., 2003). These are described by the American Dietetic Association (ADA) (1999) as foods that provide health benefits beyond the basic nutrients they can supply due to the phytochemicals or the physiologically-active components they contain. Functional foods are potentially capable of reducing the risks of diseases and promote optimal health for the consumers. Avocado fruit has no cholesterol, is low in sodium, sugar and saturated fatty acids but is high in unsaturated, particularly monounsaturated, fatty acids (Bergh, 1992a;c; Rainey *et al*., 1994; Pieterse *et al*., 2003; Dreher and Davenport, 2013).

Table 1.1: Top 20 countries producing avocado (in tonnes) at five-year intervals. Superscripts indicate the position of each country for 2012 and 2017.

	Country	2007	2012	2017
1	Mexico	1 142 892	1 316 104 [1st]	2 029 886 [1st]
2	Chile	209 645	160 000 [8th]	133 636 [9th]
3	Indonesia	201 635	294 200 [2nd]	363 157 [4th]
4	Colombia	193 996	255 384 [5th]	314 275 [5th]
5	United States of America	193 100	238 495 [6th]	132 730 [10th]
6	Dominican Republic	183 468	290 011 [3rd]	637 688 [2nd]
7	Brazil	154 096	159 903 [9th]	213 041 [6th]
8	Peru	121 720	268 525 [4th]	466 758 [3rd]
9	Guatemala	96 524	94 605 [12th]	125 596 [11th]
10	Kenya	93 639	166 948 [7th]	194 279 [7th]
11	China	92 000	108 000 [11th]	124 110 [12th]
12	Malawi	87 620	90 711 [14th]	97 358 [15th]
13	Israel	85 913	77 500 [16th]	110 000 [13th]
14	Venezuela	83 304	116 964 [10th]	133 922 [8th]
15	Spain	82 116	76 337 [17th]	92 936 [16th]
16	South Africa	65 209	91 603 [13th]	62 840 [19th]
17	DR Congo	64 340	66 509 [19th]	65 558 [18th]
18	Haiti	58 000	80 230 [15th]	97 520 [14th]
19	Cameroon	53 000	72 000 [18th]	71 235 [17th]
20	Australia	47 238	-	-
21	Morrocco	-	54 340 [20th]	-
22	Ethiopia	-	-	57 120 [20th]

A typical ‘Hass’ avocado has minerals such as calcium, magnesium, potassium, zinc, manganese; vitamins and phytochemicals such as vitamins C, B-6, B-12, A, E, K, niacin,

folate, riboflavin, tocopherol, carotene, phytosterols and phenolics (Table 1.2) (Dreher and Davenport, 2013; USDA, 2014).

Table 1.2: Some nutritional components of avocado fruit and their human health benefits

NUTRIENTS	HEALTH BENEFITS	REFERENCES
Monounsaturated fatty acids (MUFA)	Good for cardiovascular health, reduces bad cholesterol (LDL)*, maintain/increase good cholesterol (HDL)**, good for skin health, protects against abdominal fat thus helping in weight management	Bergh, 1992a,b,c; Colquhoun *et al*., 1992; Pieterse *et al*., 2003; Dreher and Davenport, 2013; Fulgoni *et al*., 2013.
Dietary fibre	Helpful in the control of sugar levels and diabetes, improves glucose tolerance, reduces the risk of gastrointestinal diseases	Bergh, 1992a; Rainey *et al*., 1994
Minerals		
Potassium (K)	Prevents stroke and its associated diseases, promotes normal blood pressure	Bergh, 1992a; Dreher and Davenport, 2013
Magnesium (Mg)	Supports normal vascular tone, serves as co-factor for cellular enzymes needed in energy metabolism	Dreher and Davenport, 2013
Phytochemicals		
Phenolics and flavonoids	Have anticancer properties, have protective effects on the liver, reduce oxidative and inflammatory stress	Ding *et al*., 2007; 2009; Dreher and Davenport, 2013; Mahmoed and Rezq, 2013;
Phytosterols Beta-sitosterol	Promote intestinal cholesterol blocking thus reducing cholesterol absorption, enhances cardiovascular health Reduces the risk of prostate cancer in men, improves urinary system	Dreher and Davenport, 2013 Strum and Faloon, 2005
Xanthophylls e.g. Lutein and zeaxanthin	Have antioxidant properties and protective effects on DNA, protect against cartilage defects, improve eye and skin health	Dreher and Davenport, 2013
Carotenoids, terpenoids,	Have anti-carcinogenic properties	Dreher and Davenport, 2013

gluthathione, persenone		
Vitamins		
Antioxidants (vitamin A (beta carotene), C and E)	Act as defence against free radicals, prevent cholesterol oxidation, protect the skin from visible signs of aging, protect against DNA damage and osteoarthritis	Bergh, 1992a,c; Rainey *et al*., 1994; Dreher and Davenport, 2013.
Vitamin B_6 (pyridoxine)	Good for the nervous system, red blood cells, teeth and gums	Bergh, 1992a
Folate	Reduces the dangerous amino acid, homocysteine, in the blood, it is necessary for cell division especially during pregnancy and infancy	Rainey *et al*., 1994; Dreher and Davenport, 2013
Vitamin K_1	Functions as co-factor for the enzyme that facilitates blood coagulation	Dreher and Davenport, 2013

*LDL – Low Density Lipoprotein **HDL – High Density Lipoprotein

The composition of its nutrients, which in most cases are higher than those found in other fruits and vegetables, are listed in Dreher and Davenport (2013). Avocado seems to be the best source of beta-sitosterol, one of its main phytosterols, when compared with other fruits such as orange which was previously considered as the richest source of the compound (Duester, 2001). Some of the health benefits of various avocado components are detailed in Table 1.2.

The importance of avocado is not confined to the consumption of its fruits alone. Other plant parts are used for various benefits from home remedies to beauty products. Oil extracted from the pulp is used for health and beauty purposes (DAFF, 2012). Paste from the fruit pulp is used for stimulating healthy hair growth. Its powdered seeds are used to treat hypertension and the leaves have antibiotic properties and are also used in the production of dyes. Even the wood has been used for some woodwork in the past (DAFF, 2012; Bost *et al*., 2013).

1.1.3 Diseases of avocado

Although the demand for avocado increases every year due to its economic importance, it is often plagued by diseases that affect its growth, development and production. Topmost

on the list of avocado diseases is *Phytophthora* root rot caused by *Phytophthora cinnamomi* (Pérez-Jiménez, 2008; Manicom, 2011). Some other diseases of avocado are *Phytophthora* cankers caused by *Phytophthora* spp; anthracnose caused by *Colletotrichum gloeosporioides*; cercospora spot of blotch caused by *Pseudocercospora purpurea*; *Dothiorela* rot caused by *D. gregaria*; scab caused by *Sphaceloma perseae*; stem-end rot caused by multiple fungi like *Dothiorella spp*., *Phomopsis perseae*, *Thyronectria pseudotrichia* etc.; *Fusarium* rot caused by several species of *Fusarium* such as *F. pallidoroseum*; sunblotch caused by avocado sunblotch viroid (ASBVd) and many more. One bacterial disease is soft rot caused by *Erwinia carotovora* (Snowdon, 1990; Pérez-Jiménez, 2008; Ploetz, 2009; Manicom, 2011; Dann *et al*., 2013). Some diseases are more prevalent in certain countries than others; some diseases affect only the tree while others affect the fruits or both (Snowdon, 1990).

1.1.4 Flowering and breeding of avocado

The flowers of avocado are bisexual; with the male and female parts of the flowers opening at different times of day (Robbertse, 2011; Crane *et al*., 2013) hence the species rarely self-pollinates and is consequently highly heterogeneous (Cruz-Hernández *et al*., 1998; Witjaksono and Litz, 1998; Robbertse, 2011). Cross-pollination occurs when male and female flower parts of different individual plants open synchronously (Crane *et al*., 2013). A mature avocado tree can produce about one million flowers but very few flowers (less than 1%) set fruit that will develop to maturity (Blumenfeld and Gazit, 1974; Crane *et al*., 2013; Bender, 2013b), with many senescing immaturely (Witjaksono and Litz, 1998; Crane *et al*., 2013; Salazar-García *et al*., 2013). The abscission of the flowers as well as the senescence of the immature fruits is linked to the various developmental processes, which must be successfully transited, from flowering to fruit set.

Usually, avocado seedlings have a long (average of 10 years) juvenile period (Cruz-Hernández *et al*., 1998; Witjaksono and Litz, 1998; Bender, 2013b; Salazar-García *et al*., 2013). Because of this and due to the commercial importance of avocado, it is usually propagated vegetatively by grafting (Ben-Ya'acov and Michelson, 1995; Bender *et al.*, 2013). Advantageously, some grafted cultivars begin flowering as early as two years after planting (Bender, 2013b). Grafting also helps growers to choose rootstocks and scions that will yield cultivars with desirable traits in the trees (such as disease tolerance and quantity of fruits produced) and in the post-harvest behaviour of fruits.

The propagation challenges of avocado such as diseases, heterogeneity, long juvenile period and the demand for qualitative fruits of high market value, call for alternative breeding strategies for improving the propagation of avocado. *In vitro* procedures such as somatic embryogenesis, *in vitro* mutagenesis and protoplast cultures are some of the biotechnological methods used to incorporate desired traits into the breeding of avocado (Litz *et al*., 2005a). In this regard, somatic embryogenesis can be used for genetic transformation so as to introduce desirable characteristics, particularly disease-resistance, into crops.

1.2 Somatic Embryogenesis

Somatic embryogenesis is an asexual process whereby a single somatic cell or a group of somatic cells develop, without the fusion of gametes, into non-zygotic, bipolar embryos otherwise known as somatic embryos, which are capable of developing into whole plants (Hirimburegama and Gamage, 1997; Vicient and Martinez, 1998; Kärkönen, 2001; Razdan, 2003). Somatic embryogenesis can occur in nature as well as *in vitro* (Litz and Gray, 1995). Since somatic embryos are formed without the process of fertilization, they are genetically identical to the parent tissues from which they are derived and hence they are clones (Deo *et al*., 2010). The somatic embryogenic process is made possible by the fact that plants possess cellular totipotency – the genetic ability of a plant somatic cell to develop into a whole plant (Solís-Ramos *et al*., 2012). Although the initiation of embryogenesis in zygotic and somatic embryogenesis is different, there are similarities in structure and in the subsequent developmental pathways (Litz and Gray, 1995; Dodeman *et al*., 1997; Von Arnold, 2008). In dicotyledonous plants, both zygotic and somatic embryos undergo the four developmental stages: globular, heart, torpedo and mature (cotyledonary) (Hirimburegama and Gamage, 1997).

1.2.1 Pathways in somatic embryogenesis

There are two pathways in somatic embryogenesis: the direct and the indirect pathways. In direct somatic embryogenesis, somatic embryos are formed directly from cells within the explants. In indirect somatic embryogenesis, the explants first develop an undifferentiated mass known as callus as an intermediary phase, and then cells within the callus differentiate to form somatic embryos (Ji *et al*., 2011). It is presumed that the somatic embryos that are formed directly originate from 'pre-embryogenic determined cells' (PEDCs). These are cells that are pre-destined to develop into embryos and would do so

under appropriate *in vitro* conditions. Such cells are found in the tissues associated with zygotic embryos such as the suspensors, nucellus, megagametophyte and embryo sac. On the other hand, those formed indirectly are formed from 'induced embryogenic determined cells' (IEDCs). Naturally, the IEDCs are not modeled to develop into embryos; hence they have to be re-directed to the embryogenic state through dedifferentiation (to form callus) and re-differentiation to form somatic embryos. These processes occur under exposure to specific plant growth regulator treatments like the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D). The IEDCs are found in somatic tissues such as leaf, cotyledon and stem explants (Minocha and Minocha, 1995; Kärkönen, 2001; Razdan, 2003; Von Arnold, 2008; Hartmann, *et al*., 2011). However, an intermediary callus phase can be generated from PEDCs such as was observed in the somatic embryogenesis of avocado using immature zygotic embryos as explants (Mooney and Van Staden, 1987). In the same vein, somatic embryos were formed directly, without intermediate callus formation, from IEDCs in the leaf explants of *Stevia rebaudiana* (Filho *et al*., 1993). Hence the direct and indirect somatic embryogenesis pathways are not fixed with PEDCs and IEDCs, respectively.

1.2.2 Factors influencing successful somatic embryogenesis

The induction of somatic embryogenesis is influenced by the source, developmental status of the explant, genotype, plant growth regulators, and basal nutrient media (Litz and Gray, 1995; Radojevic *et al*., 1999; Kärkönen, 2001; Deo *et al*., 2010; Ji *et al*., 2011).

Explants can be sourced from different organs and tissues of a plant like the hypocotyl, cotyledons, roots, (Zhang *et al*., 2001); flower buds (Capuana and Debergh, 1997); immature zygotic embryos (Witjaksono and Litz, 1999a; 1999b) and leaves (Filho *et al*., 1993). Although explants can be sourced from different plant tissues, embryonic or highly juvenile and meristematic tissues are preferred as explants for somatic embryogenesis because these types of tissues are more amenable to produce embryogenic cells than older, highly differentiated tissues (Litz and Gray, 1995; Zhang *et al*., 2001). Mooney and Van Staden (1987) also emphasized the importance of the correct dimension of the explant, as it should not be too small or too large. This is because if the explant is too small, it can lead to death and if it is too large, it can lead to maturation of the explant (in the case of immature zygotic embryo).

The source of explants also includes consideration of the donor plants. The donor, stock or parent plants are influenced by the prevailing environmental factors of their growth conditions. Such environmental factors trigger the expression of phenotypic plasticity in the donor plants (Bradshaw, 1965; Sultan, 2000; 2003). Thus, species of the same genotypes but grown under different conditions would express differences in their morphology (e.g. plant height), anatomy (e.g. cell size), biochemical activities (e.g. starch accumulation) and physiology (e.g. photosynthesis) among other traits (Sultan, 2003; Gratani, 2014; Qi *et al*., 2020). Different growth conditions can be shade, greenhouse, *in vitro*, light, among others. These conditions are controlled by factors such as relative humidity, temperature, irradiance, altitude, water and nutrient availability (Gratani, 2014). Hence, by implication, explants sourced from physiologically different parent plants may also respond differently to *in vitro* procedures.

Different genotypes of the same species can have differing responses to somatic embryogenesis procedures. According to Stasolla and Yeung (2003), the quality of somatic embryos produced and their conversion to plantlets is highly dependent upon the genotype of the originating explants. Such genotypic variation has been shown to be significant in *Glycine max* (Tian and Brown, 2000), *Arachis hypogaea* (Radhakrishnan *et al*., 2001), rose (Kim *et al*., 2004) and cocoa (Quainoo and Dwomon, 2012). In fact, some genotypes of certain species, such as cotton, are not amenable, or show very slow response to somatic embryogenesis (Zhang *et al*., 2001). In this regard, Zhang *et al*., (2001) suggested that different levels of endogenous plant hormones, especially cytokinins, may be responsible for the differential responses of genotypes to somatic embryogenesis.

Litz and Gray (1995) listed the commonly used tissue culture media as those formulated by Murashige and Skoog (MS) (1962), Schenk & Hildebrandt (1972), Nitsch and Nitsch (1969) or B5 (Gamborg *et al*., 1968). The work of Perán-Quesada *et al*. (2004) on avocado showed that the different stages of somatic embryogenesis require different media for optimization. Those authors found that while cultures on B5 major salt medium yielded higher quality, white-opaque somatic embryos than cultures on MS medium, MS medium was more suited for the maturation of the embryos. However, the optimum medium for conversion of the somatic embryos into plantlets was B5 major salt plus coconut water (Perán-Quesada *et al*., 2004).

1.2.3 Stages involved in somatic embryogenesis

The phases of somatic embryogenesis include callus initiation, maintenance/proliferation, histodifferentiation/development, maturation and plant regeneration through plantlet conversion (Kärkönen, 2001; Von Arnold, 2008).

1.2.3.1 Callus initiation

The first stage of callus initiation or induction entails culturing the explants in a medium that will enable the explant to show its latent ability for somatic embryogenesis by producing embryogenic callus (also known as proembryogenic masses – PEMs (Von Arnold *et al*., 2002; Hartmann *et al*., 2011)). The induction of callus for embryogenesis is usually in a medium enriched with auxin (Deo *et al*., 2010) with low concentrations of, or no, cytokinin. According to Hartmann *et al*. (2011), the most effective and common synthetic auxin is 2,4-D, but oftentimes each plant species and explant requires specific auxins, other than 2,4-D, for optimization. For example, picloram is the auxin that is often used for the induction of somatic embryogenesis in avocado (Witjaksono and Litz, 1998; 1999a; Efendi, 2003). According to Mooney and Van Staden (1987), the optimal concentration of picloram for the induction medium is 0.1 mgL^{-1}; concentrations lower than this level did not result in callus induction and concentrations above this optimal level were either less effective or toxic to the explants. In some cases however, cytokinins instead of auxins are used to induce callus (Hartmann *et al*., 2011). An example is the use of zeatin by Zhang *et al*., (2001) in cotton embryogenesis.

The subsequent development and establishment of somatic embryogenesis occurs in a medium with lower concentrations, or no, auxin. This is because auxin promotes the proliferation of callus but inhibits the differentiation of cells towards organized growth (Von Arnold *et al*., 2002).

1.2.3.2 Maintenance and proliferation of callus

At the maintenance and proliferation phase, embryogenic calli are subcultured onto fresh media. Embryogenic and non-embryogenic callus usually grow together in a medium and the latter tends to outgrow the former, hence it is necessary that embryogenic calli are selected to be subcultured for maintenance and proliferation (Fei, 1997). Usually, the embryogenic callus is transferred to a medium similar to, or with a lower concentration of auxin than, the induction medium (Von Arnold *et al*., 2002; Stasolla and Yeung, 2003; Hussein *et al*., 2006; Hartmann *et al*., 2011). For example in papaya, while the induction

medium contained 15 mgL^{-1} 2,4-D for embryogenic callus to be formed, the embryogenic cells had to be transferred to a proliferation medium containing a reduced concentration (2 mgL^{-1}) of 2,4-D with 4 mgL^{-1} zeatin and 60 gL^{-1} sucrose before they could develop into the globular stage of embryo development (Jattana *et al*., 2008). It is important that cultures are kept at low pH i.e. less than 6, for proliferation (Von Arnold *et al*., 2002).

1.2.3.3 Histodifferentiation/developmental phase

The histodifferentiation/development phase, also referred to as prematuration phase by Von Arnold *et al*. (2002), entails transferring the somatic embryos onto a medium free of growth regulators (Von Arnold *et al*., 2002; Hussein *et al*., 2006; Hartmann *et al*., 2011). It is necessary that the medium is auxin-free because auxin can inhibit the growth and development of shoot meristems (Razdan, 2003). Apical, shoot and root meristem differentiation occur at this stage. The lack of plant growth regulators allows the somatic embryos to develop further through the various stages of heart, torpedo and cotyledonary.

1.2.3.4 Maturation of somatic embryos

Maturation is the intermediate, transitory and key phase between somatic embryo development and conversion to plantlet. This phase prevents premature conversion of the somatic embryos to plantlets. Without this phase, the somatic embryos formed tend to be abnormal; without functional shoot and root systems (Hartmann *et al*., 2011). Complete maturity implies that somatic embryos are not only morphologically mature, but have also acquired certain physiological conditions, such as desiccation tolerance (Stasolla and Yeung, 2003). In addition, synthesis and accumulation of storage substances like starch, proteins and oils usually take place at this phase. In the laboratory, abscisic acid (ABA) is usually added to the medium to induce maturation (Hoekstra *et* al., 2001; Von Arnold *et al*., 2002; Perán-Quesada *et al*., 2004; Hartmann *et al*., 2011; Quainoo and Dwomon, 2012).

In addition to promoting embryo maturation, ABA is often used in somatic embryogenesis, to promote desiccation tolerance and to enhance the synthesis and deposition of storage reserves within the embryo during maturation. It also prevents the precocious 'germination' of the somatic embryos, allowing them to develop into 'normal' plants and reduces the possibility of the process of secondary embryogenesis (Thomas, 1993; Dodeman *et al*., 1997; Von Arnold *et al*., 2002). It has also been shown to induce somatic embryogenesis in some species such as *Daucus carota* (Nickle and Yeung, 1994). Tian and

Brown (2000) showed that ABA increased the number of somatic embryos formed in soybeans and also enhanced 'normal' somatic embryos development through various stages. Other potential roles of ABA, as outlined in Rai *et al*. (2011), include (i) it helps with the synchronization of the development of somatic embryos and to increase stress tolerance, (ii) it serves as anti-transpirant when plantlets from tissue cultures are being acclimatized, (iii) it stimulates gene expression during somatic embryogenesis and (iv) it reduces programmed cell death in cultured cells and tissues. In addition, when ABA is supplied exogenously, it can induce a certain degree of freezing tolerance (Gusta *et al*., 2005).

However, Rai *et al*. (2011) stated that the addition of ABA alone to maturation medium might not promote somatic embryo maturation in most plant species unless it is used in combination with an osmolyte such as polyethylene glycol (PEG). As reported in the previous phase, the maturation culture medium is also auxin-free because of its inhibitory role in maturation (Litz and Gray, 1995). Apart from ABA, the process of desiccation, osmoticants (such as sucrose) and gelling medium can also be used to induce or control maturation of somatic embryos (Hussein *et al*., 2006). In Witjaksono and Litz (1999b), maturation medium for avocado somatic embryos was made up of MS basal medium supplemented with 1.0 mg/L 6 – benzyl aminopurine (BAP), 1.0 mg/L gibberellins (GA_3), 4.0 mg/L thiamine HCl, 100 mg/L myo-inositol, 30 g/L sucrose and 8 g/L agar.

1.2.3.5 Plant regeneration/Plantlet conversion

Plant regeneration entails root growth and elongation, and plant conversion (which is the growth of functional root and shoot systems) of mature somatic embryos (Stasolla and Yeung, 2003; Hussein *et al*., 2006). This also normally takes place in a medium free of plant growth regulators. Normal plants will only develop from mature somatic embryos that have acquired desiccation tolerance, have normal morphology and with sufficient storage materials in their cotyledons (Von Arnold *et al*., 2002; Stasolla and Yeung, 2003).

However, the major challenge of *in vitro* somatic embryogenesis is that the number of mature embryos obtained at the end of the procedures is usually few. In addition, the frequency of conversion of mature embryos to viable plantlets is usually low as well (Stasolla and Yeung, 2003). Hence, the culture conditions for each stage of somatic embryogenesis need to be manipulated and optimized empirically, in order to obtain optimal overall results in terms of the quantity and the quality of the somatic embryos produced (Stasolla and Yeung, 2003).

1.2.4 Secondary somatic embryogenesis

It is often very difficult to obtain normal plantlets from somatic embryos due to low conversion rates, and the fact that embryos differentiate and mature at different rates from the same embryogenic tissue (Capuana and Debergh, 1997). In these cases, secondary somatic embryogenesis may be necessary to mitigate the setbacks highlighted above. Secondary somatic embryogenesis is a process in which nascent somatic embryos at the PEMs of globular stage of development are used as the starting material (explant) for the differentiation and maturation of somatic embryos (hence – secondary) (Raemakers *et al*., 1995). The advantage of secondary over primary somatic embryogenesis was demonstrated by Quainoo and Dwomon (2012). According to those authors, the percentage of somatic embryos obtained and the conversion to plantlets was significantly higher from secondary rather than primary somatic embryogenesis. One of the major benefits of secondary embryogenesis is its high multiplication rate when compared with primary embryogenesis.

1.2.5 Importance and uses of somatic embryogenesis

Somatic embryogenesis is one of the biological processes highlighted by Park and Bonga (2010) as being useful in meeting the challenges facing modern breeding programmes such as climate change, pest susceptibility and resistance, conservation and re-establishment of endangered species. It is also a useful process that enables the integration of valuable genes into desirable genotypes through genetic engineering (Park and Bonga, 2010). Similarly, somatic embryos are used in the production of synthetic seeds, germplasm conservation through cryopreservation (Hartmann *et al*., 2011) and mass propagation of desired species/cultivars (Corredoira *et al.*, 2003). Other advantages, especially for crop improvement, are highlighted in Vicient and Martínez (1998) as cell selection, somatic hybrid regeneration, polyploidy plant production, elimination of viruses, synthesis of metabolite and mycorrhizal initiation.

1.3 The Present Study

It is important to note that avocado is a highly recalcitrant tropical tree species due to its post-harvest seed behaviour (Sánchez-Romero *et al*., 2002). Intolerance to desiccation and sometimes chilling, large seed size, continued metabolic activities from seed development to seed germination and high water content at shedding are some of the characteristics of such seeds (Daws *et al*., 2005) – all of which are found in avocado seeds (Walters *et al*., 2013). Since processes that lead to acquisition or lack of desiccation tolerance are part of

the physiological mechanisms of seed development, the recalcitrance of such species may not be limited to post-harvest seed behaviour alone but also to the whole plant physiology (Benson, 2000). Thus, recalcitrance can also reflect in the responses obtained from *in vitro* propagation manipulations as seen in avocado (Wolstenholme and Whiley, 1999; Hiti-Bandaralage *et al*., 2017).

Various types of avocado explants have been used for the micropropagation of the crop as outlined by Mohamed-Yasseen (1993). However, since 1987 when Mooney and Van Staden first documented somatic embryogenesis in avocado, until date, the zygotic embryo seems to be the only documented explant of choice for somatic embryogenesis of avocado (Mooney and Van Staden, 1987; Pliego-Alfaro and Murashige, 1988; Witjaksono and Litz, 1999a; 1999b; Efendi, 2003; Perán-Quesada *et al*., 2004; Guzmán-García *et al*., 2013b; Encina *et al*., 2014). This is because zygotic embryos are generally made up of highly and rapidly dividing meristematic cells. However, zygotic embryos have already gone through sexual recombination through fertilization, hence the somatic embryos generated will not be clones of the mother plants (Hartmann *et al*., 2011).

In the present study, the explants of choice for the induction of callus and somatic embryos were avocado leaves. Leaf materials were always available in comparison with fruits from which zygotic embryos are obtained. The use of leaves as explants would ensure that the somatic embryos are true to type of the mother plant, provided somatic variation does not occur. Leaf explants would also be less destructive to the parent plants and could potentially be good explants like the zygotic embryos, given that leaf tissues contain meristematic cells too. However, since leaf growth is not indefinite but determinate, young leaves would be more meristematic than mature leaves, thus were preferred over fully-grown matured leaves.

It is known that various factors – endogenous and exogenous – influence the callogenic competence of explants and the type of callus formed. This study aimed at elucidating the interactions between these factors and how these factors promote or inhibit callus formation. It investigated the influence of the growth conditions of stock plants on the developmental status of the explants. A morphological study traced the callogenic competence of the component leaf tissues of avocado so as to understand the underlying developmental process that culminated in its callus formation.

CHAPTER TWO: OPTIMIZING EXPLANT SOURCES FOR THE INDUCTION OF CALLUS FROM AVOCADO (*Persea americana*)

2.1 Introduction

Callus is an aggregate of undifferentiated cells often induced from isolated plant parts known as explants and its formation is the first stage in the indirect pathway for somatic embryogenesis (George 2008). Explants can be leaf pieces such as were used in investigation with jojoba (Kumar *et al*., 2013), root in Indica rice (Hoque and Mansfield, 2004), zygotic embryos in onion (Sivanesan *et al*., 2015) and others (Kawochar *et al*., 2017). The explant physiological status, the types of tissues found in the explant, the genotype of the parent plant, among others, have an effect on the status and competence for callus induction and subsequent somatic embryo development (Hoque and Mansfield, 2004; Finer, 1994). Whatever the explants, it is important to note that their cells must acquire cellular totipotency, i.e. the ability of plant cells to reprogramme and to develop into other cell types and ultimately to a whole plants (Su *et al*., 2021). This is because recent studies have suggested that not all plant cells are totipotent (Fehér, 2019).

In avocado, the explant that has been widely reported for its success in callus or proembryo mass induction and subsequently for the formation of somatic embryos is the immature zygotic embryo (Mooney and Van Staden, 1987; Perán-Quesada *et al*., 2004; Litz *et al*., 2005a; Encina *et al*., 2014). However, given the context of totipotency, there are other explants of *Persea americana* that have the potential of forming callus such as the component tissues of flower, leaf, petiole, protoplast, stem, cotyledons, peduncle, fruit mesocarp and buds (Young, 1983; Mohamed-Yasseen, 1993). The young, actively growing individuals of most plant species, including avocado, are believed to have higher meristematic competence than the older plants (Hoque and Mansfield, 2004; Pliego-Alfaro, *et al.*, 2013). Hence they are the preferred source of explants.

Meristematic competence is the combined ability of cells to both grow (due to cell expansion) and proliferate (due to cell division) (Risopatron *et al*., 2010; Manzano *et al*., 2013; Herranz *et al*., 2014), and this is evident in young and expanding leaves. In *Arabidopsis thaliana*, molecular markers were used to trace proliferative regions in the leaves. These regions were found at the mesophylls of the petiole/blade junction, at the leaf margins and at the middle of adaxial and abaxial sides of the leaves. The meristems at these regions were named plate, marginal and intercalary meristems, respectively (Donnelly *et al*., 1999; Ichihashi and Tsukaya, 2015); these meristems have been earlier described by the renowned botanist, Katherine Esau (1953; 1977). However, in matured

leaves that have undergone determinate growth, the activities of the meristems are capped by negative regulators (Ichihashi and Tsukaya, 2015).

The parent plants (also referred to as mother or stock plants) from which explants are isolated play a crucial role in the response of the explants *in vitro.* The primary factor to consider is that the growth of the stock plant is robust and the plants are free from disease (Preece, 2008). In addition, the availability of nutrients, the environmental and growth conditions of the stock plants as well as any pre-treatment regimes have effects on the isolated explants (Read and Economou, 1987; George and Deberg, 2008; Preece 2008). Seedlings that serve as stock plants are not only sourced in the field where they are grown conventionally, but can also be raised *in vitro*. The work of Yadav *et al*. (2015) on *Stevia rebaudiana* suggested that *in vitro* grown plants had more vigour in terms of weight, height, number of leaves and leaf dimension when compared with those grown conventionally in the field. Also, explants derived from the field and the *in vitro* mother plants of *Syringa vulgaris* and *Prunus serrulata* responded differently to rooting, with those developed *in vitro* having higher percentage rooting than those from the field (Plietzsch and Jesch, 1998 *loc sit* Preece, 2008).

Mother plants, especially growing *ex vitro*, naturally harbour microbes mostly on the surface and sometimes, systemically (George, 2008). The stock plants must therefore be pretreated to reduce/eliminate the associated microbes before explants can be isolated for aseptic tissue culture procedures. This is achieved by treating the stock plants with decontaminants such as fungicides. The plants may also be treated with pesticides to reduce insect damage and microorganism transmission (George, 2008). Although *ex vitro* parent plants are usually routinely treated with fungicides and pesticides in the field (or in the greenhouse), microbial contaminants persist in the soil and in the environment. These microorganisms still pose as systemic and surface infection of the stock plants (da Silva *et al*., 2015; Lazo-Javalera *et al*., 2016). Thus, the explants obtained from these seedlings are not always ‘clean’ enough to be used for aseptic plant tissue culture.

Without the decontamination of the explants, the nutrient-rich culture media oftentimes encourage the growth and proliferation of microorganisms, which are present within and on the surface of the explants, resulting in contamination (George, 2008). Hence, it is necessary to optimize a decontamination protocol that is potent enough to eliminate all the potential contaminants without significantly destroying the cells and tissues of the explants.

While there are numerous reports (e.g. Sairam *et al*., 2003; Hoque and Mansfield, 2004; Gandonou *et al*., 2005; Bilal *et al*., 2016) on the differing responses of explants from different genotypes of a plant to the *in vitro* environment, there is a paucity of information about how the explants that are sourced from the same plant genotype but grown in different ways – *ex vitro* and *in vitro* – respond to plant tissue culture. The aim of this chapter was to establish the two sources of the stock plant of *Persea americana*, from which leaf pieces to be used as explants were harvested. Leaf, as the explant, is readily available, less invasive on the mother plant and it is true-to-type of the mother plant.

2.2 Materials and Methods

2.2.1 Greenhouse parent plant materials

Seedlings of two avocado cultivars, 'Edranol' and 'Fuerte', were obtained from Rietvlei nursery in Tzaneen, Limpopo province, South Africa and were maintained, first in the phytotron and later in the greenhouse (Figure 2.1) under controlled environmental conditions (26 – 28 °C, 40% humidity). The seedlings were routinely treated with pesticide, fungicides and fertilizers as shown in Table 2.1. The pesticide used was Malasol while the fungicides were Previcur®N and Odeon. The fertilizers were LAN bio-carbon enriched granules, 3:1:5 (26) SR and bone meal. The fertilizers were applied in alternating months with LAN applied in the first month while 3:1:5 (26) SR and bone meal were applied together in the following month. The protocols and schedules of application of the fertilizers, pesticide and fungicides were modifications of Mansoor (2018).

Table 2.1: The application routine of pesticides, fungicides and fertilizers used to treat avocado seedlings ('Edranol' and 'Fuerte' cultivars) maintained in the greenhouse.

Treatments	Products	Active ingredients	Strength	Brands	Conc. Used	Frequency of use
Pesticide	Malasol	Mercaptothion	500 g/L	Efecto, South Africa	2.5 ml/L	Once every two months
Fungicides	(i) Previcur®N	Propamocarb – HCl	722 g/L	Bayer, Germany	1 ml/L	Once every month
	(ii) Odeon	Chlorothalonil	720 g/L	Adama, South Africa	2 ml/L	Once every month
Fertilizers	(i) LAN	Limestone, Ammonium and Nitrate	-	WONDER™, South Africa	5 g/20 L seedling bag	Every other month
	(ii) 3:1:5 (26) SR	Nitrogen, Phosphorus and Potassium	N: 87 g/Kg; P: 29 g/Kg; K: 144 g/Kg	WONDER™, South Africa	5 g/20 L seedling bag	Every other month
	(iii) Bone meal	Nitrogen and Phosphorus	N: 40 g/Kg; P: 100 g/Kg	WONDER™, South Africa	5 g/20 L seedling bag	

Figure 2.1: Avocado seedlings maintained in the (A) phytotron and (B) greenhouse. Black arrows indicate young leaves.

2.2.2 Determination of decontamination method for leaf explants from greenhouse parent plants

Fifty explants for each of six different surface decontaminants were tested. These included: (I) 70% ethanol for 30 secs (II) 1% sodium hypochlorite (NaOCl) for 20 minutes (III) 2% NaOCl for 10 minutes (IV) 1% calcium hypochlorite ($Ca(OCl)_2$) for 20 minutes (V) 2% $Ca(OCl)_2$ for 10 minutes and (VI) 1% NaOCl with 20 drops/L of Tween 20© for 5 minutes.

In all the treatments, the chemicals were removed by washing with sterile ultrapure water. Young avocado leaves (chosen between the third to sixth leaves from the apex) were cut from the seedlings in the greenhouse and transported to the laboratory in a beaker of tap water. In the laboratory, the leaves were left under running tap water for 30 minutes after which they were treated with each decontaminant. This was followed by rinsing three times in sterile ultrapure water. Leaves were cut into small discs of 6mm in diameter with a sterile punch, with each disc necessarily containing some vascular tissues. For this trial, leaves were either punched into discs before, or after, decontamination.

2.2.3 Explant sources for callus induction

The explants of choice for callus induction were the leaves of avocado and these were either chosen from (i) the greenhouse seedlings or (ii) plantlets/shoots grown *in vitro*.

2.2.3.1 *Greenhouse seedlings*

This entailed cutting leaf explants directly from the avocado seedlings that had been growing in the greenhouse (as described under section 2.2.1). The details of the preparation of the leaves for tissue cultures are discussed in a later chapter (Chapter Three).

2.2.3.2 *In vitro plantlets/shoots*

In vitro plantlets were obtained in three ways: through whole seeds, axillary buds and embryonic axes.

2.2.3.2.1 Whole seeds

To generate plantlets *in vitro* from whole seeds, fresh avocado fruits, cv. 'Fuerte', were purchased from Johannesburg Fresh Produce market. The seeds (Figure 2.2A) were separated from the pulp, wiped clean, coated with Benomyl powder (active ingredient: benzimidazole; 500 g/Kg) and sealed in Ziploc bags. The seeds were processed for tissue culture procedures within 24 hrs of removal from the fruits as follows: The seed coats of

40 seeds were removed (Figure 2.2B) and the seeds were pre-treated with 0.5% (w/v) ascorbic acid for 10 minutes. They were surface-decontaminated by immersion in 1% NaOCl with 20 drops/L Tween 20© for 10 minutes and rinsed three times in sterile, ultra-pure water. The materials were cultured, one seed per box, in Magenta boxes that each contained 50 ml full strength MS (Murashige & Skoog), supplemented with 30 g/L sucrose and solidified with 7 g/L agar. The medium had been autoclaved for 20 minutes at 120°C and 121 kPa prior to being used. The cultures were kept in the growth room under 14-hour light and 10-hour dark photoperiod at 25°C ± 2°C with a photon flux density of 100 $\mu mol.m^{-2}.s^{-1}$.

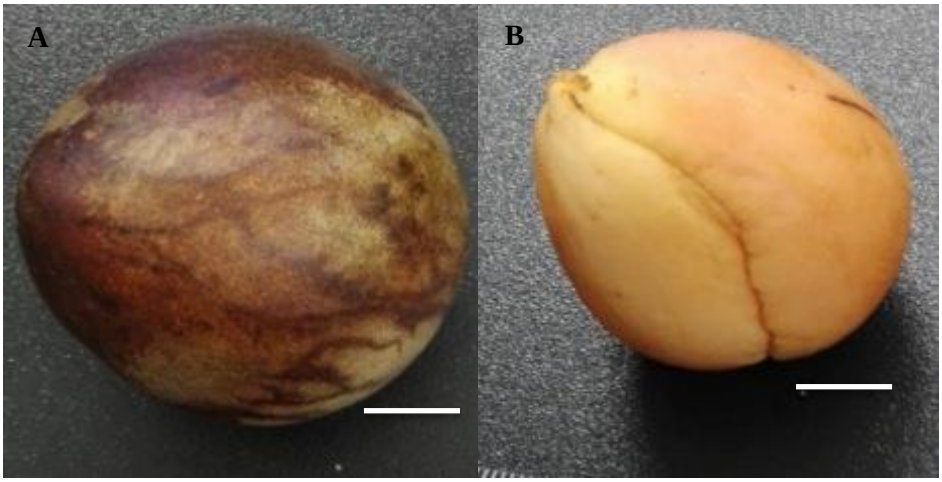


Figure 2.2: Avocado seed (A) with intact seed coat and (B) with seed coat removed. Scale bar = 1cm.

2.2.3.2.2 Axillary buds

Shoots that were approximately 5 – 10 cm in length were cut from the greenhouse seedlings and the leaves removed (Figure 2.3). The shoots were left under running tap water for 20 minutes in the laboratory after which they were dipped in 70% ethanol for 30 seconds and subsequently rinsed again under running tap water. The shoots were then surface-decontaminated by soaking in 1% (w/v) Sodium hypochlorite (NaOCl) with Tween 20© (10 drops/Litre) for 10 minutes and were rinsed three times in sterile, ultra-pure water. The shoots were, thereafter, cut into 40 nodal segments of 1 – 2cm in length; each segment served as an explant necessarily containing at least one axillary bud. The explants were plated on a growth medium that had been previously established by Mansoor (2018), with two explants per Magenta box. The cultures were maintained in the growth room under 14-hour light and 10-hour dark photoperiod at 25°C ± 2°C with a photon flux density of 100 $\mu mol.m^{-2}.s^{-1}$.

Figure 2.3: Avocado shoot isolated from greenhouse grown material. The segment shows a node (red circle) and an axillary bud (red arrow). Scale bar = 1 cm.

The growth medium, for the nodal explants, comprised full strength MS with vitamins. This was supplemented with 30 g/L sucrose and 1.0 mg/L BAP (6-Benzylaminopurine); and solidified with 3.0 g/L gelrite at pH 5.70 ± 0.05. The growth medium was dispensed, 50 ml in each Magenta box, and autoclaved (as above).

2.2.3.2.3 Isolated embryonic axes

Eighty embryonic axes were excised from avocado seeds with ca. 2 mm portion of the cotyledons still attached. This was to prevent injury of the axes during excision (Figure 2.4C). The embryonic axes were pre-treated with 0.5% (w/v) ascorbic acid for 5 minutes, after which they were surface-decontaminated in 1% NaOCl for five minutes. Embryonic axes (n = 40) were cultured in tubes (10 cm in height and a diameter of 2.5 cm), each containing 10 ml medium. Also, forty embryonic axes were plated in Magenta boxes (9 × 7 × 7 cm) each containing 50 ml culture medium and two axes per box. The medium consisted of full strength MS, supplemented with 30 g/L sucrose and solidified with 7 g/L agar. The culture vessels with the dispensed medium, sans explants, were autoclaved as described. After plating, the cultures were maintained in the growth room under 14-hour light and 10-hour dark photoperiod at 25^{o}C ± 2^{o}C with a photon flux density of 100 µmol.m^{-2}.s^{-1}.

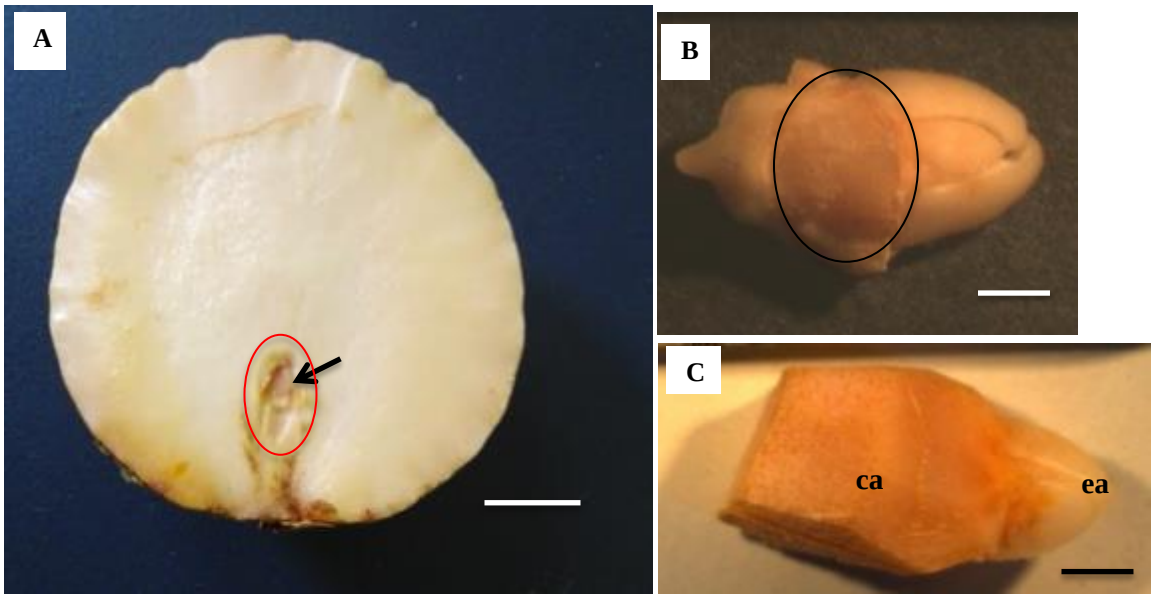


Figure 2.4: Embryonic axis of avocado. (A) Halved avocado seed showing the position (enclosed circle in red) of the embryonic axis. Black arrow indicated the point of firm attachment of the embryonic axis to the cotyledons, Scale bar = 1 cm. (B) Isolated embryonic axis showing the region of its attachment (black circle) to the cotyledons. Scale bar = 2 mm. (C) An isolated embryonic axis (ea) with cotyledonary attachment (ca). Scale bar = 2 mm.

2.2.4 Multiplication of *in vitro* shoots by subculture

After 8 – 12 weeks, well-developed shoots and plantlets (referred to as first generation *in vitro* parent plants) from the cultured greenhouse axillary buds and whole seeds, respectively, were isolated and cut into nodal segments that necessarily had axillary buds. These were then plated on the same growth medium as described for axillary buds in section 2.2.3.2.2 but without prior surface decontamination. These were expected to give rise to second generation *in vitro* parent plants.

2.2.5 Statistical analysis

Using excel data analysis add-in, A One-Way Analysis of Variance (ANOVA) was used to test for statistically significant difference at 95% confidence level (significant level $\alpha = 0.05$) when comparing more than two treatments. A post-hoc test using independent sample t-test correction was used to test pair-wise significant difference. A t-test was used to compare means of two treatments.

2.2.6 Imagery

All images were captured with a Huawei ALE-L21 13.0 megapixel camera.

2.3 Results

2.3.1 Greenhouse parent plant materials

More than 50% of the first batch of seedlings died despite the routine treatments with fertilizers, pesticides and fungicides in a controlled environment. Uprooting the seedlings revealed that the primary taproots were no longer viable and the soil was waterlogged and poorly aerated. A concurrent research by Mansoor (2018) improved on the composition of the potting medium for the second batch of seedlings. The composition comprised perlite, river sand and pine bark or peat and these were combined in equal ratio. The composition mitigated the problem of poor aeration and facilitated proper drainage of the soil. The avocado seedlings from this batch (Mansoor, 2018) were subsequently used for the experiments reported here. Since the problems of water logging and poor aeration were solved, adherence to the treatments described earlier (Table 2.1) helped to reduce the microbial and fungal load on the seedlings.

2.3.2 Determination of decontamination method for leaf explants

In the leaves of avocado used in this study, the efficiency of the various decontaminants was inversely related to the level of contamination of the cultures and the colour of the leaf explants. The level of contamination was lower when the avocado leaves were cut into discs after they had been decontaminated, than when they were disked prior to decontamination procedures (Table 2.2A).

Contamination in tissue cultures readily shows within two to three weeks after incubation. If the contamination is associated with the explants, it will seldom appear after the window period of the first two - three weeks (except in some few cases where some endophytic contaminants appear long after 2 – 3 weeks). In the decontamination of avocado leaf explants investigated, there was no difference in the level of contamination after two and four weeks of culture. However, the number of leaf explants that changed colour from green to brown increased with time and by the fourth week, a large proportion of the cultures had turned brown in all the decontaminants tested except for treatment VI (Table 2.2B). The browning of explants (Table 2.2) was a consequence of harshness of the surface decontaminants used rather than the result of oxidative activities. This was because, unlike browning due to phenolic leakage, there was no noticeable discolouration around the cut surfaces of the explants or in the culture medium (Figure 2.5).

Table 2.2: The percentage of contaminated and browned explants after treatment with different decontaminants (A) two weeks in culture (B) four weeks in culture.

A

TREATMENTS	DECONTAMINANTS	LEVEL OF CONTAMINATION (%)		BROWNING (%)	
		LEAVES DISCS		LEAVES DISCS	
		BEFORE STERILIZATION	AFTER STERILIZATION	BEFORE STERILIZATION	AFTER STERILIZATION
I	70% ethanol for 30 secs	0	0	100	100
II	1% NaOCl for 20 min.	20	0	12	8
III	2% NaOCl for 10 min.	0	0	12	10
IV	1% $Ca(OCl)_2$ for 20 min.	25	4	12	28
V	2% $Ca(OCl)_2$ for 10 min.	25	0	15	8
VI	1% (NaOCl) with Tween20 (20 drops/L) for 5 min	10	4	8	0

B

	DECONTAMINANTS	LEVEL OF CONTAMINATION (%)		BROWNING (%)	
		LEAVES PUNCHED		LEAVES PUNCHED	
		BEFORE STERILIZATION	AFTER STERILIZATION	BEFORE STERILIZATION	AFTER STERILIZATION
I	70% ethanol for 30 secs	0	0	100	100
II	1% NaOCl for 20 min.	20	0	40	36
III	2% NaOCl for 10 min.	0	0	52	52
IV	1% $Ca(OCl)_2$ for 20 min.	25	4	44	36
V	2% $Ca(OCl)_2$ for 10 min.	25	0	60	36
VI	1% (NaOCl) with Tween20 (20 drops/L) for 5 min	10	4	12	8

With leaf discs isolated after decontamination, the use of 70% ethanol for 30 seconds was effective in the elimination of all surface contaminants but resulted in browning of all explants (Table 2.2.A; treatment I). Exposure of the leaf explants to a high concentration (2%) of NaOCl for a short period (10 min.) or low concentration (1%) of NaOCl for a longer period (20 min.) also resulted in contamination-free explants but these treatments were not mild enough as browning was 52% and 36%, respectively (Table 2.2.A; treatment

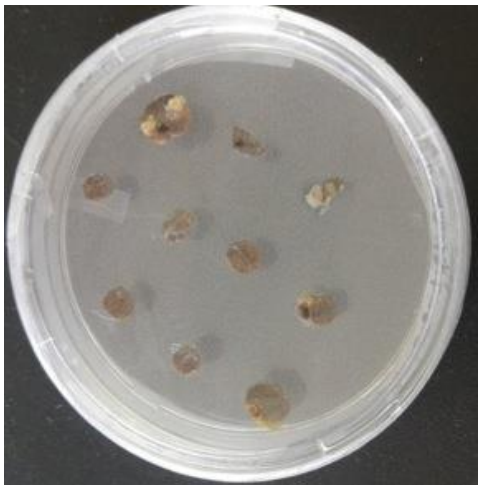


Figure 2.5: Browned explants after 4 weeks in culture. The material had been decontaminated with 2% NaOCl for 10 minutes.

II and III). Similar results were obtained when $Ca(OCl)_2$ was used at the same concentration and duration (Table 2.2.A; treatment IV and V). However, decontamination for 5 minutes in a solution of 1% NaOCl with a few drops of Tween®20 (Table 2.2.A; treatment VI) reduced contamination by 96% and browning by 92%. Hence, this was deemed optimal for the decontamination of avocado leaf explants.

2.3.3 Growth of *in vitro* plantlets/shoots

The main purpose of growing *in vitro* plantlets, in this study, was to have stock mother plants from which 'clean', microbe-free, leaf explants could be obtained when required for callus formation. Hence, the optimal method of growing *in vitro* plantlets/shoots that ensured good foliage, in terms of number of leaves per shoot and the size of the leaf lamina, was investigated.

2.3.3.1 *Whole seeds*

When *P. americana* seeds were cultured *in vitro*, (in which nutrients were also supplied exogenously), they developed healthy shoots (4.23 cm long and 1.46 cm wide) and roots (20 cm long) with each primary root having many lateral roots (Figure 2.6). The presence of the root in the seed-generated plantlets further facilitated the growth of the plantlets.

Figure 2.6: Normal plantlet obtained from *P. americana* seed.

2.3.3.2 *Axillary buds*

In this study, shoots generated from axillary buds were healthy (Figure 2.7), producing on average 7 well-expanded (Table 2.3) leaves per shoot with no noticeable anomalies on the leaves such as tissue browning or necrosis. Average leaf length and breadth were 3.8 cm and 1.02 cm, respectively (Table 2.3).

Figure 2.7: *In vitro* shoot of avocado obtained from axillary buds.

2.3.3.3 *Isolated embryonic axis*

In avocado seeds, the embryonic axes are situated towards the centre of the posterior ends of the seeds (Figure 2.4A) and were firmly attached to the cotyledons (Figure 2.4A and B). As such, the embryonic axes could not be completely isolated without considerable damage, with a consequent adverse effect on the shoot and root growth of the apices. In

order to avoid this adverse effect, the embryonic axes were isolated with a block of cotyledons attached (Figure 2.4C).

The first batch of excised embryonic axes that were plated into culture tubes developed shoots and roots. However, these organs lacked vigour, were stunted and a number of growth variations were observed (Figure 2.8). These variations ranged between shoot production only, root production only, shoot with needle-like leaves, enlarged axes with no well-defined shoot/root to proper shoot/root production (Figure 2.8).

Figure 2.8: The growth of plantlets from embryonic axes of avocado showing various growth forms *viz* root only, shoot only, shoot with small leaves and normal root and shoot development.

The lack of vigour and stunted growth was less prominent in the second batch of embryonic axes that were cultured in Magenta boxes. Plantlets from this batch developed shoots and roots without any anomalies.

Table 2.3: Effect of the source of *in vitro* shoots on number of leaves per shoot, leaf length and breadth.

Source of *in vitro* Plantlets	Percentage Growth/Germination (%)	Numbers of Leaves per Shoot	Leaf Length (cm)	Leaf Breadth (cm)
Axillary buds	90	7	3.8 ± 1.41^{a}	1.02 ± 0.51^{ab}
Embryonic axes (In culture tubes)	90	4	1.02 ± 0.23^{b}	0.31 ± 0.1^{c}
Embryonic axes (In Magenta boxes)	85	7	2.93 ± 0.52^{a}	0.73 ± 0.23^{b}
Whole seeds	80	9	4.23 ± 1.85^{a}	1.46 ± 0.73^{a}

Different superscripted letters in each column indicated significant difference.

However, the leaf length and breadth were still smaller (2.93 cm and 0.73 cm, respectively) in comparison with those obtained from whole seeds and axillary buds (Table 2.3). These growth forms portray the importance of the cotyledonary tissues in seed germination and the implication of their total or partial removal. *In vitro* plantlets obtained from whole avocado seeds were the best source for *in vitro* stock mother plants for leaf explants. This was followed by those obtained through the axillary buds. The shoots derived from the isolated embryonic axes were the least desirable (Table 2.3).

2.3.4 Multiplication of *in vitro* shoots by subculturing

In this study, the use of first generation *in vitro* parent plants to generate axillary bud explants for second generation *in vitro* shoots, was not favourable. This was evinced by the significant reduction in the shoot height and number of leaves per shoot (Figure 2.10). While the first generation of *in vitro* shoots, grown from explanted greenhouse nodal segments, had an average of 7 well-expanded leaves per shoot (Figure 2.9d), second generation *in vitro* shoots from subcultured samples had fewer (±2) leaves per shoot that were not as expanded as their primary *in vitro* mothers (Figure 2.9 a – c and Figure 2.10). The height of the shoots from the second-generation samples was significantly reduced and they were, sometimes, characterized with negligible, small and necrotic leaves (Fig. 2.9b).

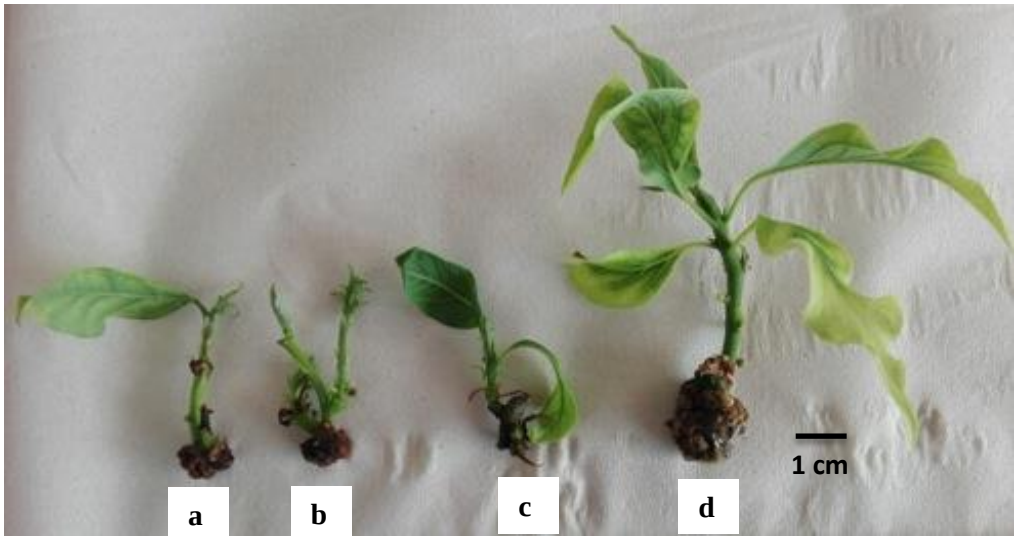


Figure 2.9: Second generation *in vitro* shoots from subcultured nodal segments with axillary bud (a - c) and first generation *in vitro* shoot from primary explanted greenhouse nodal segment (d).

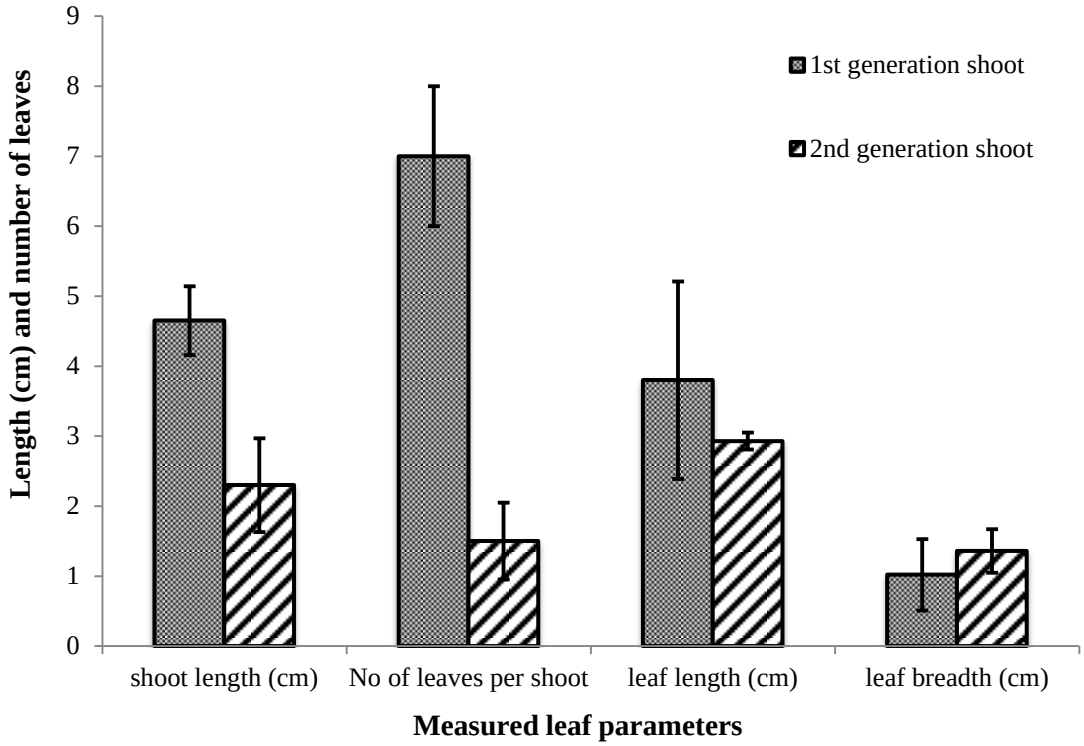


Figure 2.10: Effect of subculture on the shoot length, number of leaves per shoot, leaf length and breadth on the axillary bud (AB) of avocado.

2.4 Discussion

2.4.1 Greenhouse parent plant materials

The type of soil used for the first batch of avocado seedlings was waterlogged and poorly aerated. This type of soil provided a conducive medium for the fungus, *Phytophthora cinnamomi*, to thrive; resulting in root rot of the avocado seedlings (Ploetz and Schaffer, 1989; Wolstenholme, 2011). It thus becomes imperative that, avocado seedlings are grown in well-drained soil with high oxygen level for proper growth. Therefore, a conscientious effort is required for the selection of appropriate soil type as well as soil preparation for healthy avocado growth (Wolstenholme, 2011).

The organic composition of the potting medium, used concurrently in both Mansoor (2018) and this study for the growth of avocado seedlings, was either pine bark or peat. Both components are good inclusion in potting medium because they can facilitate good drainage and facilitate good aeration (Maher *et al*., 2008; Mupondi *et al*., 2010). These organic components also add to the nutritional value of the potting medium. In addition to the organic components, the inorganic components were river sand and perlite. Perlite is obtained from volcanic glassy rock and is versatile in its uses because of its chemical and

physical properties (Alkan and Doğan, 2001). Particularly, it is a good aerating agent (Hochmuth and Hochmuth, 1996), which makes it a good component of potting mix for avocado seedlings. River sand has low water holding capacity (Peries and Everard, 1993) and thus improves the drainage of the potting medium. A well-drained potting mix can help to mitigate the problem of *Phytophthora* root rot disease (Odneal and Kaps, 1990), which is common in avocado. Collectively, these components constituted a suitable potting medium that prevented waterlogging and allowed for efficient aeration for the avocado seedling roots.

2.4.2 Determination of decontamination method for leaf explants

Decontamination of the explants for *in vitro* cultures is arguably the most important step in the process of obtaining plant tissue cultures. It can range from simple, single-step treatments to complex, multi-step procedures (da Silva *et al*., 2015). The goal was to choose a decontamination regime that is mild enough not to destroy the cellular integrity of the explants but potent enough to eliminate the associated microorganisms.

One of the signs of compromised explant cellular integrity is browning. Browning has been associated with the oxidative activities of phenolic compounds and their associated enzymes (Jones and Saxena, 2013; Chuanjun *et al*., 2015). Oxidative browning occurs as a result of wounding or excision injury; is characterized by leakage of the phenolic substances (among others) from the cut surfaces into the culture medium. This leads to discolouration of both the producing tissues and the medium. However, this study showed that browning could also occur as a result of the harshness of the surface decontaminants used on the explants (Table 2.2). This type of browning could be attributed to either (i) the high concentration of the decontaminating solution or (ii) prolonged exposure of explants to the decontaminating solution. Browning is toxic to plant cells. Consequently, it reduces the response of the explants to tissue culture manipulation and ultimately leads to cell/tissue death in most cases (Jones and Saxena, 2013). The concentrations of decontaminants and the exposure duration thus need to be optimized for an effective and successful decontamination step in plant tissue culture.

Some of the common decontaminants are ethanol, hypochlorite compounds, mercuric chloride, silver nitrate; among others (Razdan, 2003). Ethanol is a powerful decontaminant, however, it is highly toxic to the plant cells (Oyebanji *et al*., 2009; Bello *et al*., 2018). Hence it was not suitable for the avocado leaf explants (Table 2.2, Treatment I).

In this regard, Oyebanji *et al*., (2009) and Abbasi *et al*. (2017) had suggested the use of ethanol, not on direct tissues that will be explanted, but on such explants in which the tissue of interest is hidden such as embryonic axes within seeds and unopened flower buds.

Sodium hypochlorite (NaOCl) is versatile in its uses; one of which is its effectiveness as a surface decontaminant. Calcium hypochlorite ($Ca(OCl)_2$) is also frequently used as a decontaminant in plant tissue culture procedures (Brownleader and Dey, 1997; da Silva *et al*., 2015). Upon dissociation, both NaOCl and $Ca(OCl)_2$ produce OCl^-, which form hypochlorous acid in solution. This acid interferes with, and disrupts the components of the cell wall of the microorganisms, causing their death (Bello *et al*., 2005; Bello *et al*., 2019). Brownleader and Dey, (1997) recommended 1-2% NaOCl and 9-10% $Ca(OCl)_2$ for an exposure period of 10-40 minutes. However, concentration and duration are species-dependent (Sweet and Bolton, 1979; Bello *et al*., 2018). $Ca(OCl)_2$ is milder than NaOCl (Abbasi *et al*., 20017; Bello *et al*., 2018), but NaOCl is more easily accessible and it is cheaper to buy commercial JIK; hence it was preferred as a decontaminant in this research.

It is imperative that optimal concentrations of decontaminants are used for maximum decontamination without side effects. Zinabu *et al*. (2018) showed that when the concentration of NaOCl was too low or too high, it was not optimum in the surface-decontamination of *Ensete ventricosum*. Likewise, Sweet and Bolton (1979) found that increasing the concentration of $Ca(OCl)_2$ did not reduce contamination but adversely affected seed germination. This is because high concentrations of hypochlorite are phytotoxic (Bello *et al*., 2019) and low concentrations will lead to unsaturated decontaminating solution. These observations, as well as the results of Abbasi *et al*. (2017), were in accordance with the results obtained in this study.

Soaking avocado leaf explants for 5 minutes in a 1% NaOCl solution that contained Tween®20 (Treatment VI) was potent enough to eliminate microbial contamination from the explants without compromising their cellular integrity. Tween®20 (Polysorbate20) is a non-ionic detergent that plays multiple roles (National Center for Biotechnology Information). Particularly in this study, its inclusion in the decontaminating solution helped to break surface tension of the explants thus improving the effectiveness of the decontaminant within a shorter time (5 min.). Similarly, effective decontamination was achieved in shorter time when Tween®20 was added to decontaminating solution of NaOCl (da Silva *et al*., 2015; Abbasi *et al*., 2017). Collectively, these emphasized the

importance of both the optimal concentration of the decontaminant and duration of exposure of explants to decontaminating solutions.

2.4.3 Growth of *in vitro* plantlets/shoots

2.4.3.1 *Whole seeds*

A seed, without the removal of any part, is capable of developing and growing into a new plant, on the condition that the necessary factors needed for germination are met (Hopkins and Hüner, 2008). The cotyledons of seeds serve as food reserve (Hopkins and Hüner, 2008) and in some instances have photosynthetic abilities (Chandler, 2008; Zhang *et al*., 2008). These make them a source of energy and nutrients for germinating seeds. It is believed that the bigger the cotyledons (which is ca. 28.62 g in avocado), the more nutrients are reserved therein, which corresponds to the amount of food reserved for germination (Milberg and Lamont 1997, Milberg *et al*., 1998). On that premise, the avocado seeds already have sufficient food reserve for germination; in addition, the culture medium for avocado seeds was enriched with nutrients. These provided ample nutrients for the seeds, which resulted in healthy plantlets from the seeds (Figure 2.6).

A plantlet from a seed represents a different genotype (due to sexual reproduction) from the parent plant (Bewley and Black, 1985; Hopkins and Hüner, 2008). Thus, sourcing leaf explants from as many as hundreds of such plantlets will introduce genetic variation. Genetic variation is not necessarily disadvantageous as it can improve adaptation, fitness and resistance to pests and diseases (Lacy, 1997; Bürger, 1999). However, for the purpose of this study, genetic variation would have made it difficult to truly analyze the obtained results from leaf explants of such plantlets. In addition, seeds are not produced all year round but are seasonal; hence their availability for growth of *in vitro* mother plants will be dependent on availability each season. Each season is accompanied by variations (both biological and environmental) and these impact the quality and quantity of seed produced. Therefore for this study, generation of *in vitro* stock plants from seeds was not a preference.

2.4.3.1 *Axillary buds*

According to George and Deberg (2008), shoot production from axillary buds is the most tested and dependable method of micropropagation, as it ensures that the resultant shoots are true-to-type. In the well-established avocado seedlings maintained in the greenhouse,

many nodal segments were obtained from one seedling. Thus only a few seedlings were required to obtain the nodal segments required to establish *in vitro* shoots. Consequently, the level of genetic variation in the leaf explants was also reduced when compared with those from seed-derived *in vitro* plantlets. Importantly, the availability of the vegetative nodal segments was not seasonal and material could be obtained all-year round. Hence, generation of *in vitro* stock plants from the axillary buds was preferred in this study.

2.4.3.2 *Isolated embryonic axis*

The embryonic axes of seeds, especially in dicotyledonous plants, are fully developed bipolar structures, with distinct root and shoot meristematic apices (Bewley and Black, 1985). When isolated from the seeds, the embryonic axes can develop into a whole plant if provided with appropriate growth conditions (Normah and Makeen, 2008). However, in this study, the embryonic axes were isolated with bits of cotyledonary attachment. The requirement, to isolate embryonic axes with blocks of cotyledonary attachement, was demonstrated in the work of Goveia (2007) where embryonic axes of *Trichilia dregeana* were isolated with none-, basal- and 2 mm block- cotyledonary attachments. Those results showed that, while the *Trichilia dregeana* axes from all categories of excision had 100% root production, the axes with no attachment produced no shoot while those with either basal or block attachment had more than 50% shoot production. Thus, the cotyledonary attachments protect the apices of the embryonic axes from excision injury.

In spite of the excision of embryonic axes of avocado with cotyledonary attachment in this study, the embryonic axes still developed poorly in comparison with the germination of intact seeds (Table 2.3). This is because in avocado seeds, the separation of the cotyledons (ca. 28.62 g) from the axes (ca. 0.13 g) deprived the germinating tissue the necessary nutrients and energy required for growth. Hence, the only available nutrients in the *in vitro* cultures were those supplied exogenously but these may not have been sufficient to replace the natural source of nutrients found in the cotyledons.

In addition to nutrients, the size of the culture vessels used to grow the embryonic axes, i.e. the culture tubes and the Magenta boxes, had a direct proportional effect on their growth (Table 2.3). This has been shown by numerous authors that the size, type (George, 1993), and lid of culture vessels (McClelland and Smith, 1990; George, 1993) have an effect on the growth of the cultured tissues or cells. In particular, Tisserat and Silman (2000) showed that the response of shoot growth of lettuce and spearmint was directly proportional to the

culture vessel size. Additionally, according to George (1993) as well as Nguyen and Kozai (1998), various interactions take place between the explants, the culture medium, the air in the microenvironment and the absorbent ability of the culture vessels to external environmental factors such as light and temperature. Consequently, the size of culture vessels will affect the ratio of these parameters. While smaller vessels such as the presently used culture tubes did not favour the growth of the embryonic axes, they reduced the spread of contamination as one explant was plated per culture tube. Bigger culture vessels can house more than one explant, which can facilitate the spread of infection among the explants (George, 1993).

2.4.4 Multiplication of *in vitro* shoots by subculturing

Subculturing of *in vitro* materials is necessary for two reasons: firstly, it increases the number of *in vitro* samples (micropropagation) and allows for the proliferation of other materials such as callus, which can be used in other applications. Secondly, it is for maintenance. This is required because after some weeks, the nutrients in culture media are depleted, thus depriving the cultured samples of the nutrients required for growth (George, 2008). In addition, some cultures, as in this study, could outgrow the culture vessels thus necessitating the need for subculture. Furthermore, culture media can become toxic to tissues and also dry out with time, especially if the culture vessels have a large surface area. Thus, this calls for subculturing of samples to fresh medium (George, 2008). However, subculturing is not cost effective and is time consuming. In addition, continuous subculturing can make tissues less vigorous and lead to shoot necrosis, which can ultimately lead to death (George, 2008). Such was observed in the tropical tree *Gmelina arborea* where rooting and multiple shoot formation decreased with each subculture (Naik *et al*., 2003). Similarly, reduced growth associated with subculturing has been reported in *Persea americana* by Litz *et al*. (2005b).

Less vigorous tissue growth was also observed in this study, where second generation shoots were stunted with reduced leaf number, leaf blade expansion and shoot height. The second generation *in vitro* shoots were raised from nodal segment of first generation *in vitro* shoots, whereas the latter were raised from greenhouse nodal segments. A morphological comparison (Chapter Four) between *in vitro* and greenhouse avocado materials showed that *in vitro* materials were not as fully differentiated as their greenhouse counterparts. Hence, the use of not-fully-differentiated materials as explants for generating

shoots (organogenesis) was presumably responsible for the less vigorous growth. This was because the *in vitro* materials had not attained sufficient physiological maturity to support such process ((Nhut *et al*., 2007). Similar results were obtained by Mansoor (2018) where each subculture of avocado shoots led to reduced number of shoots and decreased growth parameters.

2.5 Concluding Remarks

While nursery-bought seedlings that have been established in the greenhouse were routinely treated to eliminate systemic and surface contaminants, it was still imperative to establish a decontamination procedure that was suitable for leaf explants that were meant for tissue culture. *In vitro* shoots could be grown in many ways but for the purpose of foliage production, growing them through the axillary buds was more desirable than through the whole seeds. This is because that method minimized the genetic variations in the population and ensured continuous supply of shoots all year round. It also eliminated the need for subculturing axillary buds for second generation shoot growth. Investigation on the role of culture vessels on *in vitro* shoot growth can also be considered in the future. The developmental status of explants appeared to have different level of competence towards organogenesis and callogenesis. This may require further research to establish this opinion.

CHAPTER THREE: CALLUS INDUCTION FROM THE LEAF EXPLANTS OF AVOCADO (*Persea americana*) AND THEIR DEVELOPMENT

3.1 Introduction

Callus is a mass of unspecialized dedifferentiated cells stimulated to grow from small explants of whole plants (George, 1993). Some researchers (e.g. Sugimoto *et al*., 2011) have refuted this claim and are of the opinion that callus cells are not dedifferentiated cells but are developed from pre-existing cells. Nevertheless, it is generally accepted that callus is the result of the ability of differentiated plants to dedifferentiate (George, 1993; Efferth, 2019).

In nature, callus forms as a result of infestation, infection and in response to wounding where it helps to seal wounded tissue. In plant tissue culture, callus is often intentionally induced *in vitro* for the purpose of biotechnological procedures. Efferth (2019) highlighted a few, out of the many, uses of callus. These include regeneration of agricultural (fruits, grains, legumes, herbs) and horticultural plants and the production of therapeutic antibodies, recombinant proteins and secondary metabolites.

3.1.1 Types of callus

Callus can be grouped using various morphological parameters. The common parameters are colour, embryogenic competence and texture. In passion fruit for example, various concentrations of the inducing plant growth regulators (PGRs) produced callus of different colours ranging from translucent, light- white- and dark-yellow to white-brown (Carvalho *et al*., 2013). Thus, the colour of callus is not restricted to cream-yellow and white (as is often reported e.g. George, 1993) but rather can have a range of colours (Efferth, 2019).

In addition to colour, callus can be categorized as embryogenic or non-embryogenic. The former is made up of cells that are competent to develop and regenerate whole plants. These cells are generally isodiametric with prominent nuclei, large numbers of isodiametric mitochondria and tightly packed cytoplasm, they also accumulate starch and are less vacuolated. On the other hand, elongated and highly vacuolated cells with small nuclei are characteristics of the non-embryogenic callus. These cells are not competent for whole plant regeneration (Finer, 1994; Srivastava, 2002; Karami and Ostad-Ahmadi, 2008 and Carvalho *et al*., 2013). Nevertheless, non-embryogenic callus can be used to produce secondary metabolites, which are useful in the pharmaceutical and cosmetic industries (Efferth, 2019).

The other common parameter of describing callus is texture. Broadly, calli are categorized into two textural groups: compact or friable. The cells of the compact type of callus are

densely and tightly packed, resulting in hard callus while friable calli are soft and easily crumbled because they are made up of cells that are only loosely associated. According to the general description by Franklin and Dixon (1994), embryogenic calli are compact and slow-growing, while fast-growing and friable callus is not embryogenic. Similarly, George (1995) noted that embryogenic calli could be distinguished by their nodular structure. Nothwithstanding this, Franklin and Dixon (1994) gave an exception to grasses where both compact and friable calli were considered embryogenic. There is however, tremendous variation in texture related to competence (Table 3.1).

Table 3.1: The relationship between the texture of callus and its embryogenic competence

Species/Common Name	Callus Texture	Embryogenic Competence	Authors
Carnation	Soft and succulent	Non-embryogenic	Karami and Ostad-Ahmadi, 2008
	Hard, nodular and slow-growing	Embryogenic	
Sweet potato	Nodular and compact, slow-growing	Embryogenic	Mukherjee *et al.*, 2001
	Fragile and fast-growing	Non-embryogenic	
Muscari armeniacum	(i) Nodular and compact (ii) friable	Embryogenic	Suzuki and Nakano, 2001
Hevea brasiliensis	(i) Nodular and compact (ii) friable	Embryogenic	Piyatrakul *et al*., 2012
Maize	Friable	Embryogenic	Vasil and Vasil, 1986
Avocado 'anaheim'	Friable and translucent	Embryogenic	Guzmán-García *et al*., 2013
	Amorphous	Non-embryogenic	
Cassava	Friable	Embryogenic	Yandia *et al*., 2016

Friable calli may be soft and break apart easily but that does not necessarily imply they are non-embryogenic, rather, they are useful in isolating single cells from liquid medium (George, 1993; Efferth, 2019).

It is thus safe to conclude that callus morphological parameters do not suffice in the determination of regenerative competence. Other factors, such as physiological, biochemical, cytological as well as genetic responses are required to validate the embryogenic capacity of callus (Ribeiro *et al*., 2012).

3.1.2 Tissue culture media and the induction of callus

To induce callus and its subsequent manipulations (towards development, differentiation, maturation and plant regeneration) requires tissue culture media (Franklin and Dixon, 1994). The medium usually comprises a combination of basal nutrients, a carbon source

(most often supplied by sucrose) and plant growth regulators (PGRs), with or without a gelling agent.

The basal nutrient medium usually comprises macro- and micro- inorganic nutrients/elements, some amino acids and vitamins. The most commonly used basal nutrient media are Murashige and Skoog (MS) medium (Murashige and Skoog, 1962); B5 medium (Gamborg *et al*., 1968), Schenk and Hilderbrandt (SH) medium (Schenk and Hilderbrandt, 1972), Woody Plant Medium (WPM) (Lloyd and McCown, 1980), White's medium (White 1943) and Nitsch and Nitsch medium (Nitsch and Nitsch, 1969). However, there are derivatives of these nutrient media obtained by addition and/or subtraction of components or by the alterations of the concentrations and ratios of the components. Sometimes, the macronutrients of one nutrient medium may be added to the micronutrients of the other, for example $B5^{+}$ (Encina *et al*., 2014).

The other components of the culture media are PGRs. These control the formation, subsequent differentiation and development of callus. PGRs have been divided into five classes, *viz* the auxins, the gibberellins, the cytokinins, abscisic acid and ethylene (George, 1993; Franklin and Dixon, 1994; Srivastava, 2002; Efferth, 2019); although recent investigations have shown the influence of other compounds on growth and development (Kumar *et al*., 2016; Wiszniewska *et al*., 2018; Aremu *et al*., 2019; Dias, 2019). The auxins and the cytokinins are the most commonly used to direct the developmental pathways of plant materials *in vitro* (George, 1993; Gaspar *et al*., 1996; Machakova *et al*., 2008). Auxins are used in embryogenesis to stimulate dedifferentiation of differentiated cells while cytokinins stimulate cell division.

There are naturally-occurring as well as synthetic auxins. Indole-3-acetic acid (IAA) is the most commonly-used and researched auxin that is found naturally. Others are Indole-3-butyric acid (IBA), 4-Chloroindole-3-acetic acid (4-CI-IAA) and Phenylacetic acid (PAA) (George, 1993; Franklin and Dixon, 1994; Gaspar *et al*., 1996; Srivastava, 2002; Machakova *et al*., 2008; Korasick *et al*., 2013). Synthetic auxins that are commonly used are 2,4-Dichlorophenoxyacetic acid (2,4-D) and 1-Naphthaleneacetic acid (NAA). Others include *p*-Chlorophenoxyacetic acid (pCPA), 4-Amino-3,5,6-trichloropicolinic acid (Picloram), 3,6-Dichloro-2-methoxybenzoic acid (Dicamba), 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T). Synthetic auxins are used in lower concentrations to dedifferentiate cells; however, they have herbicidal effect on plants when the concentrations are higher.

Similar to auxin, several cytokinins are known to occur naturally in plants. However, the first one to be discovered, 6-Furfurylaminopurine (kinetin), was not found *in planta* but as a product of degraded DNA of herring sperm (Srivastava, 2002; Hurný and Benková, 2017). This compound is now synthesized and used in plant tissue culture. Others are Zeatin (isolated from the endosperm of young maize) (Razdan, 2003), 6-Benzylaminopurine (BAP) (Srivastava, 2002) and *N*-Isopentenylaminopurine (2iP). Addtionally, there are compounds that are not cytokinins but have cytokinin-like characteristics. Examples are Thidiazuron (TDZ) (Srivastava, 2002) and compounds found in endosperms such as liquid endosperm of coconut (Razdan, 2003). BAP and kinetin are the mostly-used cytokinins in plant tissue cultures because they are less prone to oxidation/degradation by cytokinin oxidase. In addition, BAP is more accessible and affordable (Srivastava, 2002).

3.1.3 Callus initiation and induction

Auxins incorporated into basal media are often used to induce callus from explants. The auxins of choice can be one type e.g. 2,4-D or combinations of two or more types e.g. 2,4-D and NAA. However, some plants require a balance between cytokinin and auxin for optimal induction of callus from explants (Franklin and Dixon, 1994). Thus, the *in vitro* requisites for callus formation are species dependent and have to be extensively detailed and optimized for each species (Efferth, 2019).

To induce callus, the plant cells must be capable of active cell division – an attribute that can be lost from differentiated cells (Ikeuchi *et al*., 2013) and thus hinder callus induction. Regulators such as cyclins and cyclin-dependent kinases have to be activated in order for the plant cells to regain their suppressed ability to divide and multiply. The molecular regulators that are involved in the formation of lateral roots in the model plant *Arabidopsis* are also believed to be involved in callus induction. Since auxins initiate the formation of lateral root in that model plant, it is believed that an understanding of the regulators involved in that process will give an insight into how auxin initiates callus formation *in planta* (Ikeuchi *et al*., 2013; Xu *et al*., 2018).

The pathways of callus induction in media that contain cytokinins is not well elucidated. It is, however, known that cytokinins stimulate ARRs (*Arabidopsis* Response Regulators), and the upregulation of these transcription factors (ARRs) results in callus formation (Ikeuchi *et al*., 2013). ARRs stimulate the expression of cyclins, which help with the

resumption of the cell cycle. During callus formation, it is important to note that the process of cell wall formation during cell division is by a process called 'free wall formation' and not through the conventional mechanisms that involve the mitotic spindle (Srivastava, 2002).

Since the activity of auxins affects DNA replication and cytokinins influence pre-mitotic events, it is important that the combination of both PGRs is well regulated in culture media for optimal induction; as there is the possibility for both compounds to inhibit the activities of each other (Gaspar *et al*., 1996; Machakova *et al*., 2008). Such inhibition was seen in Bambara groundnut where the combination of cytokinin and auxin significantly reduced callus induction and growth (Konate *et al*., 2013).

3.1.4 Proliferation and maintenance of embryogenic callus

Callus formation is usually followed by a proliferation/maintenance stage where the calli formed are subcultured to fresh proliferation media. This is required in order to stimulate further cell division thus increasing the quantity of the calli formed. In addition, the auxins in the induction media tend to become depleted thus also necessitating the need for subculturing to fresh medium (Von Arnold *et al*., 2002). Another reason for this phase is to separate and subculture the embryogenic calli from the non-embryogenic type as both types tend to develop and grow together (Fei, 1997). Proliferation media are usually the same or similar to the induction media but can be modified if necessary (Von Arnold *et al*., 2002). Sometimes, calli can proliferate on auxin-free media if there is sufficient endogenous auxin in the calli. Thayami *et al*. (2007) reported the proliferation medium for the callus of *Camellia sinensis* (L.) to be slightly different from the induction medium by the reduction of agar concentration from 8% (in induction medium) to 7%. Similarly, Von Arnold *et al*. (2002) found that liquid proliferation medium resulted in more synchronous growth of calli than those on semi-solid medium. While the subculturing to maintenance/proliferation media can have advantages, repeated subculturing may lead to somaclonal variations and reduced and/or loss of embryogenic competence (Von Arnold *et al*., 2002; Hussein *et al*., 2006).

3.1.5 Conversion to somatic embryos

The developmental stages after callus formation, proliferation and maintenance are geared towards the differentiation of the unorganized mass of cells and their subsequent

development into somatic embryos (SEs). It is important to identify and use embryogenic cells for these developmental stages (Zimmerman, 1993).

The first of the developmental stages is the histodifferentiation or prematuration stage. It is also considered as the transition phase between the callus and the somatic embryos (Von Arnold *et al*., 2002). In this phase, callus growth through cellular proliferation ceases and cellular differentiation is initiated (Stasolla and Yeung, 2003). During induction, exogenously supplied auxins are indispensable in callus formation. However, its activities do not include directing embryogenic callus cells towards further development into SEs. Hence, auxins are gradually reduced and/or removed in the histodifferentiation stage (Zimmerman, 1993; Srivastava, 2002). According to Zimmerman (1993), the developing somatic embryos are able to synthesize their own endogenous auxin, the polar transport of which helps to direct the polarity of the SEs. The polarity of the SEs through differentiation will eventually represent the root and shoot apical meristems.

Once the calli have begun embryonic development in the histodifferentiation phase, the SEs are directed towards maturation. More often, abscisic acid (ABA) is incorporated into the maturation medium to stimulate the maturation of the SEs. Also, accumulation of storage products, accompanied by biochemical changes, takes place (Von Arnold *et al*., 2002; Stasolla and Yeung, 2003). Morphologically, the somatic embryos, which are globular in structure at the differentiation phase, morph through the various stages of heart, torpedo and cotyledonary during the maturation phase. This phase culminates with the acquisition of desiccation tolerance by the SEs (Von Arnold *et al*., 2002). Desiccation tolerance is a physiological state, acquired by orthodox-type seeds, that prepares the embryo for over-wintering and subsequent germination. Seeds of recalcitrant species do not undergo this step (also referred to as maturation drying) in their developmental pathway and thus can germinate precociously. All somatic embryos (Litz and Gray, 1995), and especially those of recalcitrant species origin (Von Arnold *et al*., 2002), follow the same path of premature germination without acquisition of desiccation tolerance (Litz and Gray, 1995; Von Arnold *et al*., 2002). However, the survival rate of the resultant plantlets can be low (Stasolla and Yeung, 2003). It does seem imperative that acquisition of desiccation tolerance be imposed on the SEs and this can be achieved in various ways. These include partial desiccation and the inclusion of osmotic agents such as inorganic salts, sugars or Polyethylene glycol in the maturation medium (Von Arnold *et al*., 2002).

Increased gelling agent in the maturation medium can also impose water stress on the somatic embryos and trigger the acquisition of desiccation tolerance.

A successful maturation phase (and other preceding phases), that produces morphologically- and physiologically-competent somatic embryos, results in their successful conversion to plantlets (Von Arnold *et al*., 2002). Generally, the regeneration/conversion medium is without any plant growth regulators. However, the composition of the medium is species-dependent. Thus, it may be required that the regeneration medium be modified with auxins, cytokinins and additives such as casein hydrolysate and glutamine before the SEs develop into plantlets (Von Arnold *et al*., 2002).

3.1.6 Somatic embryogenesis in avocado

Somatic embryogenesis has been reported, with some level of success, in avocado (*Persea americana*) (Table 3.2). However, in almost all of those reports, immature zygotic embryos were the explants of choice (Table 3.2, see from Mooney and Van Staden (1987) to Encina *et al*. (2014)). Additionally, picloram, at 0.1 mg/L, was the auxin that was mostly used to obtain those embryogenic culture lines (Table 3.2). Researchers used the protocols established by Witjaksono and Litz (1999a, b) and classified their obtained calli according to those authors. This was either proembryonic mass (PEM)-type of callus or callus that showed a distinctive growth pattern such embryo initials, which were referred to as somatic embryo-type of callus.

The use of zygotic embryos as explants for somatic embryogenesis was not limited to only avocado in the Lauraceae. These explants were also used for other species in the family e.g. *Laurus nobilis* L. (Canhoto *et al*., 1999); *Cinnamomum pauciflorum* Nees (Kong *et al*., 2009); *C. camphora* L. (Shi *et al*., 2009; 2010; 2016b); *C. kanehirae* (Chang *et al*., 2015); *Sassafras randaiense* (Chen and Wang, 1985); *Ocotea catharinensis* (Moura-Costa *et al*., 1993; Viana and Mantell, 1999) and *O. porosa* (Pelegrini *et al.,* 2013).

Table 3.2: Summary of the previously-reported somatic embryogenesis research on avocado (*Persea americana*)

Explant used	Cultivars	Optimum basal nutrient medium and additives	Optimum PGR	Description of callus	Comments	Authors
Leaf and stem	'Lula' and 'Waldin'	Anderson's mineral salt and vitamins; 1 g/L casein hydrolysate; 30 g/L sucrose	1 mg/L 2,4-D for leaves; 1 mg/L BAP for stems	White and friable; dense, greenish white.	Callus formed were not developed further.	Young, 1983.
Immature zygotic embryos	'Fuerte' and 'Duke 7'	MS medium; 0.4 mg/L thiamine HCl, 100 mg/L myo-inositol, 30 g/L sucrose, 1 g/L charcoal	0.1 mg/L picloram	Callus was friable, composed of small nodular structures that were loosely attached to each other. Colour was between white and chocolate brown.	Callus developed into somatic embryos following all developmental stages. Plantlet was regenerated from the somatic embryos.	Mooney and Van Staden, 1987.
Immature zygotic embryos	'Hass'	MS salts; 0.4 mg/L thiamine HCl, 100 mg/L myo-inositol, 30 g/L sucrose	0.1 mg/L picloram	Two types of calli: amorphous, tan to grayish-coloured callus and spherical, glossy and light-cream coloured callus with distinct growth.	Some developed to somatic embryos with few developing shoots.	Pliego-Alfaro and Murashige, 1998.
Protoplasts obtained from embryogenic suspension culture	'T362'	MS basal salt; 1 g/L thiamine HCl, 100 mg/L myo-inositol, 51.34 g/L sucrose varied concentration of mannitol and	-	Explanted protoplasts formed proembryonic masses.	PEMs developed into somatic embryos. The regeneration to plantlet was less than 1%.	Witjaksono *et al.*, 1998.

		8P*				
Immature zygotic embryos	'Thomas'	MS medium; 30 g/L sucrose and 0.4 mg/L thiamine HCl, 100 mg/L myo-inositol.	0.1 mg/L picloram	Callus was described as proembryonic masses.	Proembryonic mass developed to somatic embryos and subsequently genetically transformed.	Cruz-Hernández *et al.*, 1998.
Immature zygotic embryos	'Booth 8 and 8a', 'Esther', 'Isham', 'M25864', 'T362', 'Thomas' and 'Yon'	B5 major salt with MS minor salt; and 0.4 mg/L thiamine HCl, 100 mg/L myo-inositol, 30 g/L sucrose	0.1 mg/L picloram	Explants generated proembryonic masses (PEMs) and direct formation of somatic embryos.	Genotypes determined whether explants formed PEMs or Somatic embryo-type.	Witjaksono and Litz, 1999a,b.
Nucellar tissue of immature fruits	'Hass'	Half strength MS mineral salts	1 mg/L 2,4-D; 4 mg/L picloram + 0.4 mg/L IBA; 4 mg/L picloram + 0.2 mg/L IBA	Creamy to white callus with decreased quality after prolonged induction phase causing callus colour change to coffee.	Somatic embryos were developed from callus and developed through to germination stage but with low recovery.	Vidales-Fernandez *et al.*, 2003.
Immature zygotic embryos	'Suarda', 'T362', and 'Nabal'	$B5^{+}$ (i.e. B5 major- and MS minor salts); 4 mg/L thiamine HCl, 100 mg/L myo-inositol.	0.1 mg/L picloram	Calli comprised the proembryonic- and somatic embryo-types.	Callus was generated for the purpose of genetic transformation.	Efendi, 2003.
Immature zygotic embryos	'anaheim'	MS medium	0.1 mg/L picloram	Embryogenic callus formed.	Callus formed white opaque somatic embryos that were further developed	Peran-Quesada *et al.*, 2004.

					to plantlets.	
Immature zygotic embryos	-	B5 major salts + MS minor salts; 30 g/L sucrose and 4 mg/L thiamine HCl, 100 mg/L myo-inositol.	0.1 mg/L picloram	Explants formed proembryonic masses.	Embryogenic cultures formed somatic embryos that were subsequently genetically transformed; but were not germinated.	Litz *et al*., 2005a.
Immature zygotic embryos	'Hass'	B5 major salts + MS minor salts with organics; 30 g/L sucrose	0.1 mg/L picloram	White opaque somatic embryos formed from embryogenic cultures.	Shoots germinated from embryos and were subsequently used for micrografting.	Raharjo and Litz, 2005.
Nucellus of fruits from sunblotch viroid-infected tree	'Vero Beach' SE2	B5 major salts + MS minor salts with organics; 30 g/L sucrose and 4 mg/L thiamine HCl, 100 mg/L myo-inositol.	0.1 mg/L picloram	Callus were embryogenic masses that later developed to white opaque somatic embryos.	Somatic embryos developed through to plant regeneration.	Suarez *et al*., 2006.
Immature zygotic embryos	'Hass'	B5 major salts + MS minor salts with organics; 30 g/L sucrose and 0.1 mg/L thiamine HCl, 100 mg/L myo-inositol.	0.1 mg/L picloram	Callus was embryogenic.	Embryogenic culture developed to somatic embryos that were subsequently transformed genetically with antifungal plant defensin gene.	Raharjo *et al*., 2008.
Immature zygotic embryos	'Duke-7'	MS medium	0.1 mg/L picloram	Callus were friable, considered to be embryogenic.	Somatic embryos developed through to plant regeneration; the effect of	Márquez-Martín *et al*., 2011.

					gelling agents and osmotic agents tested.	
Immature zygotic embryos	'Anaheim'	MS medium	0.1 mg/L picloram	Callus classified based on embryogenic cell line as Somatic Embryo-type and Pro Embryogenic Mass-type.	Somatic embryos developed through to shoot formation. Duration in suspension culture, density of inoculum and different maintenance media tested.	Márquez-Martín *et al*., 2012.
Immature zygotic embryos	'Duke 7'	MS medium	0.1 mg/L picloram	Callus was embryogenic.	Callus developed to somatic embryos that were further genetically transformed and developed into transgenic plantlet.	Palomo-Ríos *et al*., 2012.
Immature zygotic embryo	'Duke 7'	MS medium	0.1 mg/L picloram	White opaque somatic embryos formed from embryogenic callus.	Somatic embryos developed into whole plants through improved maturation treatment.	Palomo-Ríos *et al*., 2013.
Immature zygotic embryos	'Anaheim'	MS medium	0.1 mg/L picloram	Embryogenic callus was friable, transluscent and beige in colour; non-embryogenic was amorphous and grayish.	Embryogenic callus developed to white opaque somatic embryos; both group of calli used for proteomics study.	Guzmán-García *et al*., 2013.
Immature zygotic embryos	'Reed', 'Hass', 'Duke 7' and	B5 major salt without NH_4NO_3 with MS minor salt;	0.1 mg/L picloram	White opaque somatic embryos formed from	Somatic embryos formed from only 'Duke 7' were	Encina *et al*., 2014.

	'A10'	and 4 mg/L thiamine HCl, 100 mg/L myo-inositol.		induction medium.	developed further till plant regeneration.	

*****8P is made up of (in mg/L) 1.0 calcium panthothenate, 2.0 ascorbic acid, 1.0 choline chloride, 0.002 *p*-aminobenzoic acid, 0.4 folic acid, 0.2 riboflavin, 0.01 biotin, 0.01 retinol, 0.01 cholecalciferol, 0.02 cyanocobalamine, 20 sodium pyruvate, 40 citric acid, 40 malic acid, 40 fumaric acid, 250 fructose, 250 ribose, 250 xylose, 250 mannose, 250 rhamnose, 250 cellobiose, 250 galactose, 250 mannitol, 1000 malt extract, 250 casein hydrolysate and 2% coconut water.

3.1.7 Use of leaf as an explant

Young (1983) attempted callus induction from leaf explants of avocado. The major findings from that investigation were (i) 2,4-D stimulated more callus induction than NAA and IBA (ii) the leaves retained the highest level of 'viability' (i.e. retention of green colouration and no visible sign of injury on the leaves) in NAA treatment when compared with other auxins (iii) more callus was formed using older leaves than younger leaves (iv) the portion (tip, mid and basal) of the older leaves used as explant influenced leaf viability and callusing, with the basal portion being preferable (v) the orientation of the explanted leaves on the medium affected the viability and callusing ability of the leaves; the abaxial or adaxial surface was more favourable than the distal or proximal ends (vi) callusing was mainly found at the cut ends of prominent veins (vii) white friable callus was mainly formed while compact, greenish-white calli were formed occasionally by the leaf explant.

The leaves have also been used as an explant for somatic embryogenesis in other species of the Lauraceae, albeit with little or no success. Examples include *Cinnamomum tamala* (Sharma and Nautiyal, 2009) and *Eusideroxylon zwageri* (Gibson and Rebicca, 2016). However, successful somatic embryogenesis with acclimatized plantlets has been achieved in other plant species from various families and different growth forms (Table 3.3). The successful use of leaf as explant in other plant species suggests the possibility of its use as an explant in avocado especially with the initial work from Young (1983).

The main aim of the current investigation was to develop somatic embryos from the leaf explants of avocado. In order to achieve this, it was imperative to (i) empirically determine the optimal media for the induction of callus from leaf explants (ii) establish the effect of the source of the donor plants on the competence of the leaf to form callus (iii) determine which avocado cultivar was amenable to tissue culture manipulation towards somatic embryo formation and (iv) determine suitable media needed for further growth and development of the formed callus.

Table 3.3: Summary of some successful somatic embryogenesis (SE) from various species using leaves as explants (D – Direct; I – Indirect)

Species	Growth form & Family	SE pathway	Stage reached	References
Dendranthema grandiflora	Perennial herb (Asteraceae)	Direct	Plant recovery from five out of 23 cultivars	May and Trigiano, 1991
Vitis rotundifolia M ichx.	Climbing, deciduous (Vitaceae)	Indirect	Flowered regenerated plants	Robacker, 1993
Stevia rebaudiana	Perennial herb (Asteraceae)	Direct	Formation of somatic embryos	Filho *et al*., 1993
Quercus robur L.	Tree (Fagaceae)	Indirect	Conversion of somatic embryo to plantlets with root and shoot formation	Cuenca *et al*., 1999
Saintpaulia ionantha Wendl.	Perennial herb (Gesneriaceae)	Direct	Formation of somatic embryos	Mithila *et al*., 2003
Rosa hybrid and *R. multiflora*	Perennial shrub (Rosaceae)	Indirect	Acclimatization of regenerated plants with more than 90% survival rate.	Kim *et al*., 2004
Arachis glabrata	Perennial herb (Fabaceae)	Indirect	Plant regeneration	Vidoz *et al*., 2004
Saccharum officinarum	Perennial grass (Poaceae)	Direct and indirect	Somatic embryo formation	Ali *et al*., 2007b
Prunus incisa (Thunb.)	Deciduous shrub (Rosaceae)	Indirect	Somatic embryos at different stages of development formed from callus	Kaouther *et al*., 2011
Onobrychis sativa	Perennial herb (Fabaceae)	Direct and indirect	Shoot formation from explanted leaf	Mohajer *et al*., 2012
Nothapodytes foetida	Evergreen tree (Icacinaceae)	Direct	Plant regeneration	Khadke and Kuvalekar, 2013
Sapindus emarginatus Vahl	Tree (Sapindaceae)	Indirect	Acclimatization of regenerated plants with 75% survival rate.	Devaraju and Reddy, 2013
Tolumnia Louise Elmore 'Elsa'	Epiphyte (Orchidaceae)	Direct	Acclimatization of regenerated plants with 100% survival rate.	Shen *et al*., 2018
Acrocomia aculeate	(Palm) Tree (Arecaceae)	Indirect	Formation of somatic embryos	Meira *et al*., 2019
Haworthia retusa	Perennnial succulent (Asphodelaceae)	Direct	Acclimatization of regenerated plants with 100% survival rate.	Kim *et al*., 2019
Daucus carota L.	Biennial herb (Apiaceae)	Indirect	Plant regeneration	Sundararajan *et al*., 2019
Eusideroxylon zwageri	Tree (Lauraceae)	Indirect	Cotyledonary somatic embryos	Gibson and Rebicca, 2016
Cinnamomum tamala	Tree (Lauraceae)	Indirect	Shoot formation	Sharma and Nautiyal, 2009

3.2 Materials and Methods

3.2.1 Callus induction

3.2.1.1 Source of explants

Avocado leaf fragments from three cultivars *viz* 'Edranol', 'Fuerte' and 'Hass', were used to induce callus. While leaf explants were obtained from all the three cultivars from *in vitro*-grown shoots, explants from only 'Edranol' and 'Fuerte' cultivars were sourced from greenhouse plants.

Young leaves from greenhouse seedlings were selected between the 3rd and 6th position from the apex. In addition to this criterion, other factors that determined the selection of the young leaves were that they (i) were light green and (ii) had softer, smoother and a glossy texture when compared with the older leaves (Chapter 2, Figure 2.1). The young leaves were carefully detached from the seedlings with a pair of scissors and were transported to the laboratory in a beaker, filled with tap water. In the laboratory, they were rinsed under running tap water for 15 minutes after which they were soaked in a solution that comprised 1% sodium hypochlorite and 20 drops per litre Tween 20© for five minutes. The leaves were rinsed three times in sterile ultra-pure water and were pat-dried with sterile paper towel. Using a sterilized puncher, the leaves were then cut into discs of approximately 0.5 cm in diameter (0.196 cm^2 surface area), with each leaf disc necessarily containing some vascular tissues i.e. the veins but not the midrib. Leaf explants of ca. 1 cm^2 surface area were also used but these were cut aseptically in the laminar airflow.

Leaves obtained from *in vitro* seedlings (Chapter Two, section 2.3.3.2) were not surface-decontaminated as they were obtained from 'clean' *in vitro* materials. The leaves were cut aseptically into rectangles of approximately 3 mm by 4 mm.

3.2.1.2 Nutrient media and plant growth regulators (PGRs)

Four different basal nutrient media were tested: Murashige and Skoog (MS), Gamborg's B5 (B5), $B5^+$ (Encina *et al*., 2014) and mango medium for somatic embryo induction (MMSE) (Pateña *et al*., 2002). Various concentrations of picloram and 2,4-D were added to $B5^+$ and MMSE, respectively with other additives (Table 3.4). MS and B5 nutrient media were each supplemented with three different auxins with varying concentrations and with/without varying concentrations of the cytokinin 6-Benzylaminopurine (BAP). Other components that were constantly added to the MS and B5 nutrient media were shown (Table 3.4). The pH of the media was adjusted to 5.70 ± 0.05 and solidified with 3.0 g/L gelrite.

Table 3.4: The composition of, and additives to, the basal nutrient media used for callus induction from the leaf explants of avocado.

Basal nutrient medium	Code	Component	PGRs	Other additives
Murashige and Skoog	MS	MS macro- and micronutrients, MS vitamins	***Auxin:*** 2,4-D (in mg/L: 0.5, 1.0, 2.0, 4.0 and 8.0). Picloram (in mg/L: 0.05, 0.10, 0.15 and 0.20) NAA (in mg/L: 0.5 and 1.0) ***Cytokinin:*** BAP (in mg/L: 0.1, 0.2, 0.5, 1.0, 1.5 and 2.0)	4.0 mg/L thiamine-HCl, 100 mg/L myo-inositol, 30 g/L sucrose and 3.0 g/L gelrite
Gamborg's B5	B5	B5 major and minor salts		
Gamborg's B5 Plus	$B5^{+}$	B5 macronutrients and MS micronutrients	***Auxin:*** Picloram (in mg/L: 0.05, 0.10, 0.15 and 0.20)	4.0 mg/L thiamine-HCl, 100 mg/L myo-inositol, 30 g/L sucrose and 8 g/L agar
Mango Medium for Somatic Embryogenesis	MMSE	B5 macronutrients, MS micronutrients, MS vitamins and MS-FeEDTA,	***Auxin:*** 2,4-D (in mg/L: 0.5, 1.0, 2.0, 4.0 and 8.0)	0.4 g/L glutamine, 100 ml/L coconut water, 60 g/L sucrose and 2.5 g/L gelrite

The media were autoclaved for 20 minutes at 120°C and 121 kPa, and were subsequently dispensed, 40 ml each, into Petri dishes (90 mm in diameter and 15 mm high), sealed in plastic bags and stored at 4°C until use. Media that were selected as optimal for callus induction and used for subsequent experimentation were each coded with a Callus Induction Medium (CIM) number.

3.2.1.3 Induction

Either 5 or 10 leaf explants were plated per plate with either the abaxial or adaxial surface making contact with the culture medium. The number of plates varied for each cultivar; ranging from 10 to 20 plates per cultivar. In each case, the plates were divided into two, with one half incubated under light conditions and the other half in the dark. Darkness was achieved by wrapping the Petri dishes in aluminium foil. The cultures were kept in the growth room under 14-hour light and 10-hour dark photoperiod at 25°C ± 2°C with a photon flux density of 100 $\mu mol.m^{-2}.s^{-1}$.

3.2.2 Callus proliferation

Six weeks after the explants were plated on the various induction media, the explants that had developed calli were sub-cultured on to fresh media in Petri dishes. The fresh media were chosen to be the same as those that favoured callus induction in the explants.

3.2.3 Callus development/pre-maturation

In order to stimulate and initiate histodifferentiation after four weeks in proliferation media, the calli (usually as a whole clump, without fragmentation) were transferred to media with different compositions, adapted from Encina *et al*. (2014). All media had the following as the constant part of their composition: full strength (4.4 g/L, Sigma) MS nutrient medium, 4.0 mg/L thiamine-HCl, 100 mg/L myo-inositol, 30 g/L sucrose, and 3.0 g/L gelrite (Encina *et al*., 2014). Then they were modified as follows:

i. No modification to the constant compositions above i.e. Full strength MS without PGRs.
ii. As (i) above but sucrose was increased from 30 g/L to 60 g/L.
iii. As (i) above but gelling agent was increased from 3.0 g/L to 6.0 g/L gelrite.
iv. BAP at 2.0 mg/L was added to the composition of (i).
v. Activated charcoal at 4.0 g/L was added to the composition of (iii).
vi. As per medium (iii), plus 1.0 mg/L gibberellic acid (GA_3) and 0.5 mg/L BAP.
vii. (vi) but with activated charcoal at 4.0 g/L.
viii. (vi) but BAP was increased to 2.0 mg/L.
ix. Activated charcoal (4.0 g/L) was added to the composition of (viii) above.
x. As per medium (i), plus 1.0 mg/L GA_3, 2.0 mg/L BAP, 45 g/L sucrose, 100 ml/L coconut water (CW) and 6 g/L gelrite.
xi. As per (x) but CW was increased to 200 ml/L.

The pH of the media were adjusted to 5.7 ± 0.05, and 25 ml of each medium was dispensed into 100 ml culture bottles (5.3 cm diameter and 8 cm high), respectively.

3.2.4 Maturation of globular structures

Globular structures that had developed and were loosely attached to the surface of, and embedded within, callus cells were separated. The larger ones were manually separated with a pair of forceps. The relatively smaller ones were separated as follows: the lumps that contained the small globules and callus cells were immersed in sterile Millipore ultra-pure water and were agitated on a shaker. Subsequently, the cell debris was decanted leaving the off-white/cream-coloured globules at the bottom of the conical flask and the suspension liquid at the top. All the globular structures were then soaked for one minute in a solution that contained 1.0 ml/L SIGMA® antibiotic antimycotic solution (100X). One millilitre (1.0 ml) of SIGMA® antibiotic antimycotic solution (100X) comprises 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B). The structures were then

rinsed three times with sterile, Millipore ultra-pure water. They were blotted dry on sterile paper towel and were plated on either the solid or liquid medium of two maturation media with different compositions. One maturation medium was made up of full strength (4.4 g/L) MS, 4.0 mg/L thiamine-HCl, 100 mg/L myo-inositol, 1.0 mg/L BAP, 1.0 mg/L GA_3, 100 ml/L CW, 2.0 mg/L Abscisic acid (ABA) and 45 g/L sucrose (plus 6.0 g/L gelrite for the solid medium). The second maturation medium was similar to the first one but CW was increased to 200 ml/L.

One millilitre of the liquid suspension was gently dispensed on to 40 ml solidified medium in 90 mm Petri dish; another 1.0 ml suspension was also dispensed per 40 ml liquid medium and left to agitate gently on a shaker. For the globular structures, 10 were plated on 40 ml solidified medium in each 90 mm Petri dish while another 10 were placed in each conical flask containing 100 ml liquid medium and left to agitate on the shaker.

3.2.5 Morphological observation of fresh leaf

Fresh avocado cv. 'Edranol' leaves were fixed, sectioned and viewed using light microscopy as described later in Chapter Four.

3.2.6 Statistical analysis

In experiments that involved 2 sets of experimental parameters, t-Tests for Paired Samples were used to ascertain significant differences between the means. Where there was a significant difference, one-way and/or two-way Analysis of Variance (ANOVA) was used to check for significant differences within the various components of the experimental parameters. Tukey's Honest Significant Difference (Tukey's HSD) was used to test the level of significant difference between each component of an experimental parameter. All tests were conducted at *0.05* significant level and were carried out using Data Analysis ToolPak add-in on Microsoft Excel and SPSS statistical packages.

3.3 Results

3.3.1 Callus induction

3.3.1.1 'Edranol' cultivar

3.3.1.1.1 The importance of leaf surface contact with the callus induction medium

The culture medium that contained 0.5 mg/L 2,4-D with 0.1 mg/L BAP on Gamborg's B5 nutrient medium was used to test the impact of which leaf surface making contact with induction medium was better.

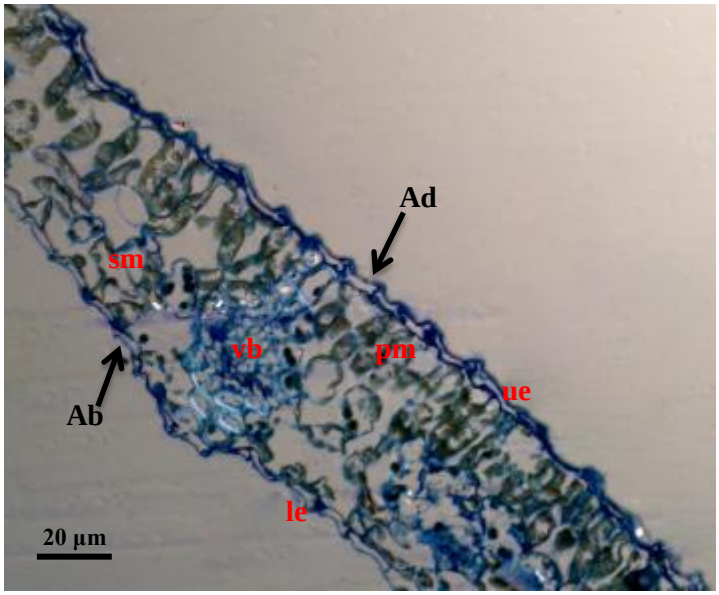


Figure 3.1: A cross section through an avocado (cv. 'Edranol') leaf obtained from the greenhouse. Adaxial (ad) and abaxial (ab) surfaces, the lower (le) and upper (ue) epidermis, vascular bundle (vb), palisade (pm) and spongy mesophyll (sm).

Twenty two percent of the explants with the abaxial surface in contact with the induction medium produced callus while the response was significantly higher (54%) in explants with adaxial surface making contact with the medium.

The avocado leaf, obtained from the greenhouse material, has one layer of cylindrical palisade mesophyll cells while the spongy mesophyll comprises cells of different shapes, interspersed with air spaces (Figure 3.1). The palisade mesophylls are perpendicular to the upper epidermal layer while the spongy mesophylls are found in the middle of the leaf and towards the lower epidermal layer. Cells of both the epidermal layers of the adaxial and abaxial surfaces are concave towards the mesophylls. The vascular bundle is located among the spongy mesophyll cells.

3.3.1.1.2 Light and dark incubation

The incubation of the explants in the dark significantly favoured more callus formation than incubation in the light in both 2,4-D- and picloram-based media (Table 3.5 and 3.6). However, in three (out of the 20) of the tested picloram-based media, that contained 0.05, 0.10, and 0.20 mg/L picloram in combination with 0.1 mg/L BAP, callus formation was higher in the light (20, 15 and 5%, respectively) than in dark incubation where there was no callus formation (Table 3.6).

Table 3.5: The percentage of leaf explants of avocado cv. 'Edranol' grown under greenhouse conditions that produced callus on Gamborg's B5 medium with different combinations of 2,4-D and BAP and incubated under light and dark conditions (n = 10 with three replicates).

	LIGHT					DARK					
	6-BENZYLAMINOPURINE (BAP) (mg/L)										
2,4-D (mg/L)	**0**	**0.1**	**0.2**	**0.5**	**1.0**	**0**	**0.1**	**0.2**	**0.5**	**1.0**	***p* Value**
0.5	0	0	48	30	30	0^{a}	0^{a}	56^{de}	48^{cde}	47^{cd}	*0.001681*
1.0	0	0	40	12	24	0^{a}	0^{a}	60^{de}	72^{e}	70^{de}	*0.000079*
2.0	0	0	40	20	5	0^{a}	0^{a}	60^{de}	55^{de}	30^{bc}	*0.000108*
4.0	0	0	0	4	0	0^{a}	0^{a}	16^{ab}	24^{abc}	0^{a}	*0.012383*
8.0	0	0	0	0	0	0^{a}	0^{a}	0^{a}	12^{abc}	0^{a}	*0.016704*

Paired t-Test showed significant differences between dark and light incubation ($p = 0.001242$). Subsequently, one-way ANOVA showed significant differences among the various media in the dark (1.11×10^{-16}). Different superscripts showed significant difference between the means of all the 25 media incubated in the dark.

Table 3.6: The percentage of leaf explants of avocado cv. 'Edranol' grown under greenhouse conditions that produced callus on Gamborg's B5 medium with different combinations of the auxin picloram and BAP and incubated under light and dark conditions (n = 10 with three replicates).

	LIGHT					DARK					
	6-BENZYLAMINOPURINE (BAP) (mg/L)										
Picloram (mg/L)	**0**	**0.1**	**0.2**	**0.5**	**1.0**	**0**	**0.1**	**0.2**	**0.5**	**1.0**	***p Value***
0.05	0	20	0	0	20	0^{a}	0^{a}	28^{bc}	36^{bcd}	36^{bcd}	*0.001346*
0.10	0	15	0	8	8	0^{a}	0^{a}	32^{bcd}	44^{cde}	52^{de}	*0.0000007*
0.15	0	0	0	20	16	0^{a}	0^{a}	20^{ab}	44^{cde}	32^{bcd}	*0.000296*
0.20	0	5	4	0	8	0^{a}	0^{a}	36^{bcd}	36^{bcd}	60^{e}	*0.000228*

Paired t-Test showed significant differences between dark and light incubation ($p = 0.00182$). Subsequently, ANOVA showed significant differences among the various media in the dark (9.37×10^{-13}). Different superscripts showed significant differences between the means for the 20 media tested in the dark.

3.3.1.1.3 Effect of basal nutrient media

When incubated on Gamborg's B5 medium under dark conditions (Table 3.5 and 3.6), the majority of explants formed callus; especially those on media that contained BAP in the range of 0.2 – 1.0 mg/L. However, the use of MS as a basal medium did not result in significant callusing (Table 3.7a and b).

Table 3.7: The percentage of leaf explants of avocado cv. 'Edranol' taken from plants grown in the greenhouse, producing callus on full strength MS with vitamins medium containing different combinations of the auxins (a) 2,4-D or (b) picloram and with BAP under dark incubation (n = 25). (Blank cells in the tables signified untested media).

(a)

	6-BENZYLAMINOPURINE (BAP) (mg/L)					
2,4-D (mg/L)	**0**	**0.1**	**0.2**	**0.5**	**1.0**	**1.5**
0.5	0	0	6	6.67	0	14
1.0	0	0	0	0	8	0
2.0	5	0	-	-	-	-
4.0	0	0	-	-	-	-
8.0	0	0	-	-	-	-

(b)

	6-BENZYLAMINOPURINE (BAP) (mg/L)					
Picloram (mg/L)	**0**	**0.1**	**0.2**	**0.5**	**1.0**	**1.5**
0.05	0	0	2	0	0	4
0.10	5	0	0	0	0	1.11
0.15	5	0	-	-	-	-
0.20	0	0	-	-	-	-

Callus induction was less than 10% (except for the medium that contained 0.5 mg/L 2,4-D in combination with 1.5 mg/L BAP) in the tested media (Table 3.7).

Mango Medium for Somatic Embryo induction (MMSE) was used and supplemented with five varying concentrations of the auxin 2,4-D (Table 3.4). All the explants were incubated in the dark and both the adaxial and abaxial surfaces of the leaf were tested. Contamination was not prevalent in the cultures (results not shown), however none of the explants induced callus. The medium was therefore not used in any further experimentation.

$B5^+$ medium was also supplemented with four concentrations of the auxin picloram (Table 3.4) and both the adaxial and abaxial surfaces of the leaf were tested. One of the four media did not stimulate callus induction. The remaining three stimulated between 4 and 8% (result not shown) callus. Hence $B5^+$ and its variants were not deemed optimal for callus induction from avocado leaf explants and their use was discontinued.

3.3.1.1.4 Effect of explant size

The small explants formed more callus (total mean of 43.45 ± 14.3%) than the larger explants (total mean of 32.8 ± 23%). The response of small explants was higher in five out of the nine media tested, of which three were significantly different (Table 3.8).

Table 3.8: The percentage of greenhouse-derived avocado (cv 'Edranol') leaf explants of different sizes that produced callus on Gamborg's B5 with nine different combinations of 2,4-D and BAP (n = 25 with three replicates).

Induction Media	CIM No	Large explants (Leaf squares)	Small explants (Leaf disc)	*P* value
0.5 mg/L 2,4-D; 0.2 mg/L BAP	CIM1	0^{a}	66.67^{e}	*0.002063*
0.5 mg/L 2,4-D; 0.5 mg/L BAP	CIM2	46.67^{bcde}	35^{bc}	*0.397917*
0.5 mg/L 2,4-D; 1.0 mg/L BAP	CIM3	65^{de}	31.43^{bc}	*0.034244*
1.0 mg/L 2,4-D; 0.2 mg/L BAP	CIM4	56^{cde}	25.33^{ab}	*0.005231*
1.0 mg/L 2,4-D; 0.5 mg/L BAP	CIM5	30^{bc}	66.15^{e}	*0.000524*
1.0 mg/L 2,4-D; 1.0 mg/L BAP	CIM6	45^{bcde}	41.43^{bcde}	*0.754462*
2.0 mg/L 2,4-D; 0.2 mg/L BAP	CIM7	0^{a}	46.15^{bcde}	*0.001418*
2.0 mg/L 2,4-D; 0.5 mg/L BAP	CIM8	20^{b}	41.33^{bcde}	*0.044101*
2.0 mg/L 2,4-D; 1.0 mg/L BAP	CIM9	32.45^{bc}	37.78^{bcd}	*0.582574*

Rectangular, large explants and leaf discs had surface areas of ca. 1.0 cm^2 and 0.196 cm^2, respectively. Paired t-Test showed no significant differences between large and small explants (p = 0.380794). Different superscripts showed significant differences between all the means.

The large explants formed more callus in four out of the nine media, of which two were significantly different (Table 3.8). Unlike the small explants that formed callus in all of the nine media, the large explants did not form any callus in two (CIM1 and CIM7) of the media tested (Table 3.8). Cumulatively, however, the size of the explants did not have a significant effect on the number of explants that produced callus (p = 0.380794).

3.3.1.1.5 Effect of the source of explant

Leaf explants sourced from *in vitro* shoots produced more callus than the explants obtained from the greenhouse (Figure 3.2). The difference in percentage callusing between the *in vitro* and greenhouse-derived materials ranged from 1.29% (CIM4) to 41.43% (CIM9). Cumulatively, percentage callusing was significantly higher (p = 0.008) in *in vitro*-derived material (72.7 ± 10.35%) than greenhouse-derived (54.2 ± 15.40%) explants.

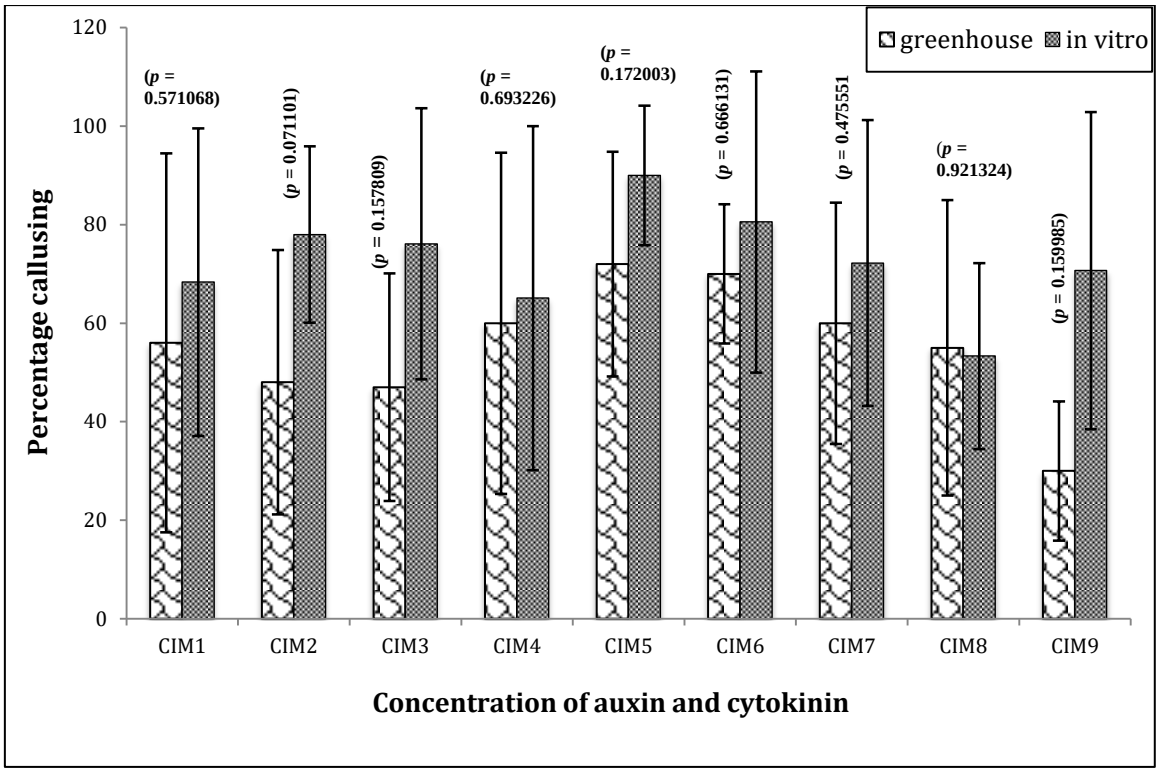


Figure 3.2: Percentage of callused explants from material grown in the greenhouse and that obtained from *in vitro* 'Edranol' leaf materials on Gamborg's B5 with nine different combinations of 2,4-D and BAP.

There was a statistical relationship between the two experimental parameters of explant sources (Paired t-Test, $p = 0.008081$).

However, in individual cases, the differences in both sources were not statistically significant ($p \geq 0.05$) (Figure 3.2). In both greenhouse- and *in vitro*-derived explants, CIM5 (i.e. the induction medium that contained 1.0 mg/L 2,4-D and 0.5 mg/L BAP) induced the highest percentage of callus.

Similar to the responses of materials cultured on Gamborg's B5 as basal nutrient medium (Figure 3.2), the explants sourced from the *in vitro* shoots and cultured on MS basal nutrient medium also produced more callus than those obtained from greenhouse material (Figure 3.3). The difference ranged from 2.5% (CIM14) to 23% (CIM13).

Using the auxin 2,4-D in combination with BAP, the data represented in both figures (Figures 3.2 and 3.3) showed the same trend of more callus induction from *in vitro* explants than from explants of plants grown in the greenhouse. However, the nutrient medium, MS, was less favourable for callus induction (Figure 3.3) than Gamborg's B5 basal medium (Figure 3.2).

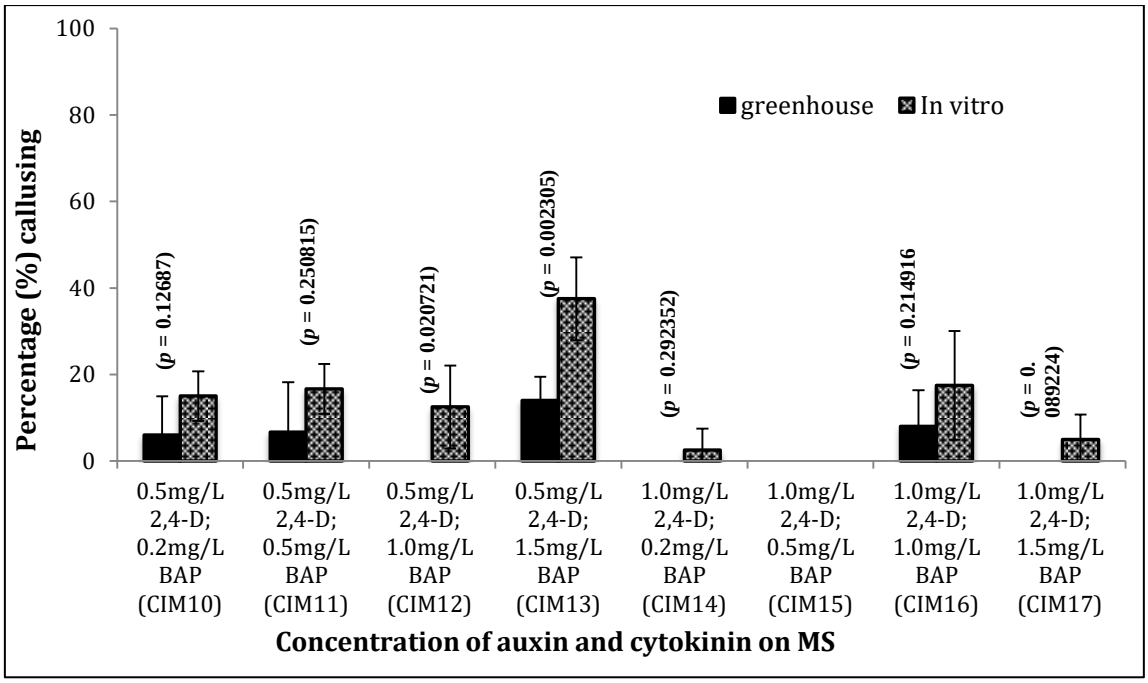


Figure 3.3: The percentage of callused leaf explants obtained from the *in vitro* and greenhouse 'Edranol' shoots. Leaf explants were cultured on MS media supplemented with different combination of 2,4-D and BAP.
There was a statistical relationship between the sources of explants (Paired t-Test, $p = 0.009534$).

With the use of the auxin NAA, in combination with BAP in MS basal nutrient media, all the media tested with both leaf explants from the *in vitro*- and the greenhouse-grown plant materials produced callus (Table 3.9).

Table 3.9: The effect of different combinations of BAP and NAA in MS medium, on callus induction (percentage) from leaf explants of 'Edranol' obtained from the greenhouse and *in vitro* (n = 50 with three replications).

Induction Media	CIM No	Greenhouse	*In vitro*	*P*
0.5 mg/L NAA; 0.5 mg/L BAP on MS	CIM18	3.00^{a}	32.00^{bcd}	*0.031235*
0.5 mg/L NAA; 1.0 mg/L BAP on MS	CIM19	25.00^{abcd}	36.89^{cd}	*0.358766*
0.5 mg/L NAA; 1.5 mg/L BAP on MS	CIM20	32.67^{bcd}	28.00^{bcd}	*0.691871*
0.5 mg/L NAA; 2.0 mg/L BAP on MS	CIM21	36.67^{cd}	40.00^{cd}	*0.889064*
1.0 mg/L NAA; 0.5 mg/L BAP on MS	CIM22	4.67^{a}	12.00^{ab}	*0.296885*
1.0 mg/L NAA; 1.0 mg/L BAP on MS	CIM23	11.25^{a}	42.00^{b}	*0.025541*
1.0 mg/L NAA; 1.5 mg/L BAP on MS	CIIM24	18.00^{abc}	72.00^{e}	*0.020117*
1.0 mg/L NAA; 2.0 mg/L BAP on MS	CIM25	18.00^{abc}	78.00^{e}	*0.001833*

$p = 0.024612$ (paired t-Test for two experimental parameters). Different superscripted letters indicated significant differences among the means.

The *in vitro* derived materials, with the exception of those plated on CIM20, developed more callus than the greenhouse-derived materials. In all the eight media tested, callus induction was less than 50% in the greenhouse-derived materials and in six out of the eight media for the *in vitro* materials (Table 3.9). Explants obtained from *in vitro* materials were significantly more responsive to callus formation manipulations (total mean = 42.59 ± 22.11%) than those obtained from greenhouse-derived plants (total mean = 18.66 ± 12.29%) (Table 3.9).

3.3.1.1.6 Plant growth regulators

The use of auxins alone, either 2,4-D (Table 3.5) or picloram (Table 3.6), did not result in callus formation in the leaf explants when cultured under either light or dark conditions. However, the inclusion of the cytokinin BAP enhanced callus formation from the leaf explants. Additionally, increasing the concentration of BAP with the auxin NAA resulted in increased callus formation (Table 3.9). The optimal range for callus induction was 0.5 – 2.0 mg/L 2,4-D with 0.2 – 1.0 mg/L BAP in Gamborg's B5 basal medium, under dark incubation (Table 3.5). Within that optimal range, 1.0 mg/L 2,4-D and 0.5 mg/L BAP (CIM5) was the most effective combination of PGRs for callus formation (Tables 3.5, 3.8 and Figure 3.2).

3.3.1.1.7 Relationship between auxins and nutrient medium

When incorporated in B5 basal nutrient medium, both 2,4-D and picloram favoured callus formation from avocado leaf material under dark incubation. However, explants were more responsive and induced more calli in media that contained 2,4-D (Table 3.5) than media that contained picloram (Table 3.6). On the other hand, in MS basal nutrient medium, 2,4-D and picloram did not enhance callus formation in most cases, with callusing ranging from 1 – 14% (Table 3.7a and b). In contrast, the incorporation of the auxin NAA to the MS media stimulated more callus induction (both greenhouse- and *in vitro*-derived materials) (Table 3.9) when compared with the other auxins (i.e. 2,4-D (Table 3.7a, Figure 3.3) and picloram (Table 3.7b)) in MS media.

Increasing the concentration of BAP to 1.5 mg/L with 2,4-D in both B5 and MS nutrient media, resulted in a higher number of explants producing callus, although not significantly. However, increasing the BAP concentration in media with NAA only resulted in more callus formation in B5 nutrient medium but not in MS medium (Table 3.10).

Table 3.10: Effect of different concentrations of auxins, cytokinin and nutrient media on callus induction from avocado leaf cv 'Edranol' sourced from greenhouse-established plants (n = 50 with three replicates).

Auxin/cytokinins (mg/L)	Nutrient medium	CIM No	Percentage callusing
1.0 mg/L 2,4-D; 0.5 mg/L BAP	B5	CIM5	43.67^{bc}
0.5 mg/L 2,4-D; 1.5 mg/L BAP	B5	CIM26	59.17^{c}
1.0 mg/L 2,4-D; 0.5 mg/L BAP	MS	CIM15	24.74^{ab}
0.5 mg/L 2,4-D; 1.5 mg/L BAP	MS	CIM13	34.00^{abc}
1.0 mg/L NAA; 0.5 mg/L BAP	B5	CIM27	27.59^{ab}
0.5 mg/L NAA; 1.5 mg/L BAP	B5	CIM28	28.33^{ab}
1.0 mg/L NAA; 0.5 mg/L BAP	MS	CIM22	15.00^{a}
0.5 mg/L NAA; 1.5 mg/L BAP	MS	CIM20	12.00^{a}

Different superscripts showed means that were significantly different.

The summation of all the means showed that the Gamborg's B5 formulation significantly (p = 0.006) favoured callus induction (39.69 ± 14.95%) more than MS nutrient medium (21.43 ± 9.99%). Similarly, the auxin 2,4-D also significantly (p = 0.02) stimulated more callus induction (40.39 ± 14.71%) than NAA (20.73 ± 8.45%) (Table 3.10).

3.3.1.1.8 Comparison between 2,4-D and NAA

Using *in vitro* leaf explants, the most effective combination of PGRs for callus induction in this study, CIM5 (i.e. 1.0 mg/L 2,4-D and 0.5 mg/L BAP in B5, and incubated in the dark), was used as a reference point for comparison with MS containing NAA and BAP. The results showed some striking differences in the explants' responses (Table 3.11 and 3.12).

3.3.1.1.8.1 Contamination

Considering that explants were obtained *in vitro*, they were expected to be relatively free of contamination, or the presence of microorganisms on them to be minimal. Hence, seven out of the nine media tested were contamination-free for the six-week period. The remaining two media had only 8% and 6% contamination visible at the fourth and fifth week, respectively with no new contaminated explants observed for the remaining testing period (Table 3.11(i)).

Table 3.11: The percentage of *in vitro* 'Edranol' leaf explants that (i) were contaminated (ii) turned brown and (iii) formed callus when CIM5 was compared with the induction media that contained various concentrations of NAA/BAP in MS.

		(i) Percentage Contamination				(ii) Percentage Browning				(iii) Percentage Callusing			
Induction Media	**CIM No**	**3 wks**	**4 wks**	**5 wks**	**6 wks**	**3 wks**	**4 wks**	**5 wks**	**6 wks**	**3 wks**	**4 wks**	**5 wks**	**6 wks**
**1.0 mg/L 2,4-D; 0.5 mg/L BAP; B5	CIM5	0	8	8	8	0	15	15	15	50	80	80	90
0.5 mg/L NAA; 0.5 mg/L BAP; MS	CIM18	0	0	0	0	40	44	58	58	4	18	20	32
0.5 mg/L NAA; 1.0 mg/L BAP; MS	CIM19	0	0	0	0	28.5	46.9	77.5	77.5	2	12.2	32.65	36.73
0.5 mg/L NAA; 1.5 mg/L BAP; MS	CIM20	0	0	0	0	32	48	78	78	8	10	16	28
0.5 mg/L NAA; 2.0 mg/L BAP; MS	CIM21	0	0	0	0	48	66	74	74	4	18	36	40
1.0 mg/L NAA; 0.5 mg/L BAP; MS	CIM22	0	0	0	0	64	78	90	90	0	2	2	12
1.0 mg/L NAA; 1.0 mg/L BAP; MS	CIM23	0	0	0	0	32	58	66	66	4	8	22	42
1.0 mg/L NAA; 1.5 mg/L BAP; MS	CIM24	0	0	6	6	12	24	38	38	8	36	54	72
1.0 mg/L NAA; 2.0 mg/L BAP; MS	CIM25	0	0	0	0	6	6	14	14	10	46	66	78

**highlight indicated the reference medium, CIM5, for comparison.

3.3.1.1.8.2 Browning

Almost all the explants experienced browning during culture in all the media tested. This browning was not as a result of the harshness of the decontaminating solutions as the explants were obtained *in vitro* and thus were not decontaminated. This indicated that the constituents of the media imposed the experienced stress on the explants. In this regard, the explants cultured on CIM5 had 15% browning and 90% callusing while the explants that were in CIM22 had 90% browning and 12% callusing (Table 3.11(ii)). Occasionally, a browned explant produced callus (arrow, Figure 3.4). This usually happened if the yet un-browned part of the explant initiated callus induction before the deteriorating browning spread to the initiation point.

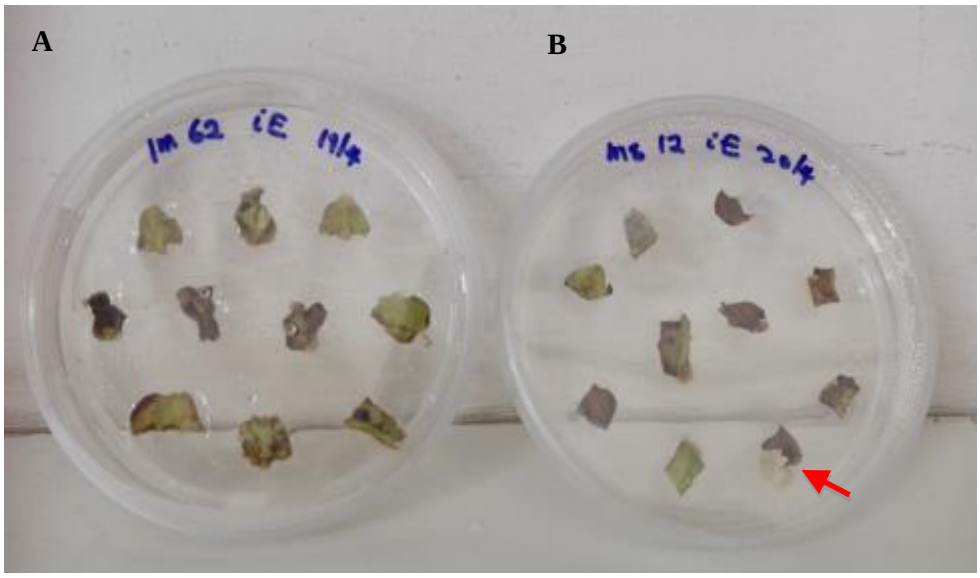


Figure 3.4: Browning of *in vitro* 'Edranol' leaf explants when cultured in (a) CIM5 (b) CIM21 after four weeks in culture. Red arrow indicated a callused browned explant.

3.3.1.1.8.3 Callusing

Fifty percent of the explants that were plated on CIM5 (i.e. reference medium) had produced callus by the third week in culture while the callus induction rate in MS media that contained NAA and BAP was lower (maximum of 10%) by the third week. The explants on CIM5 had 90% callusing by the sixth week while only two (CIM24 and CIM25) out of the eight MS media with NAA and BAP produced more than 50% callus by the same time (Table 3.11(iii)).

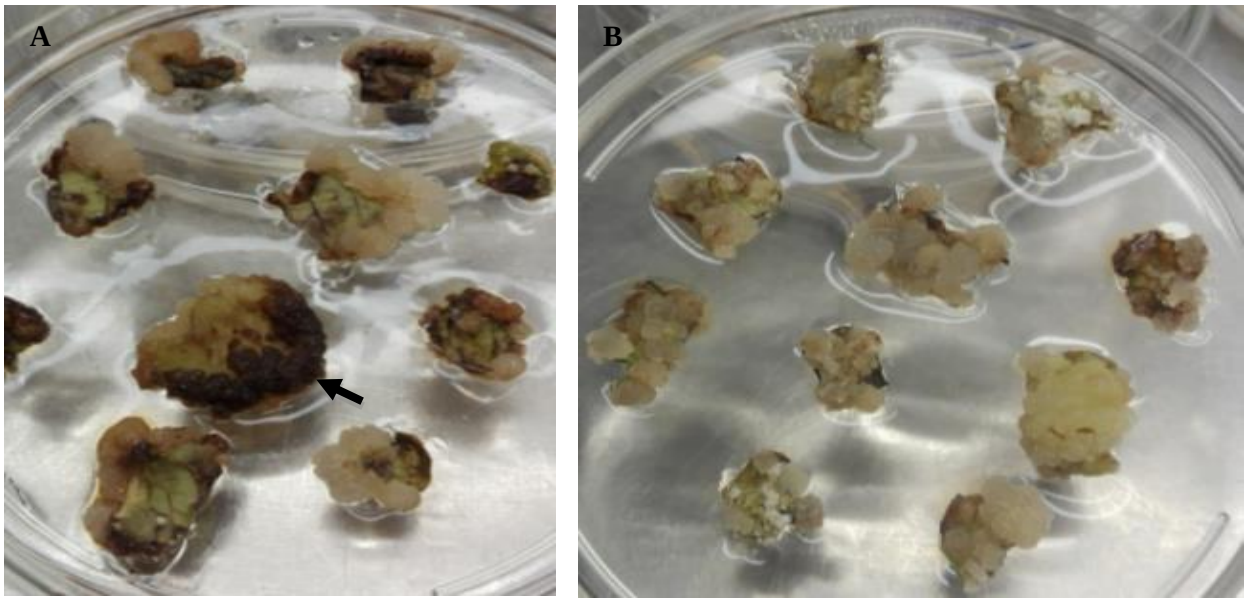


Figure 3.5: Six-week-old cultures showing browned/dying callus (black arrow) in CIM5 (A) and calli that appeared healthy in an induction medium containing CIM25 (B).

While CIM5 was the most favourable (percentage response and rate) for callus induction in 'Edranol' avocado leaf explants (Table 3.11(iii)), the lifespan of the callus formed was short as they started to brown and die before the sixth week of their formation (Figure 3.5A). On the other hand, those calli formed on the MS induction media containing NAA and BAP combinations still retained their colour, texture and growth within the same duration (e.g. Figure 3.5B).

Most commonly, the calli were cream and white, which were either compact, grainy or soft and crumbled easily. The arrangement of the callus on the explants were sometimes in clusters (small to large) that covered most or all the explants, while others were aligned in a chain-like manner following underlying vascular tissue (Table 3.12 and Table 3.13).

Table 3.12: Description of the callus formed on CIM5 compared with the MS induction media that contained NAA and BAP combinations.

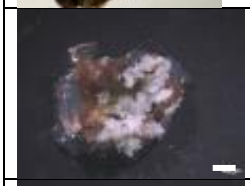
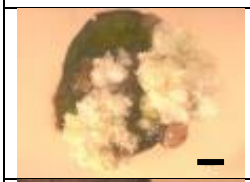
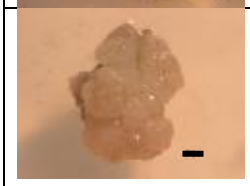
Induction Media	CIM No	Description of callus on explants.
**1.0 mg/L 2,4-D; 0.5 mg/L BAP	CIM5	44.44% explants had callus that were compact, cream-coloured and round; 55.56% callus was grainy, light cream-coloured, appeared in large clusters but not compact
0.5 mg/L NAA; 0.5 mg/L BAP	CIM18	62.5% callus were tiny (ca. 0.3 mm) globular structures aligned in chain-like manner on explants; 25% had callus that were white-coloured; 12.5% undefined
0.5 mg/L NAA; 1.0 mg/L BAP	CIM19	33.33% had cream coloured clusters of tiny/medium nodules; 44.44% had callus with white patches; 22.22% undefined
0.5 mg/L NAA; 1.5 mg/L BAP	CIM20	28.57% had cream-coloured callus in moderate clusters; 28.57% had white callus; 42.86% in small clusters
0.5 mg/L NAA; 2.0 mg/L BAP	CIM21	20% had soft, easily crumbled, off-white, translucent callus; 45% had cream-coloured compact clusters; 35% tiny callusing
1.0 mg/L NAA; 0.5 mg/L BAP	CIM22	All had small clusters (ca. 1 mm) of tiny nodules
1.0 mg/L NAA; 1.0 mg/L BAP	CIM23	19.05% had white callus; 47.62% had a chainlike clusters of nodules; 33.33% had small clusters
1.0 mg/L NAA; 1.5 mg/L BAP	CIM24	2.78% had soft callus; 13.89% in large clusters; 61.11% had cream-coloured callus; 22.22% were undefined
1.0 mg/L NAA; 2.0 mg/L BAP	CIM25	56.41% had cream-coloured callus; 20.51% had cream-coloured that contained white patches; 23.08% were undefined

**highlight indicated reference medium (CIM5).

Ten different types of calli (Table 3.13, A – J) were formed from the leaf explants of avocado cv 'Edranol'. The different types of calli were characterised by different colours and textures. The induction of callus from the leaf explants was from different parts of the leaf such as the end of a prominent vein (e.g. type A, G) the cut edges of the leaf explants (e.g. type B, J) and the surface of the leaf explants i.e. lamina (e.g. type I, F).

Table 3.13: Description of the different types of calli formed during callus induction in the leaf explants of avocado cv 'Edranol'. Scale bar = 1mm

Callus pictures	Description	Type

	‘Callus’ did not look like an aggregation of cells but the enlargement of one cell from the main vein of the leaf explant or the enlargement of tissue of main vein. Callus was formed at the end of a prominent vein. The texture of the structure was hard.	A
	Loosely attached white callus formed around dead (brown) leaf. Callus was formed at the cut edges of the leaf explant and spread to the surface of the explants.	B
	An enlarged green and compact callus, completely overlaid with ‘coating’ of greenish-white callus. Callus was initially formed at the end of a prominent vein and spread over the entire explant.	C
	Callus was a mixture of three types: at the left end was clump of soft and brown aggregates; at the middle was a mixture of white globules and amorphous callus and at the right end was an aggregate of brown nodules. Callus appeared to be formed primarily at the distal end and secondarily at the middle.	D
	Callus was formed around the cut edge of the leaf explant. Two types of callus were seen: on one side was nodular aggregate and on the other side was fine, and soft aggregate.	E
	White, loosely attached callus that was formed primarily from the explant around the cut edges and on the surface of the explants.	F
	Callus formed at the end of a prominent vein of the leaf explants; the callus cells were globular at the periphery and brown amorphous aggregate at the centre. White callus formed secondarily around the primary callus.	G
	Cream-coloured callus with uniform globular cells that were compacted together. Callus was formed across the length of the prominent vein of the leaf explant and cut edges.	H
	Compact, cream-coloured callus on one end and translucent callus at the other. Callus originated from the end of a prominent vein and on the surface of the explant.	I
	Light brown, soft callus with the ‘absorbent’ nature of sponge, formed at the cut edges of the explants and growing towards the centre and entire surface of the explant.	J

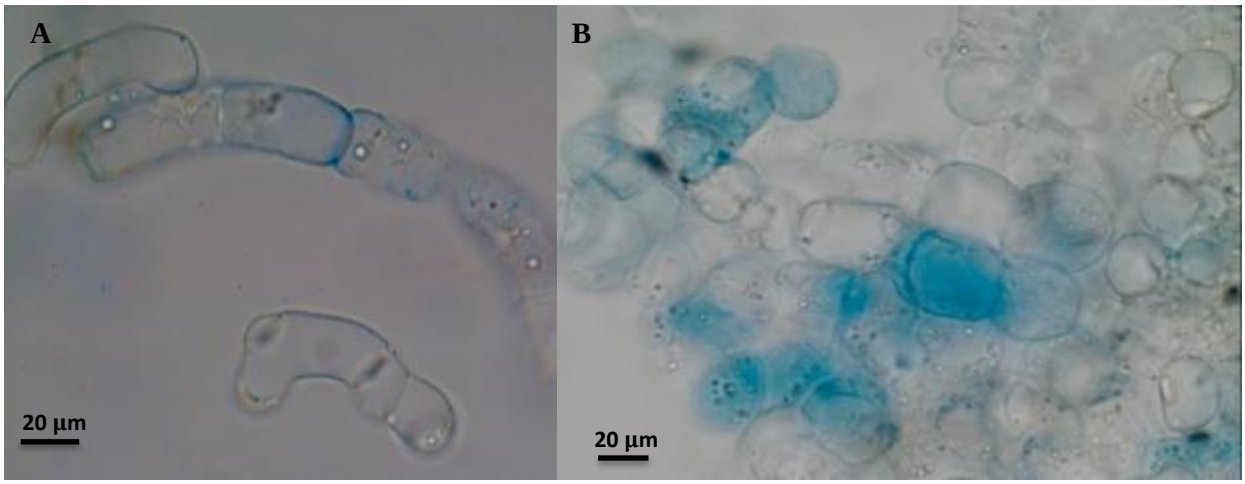


Figure 3.6: The different cells of the callus (A) elongated cell (B) spherical cells

The callus formed from each explant and for each medium did not always fall, exclusively, into one of the types of callus highlighted (Table 3.13); rather an explant usually produced two or more types of callus. The cells of the callus on each explants were either elongated, spherical or a mixture of both (Figure 3.6).

3.3.1.2 'Fuerte' cultivar

The optimal range for callus induction for *Persea americana* cv 'Edranol' (CIM1 – CIM9 *viz* 0.5 – 2.0 mg/L 2,4-D with 0.2 – 1.0 mg/L BAP in Gamborg's B5 basal medium, under dark incubation, Table 3.4) was used to compare 'Fuerte' leaf explant sizes, i.e. smaller leaf disc explants (0.196 cm^2 surface area) with the larger leaf explants (ca. 1.0 cm^2 leaf area).

There was no relationship between the 'Fuerte' leaf explant sizes and their ability to form callus (p = 0.7946). Also, in two (out of the nine) media tested, both small and large explants did not form callus. The large explants formed more callus than small explants in four of the tested media while small explants produced more callus than large explants in three media (Table 3.14). Only in three (out of 18) cultures did both large and small 'Fuerte' leaf explants produce ≥ 50% callus. These included the CIM3 for small explants and CIM6 for both large and small explants (Table 3.14). The comparison of both cultivars (i.e. 'Edranol' and 'Fuerte') showed that the media used significantly favoured callus induction in 'Edranol' more than 'Fuerte' (p = 0.006) (Table 3.14).

Table 3.14: Comparison of percentage callusing between leaf explants of different sizes from 'Edranol' and 'Fuerte' cultivars obtained from greenhouse-grown avocado plants (n = 25 with three replications).

Induction Media	CIM No	'Fuerte' Large explants	'Fuerte' Small explants	'Edranol' Large explants	'Edranol' Small explants
0.5 mg/L 2,4-D; 0.2 mg/L BAP	CIM1	0	15	0	66.67
0.5 mg/L 2,4-D; 0.5 mg/L BAP	CIM2	20	0	46.67	35
0.5 mg/L 2,4-D; 1.0 mg/L BAP	CIM3	13.33	50	65	31.43
1.0 mg/L 2,4-D; 0.2 mg/L BAP	CIM4	0	0	56	25.33
1.0 mg/L 2,4-D; 0.5 mg/L BAP	CIM5	20	11.11	30	66.15
1.0 mg/L 2,4-D; 1.0 mg/L BAP	CIM6	53.33	64	45	41.43
2.0 mg/L 2,4-D; 0.2 mg/L BAP	CIM7	0	0	0	46.15
2.0 mg/L 2,4-D; 0.5 mg/L BAP	CIM8	33.33	4	20	41.33
2.0 mg/L 2,4-D; 1.0 mg/L BAP	CIM9	33.33	12.50	32.45	37.78
Total Mean		**19.26 ± 18.39^{b}**	**17.40 ± 23.43^{ab}**	**32.8 ± 23.0^{abd}**	**43.45 ± 14.3^{d}**

Induction media comprised Gamborg's B5 with nine different combinations of 2,4-D + BAP. Rectangular, large explants and small, disc explants had surface areas of ca. 1.0 cm^2 and 0.196 cm^2, respectively. 'Edranol' data were imported from Table 3.7 of this chapter for the purpose of comparison.

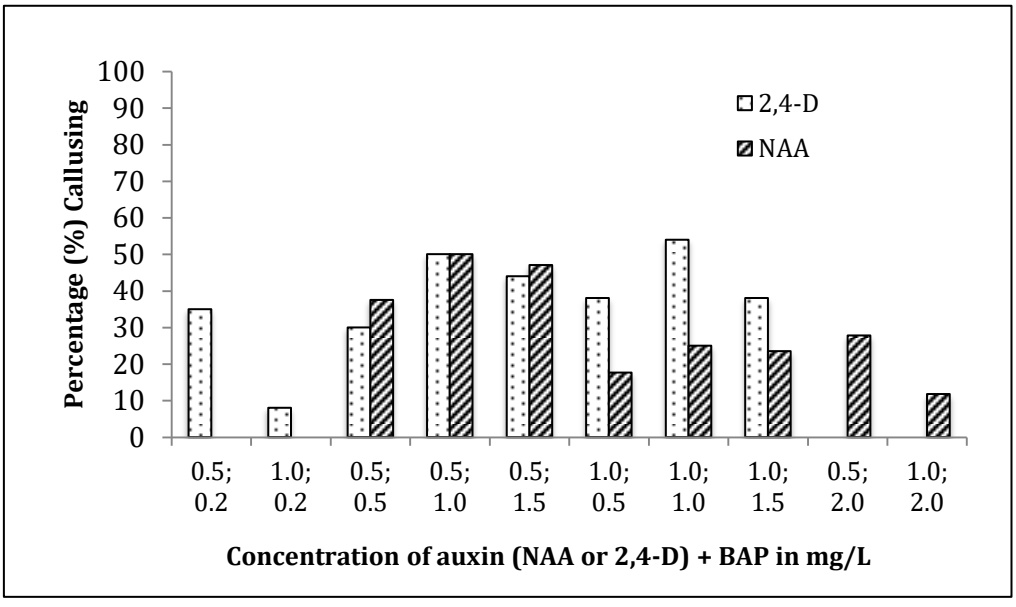


Figure 3.7: The percentage of *in vitro* 'Fuerte' avocado leaf explants producing callus on media that contained MS supplemented with different combinations of each of two auxins (NAA and 2,4-D) and BAP (n = 50 without replication).

The percentages of explants that formed callus did not follow a normal distribution for both 2,4-D and NAA. However, the concentrations of 0.5 mg/L for both 2,4-D and NAA, in combination with 1.0 mg/L BAP were optimal for callus induction for both auxins (without considering the 'outlier' result) (Figure 3.7). As previously reported for the 'Edranol' cultivar (in this chapter), the auxin 2,4-D stimulated more callus induction than picloram or NAA. Media that contained NAA only had higher (than 2,4-D) callus induction in two out of the six media with a maximum difference of 7.5%. On the other hand, 2,4-D-enriched media had higher callus induction in three media with a difference as high as 19%, in the medium that contained 1.0 mg/L 2,4-D + 1.0 mg/L BAP (Figure 3.7).

It is noteworthy that the kind of callus formed by *in vitro* 'Fuerte' leaf explants and across all the media tested was white with a fluffy texture (Figure 3.8); and the callus did not survive subculturing. Furthermore, the *in vitro* leaf explants of 'Fuerte', after weeks in culture, were enlarged and remained green.

Figure 3.8: Callus from *in vitro* 'Fuerte' leaf explants at 6 weeks in a medium containing 0.5 mg/L 2,4-D and 1.0 mg/L BAP on MS. The calli were mainly white and fluffy.

3.3.1.3 'Hass' cultivar

As with the 'Fuerte' cultivar, the 9 media that were empirically deemed optimal for the initiation of callus in 'Edranol' were also used to test for the induction of callus from the 'Hass' cultivar. Similar to the 'Edranol' cultivar, CIM5 (1.0 mg/L 2,4-D; 0.5 mg/L BAP on B5) was one of the two most favourable media for inducing callus from the leaves of *Persea americana* 'Hass' (Table 3.15). Although explants were sourced *in vitro*, six out of the nine cultures were contaminated (Table 3.15). Browning was visible in four cultures and was minimal in each case, having a maximum of 20%. All the explants in all the media formed callus. However, callus formation was not vigorous and comprised clusters of

small nodules (pictures not shown). These turned browned within a few weeks and eventually died. The use of the 'Hass' cultivar was therefore discontinued.

Table 3.15: The percentage of leaf explants from 'Hass' avocado shoots *in vitro* that were callused, contaminated and browned after six weeks in culture.

		Percentage		
Induction Media on B5	**CIM No**	**Callusing**	**Contamination**	**Browning**
0.5 mg/L 2,4-D; 0.2 mg/L BAP	CIM1	10	0	10
0.5 mg/L 2,4-D; 0.5 mg/L BAP	CIM2	32.86	0	0
0.5 mg/L 2,4-D; 1.0 mg/L BAP	CIM3	38.57	14.29	7.14
1.0 mg/L 2,4-D; 0.2 mg/L BAP	CIM4	20	20	20
1.0 mg/L 2,4-D; 0.5 mg/L BAP	CIM5	37.14	14.29	2.86
1.0 mg/L 2,4-D; 1.0 mg/L BAP	CIM6	11.43	14.29	0
2.0 mg/L 2,4-D; 0.2 mg/L BAP	CIM7	10	0	0
2.0 mg/L 2,4-D; 0.5 mg/L BAP	CIM8	11.43	28.57	0
2.0 mg/L 2,4-D; 1.0 mg/L BAP	CIM9	11.43	14.29	0

3.3.2 Callus proliferation

In order to increase the size i.e. proliferate the callus induced, the explants that formed callus were subcultured after 6 weeks on to fresh media of the same compositions as the induction media.

The subcultured calli did not all respond as hypothesized, with the materials showing one or many of the following: callus that responded positively by proliferating (e.g. CIM20); callus browning followed by death (e.g. CIM5 and CIM22); secondary callus formation with calli that were of different types from the primary callus (e.g. CIM24) or callus that remained unchanged (e.g. CIM23 and CIM25). In most cases, some portions of the calli changed colour from white and/or cream in the first primary induction media, to green in the secondary induction/proliferation media (Table 3.16). Unfortunately, some cultures were contaminated (e.g. CIM23 and CIM25).

Materials that were in CIM18 – CIM21 responded more positively to subculture than those in CIM22 – CIM25; both groups had MS as the basal medium. On the other hand, materials cultured on CIM5 (1.0 mg/L 2,4-D + 0.5 mg/L BAP in B5) responded poorly to transfer to a fresh induction medium with a decline of more than 70% of the inoculated callus. Generally, the callus proliferation stage did not effectively result in an increase in callus mass, especially in the medium enriched with 2,4-D.

Table 3.16: Response (after 5 weeks) of *Persea americana* 'Edranol' callus to subculture onto the various fresh induction media.

Induction Media	CIM No	No of Subcultured Calli with Explants Attached	Proportion of Callus Responding to Fresh Induction Media.
1.0 mg/L 2,4-D; 0.5 mg/L BAP; B5	CIM5	18/20	27.78% of the callus survived.
0.5 mg/L NAA; 0.5 mg/L BAP; MS	CIM18	12/50	50% of the callus survived and were green
0.5 mg/L NAA; 1.0 mg/L BAP; MS	CIM19	16/49	37.5% of callus were green (previously in white patches); 62.5% were cream turning light-green
0.5 mg/L NAA; 1.5 mg/L BAP; MS	CIM20	14/50	28.57% (previously cream callus in moderate clusters) were enlarged and green; 28.57% were green (previously white); 42.86% that were previously in small clusters were slightly enlarged
0.5 mg/L NAA; 2.0 mg/L BAP; MS	CIM21	20/50	10% were moderately enlarged and green; 15% were massively enlarged, fluffy and white; 35% had changed from cream compact clusters to moderately grown green callus; 5% were dead; 35% (previously with very tiny callusing) had grown moderately with cream/green callus
1.0 mg/L NAA; 0.5 mg/L BAP; MS	CIM22	6/50	All dead (previously had very small clusters of tiny nodules)
1.0 mg/L NAA; 1.0 mg/L BAP; MS	CIM23	21/50	9.52% contaminated; 42.85% were dead; 14.29% were partially alive while another 14.29% remained unchanged; the size of 19.04% of the callus remained unchanged but slightly green
1.0 mg/L NAA; 1.5 mg/L BAP; MS	CIM24	36/50	25% were dead; 8.33% formed secondary callus after the browning/death of the primary callus; 2.78% had secondary callus without the death of the primary; 13.89% were contaminated; 38.89% remained cream-coloured, turning light green; 11.11% were green callus
1.0 mg/L NAA; 2.0 mg/L BAP; MS	CIM25	39/50	28.2% were dead; 10.26% were contaminated; 7.7% growing massively with white/green fluff; 20.51% developed secondary callus; 33.33% remained unchanged.

3.3.2.1 Extension of incubation period for callus induction

This callus induction experiment was carried out using only two of the induction media: CIM5 (1.0 mg/L 2,4-D + 0.5 mg/L BAP) and CIM6 (1.0 mg/L 2,4-D + 1.0 mg/L BAP) both in Gamborg's B5 basal nutrient medium.

Table 3.17: Effect of increased subculturing period (8 rather than 6 weeks) on callusing from greenhouse *Persea americana* 'Edranol' leaf explants (n = 100 with four replicates).

	Percentage callusing		
Induction media in B5	**6 wks**	**8 wks (without subculture)**	**12 wks (after subculturing at 8 wks)**
CIM5	46.06 ± 9.90^{ab}	49.83 ± 7.90^{ab}	19.14^{a}
CIM6	29.17 ± 4.71^{a}	45.70 ± 2.40^{ab}	21.89^{a}
***P* value**	**0.078404**	**0.754363**	

Callusing from the leaf materials increased in both of the media when left for 8 weeks without subculture. Subculturing was done at the eighth week. After subculturing to fresh induction media of the same composition, more than half of the calli were lost to browning (Table 3.17).

3.3.3 Callus development

Developmental media (ii), (x) and (xi) (Table 3.18) did not result in any changes in the calli and the calli eventually died. Media (iv) and (v) had the highest positive effect on the calli from six (out of the eight) induction media; with the calli turning green. Further development and longevity in culture was noticeable and more favourable in those calli that were induced on NAA-incorporated induction media than 2,4-D-incorporated media (Table 3.18). Similarly, the responses of the callus induced on B5 basal nutrient formulation, in developmental media was less than the response of MS-based induced callus in the same developmental media.

Table 3.18: The effect of nutrient media and auxins on the developmental path of callus previously obtained from eight different induction media subcultured to 11 different developmental media.

All media had the following as constant part of their composition: full strength (4.4 g/L) MS nutrient medium, 4.0 mg/L thiamine-HCl, 100 mg/L myo-inositol, 30 g/L sucrose, and 3.0 g/L gelrite and modified from (i) to (xi). n ranges from 2 to 8.

∗ indicates the lower number of subcultured explant-bearing callus: n = 2. n was small because the number of explants reduced with each subculture.

Auxin/cytokinins (mg/L)	CIM No	Percentage Callusing	Percentage of Callus that are still Alive and 'Greening' in Callus Development Media										
			(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)	(ix)	(x)	(xi)
1.0 mg/L 2,4-D; 0.5 mg/L BAP; B5	CIM5	43.67	0	0	0	12.5	0	0	0	0	0	0	0
0.5 mg/L 2,4-D; 1.5 mg/L BAP; B5	CIM26	59.17	0	0	0	0	0	0	0	0	0	0	0
1.0 mg/L 2,4-D; 0.5 mg/L BAP; MS	CIM15	31.43	0	0	0	0	50 ∗	0	0	0	0	0	0
0.5 mg/L 2,4-D; 1.5 mg/L BAP; MS	CIM13	34.00	0	0	0	25	50	0	0	0	0	0	0
1.0 mg/L NAA; 0.5 mg/L BAP; B5	CIM27	27.59	60	0	40	20	20	66.66	66.66	100	66.66	0	0
0.5 mg/L NAA; 1.5 mg/L BAP; B5	CIM28	28.33	33.33	0	0	33.33	33.33	25	40	40	0	0	0
1.0 mg/L NAA; 0.5 mg/L BAP; MS	CIM22	20.00	60	0	100	80	80	100	100	50	66.66	0	0
0.5 mg/L NAA; 1.5 mg/L BAP; MS	CIM20	12.00	33.33	0	66.66	100	100	0 ∗	100 ∗	100 ∗	100 ∗	0	0

(i) MS basal medium plus vitamins without PGRs (plant growth regulators)

(ii) As (i) above but sucrose increased from 30 g/L to 60 g/L.

(iii) As (i) above but gelling agent increased from 3.0 g/L to 6 g/L gelrite.

(iv) BAP at 2.0 mg/L was added to the composition of (i)

(v) Activated charcoal at 4.0 g/L was added to the composition of (iii)

(vi) Medium (iii) plus 1.0 mg/L gibberellic acid (GA_3) and 0.5 mg/L BAP.

(vii) Medium (vi) plus activated charcoal at 4.0 g/L

(viii) Medium (vi) plus 2.0 mg/L BAP

(ix) Medium (viii) plus activated charcoal

(x) Medium (i) plus 1.0 mg/L gibberellic acid, 2.0 mg/L BAP, 45 g/L sucrose, 100 ml/L coconut water (CW) and gelrite increased at 6 g/L.

(xi) Medium (x) plus 200 ml/L CW.

All the callus from the four induction media containing 2,4-D responded poorly to the developmental media. These included callus from CIM5 with 12.5% development on only one of the eleven developmental media (Medium iv); callus from CIM15 with 50% callus development only on media No. (v) while callus produced on CIM13 responded to media No. (iv) and (v) with 25% and 50% callus development, respectively. Callus from CIM26 responded to none of the 11 developmental media (Table 3.18).

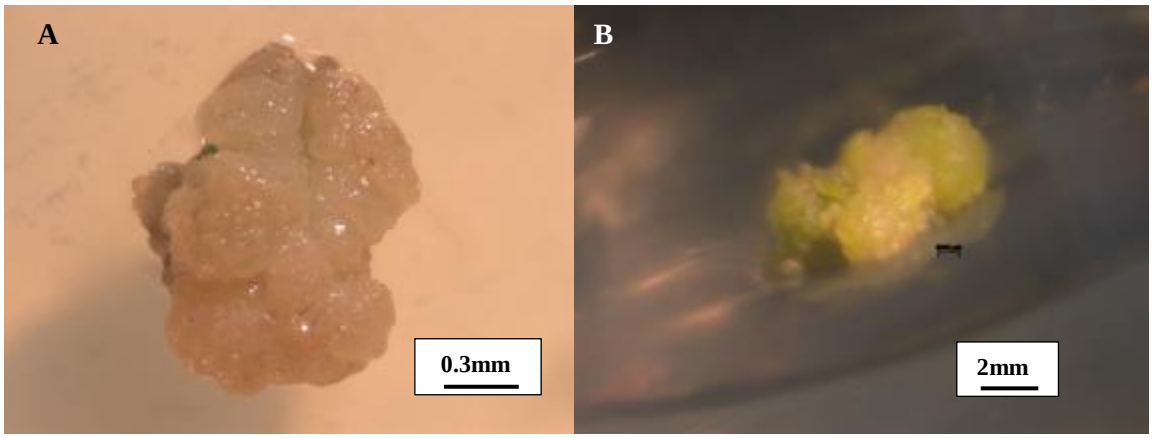


Figure 3.9: Callus generated from leaf explants on 1.0 mg/L NAA + 0.5 mg/L BAP. A: callus at six weeks B: callus at 10 weeks, which was transferred to full strength MS without PGRs but increased gelling agent at six weeks. NB: 'greening' after subculture

In contrast, all callus from the four NAA-incorporated induction media had as high as 100% positive responsiveness to the developmental media, the lowest being 12%. Callus from each of the media responded to either seven or eight out of the 11 developmental media (Table 3.18). Positive response indicated that the calli were still alive, but there was neither visible growth nor visible sign of somatic embryo development; rather, a change of colour from cream to green was predominant (Figure 3.9). Thereafter the material was subcultured, after six weeks, to fresh developmental media of the same composition. However, all the calli deteriorated, with rapid browning, which eventually led to death.

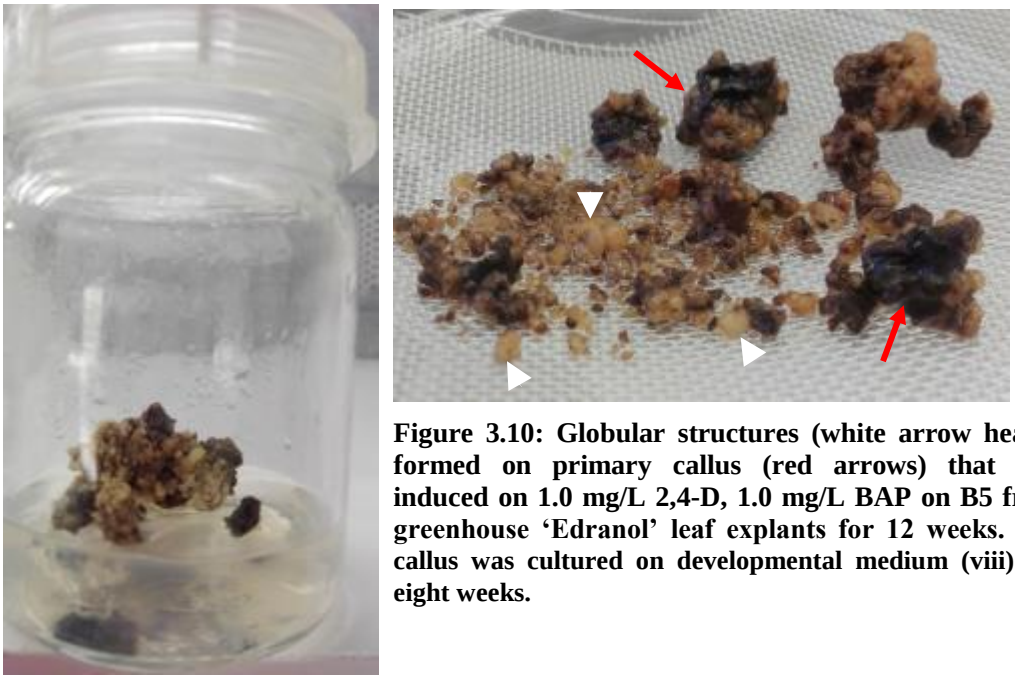


Figure 3.10: Globular structures (white arrow heads) formed on primary callus (red arrows) that was induced on 1.0 mg/L 2,4-D, 1.0 mg/L BAP on B5 from greenhouse 'Edranol' leaf explants for 12 weeks. The callus was cultured on developmental medium (viii) for eight weeks.

Calli induced from greenhouse-derived 'Edranol' leaf explants (Table 3.17) were transferred to developmental medium (viii) and left without subculturing for eight weeks. Only two callus masses (from two explants) developed further (Figure 3.10). The primary calli had browned, dried and appeared dead; but loosely attached with, and embedded within the callus were distinct, firm and cream-coloured globular structures that were easily separated from the brown primary callus debris (Figure 3.10).

3.3.4 Maturation of globular structures

Efforts to stimulate further development in the globular structures obtained above (Figure 3.10) proved futile as none of them survived in the new culture medium meant for maturation. The globular structures were not responsive when transferred to either the liquid or solidified maturation media (Section 3.2.4). They eventually turned brown and died.

3.4 Discussion

There is a paucity of information and lack of reference to any previous work associated with avocado leaves. The only available published data is that of Young (1983). Therefore, the present study investigated callus induction and development from avocado leaf explants. Leaves have various meristematic regions (Donnelly *et al*., 1999; Ichihashi and Tsukaya,

2015), are least restricted by seasonal changes (Siwach and Gill, 2014) and the removal of a few leaves is less invasive on the stock plant. Thus, leaves are suitable as explants for callus induction.

3.4.1 Callus induction

The formation of callus is the intermediary stage in the indirect pathway of somatic embryogenesis and its successful induction depends on a balanced combination of PGRs, principally the auxins and the cytokinins (Konate *et al*., 2013). Not only the combination of PGRs but the types of auxins used also determine the success of the callus induction and the type of callus formed (Suzuki and Nakano, 2001; Ojha *et al*., 2012; Konate *et al*., 2013; Habibah *et al*., 2018). This aspect of the study compared the various treatments that explants were subjected to in order to induce callus formation. The tasks were to empirically work out a range of media that favoured high percentage of callus formation, and the factors that facilitated such performance.

3.4.1.1 'Edranol' cultivar

3.4.1.1.1 The importance of the leaf surface in contact with callus induction medium

The leaf is one of the vegetative organs (alongside root and stem) of a plant. The leaves are mostly composed of the photosynthetic mesophyll layers and vascular tissues in between the epidermal layers (Evans, 2009). While leaves may be simple or compound and are often described based on their shape, margin, apex, base, arrangement and venation pattern, the lamina was the point of interest in this research. This flat, expanded blade has three-dimensional axes in its growth pattern: the midrib to margin (medio-lateral) axis, the base to tip (proximal-distal) axis and the dorsi-ventral axis. The latter is denoted as the adaxial (upper, facing the sky) and abaxial (lower, facing the ground) surfaces of the leaf (Ichihashi and Tsukaya, 2015). The dorsi-ventral orientation allows the adaxial surface to specialize in capturing photons from the sun for photosynthesis while the abaxial surfaces function primarily in gaseous exchange (Kidner and Timmermans, 2010; Hayakawa *et al*., 2016).

In this research, the adaxial surface of avocado explants in contact with the culture medium significantly favoured more callus formation than the abaxial surface (54% and 22%,

respectively). This was believed to be as a result of the cellular components associated with the dorsi-ventral axis of the leaf.

Like most leaves of higher plants, avocado leaves are asymmetrical in the dorsi-ventral axis (Figure 3.1) (Juarez *et al*., 2004). The palisade mesophyll cells were tightly arranged perpendicular to the epidermal layer of the adaxial surface. On the other hand, the spongy mesophylls were loosely attached within and towards the abaxial surface and were interspersed with airspaces (Esau, 1977; Nakata and Okada, 2013; Patay *et al*., 2016). Despite the seemingly tight arrangement of the palisade mesophyll cells, their exposure to intercellular spaces can be up to four times more than in the spongy mesophyll cells, thus having a larger surface area (Esau, 1977; Pallardy, 2008). The epidermis of abaxial surfaces, in most plants (and in avocado, Chapter Four), is thinner than the upper epidermis (Osborn and Taylor, 1990; Benitez de Rojas and Ferrarotto, 2009). Therefore, the adaxial surface making contact with the induction medium while the abaxial surface was exposed implied that: one, the abaxial surface was not restricted, facilitating gaseous exchange. Two, it was easier for the abaxial epidermis to rupture due to pressure, for callus to bud out of the leaf (Laparra *et al*., 1997; Yusoff *et al*., 2012; Gomes *et al*., 2017; Meira *et al*., 2019). Three, according to George (1993), the adaxial surface absorbs more nutrients than the abaxial surface. Thus, more palisade mesophyll cells were receptors of the induction signals from the medium therefore stimulating callogenesis. While characteristic airspaces surrounding the spongy mesophylls would facilitate better diffusion and interaction of the PGRs with the component cells, there were still fewer spongy mesophyll cells that would receive induction signal. Four, the palisade mesophyll cells of avocado could be more meristematic than spongy mesophyll cells as suggested by Juarez *et al*. (2004).

This shows that the orientation of explants in tissue culture media has an effect on their response to the media. Such were the results obtained in Bambara groundnut by Konate *et al*. (2013) and their results were similar to those obtained in this research.

3.4.1.1.2 Light and dark incubation

In nature, light is an important factor in plant development as it supplies all the energy required for the plant's metabolism. However, in the *in vitro* environment, the role of light is

dependent on the type of tissue culture being carried out. For example, in *Barringtonia racemosa*, light incubation did not favour callusing of the leaves over dark incubation (Behbahani *et al*., 2011). However, the purpose of generating the callus by those authors was for metabolite (lycopene) production, which was induced more under light conditions. According to Summart *et al*. (2008), the stimulatory or inhibitory role of light in callus induction is particularly dependent on the cell types and the genotypes of the materials being tested (e.g. Kintzios *et al*., 2002).

Dark incubation significantly favoured more callus formation from leaf explants of avocado cv. 'Edranol' than light incubation (Table 3.4 (p = 0.001242) and Table 3.5 (p = 0.00182)). In this respect, Mazumdar *et al*. (2010) highlighted the benefits of dark incubation such as (i) it slows the rate at which plant growth regulators (endogenous or exogenous) are degraded in the explants and (ii) it enhances their transport to and within the cells. On the other hand, it has been shown that incubation under light conditions can restrain cell division (Behbahani *et al*., 2011) – a process that is necessary during callogenesis. In addition, Habibah *et al*. (2018) believed that incubation under light conditions promoted the production of phenolics in the explants and this reduced callus formation. This phenomenon was observed in this research (result not shown), as colorations of induction media were more apparent in cultures incubated in the light than those in the dark. Osorio *et al*. (2018) also observed the accumulation of phenolics in avocado tissues that were incubated under light conditions. In this regard, exposure of culture media to light has been shown to result in the photochemical changes of the media and the degradation of the auxins, among other constituents of the media (Nissen and Sutter, 1990; Stasinopoulos and Hangarter, 1990).

Similar to the present results, the work of Behbahani *et al*. (2011) not only showed that callus was formed earlier, but also that the dry weight of the callus formed and the percentage of formation was higher under dark conditions. Similar results were also found in *Stelechocarpus burahol* (Habibah *et al*., 2018), *Lavandula vera* (Kintzios *et al*., 2002), *Cucumis melo* (Stipp *et al*., 2001), common mallow (*Malva sylvestris* L.) (Konstas *et al*., 2003) and *Oncidium* (Chen and Chang, 2000). However in garlic, there was no significant difference in callus formation under light and dark incubation (Martín-Urdíroz *et al*., 2004).

3.4.1.1.3 Effect of basal medium nutrient compositions

The formulation of the basal nutrient medium, i.e. the combination of minerals, major and minor nutrients, is not only important in the development, but also in the morphogenesis of plant cells, tissues and organs *in vitro* (Ramage and Williams, 2002). Most basal nutrient media were initially designed for specific species. For example, MS medium was designed for tobacco (Murashige and Skoog, 1962), Gamborg's B5 for soybeans (Gamborg *et al*., 1968), Woody Plant Medium for mountain laurel, *Kalmia latifolia* (Lloyd and McCown, 1980), among others. However, it is now common practice that these media are used based on their prior success in published data, without modification, for a wide range of species (Greenway *et al*., 2012). However, Ramage and Williams (2002) posited that this practice would not yield optimal results for such a wide range of species, rather, some optimal modification would be required for a particular species. In order to accommodate such variation, customized basal nutrient media for specific species and types of tissue cultures are being explored (Phillips and Garda, 2019). Those authors showed that all the mineral nutrients in basal media may not be required for morphogenesis, but this would have to be empirically determined for each species.

For *P. americana* ('Edranol'), Gamborg's B5 promoted higher amounts of callus formation (Table 3.4 and Table 3.5) than MS (Table 3.6a and Table 3.6b). Three (of the four) variants of $B5^{+}$ medium produced callus between 4% and 8%. On the other hand, all the tested variants of Mango Medium for Somatic Embryo (MMSE) did not result in callus formation.

MS and B5 are the most commonly used basal nutrient media (Greenway *et al*., 2012). Although statistics showed that the use of MS and its derivatives was more (82%) than B5 and allies (5%) (Phillips and Garda, 2019), MS was still less favourable for callus induction in *P. americana* ('Edranol'). This was attributed to the high concentration of ammonium salts in MS when compared with either B5 or WPM nutrient medium (Behbahani *et al*., 2011). Total Nitrogen concentration is 59.92 mM, 26.75 mM and 14.70 mM in MS, B5 and WPM, respectively (Pinto *et al*., 2008). Apart from nitrate, which is common to both MS and B5, ammonium is the additional source of Nitrogen in MS (Ramage and Williams, 2002) and it has been associated with high acidity, which can suppress morphogenesis (Ramage and Williams, 2002). Ammonium toxicity was associated with undesirable explant responses in

avocado *in vitro* (Pliego-Alfaro *et al*., 2002). This, presumably, may be responsible for lower results observed in MS in the work reported here. Other species responded in a similar way, showing lower response in MS than B5, e.g. garlic (Martín-Urdíroz *et al*., 2004) and *Barringtonia racemosa* (Behbahani *et al*., 2011). On the other hand, favourable callus induction in *Eucalyptus globulus* was in the order MS > B5 > WPM (Pinto *et al*., 2008). Thus, high Nitrogen concentration may not have a deleterious effect on all species since its low concentration, as it is in B5, may lead to deficiency symptoms in some species (Phillips and Garda, 2019). In some other instances, different species of a genus may require different basal nutrient media for callus induction e.g. *Eucalyptus* (Pinto *et al*., 2008).

In line with the recommendations put forward by Ramage and Williams (2002) and practiced by others researchers (Phillips and Garda, 2019), MMSE and $B5^{+}$ used in this research were modifications of MS and B5. However, they were not suitable for callogenesis in avocado leaves. These two basal media therefore require further empirical manipulation before an optimal balance is reached. In this regard, Greenway *et al*. (2012) noted that the modifications to B5 yielded better results than the original formulation.

3.4.1.1.4 Effect of explant size

A bigger leaf explant supposedly, should result in more callus formation than the smaller explants because it contains more cells and its larger surface area is exposed to the induction media (Nhut *et al*., 2007). However, an optimal explant size is required for effective callusing, as explants that are too small or too big will result in suboptimal callus formation (Bhau, 1999). Both leaf discs (small explants) and leaf squares (big explants) of avocado cv. 'Edranol', in this research, were cut along the perimeters. The perimeter to area ratio in small explants was twice (8:1) that of big explants (4:1). Since callus usually originates from wounding (cut) sites (Webster *et al*., 2006; Konate *et al*., 2013), the explants with bigger ratio, i.e. small explants, would be expected to generate more callus (Table 3.7). However, the uptake of molecules (PGRs) by the explants from the media can be through the surface (in this case, the epidermis) and/or through the cut surfaces (Machakova *et al*., 2008). If molecule uptake is through the epidermis alone, a bigger explant would be more favoured; and vice versa if the uptake is through the cut perimeters alone. If both epidermis and cut surfaces serve

as points of uptake, there may not be a difference between small and big explants. Hence, size of explant was not of consequence in *P. americana* ($p = 0.380794$).

Various plant species responded differently to the size of explant. For example, both small and bigger explants were favoured in sunflower genotypes (Espinasse *et al*., 1989). Small explants favoured more callus formation in bermuda grass (Chaudhury and Qu, 2000), *Sorghum bicolor* (Nhut *et al*., 2003) and date palm (Othmani *et al*., 2009). In mung bean, different sizes of explants required different concentrations of growth regulators. This was associated with different levels of endogenous growth regulators in the various explant sizes (Gulati and Jaiwal, 1992).

3.4.1.1.5 Effect of the source of explant

Different growth conditions, such as in the greenhouse and the *in vitro* micro-environment in this study, can trigger the expression of phenotypic plasticity in plants of the same genotype (Bradshaw, 1965; Qi et al., 2020). The phenotypic plasticity is expressed in the biochemistry, anatomy, morphology and physiology of the plant (Gratani, 2014). The greenhouse and the *in vitro* growth environments are influenced by different environmental factors such relative humidity, light intensity and temperature (Gratani, 2014). These environmental factors give cues for the differences observed in the plants that are grown in those environments.

In this research, there were differences in the leaves of avocado 'Edranol' that were obtained from avocado shoots harvested from the greenhouse and *in vitro*. These included cell size, leaf thickness, number of chloroplasts per unit area and the presence or absence of bundle sheath extension in the leaves (details are presented in Chapter Four). Some of the differences observed were similar to those observed by other authors e.g. Noé and Bonini, (1996), Cozza *et al*. (1997) and Chirinéa *et al*. (2012); some were not.

Beyond the anatomical and morphological differences observed in avocado cv. 'Edranol' leaves harvested from both growth conditions, the results obtained also showed a difference in their callogenic competence (Figure 3.2 and 3.3) in favour of the *in vitro* leaf explants. In the next chapter (Chapter Four), the progression of cellular activities that culminated in callus formation suggested that *in vitro* leaf materials were less differentiated when compared with those obtained from the greenhouse. In addition to this, Gueye *et al*. (2009) posited that the

degree of tissue differentiation in plants has a reverse relationship on the callogenic competence of the tissues. Hence, the less differentiated tissues of the *in vitro*-derived leaf has more capacity for callus formation than the greenhouse-derived leaf (Figure 3.2 and 3.3). This result however was different from that obtained in common daisy (*Bellis perennis* L) (Karakas and Turker, 2013).

3.4.1.1.6 Plant growth regulators

Plant growth regulators are the most important factors to consider for callogenesis (Stasinopoulos and Hangarter, 1990). Their absence in tissue culture media usually results in no callus formation or any obvious cellular activity in the explants (Kiong *et al*., 2007; Konate *et al*., 2013, Abidin & Metali, 2015 and Hesami *et al*., 2018). The interactions between auxins and cytokinins are mostly considered for callus induction (Gaspar *et al*., 1996; Machakova *et al*., 2008). Auxins are present endogenously in plants but usually these are not sufficient to induce callus from explants *in vitro*. According to Khodashenas *et al*. (2015), auxin inhibitors balance out the endogenous auxins resulting in zero net auxins available within the explants, thus necessitating the supply of exogenous auxins. Plant growth regulators are required in small concentrations and cells' response increases with increased concentration of PGRs until a saturating concentration is reached, beyond which the PGRs may no longer be beneficial but inhibitory to the cells (Srivastava, 2002; Behbahani *et al*. 2011).

Synthetic auxins such as 2,4-D, dicamba, MCPA, 2,4,5-T and picloram have a herbicidal effect on plants at high concentrations and are thus used commercially as herbicides (Srivastava, 2002; Machakova *et al*., 2008). This necessitated the use of auxins at low concentrations in this research. Various plants require a specific range of optimal concentrations for callus formation. Examples include *Barringtonia racemosa* (Behbahani *et al*., 2011) and Bambara groundnut (Konate *et al*., 2013). Some species may not respond to certain auxins e.g. sugarcane (Ho and Vasil, 1983). To induce callus in trees with broad leaves, George (1993) and Machakova *et al*. (2008) suggested the use of 2,4-D in the range of 1.0 – 3.3 mg/L, while a concentration range of 0.015 – 1.0 mg/L was suggested for picloram (George, 1993). This is because picloram is a stronger auxin than 2,4-D (Habibah *et al*., 2018).

For *P. americana*, 2,4-D favoured more callus formation than either picloram or NAA (Table 3.4, Table 3.5 and Table 3.9). The use of 4 – 8 mg/L 2,4-D, which was beyond the recommended range by Machakova *et al*. (2008), resulted in significantly reduced callus formation (Table 3.4). On the other hand, picloram within the suggested range (0.05 – 0.2 mg/L) was not optimal for callus formation (Table 3.5). The use of auxin alone, either 2,4-D (Table 3.4) or picloram (Table 3.5) did not result in callus formation. However, the addition of the cytokinin BAP in the 0.2 – 1.0 mg/L range enhanced the formation of callus (Table 3.4 and Table 3.5).

The highest, but not optimal, concentration of picloram used to induce callus from avocado leaf explant in this research was 0.2 mg/L. Meanwhile 0.1 mg/L of picloram was usually used, successfully, for the development of embryogenic cultures from immature zygotic embryos of avocado (Mooney and Van Staden, 1987; Pliego-Alfaro and Murashige, 1998; Cruz-Hernández *et al*., 1998; Witjaksono and Litz, 1999a&b; Efendi, 2003; Peran-Quesada *et al*., 2004; Litz *et al*., 2005a; Raharjo and Litz, 2005; Suarez *et al*., 2006; Raharjo *et al*., 2008; Márquez-Martín *et al*., 2011, 2012; Palomo-Ríos *et al*., 2012, 2013; Guzmán-García *et al*., 2013; Encina *et al*., 2014). Immature embryos are highly meristematic and have a relatively high amount of endogenous auxin, which is needed for their polar differentiation and growth (Carnes and Wright, 1988; Delbarre *et al*., 1996). Hence, a small quantity of exogenous auxin (such as 0.1 mg/L picloram for avocado) was sufficient for optimal embryogenic callus formation. Very young leaves also have a relatively high amount of endogenous auxin (Paque and Weijers, 2016) and meristematic tissues but not necessarily embryogenic. While picloram has been established as an auxin of choice for explanted avocado immature zygotic embryos, it was not optimal for leaf explants (Table 3.5).

Machakova *et al*. (2008) also recommended the addition of cytokinin to auxin in order to stimulate callus induction, particularly in explants from dicotyledonous plants. The mechanisms of the interactions between auxins and cytokinins have not yet been fully elucidated. However, Ikeuchi *et al*., (2013) gave some ideas of how they interact together to form callus. It is hypothesized that cytokinins stimulate the expression of cyclin while auxins suppress the expression of cyclin inhibitors. Cyclins help with the resumption of cell cycle during callogenesis (Ikeuchi *et al*., 2013). However, at certain concentrations or combinations

of different types of auxins and cytokinins, the actions of both PGRs can be antagonistic thus inhibiting their abilities to stimulate callus induction (George, 1993). Example is found in (Konate *et al*., 2013).

3.4.1.1.7 Relationship between auxins and nutrient media

In this research, the efficiency of auxins, in the order 2,4-D > NAA > Picloram, was promising in Gamborg's B5 (Table 3.4, Table 3.5 and Table 3.9). These results were similar to what was obtained in Bambara groundnut (Konate *et al*., 2013). However, these auxins were not as effective in stimulating callus formation when incorporated into MS nutrients. The ineffectiveness of MS in this study could be related to its high salt concentration. Such high salt concentration was reported to be responsible for the decrease in the concentration of the naturally-occurring auxin, IAA, in culture medium. While synthetic auxins, such as 2,4-D and NAA, are more stable in culture media than IAA (Nissen and Sutter, 1990), their effectiveness could have been inhibited by the concentrations of the basal nutrient components. The basis for the interaction between basal nutrient salts and auxins is not yet known. Nevertheless, in a review by Mir *et al*. (2010), various interactions between PGRs and nutrients were documented. Some of the interactions are stimulatory to plants' growth and development while others are inhibitory. These interactions affect the physiology, biochemical activities as well as the various growth parameters of the plants (Mir *et al*., 2010). Also, Best *et al*. (2014) showed the possibility of interaction between the active compounds of PGRs and growth medium constituents by using the inhibitors of brassinosteroid and gibberellic acid biosynthesis. In cauliflower, the interaction was not only between the PGRs and the basal nutrients but also the concentration of the sucrose that was included (Vandemoortele *et al*., 1999).

3.4.1.1.8 Comparison between 2,4-D and NAA

3.4.1.1.8.1 Contamination

Contamination is one of the most challenging obstacles to overcome in plant tissue culture (Leifert and Cassells, 2001), as it is responsible for most losses of plant tissues (Leifert *et al*., 1991). If properly conducted, a decontamination protocol will result in contamination-free tissue culture. However, it is almost impossible to eliminate all the microbes, as some would

persist in the tissue culture as latent contaminants. These arise from endophytic and sometimes systemic microbes, usually bacteria, which have been shown to locally colonize many plants both inter- and intracellularly (Cassells, 2012). Latent contaminants are dormant; not showing any visible growth on *in vitro* culture and are asymptomatic (Leifert and Cassells, 2001). They can, however, become pathogenic and virulent at a later time especially during handling, such as subculture, of *in vitro*-grown materials.

Almost all the explants obtained from *in vitro* stock plant were free of contamination (Table 3.10), i.e. without any visible growth or sign of contamination, for the period of six weeks in culture. The observed contamination could have arisen from the handling techniques of the explants or as a manifestation of latent contaminants. (Leifert and Cassells, 2001; Ray and Ali, 2017).

3.4.1.1.8.2 Browning

It was likely that two events were initiated simultaneously when avocado cv. 'Edranol' leaf explants were plated: callus initiation and browning (Table 3.10(ii) and 3.10(iii)). Both events were inversely related: there was reduced browning in explants with high callus induction and vice versa. Jones and Saxena (2013) supported the inverse correlation between browning and growth of tissues.

Enzymatic browning is caused by the accumulation and oxidation of phenolic compounds (Jones and Saxena 2013; Chuanjun *et al*., 2015). The production of phenolic compounds increases in wounded or stressed tissues such as cultured explants compared with normal growing plants. This results in the accumulation of toxic compounds such as phenol oxidases, and it can eventually lead to death (Jones and Saxena, 2013) especially in isolated tissue (Hiti-Bandaralage *et al*., 2017) such as leaf explants. Avocado tissues have abundant phytochemicals. Rahman *et al*. (2018) and Castro-Lopéz *et al*. (2019) detected 18 phenolics (e.g. flavonoids, saponin, tannin and steroid) in the extract of avocado leaves. The effect of polyphenoloxidases (PPO), (the enzymes responsible for the oxidation of phytochemicals such as phenols thus resulting in browning), is not only visible in avocado fruit pulp (Toledo and Aguirre, 2017) but also in the leaves. Not only PPO but also peroxidases and air can also oxidize phenolic compounds (Hiti-Bandaralage *et al*., 2017), resulting in browning. Auxins

have been implicated in promoting the production of Reactive Oxygen Species (ROS) (Smirnoff, 2005), but the mechanisms of this are as yet unclear. The type and concentration of PGRs as well as basal media nutrient composition can also impact tissue browning such as shown by Mansoor (2018); where the cytokinin BAP was believed to contribute to tissue browning in avocado.

In this study, explants in CIM5 (1.0 mg/L 2,4-D + 0.5 mg/L BAP on B5) showed the least browning by the sixth week in culture. On the other hand, explants in all the 8 media that contained various concentrations of NAA/BAP on MS had browning in the range of 14 – 90% by the sixth week (Table 3.10(ii)). This result suggested that NAA/BAP and/or MS promoted explant browning more than 2,4-D/BAP and/or B5. However, since callus induction and browning were initiated at the same time, callus initiation/formation progressed faster in the explants that were in the 2,4-D/BAP and/or B5 medium; thus masking the extent of browning. On the other hand, on the NAA/BAP and/or MS, browning progressed faster than callus initiation.

3.4.1.1.8.3 Callusing

It was not unexpected that 2,4-D stimulated more callus formation than NAA (Table 3.10(iii)); as 2,4-D had been long shown to be ten times a stronger auxin than NAA (Overbeek *et al*., 1951). There are many instances where 2,4-D has initiated more callus formation than NAA (e.g. in Ali *et al*., 2007b; Naz and Khatoon, 2014). Also, it was postulated by Delbarre *et al*. (1996) that the uptake and transportation of NAA was mainly through diffusion while 2,4-D was transported by both diffusion and influx carriers. This leads to an increased accumulation of 2,4-D than NAA in the cells (Delbarre *et al*., 1996). In addition, the metabolism of NAA is very rapid when compared with 2,4-D, which does not readily disintegrate when absorbed (Hošek *et al*., 2012; Naz and Khatoon, 2014). The activities of peroxidase isozymes have also been linked with the degradation of auxins in the root formation of avocado microcuttings (García-Gómez *et al*., 1995). In this regard, NAA, being a 'weaker' auxin, may be impacted more by this degradation than 2,4-D. Use of MS as a basal medium might have promoted the production of phenol in the NAA/BAP media, thus contributing to browning in those media (see section 3.4.1.1.3). In the same way Osorio *et al*. (2018) showed an increase in phenol accumulation in explants cultured on MS when compared with those cultured on WPM.

It is generally believed that 2,4-D is more suitable for callus induction (evinced in Table 3.10(iii)), while NAA is appropriate for morphogenesis (Gaspar *et al*., 1996; Hesami *et al*., 2018). In this research, callus growth on 2,4-D was rapid and had reached 90% callus formation by the sixth week. On the other hand, NAA with the same concentration as 2,4-D had only 12%, but other NAA/BAP concentrations had as high as 78% (Figure 3.10(iii)). Nevertheless, the 2,4-D enriched medium could not sustain the growth of the callus by the sixth week as some calli were browning and dying off by the sixth week (Figure 3.5a). This phenomenon was not observed in the NAA-induced callus (Figure 3.5b). Naz and Khatoon (2014) correlated rapid and vigorous callus growth with poor regenerative competence.

2,4-D does not only function as an auxin but as a stress chemical as well (Zavattieri *et al*., 2010). Thus, the inability of sustained callus growth in a 2,4-D enriched medium could be associated with stress induced by the auxin, which is known to have a detrimental effect as herbicide (Kiong *et al*., 2007). Aging, coupled with browning, reduces the morphogenic ability of the affected callus (Shibli *et al*., 2001; Mukhopadhyay *et al*., 2005; Kaewubon *et al*., 2015) and the callus may eventually die as observed in this research. As a recommendation for subsequent study, callus developed on a 2,4-D-enriched medium should not be maintained on induction medium for too long; rather it should subcultured to a fresh medium, before the onset of browning and directed towards further development. Mukhopadhyay *et al*. (2005) and other authors such as Hesami *et al*. (2018) and Sofiari *et al*. (1997), successfully initiated callus with 2,4-D and subsequently stimulated root and shoot development with NAA. On the other hand, Pliego-Alfaro and Murashige (1988) were able to generate callus rapidly with a relatively high concentration (10 mg/L) of 2,4-D using immature zygotic embryos of avocado as explants. However, there was no development towards differentiation after subsequent subcultures to different media. Browning/necrosis of the callus derived from 2,4-D was also seen in *Melaleuca alternifolia* (Kiong *et al*., 2007) and *Artemisia annua* (Yann *et al*., 2012). In *Theobroma cacao*, 2,4-D-derived callus necrotized after formation but NAA-derived callus did not brown (Novak *et al*., 1985). It is crucial to note, however, that callus browning does not necessarily mean loss of viability. This was shown in the calli of *Globularia trichosantha*, which started off as yellow but turned brown after a few weeks but were still able to divide and grow new calli (Çölgeçen *et al*., 2018).

Explants in an induction medium may give rise to different types of callus (Table 3.11). All the nine media tested and represented resulted in heterogeneous calli of different sizes, colours, textures and arrangement (Table 3.11). These variations are possible because callus cells are not formed from a single cell but from tissues whose cells may not be homogenous but rather are made up of different types of cells (Efferth, 2019). Formation of different forms of callus has also been associated with different compositions of the various induction media used (Sangra *et al*., 2019). The heterogenic nature of the callus formed can go beyond the morphology to physiological, structural and genetic differences (Mukhopadhyay *et al*., 2005). The various types of calli were majorly formed around the cut edges, at the end of prominent veins and sometimes on the lamina (Table 3.11). These were the sites, commonly reported by researchers, for callus initiation (Keng *et al*., 2008; Karami and Ostad-Ahmadi, 2008; *Ojha et* al., 2012; Yann *et al*., 2012; Naz and Khatoon, 2014).

Calli obtained from the 'Edranol' avocado leaf explants were a mixture of both isodiametric and elongated cells (Figure 3.6). The former is usually considered as one of the characteristics of embryogenic callus while elongated, cylindrical cells are associated with non-embryogenic calli (Finer, 1994; Srivastava, 2002; Razdan, 2003; Karami and Ostad-Ahmadi, 2008). However, Ribeiro *et al*. (2012) did not support this consideration. Those authors recorded callus cells that were isodiametric but cytological observation confirmed them non-embryonic. Thus, beyond the callus cell shapes, the most important factor to consider for callus embryogenic competence, among other things, is a functional nucleus (Srivastava, 2002); without which the callus will only be meristematic, at best.

3.4.1.2 'Fuerte' cultivar

In the first round of experiments, the 45 auxin/cytokinin combinations in either MS or B5 that resulted in callus formation in the 'Edranol' cultivar were not favourable for the 'Fuerte' cultivar. In the second round of experiments carried out to compare explant sizes, both explant sizes tested produced callus on some of the media used (Table 3.13). In the 'Edranol' cultivar, small explants produced more callus than big explants (section 3.4.1.1.4) but the reverse was the case with the 'Fuerte'. When 2,4-D was compared with NAA, the results obtained were also similar to those obtained in 'Edranol' in that 2,4-D was preferred over NAA for callus induction (Figure 3.7). While the same media compositions (CIM1 – CIM9) were used to test

the effect of explant size on callus formation in both the 'Fuerte' and the 'Edranol' cultivars, the response of the 'Fuerte' cultivar in those media was significantly lower than 'Edranol' (Table 3.13). Different cultivars or genotypes of the same species have been shown to respond differently to the same induction environment. Examples of such differences were seen in cocoa (Li *et al*., 1998), garlic (Martín-Urdíroz *et al*., 2004), lily (Mori *et al*., 2005), tobacco (Ali *et al*., 2007a), wheat (Jasdeep *et al*., 2019) and many more. It thus becomes imperative that conditions be optimized for each cultivar in order to obtain the best result.

Callus cells that died off after formation may have insufficient accumulation of biofuels, such as starch grains, required to supply energy for continuous cell division and growth (Carvalho *et al*., 2013). Such may be the case of the distinct white, fluffy and nonviable callus that was produced by the *in vitro*-derived explants of 'Fuerte' across the media tested (Figure 3.8). The enlargement of the leaf explants in culture, as displayed by those of *in vitro* 'Fuerte', was also recorded for the *in vitro* leaves of *Fraxinus nigra* (Lee and Pijut, 2017), *Tectona randis* (Karunaratne *et al*., 2014), *Artemisia annua* (Yann *et al*., 2012), *Jatropha curcas* (Mazumdar *et al*., 2010) and *Vitex negundo* (Jawahar *et al*., 2008). This phenomenon is because the *in vitro* leaves are still growing towards the expansion of their lamina.

3.4.1.3 'Hass' cultivar

The use of the same induction media (CIM1 – CIM9) that favoured callus formation in the 'Edranol' cultivar was not favourable for the 'Hass' cultivar. 2,4-D in the 0.5 – 2.0 mg/L plus BAP in the 0.2 – 1.0 mg/L range were not optimal for callus induction in the 'Hass' cultivar (Table 3.14).

The fact that explants were sourced *in vitro* did not preclude them from contamination (Table 3.14). Of all the three cultivars tested, browning was minimal in the 'Hass'. The problem of contamination and its sources as well as explant browning have been extensively discussed in section 3.4.1.1.8. Although all media stimulated callus formation, the callus formed contained extremely tiny globules that lacked vigour and eventually died. This implied that the callus was non-embryogenic and lacked the necessary reserves and cellular components that were indispensable for callus growth (Carvalho *et al*., 2013).

The ‘Hass’ cultivar of *P. americana* is the most prevalent avocado cultivar worldwide, accounting for up to 95% of commercially produced avocado fruits on the international market (Pedreschi *et al*., 2019). Thus, an optimal method for its micropropagation through the indirect pathway will be of immense benefit. A follow up study to achieve this goal is therefore imperative.

3.4.2 Callus proliferation

The subculture of callus to fresh induction medium is aimed at maintaining and increasing the growth of callus (Nakasha *et al*., 2016). However, the period and frequency of subculture need to be optimized. This is because prolonged un-subcultured callus may eventually die and over-subcultured callus may lose its embryogenic competence (Razdan, 2003; Nakasha *et al*., 2016). The callus of *Oncidium* was, however, successfully subcultured for two years without loss of its regenerative competence (Chen and Chang, 2000). Keng *et al*. (2008) suggested that subculturing to fresh induction media should be carried out before the callus starts to brown; and it has been recommended that subculture be carried out within 4 – 6 weeks after induction (Nakasha *et al*., 2016).

In *P. americana*, subculture was carried out after five weeks (Table 3.15) and eight weeks (Table 3.16). Calli obtained from different induction media did not all thrive when subcultured. Callus obtained from some media e.g. CIM20, increased in size while callus from other media, such as CIM5 or CIM15, turned brown and did not survive after subculture (Table 3.15).

Keng *et al*. (2008) attribute callus death, after subculture, to the components of the initial induction medium. Factors that contribute to cessation of callus growth and/or death are accumulation of toxic compounds, depletion of nutrients, insufficient oxygen and metabolites from the tissues (Yuan *et al*., 2012). Coversely, there are other contributors to subculture survival such as the genotype and the cultivar of the source plant (Minhas *et al*., 1999) and composition of the induction medium (Sangra *et al*., 2019). Some callus responded positively to subculture e.g. *Melastoma malabathricum* (Keng *et al*., 2008), *Artemisia annua* (Yann *et al*., 2012) and *Chlorophytum borivilianum* (Nakasha *et al*., 2016). On the other hand, the response of the callus of *Corylus avellana* was dependent on the methods of subculture. *C. avellana* callus subcultured on a solid medium turned brown and died after first subculture

while increased viability and reduced browning was noticed in those subcultured to liquid medium (Rahpeyma *et al*., 2017). Subculture was believed to stimulate increased browning in *Camellia sinensis*, hence the callus were not subcultured (Seran *et al*., 2007).

Avocado callus derived from 2,4-D and/or B5 (e.g. CIM5) were not amenable to subculturing to fresh induction medium of the same composition (Table 3.15 and Table 3.16); whereas extended period at the induction phase was beneficial (Table 3.16). The observations in these two experiments (Table 3.15 and 3.16) containing CIM5 were conflicting. In the first experiment, 90% of callus was formed on CIM5 and they had started to brown by the sixth week (Table 3.15 and Figure 3.5), which was an indication of the need for subculture. On the other hand, in the second experiment, 46.06% and 49.83% of calli were formed on the same medium (CIM5) after six and eight weeks, respectively. Prolonging the induction phase to eight weeks without subculture resulted in more explants forming callus, without obvious signs of browning (Table 3.16). The plausible cause of the conflicted response could be linked to the different times of harvest of the leaf explants used, which consequently could impact the maturity as well as the physiological state of the explants. It does become imperative to consider the state of the explants that are harvested at a point in time, and their responses to callusing. The behaviour of the callus after formation should then serve as a guideline to determine when the callus must be transferred. Browning of callus, however, does not always equal cell death. Some browned calli are still meristematic, thus giving rise to new cell aggregates. An example was the callus obtained in CIM24 (Table 3.15). A similar result was seen for *Glolularia trichosantha* (Çölgeçen *et al*., 2018).

3.4.3 Callus development and maturation of globular embryo-like structures

In the order of somatic embryo development, the differentiation of the callus to form somatic embryos succeeds callus induction. The changes from induction to developmental media, used in this research, included reduced or no auxin (Razdan, 2003), increased cytokinin (Razdan, 2003), increased gelling agent (Encina *et al*., 2014), increased sucrose (Ho and Vasil, 1983; Encina *et al*., 2014), inclusion of activated charcoal (Ho and Vasil, 1983) and coconut water/milk (Ho and Vasil, 1983; Encina *et al*., 2014).

None of the callus responded to the developmental media that contained increased sucrose concentration (ii) and those that contained coconut water (x) and (xi). NAA-induced callus

turned green in PGR-free medium (i) (Table 3.17 and Figure 3.9). George (1993) gave a plausible explanation for the 'greening', that such calli may originate from cells/tissues that contain chloroplasts such as the leaves, which was the explant used to generate calli in this research. In addition, light incubation contributed to colour change in *Oncidium* (Chen and Chang, 2000). Thus these factors could both be responsible for the green colouration of calli observed in this research.

While NAA-supplemented media may not be optimal for callus induction as seen in this research, NAA-based calli were more responsive to developmental conditions than calli that were of 2,4-D origin. In the same vein, although NAA is considered a weaker auxin, it is often considered as a more suitable auxin for further callus development (Baker and Wetzstein, 1994). Somatic embryo formation was optimum in NAA-supplemented medium in *Oncidium* (Chen and Chang, 2000) and *Muscari armeniacum* (Suzuki and Nakano, 2001), while it only enhanced some rooting in the calli of *Cleome rosea* (Simões *et al*., 2010). The source of the callus with respect to explant types also influenced differentiation and embryo formation (Chen and Chang, 2000).

The calli that formed embryos (Figure 3.10) were incubated without subculture for eight weeks each, at induction and developmental phases. There was a distinct morphological change seen in the calli (Figure 3.10). The morphology of the somatic embryos and attachment was similar to the somatic embryo/callus attachment of *Cleome rosea* (Simões *et al*., 2010). However, the number of explant-bearing callus that ultimately developed to this stage was very negligible. Callus induction utilized 800 explants (Table 3.16) and only two explants with sustained callus growth developed until the globular embryo stage (Figure 3.10).

For unsubcultured callus, Seran *et al*. (2007) recommended the transfer of the callus to a differentiation medium before the sixteenth week of callus induction. A prolonged induction phase without subculture causes the amount of available auxin to decline and thus stimulates the callus towards differentiation (Ho and Vasil, 1983). Short induction period in the sub-tropical date palm resulted in non-embryogenic callus that did not survive the developmental phase; this was reversed when the induction phase was prolonged (Mazri *et al*., 2017). The differentiation of callus is further promoted when it is transferred to a developmental medium with reduced or no auxin such as in *Cleome rosea* (Simões *et al*., 2010), because little or no

auxin promoted somatic embryo formation (Simões *et al*., 2010). However, similar to the negligible proportion of callus that formed somatic embryos in this research, the calli from the leaf of *Cleome rosea* were not embryogenic and hence did not form somatic embryos (Simões *et al*., 2010).

All attempts to mature and germinate the somatic embryos were futile in this research, even though the medium contained components that can increase osmotic stress. These included increased concentration of sucrose and gelling agent as well as abscisic acid. Osmotic stress is expected to gear the metabolism of the embryos towards the synthesis of biomolecules, such as proteins, which are needed for germination (Simões *et al*., 2010). The conversion of somatic embryos to plantlets is still elusive in some species. Pliego-Alfaro and Murashige (1988) could generate callus from avocado immature zygotic embryos using a relatively high concentration of 2,4-D. However, those calli could not differentiate to any distinct structure beyond induction. Failure to mature and germinate could be a result of defects in the physiological and morphological development of the somatic embryos (Simões *et al*., 2010). Those authors also suggested insufficient accumulation of storage reserve as the cause for the failure recorded.

While this step seems elusive presently, future research will be aimed at optimizing the medium that will enhance successful conversion of somatic embryo to plantlet.

CHAPTER FOUR: MORPHO-HISTOLOGICAL OBSERVATION OF CALLUS FORMATION FROM THE LEAVES OF AVOCADO CV. 'EDRANOL'

4.1 Introduction.

In theory, cellular totipotency is the latent ability of any plant cell to regenerate into a whole plant (Fehér, 2019). This involves a series of regulated changes in the biochemical, metabolic, histological, and morphological mechanisms of the plants. In this aspect, the formation of somatic embryos is the highest demonstration of cellular totipotency (Gutiérrez-Mora *et al*., 2012; Bhojwani and Dantu, 2013). Due to cellular totipotency, a differentiated/specialized and sometimes mature cell can revert and become dedifferentiated i.e. through cell division from an undifferentiated and meristematic cell (Bhojwani and Razdan, 1996; Kurczynska *et al*., 2012; Bhojwani and Dantu, 2013), provided there are functional nuclear and membrane systems (Bhojwani and Razdan, 1996). This is possible because all living, differentiated and specialized plant cells retain their plasticity during development i.e. the ability of a cell to acquire a new fate and change to another type of cell (Grafi, 2004; Bhojwani and Dantu, 2013).

The dedifferentiated and meristematic cells can subsequently be directed towards organogenesis, somatic embryogenesis or result in an intermediary phase of undifferentiated, meristematic callus cells (Kurczynska *et al*., 2012; Bhojwani and Dantu, 2013). Examples of dedifferentiation/redifferentiation of differentiated cells were seen in the following: the epidermal and mesophyll cells of *Taxus baccata* leaves produced callus (Monacelli *et al*., 1995), the midrib of the leaf of the medicinal plant, *Cichorium intybus* produced callus (Dakshayini *et al*., 2016), callus production occurred in the stem of *Ranunculus sceleratus* (Konar *et al*., 1972), somatic embryos developed from the epidermal cells of pineapple guava (Canhoto and Cruz, 1996), among others. The developmental path towards organogenesis can be distinguished from somatic embryogenesis through vascular connection and polarity. In somatic embryogenesis, bipolar structures are usually formed, which often lack a vascular connection with the explanted tissues. Hence, the somatic embryos can be removed from the explanted tissues without any injury. On the other hand, unipolar structures are usually formed in organogenesis and there is a vascular continuum between the newly-formed organ and the explanted tissue (Bhojwani and Dantu, 2013; Kurczynska *et al*., 2012).

One of the markers of transition from differentiated somatic cells to dedifferentiated/redifferentiated cells, is cell division; which can be periclinal or anticlinal.

Periclinal cell division is considered to produce embryogenic cells (Kurczynska *et al*., 2012). Divided cells can be meristematic and/or embryogenic and non-embryogenic (Kurczynska *et al*., 2012). While it is easier to distinguish between non-embryogenic and embryogenic cells, it is challenging to truly decipher the distinction between meristematic and embryogenic cells. This is because all embryogenic cells are meristematic, thus embryogenic cells possess all the characteristics of meristematic cells such as being small, spherical cells with dense cytoplasm, a prominent nucleus and small vacuoles (Kurczynska *et al*., 2012). However, Verdeil *et al*. (2007) used the structure of the nucleus/nucleolus to differentiate between meristematic and embryogenic cells. According to those authors, the meristematic cells of shoot meristems possess a spherical nucleus with evenly distributed multi-nucleoli, while an irregular nucleus with one prominent nucleolus is characteristic of embryogenic cells (Verdeil *et al*., 2007). Canhoto and Cruz (1996) also used the presence of increased vacuolation to differentiate meristematic from embryogenic cells. In addition, callose deposition and high starch contents are features of embryogenic callus (Mazri *et al*., 2013).

Callose (β-1,3-glucan), which is one of the markers of embryogenic competence (Mazri *et al*., 2013), is one of the polysaccharides components of plant cell walls (Dashek, 2000a; Piršelová and Matušiková, 2013). It is often produced during developmental stages thus its presence can be temporal in cells.

In this section, a comparative study was conducted on the ontogeny of callus, between the leaf explants of *Persea americana* cv. ‘Edranol’; sourced from two different growth conditions: the greenhouse and *in vitro*. Growth conditions, under the influence of different environmental factors, trigger the expression of phenotypic plasticity in plants. The expression can be morphological, biochemical, anatomical and physiological (Gratani, 2014). Cellular components of the fresh leaf tissues from both sources were measured. In culture, weekly histological observations on the cellular activities within the leaf tissues and the types of callus cells were recorded.

4.2 Materials and Methods

4.2.1 Explants and callus formation

Leaf explants were obtained from the seedlings/shoots of *Persea americana* cv. ‘Edranol’ that were maintained in the greenhouse and from those that were grown *in vitro* (Chapter Two).

Explants from the greenhouse were decontaminated and cultured in Petri dishes for callus formation as described in Chapter Three (section 3.2.1). Leaf explants sourced from *in vitro* required no prior decontamination before they were cultured. The leaf explants were cultured on CIM5 (i.e. Gamborg's B5 supplemented with 1.0 mg/L 2,4-D and 0.5 mg/L BAP). In addition, the explants from the greenhouse were also cultured on 1.0 mg/L NAA with 2.0 mg/L BAP incorporated into Gamborg's B5. Cultures were maintained for six weeks. Fresh leaves obtained from both the greenhouse and the *in vitro* shoots were also prepared for microscopy without prior culture.

4.2.2 Light microscopy

Leaf samples (ca. 2 mm by 2 mm) from both growth conditions and representing fresh and materials from each week of culture were prepared for microscopy. Samples were fixed in 2% glutaraldehyde in 0.05 M sodium phosphate buffer for eight hours at 25°C. Thereafter, the glutaraldehyde was removed and the samples were rinsed four times with the buffer. The samples were post-fixed in 2% osmium tetraoxide for two hours, after which they were rinsed with the buffer. Primary dehydration of the sample was carried out in 10%, 25% and 50% ethanol concentrations for 15 min each. The fixed samples were then stained with 1% uranyl acetate in 75% ethanol for one hour. Secondary dehydration was carried out through 75%, 100% ethanol and propylene oxide. The samples were then embedded in Spurr's epoxy resin (Spurr, 1969) and allowed to polymerize overnight at 70°C (details in appendix). Thin sections (1.0 – 1.5 µm) of fixed leaf samples were cut using a Reichert-Jung ultramicrotome. The sections were mounted on glass slides with drops of water, which was later evaporated on a hot plate. The samples were stained with toluidine blue, washed off three times with distilled water and heat-dried. The samples were mounted in DPX and were covered with glass cover slips. Sections were subsequently viewed with a light microscope (Zeiss Olympus BX63 OM/FM) using Bright Field (BF) optics. Images were captured with an attached Olympus DP 80 camera (Olympus, Japan). ImageJ software (free downloadable application) was used to isolate and analyze chloroplasts in the leaf sections obtained from both the greenhouse and *in vitro* (Figure 4.1).

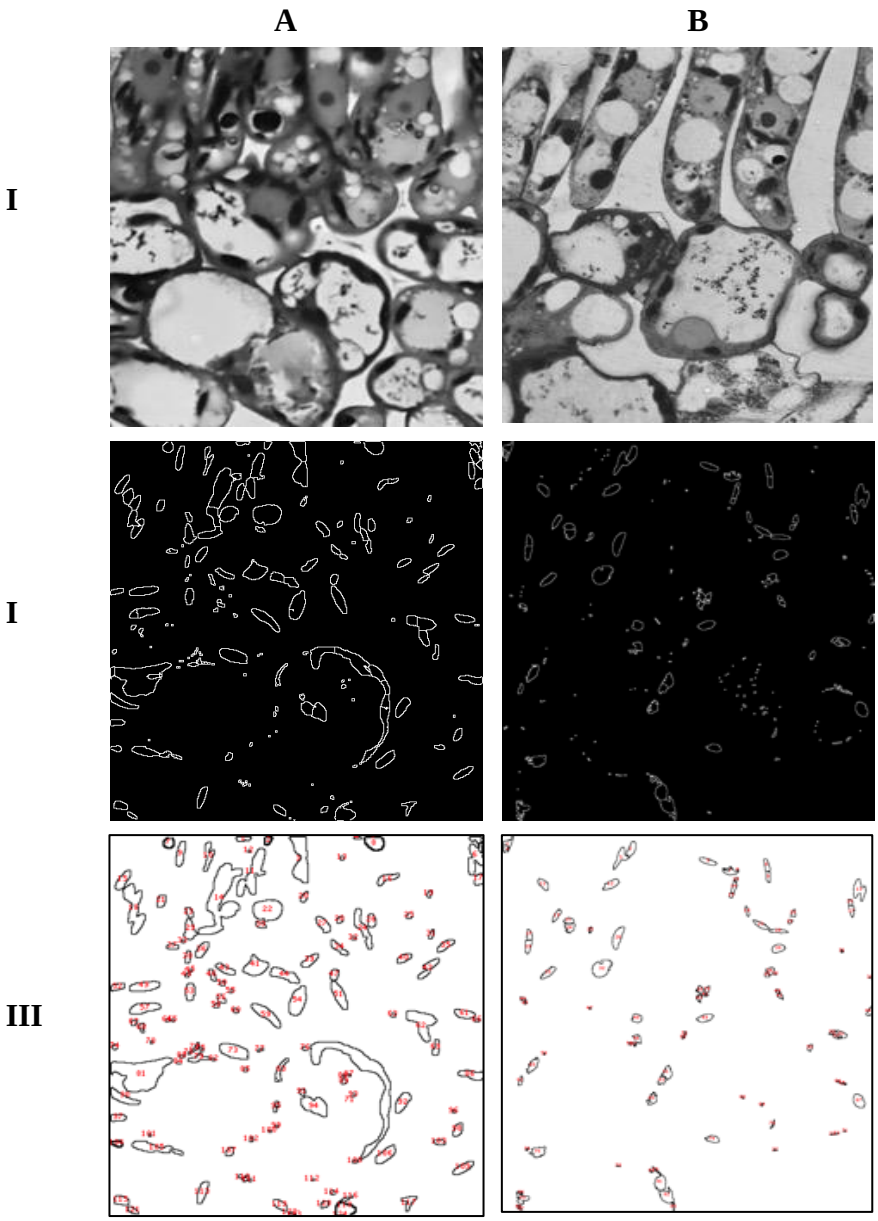


Figure 4.1: Use of ImageJ software to isolate and analyze chloroplasts in the leaf sections of avocado obtained from the greenhouse (column A) and *in vitro* (column B). Row I: portion of leaf sections to be analyzed but first, has to be changed to 8-bit image type. Row II: threshold was adjusted to isolate the chloroplasts, which were subsequently outlined. Row III: outlined chloroplasts were counted and numbered accordingly.

4.2.3 Fluorescence microscopy

Water-soluble aniline blue stain was used to detect callose deposition in callus cell walls (Dashek, 2000b). Where callose was present, it fluoresced as yellow-green (Śnieżko, 2000).

Fresh calli were soaked in aniline blue stain (Herburger and Holzinger, 2016) (Appendix) for 2 – 3 hours. The stain was rinsed off three times (15 minutes interval) with distilled water. In the dark, a small piece of callus was crushed in a drop of water on a glass slide and thereafter covered with a glass cover slip. The callus cells were viewed with a 430 nm UV light using the microscope described above. Images were captured as described above.

4.2.4 Data and statistical analysis

Where measurement of cellular components of leaves was conducted, t-Tests for Paired Two Samples for Means were used to ascertain significant difference. All tests were carried at *0.05* significant level and were carried out using Data Analysis ToolPak add-ins on Microsoft Excel.

4.3 Results

4.3.1 Fresh leaves obtained from the greenhouse and *in vitro* plants

The tissues that made up avocado leaf, either from the greenhouse or *in vitro*, were arranged in the order of upper epidermis, palisade mesophyll, spongy mesophyll with associated vascular bundles and the lower epidermis (Figure 4.2 & Figure 4.3). The cells of the fresh leaves from both sources were highly vacuolated and appeared to be devoid of intracellular components at the light microscope level (apart from the chlorophlasts that stained green in the greenhouse material (Figure 4.2) and appeared as heavily stained dark blue organelles in the *in vitro* material (Figure 4.3)).

4.3.1.1 The epidermis

The epidermal layers of *Persea americana* leaves were uniseriate i.e. single-layered and were characterized with various cellular shapes (Figure 4.2 and Figure 4.3). The upper and lower epidermal layers of greenhouse-derived leaves were made up of interlocked thin, elongated and concave cells thus giving the layer an undulating contour (Figure 4.2). The stomata were seen on the abaxial side of the leaves (i.e. hypostomatic) and were characterized by substomatal cavities (Figure 4.2).

On the other hand, the epidermis of the *in vitro*-derived leaf comprised cells of different sizes and shapes that ranged from round, isodiametric to elongated cells. The cells had smooth cell walls and were tightly packed, giving the epidermis a less contoured outline (Figure 4.3).

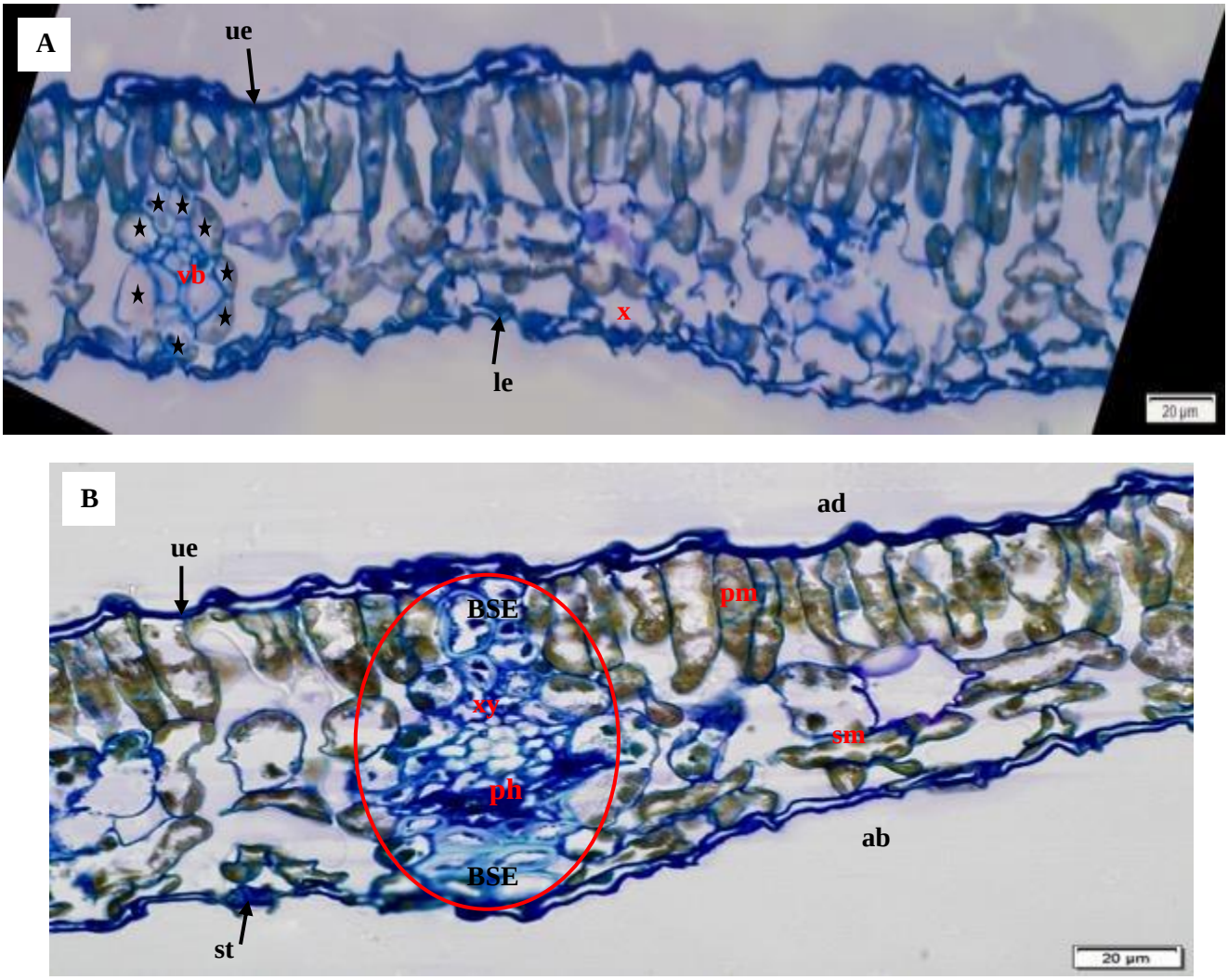


Figure 4.2: Cross section of fresh avocado leaves obtained from the shoots of the greenhouse-maintained material; observed using a Bright Field (BF) optics. A: leaf section showing vascular bundle (vb) enclosed with bundle sheath (marked with black stars). The lower (le) and upper (ue) epidermis had thick cell walls seen as deep blue stain. B: the vascular bundle (enclosed in red circle) extended to form Bundle Sheath Extension (BSE). *x* indicated substomatal cavity; *st* stoma with two guard cells; *ad* adaxial surface; *ab* abaxial surface; *pm* palisade mesophyll; *sm* spongy mesophyll.

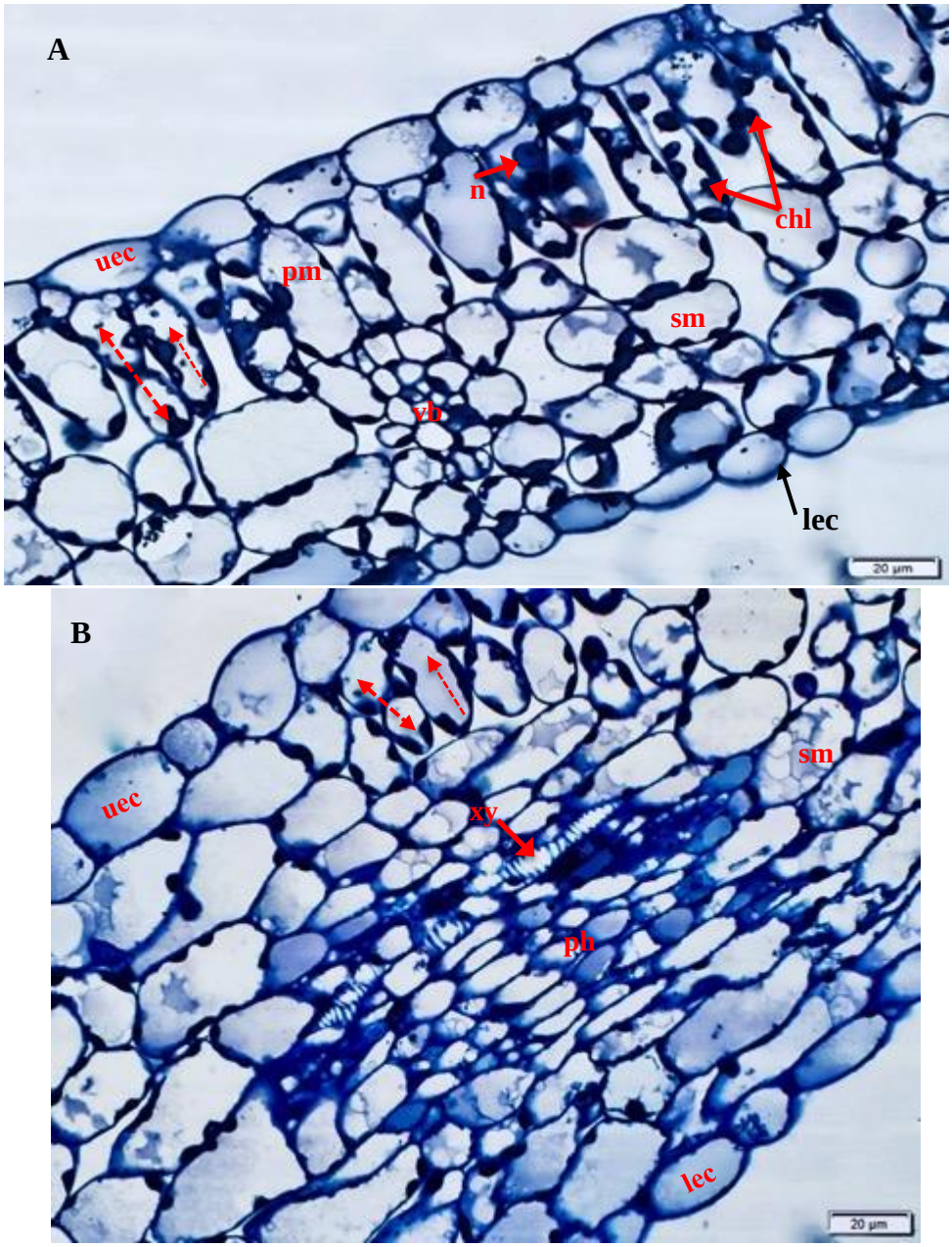


Figure 4.3: Cross section of fresh avocado leaves obtained from the material grown *in vitro*. Cells contained large central vacuoles, which confined the organelles – chloroplast (chl) and nucleus (n) – to the periphery of the cells. A: vascular bundle (vb) was shown in transverse section; while in the lateral view (B), the xylem was visible. The palisade mesophyll (pm) cells were either in a single layer (single arrowhead with broken line) or double (double arrowhead with a broken line) layers. *uec* upper epidermal cells; *lec* lower epidermal cells; *sm* spongy mesophyll.

4.3.1.2 The mesophyll

The photosynthetic layers, i.e. the mesophylls, of avocado leaves from both the greenhouse and *in vitro* shoots were distinctively separated into palisade and spongy mesophylls; with the palisade mesophyll towards the adaxial surface and the latter towards the abaxial surface (Figure 4.2 & Figure 4.3). The palisade mesophyll was a single layer of cells in the greenhouse-derived materials (Figure 4.2) whereas it was a mixture of single (red, broken and single arrowhead) and double (red, broken and double arrowheads) layers in the *in vitro* materials (Figure 4.3). The spongy mesophyll of the greenhouse-derived leaf comprised irregular-shaped cells that were randomly arranged and interspersed with intercellular air spaces (Figure 4.2). In the *in vitro*-derived leaf, the spongy mesophyll consisted of three to four layers of isodiametric cells of different sizes; these were arranged in a fairly tight and orderly manner (Figure 4.3) and separated by fewer intercellular air spaces.

There were significantly more chloroplasts in the mesophylls of greenhouse material, which, consequently, occupied more space in the leaf than those of the *in vitro* materials. However, the surface area of individual chloroplasts from both the greenhouse or *in vitro* was similar (Table 4.1).

Table 4.1: Comparison of the distribution and size of chloroplasts from the leaves of avocado derived from the greenhouse and *in vitro*.

	Number of chloroplasts/100 um^2	Surface area of chloroplast (um^2)	Total area occupied by chloroplast (%)
Greenhouse	3 ± 2^a	5.16 ± 0.98^a	8.0 ± 2.8^a
In vitro	1 ± 0.8^b	4.79 ± 2.33^a	4.4 ± 1.7^a

Different superscript within a column indicated significant difference.

4.3.1.3 Vascular bundles

Less prominent, higher order veins (vascular bundles) were enclosed with bundle sheaths in both the greenhouse and the *in vitro* leaves (Figure 4.2A and Figure 4.3A). Additionally, in the greenhouse-derived leaves, the bundle sheath of lower order, prominent veins (but not midrib) extended to the upper and lower epidermal layers, hence there were no mesophyll cells above/below the vascular bundles (Figure 4.2B). This extension is referred to as Bundle sheath extension (BSE). Cells of BSE were made up of thick-walled sclerenchyma cells. The arrangement of the components of the vascular bundles, in both the greenhouse and the *in vitro* materials, was in the general order of xylem towards the adaxial surface (Figure 4.3B)

and the phloem beneath the xylem. These tissues were both enclosed by a ring of bundle sheath cells (Figure 4.2 and Figure 4.3).

4.3.1.4 Leaf sizes

In almost all the leaf tissues, except for cell length in both the upper and lower epidermis, the cells from *in vitro* leaf tissues were significantly bigger than the cells from the greenhouse tissues.

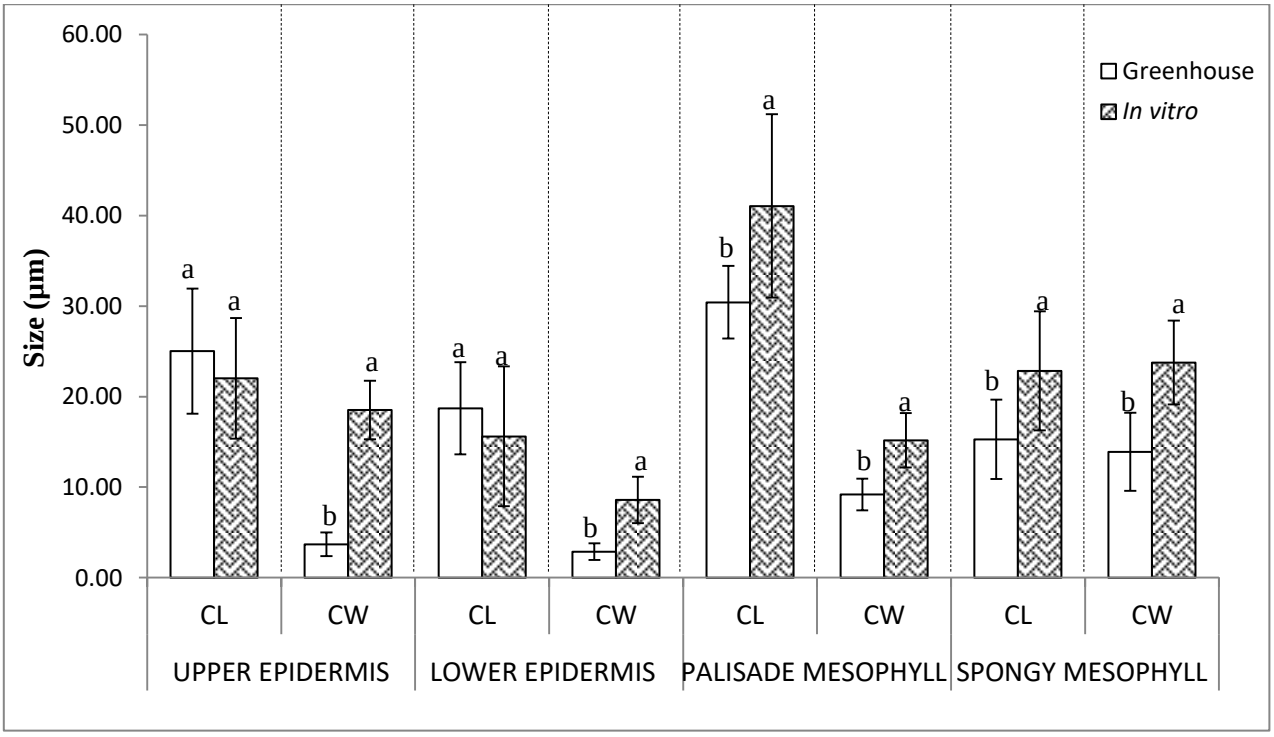


Figure 4.4: The sizes (CL – Cell length; CW – Cell width) of the different fresh leaf tissues derived from shoots obtained from the greenhouse and the in vitro environment. Different letters within a cell group denoted significant difference.

The *in vitro*-derived leaves had longer palisade and wider spongy mesophyll cells (Figure 4.4), with the latter further arranged in stacks of 3-4 cells (Figure 4.3). Both the upper and lower epidermal layers of the leaves from the greenhouse were significantly thinner than those obtained from the *in vitro* epidermal tissues (Figure 4.4).

Thus, the orderly arrangement of these tissues resulted in leaves obtained from the *in vitro*-grown shoots being significantly thicker (171.19 ± 21.55) than the leaves obtained from the greenhouse (75.98 ± 8.6). However, the epidermal cells of greenhouse leaves had relatively

thicker cell walls (deep blue stain, Figure 4.2) in comparison with the epidermal cell walls of *in vitro* leaves.

4.3.2 Responses of leaf materials cultured on callus induction medium

4.3.2.1 One week from induction

After one week in callus induction medium, cells of the leaf tissues had increased in sizs, especially in the greenhouse leaves where the increase in size was significant (Table 4.2).

Table 4.2: Comparison between the size of different cell types of fresh avocado leaves and those incubated for one week in callus induction medium (n = 20**).**

Dimension of Cell Types	Size (µm)			
	Greenhouse		*In vitro*	
	Fresh	One week	Fresh	One week
L of palisade cells	30.43 ± 4.02[a]	59.46 ± 6.39[b]	41.07 ± 10.14[a]	43.68 ± 6.33[a]
W of palisade cells	9.19 ± 1.76[a]	11.41 ± 1.82[b]	15.18 ± 3.02[a]	11.47 ± 1.67[b]
L of spongy cells	15.29 ± 4.38[a]	23.66 ± 4.96[b]	22.84 ± 6.58[a]	26.56 ± 6.10[a]
W of spongy cells	13.91 ± 4.30[a]	18.36 ± 3.42[b]	23.77 ± 4.62[a]	20.10 ± 4.39[a]
L of epidermal cells	27.69 ± 6.82[a]	21.22 ± 4.63[a]	22.52 ± 2.54[a]	24.93 ± 6.10[a]
W of epidermal cells	3.02 ± 1.06[a]	17.87 ± 1.25[b]	19.09 ± 2.41[a]	16.39 ± 1.98[a]

One-way ANOVA was used to ascertain significant difference at *0.05* significant level between fresh and one-week incubation for each cell type from each of the two sources. Different superscripts indicate significant differences. L – length; W – width.

There were visible changes in both greenhouse- and *in vitro*-derived leaves. These included (i) vacuolation of the cells, i.e. the singular, large vacuoles of the fresh leaves were fragmented to 2-4 smaller vacuoles per cell. (ii) Chloroplasts and nuclei were more discernable in the cells. The nucleoli in the nuclei were prominent (Figure 4.5). (iii) Phloem cells in the vascular bundles of the leaf materials were of meristematic nature because the cells were small and isodiametric, with relatively large nuclei and prominent nucleoli (Figure 4.5B). However, the xylem tissues of the vascular bundles typically remained unchanged because they were made up of dead cells. (iv) Etioplasts were visible in the photosynthetic layers of the greenhouse leaves (Figure 4.5A). Etioplasts are chloroplasts that have lost their green coloration because the leaf explants were incubated in the dark.

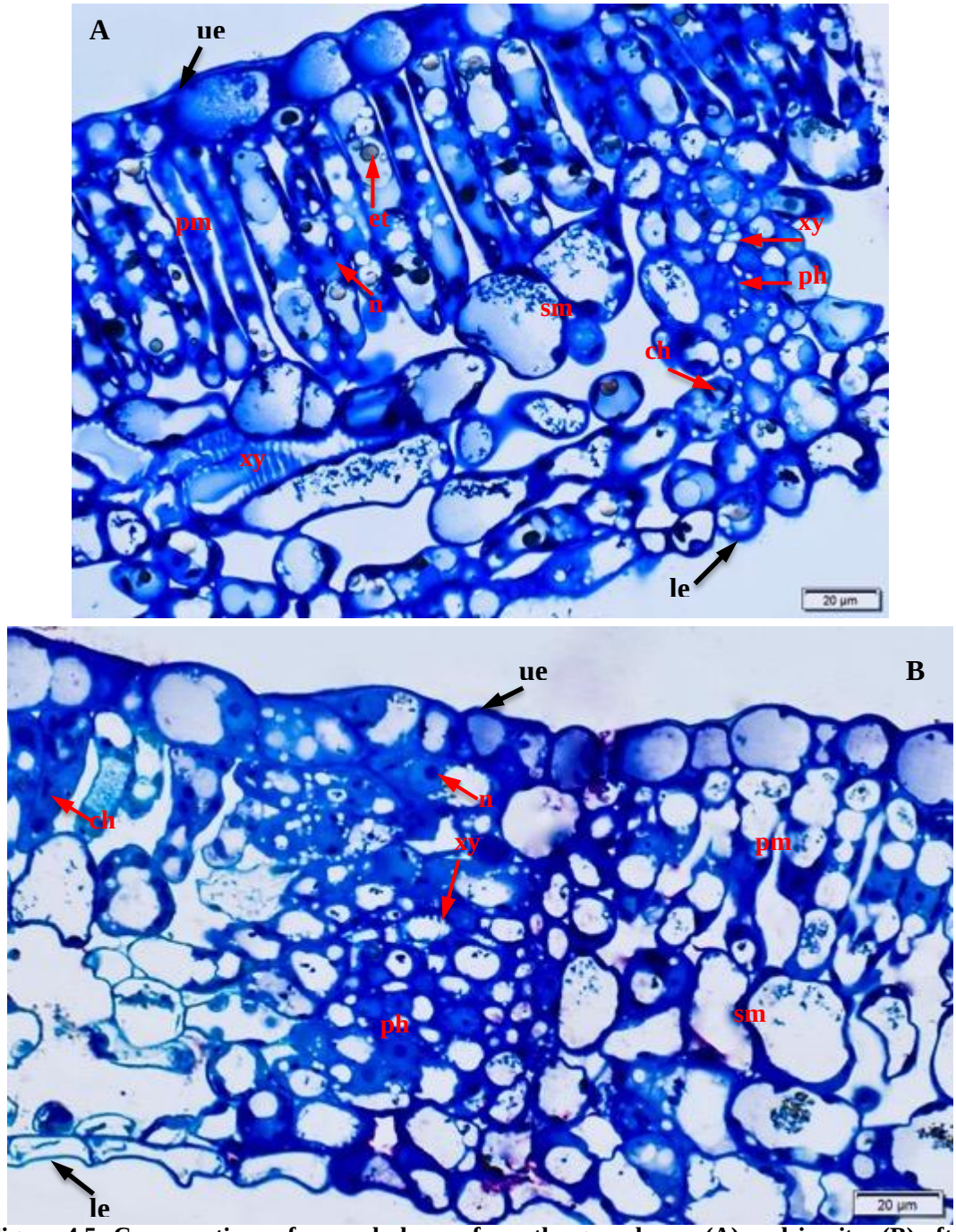


Figure 4.5: Cross sections of avocado leaves from the greenhouse (A) and *in vitro* (B) after one week in callus induction medium. In the leaves from both sources, fragmentation of vacuoles was visible in palisade mesophyll cells (pm); chloroplast (ch) and nucleus (n) with conspicuous nucleolus was more prominent. Etioplasts (et) were visible in greenhouse leaves (A). The phloem (ph) cells in the *in vitro* leaves appeared meristematic with small cells and large nucleus (B). *ue* upper epidermis; *le* lower epidermis; *sm* spongy mesophyll; *xy* xylem.

4.3.2.2 Two weeks from induction

After two weeks in culture, the arrangement of the cellular components of the leaves obtained from the greenhouse remained intact and discernible (Figure 4.6A). In the *in vitro* materials, cell division had occurred in all the internal tissues of the leaf explant (Figure 4.6B). The mesophyll tissues had undergone dedifferentiation as was evinced by the complete loss of distinction between the palisade and spongy mesophyll cells (Figure 4.6B). However, the xylem of the vascular bundles typically remained unchanged in particular in the *in vitro* material (Figure 4.6B).

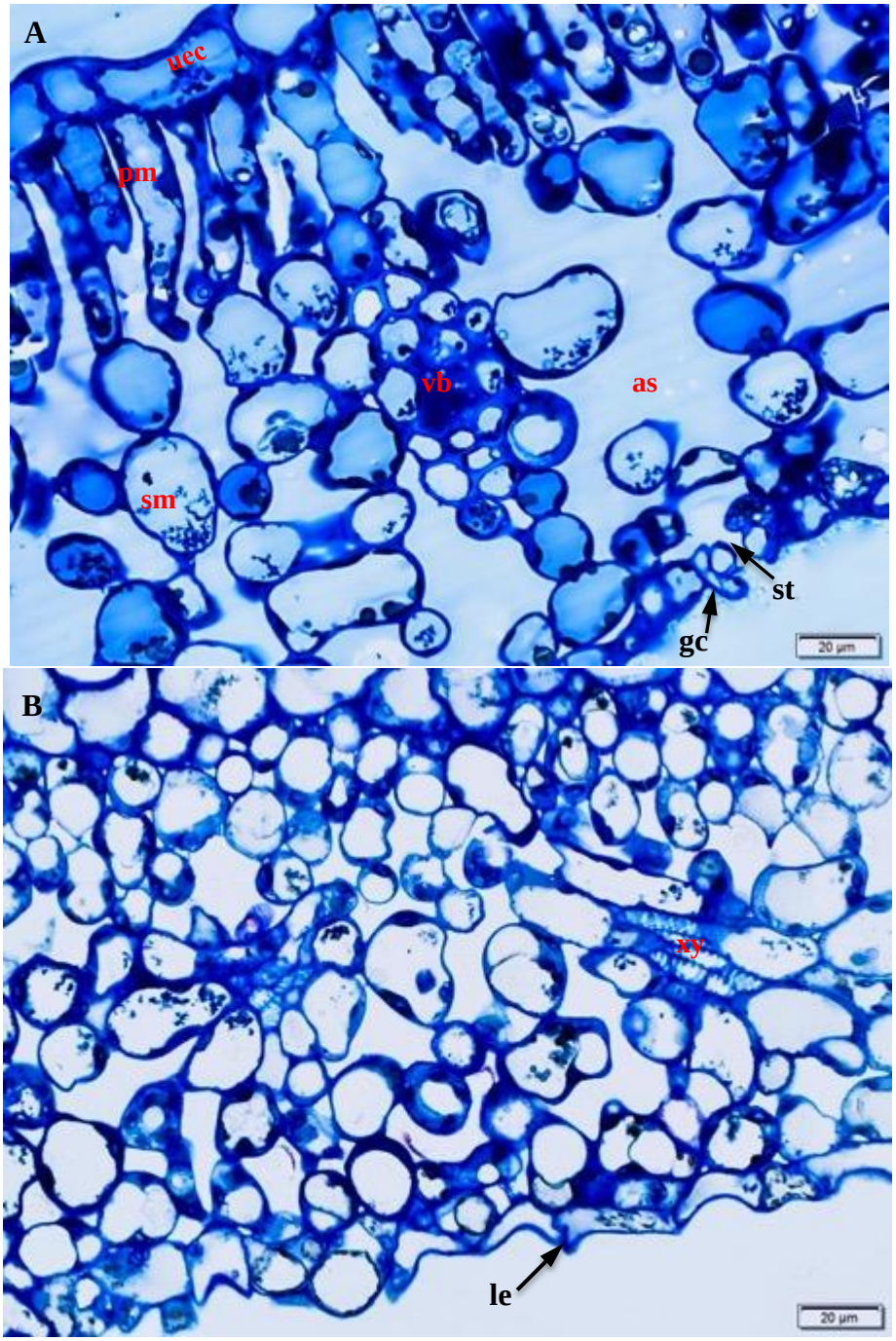


Figure 4.6: Light micrographs of avocado leaves at two weeks of incubation in callus induction medium. (A): greenhouse-derived leaf with discernible cellular components (B): *in vitro*-derived leaves with loss of distinction between the cells of the palisade and spongy mesophylls. *pm* palisade mesophyll; *sm* spongy mesophyll; *vb* vascular bundle; *as* airspace; *uec* upper epidermal cell; *gc* guard cell; *st* stoma; *xy* xylem; *le* lower epidermis.

4.3.2.3 Three weeks from induction

By the third week in culture, there was a considerable aggregation of cells forming callus in both the greenhouse and *in vitro* leaf materials (Figure 4.7 & 4.8). In the greenhouse leaves, calli were initiated from two tissues: the vascular bundle and its surrounding cells, and the epidermal layer (Figure 4.7A). The callus from the vascular bundle and associated cells comprised vacuolated cells with no visible cytoplasmic contents, held together by tannin-like substances (Figure 4.7B). The calli from epidermal cells were formed by periclinal division of individual cells to form 2-4 celled groups, and each group was bound by a common cell wall. Each cell within a group possessed its cell wall, nucleus and vacuole (Figure 4.7C). In the *in vitro* leaf material, the prominent vascular bundle comprised numerous small cells as a result of cell division. The mesophyll cells below and above the vascular bundle had dedifferentiated to form callus cells (Figure 4.8A). The increase in the number of cells, due to cell division, from both the vascular bundle and mesophyll, pushed the cells through the epidermis to form callus on the explant surface (Figure 4.8B). Callus also originated from the epidermal layer of *in vitro* leaf materials (Figure 4.8C). Aggregates of a few callus cells were located at subepidermal region of the leaf explants (Figure 4.8D), which may result in the callus bulging point (broken line, Figure 4.8A). The xylem (Figure 4.8E) typically remained unchanged after three weeks in callus induction medium, because they comprised dead cells.

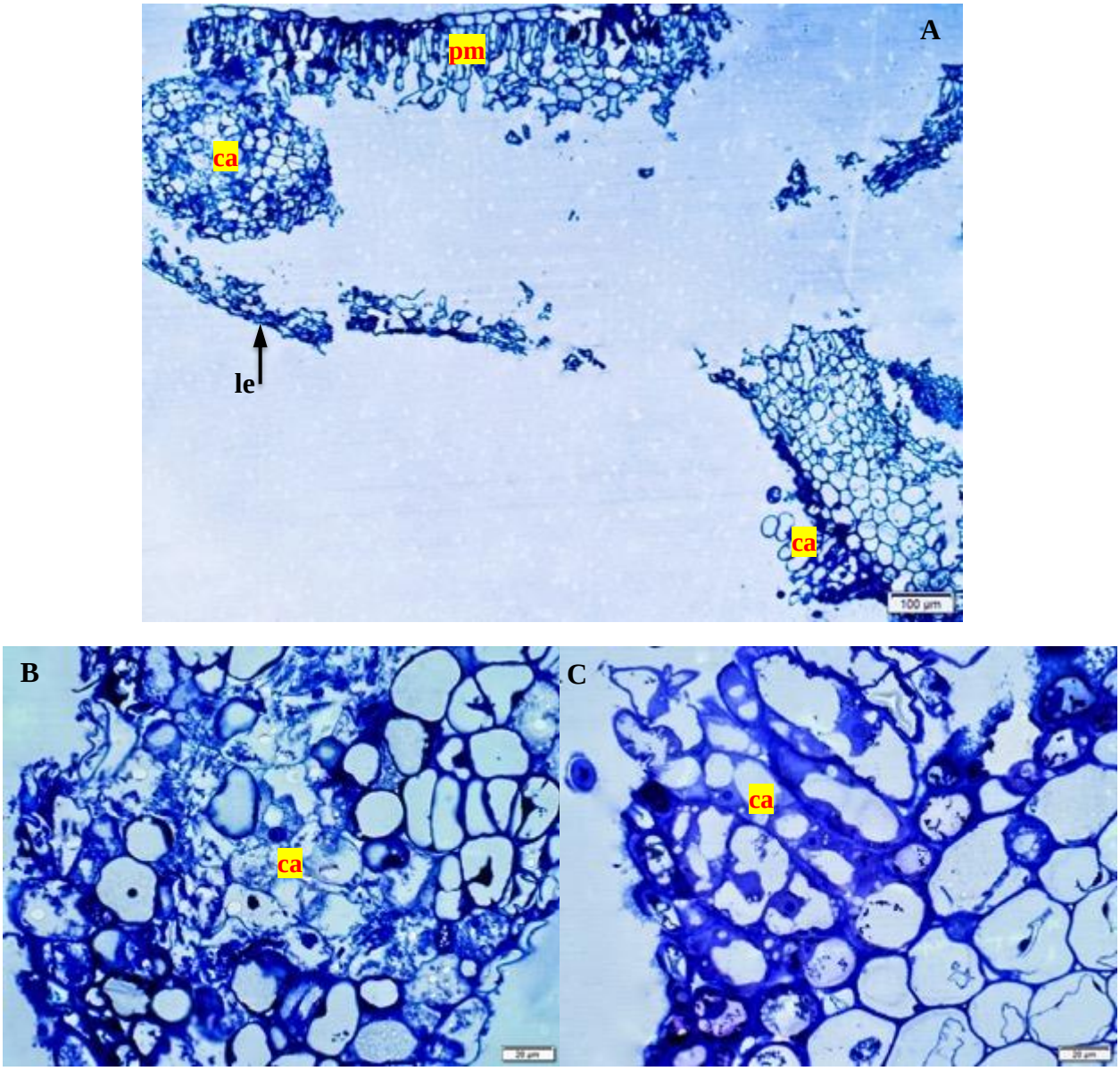


Figure 4.7: Cross sections of leaf from the greenhouse material, after three weeks in culture medium. A: transverse sectional plan of the leaf, showing callus cells aggregate (ca) around vascular bundle, expanded in (B); and from the epidermis, expanded in (C).

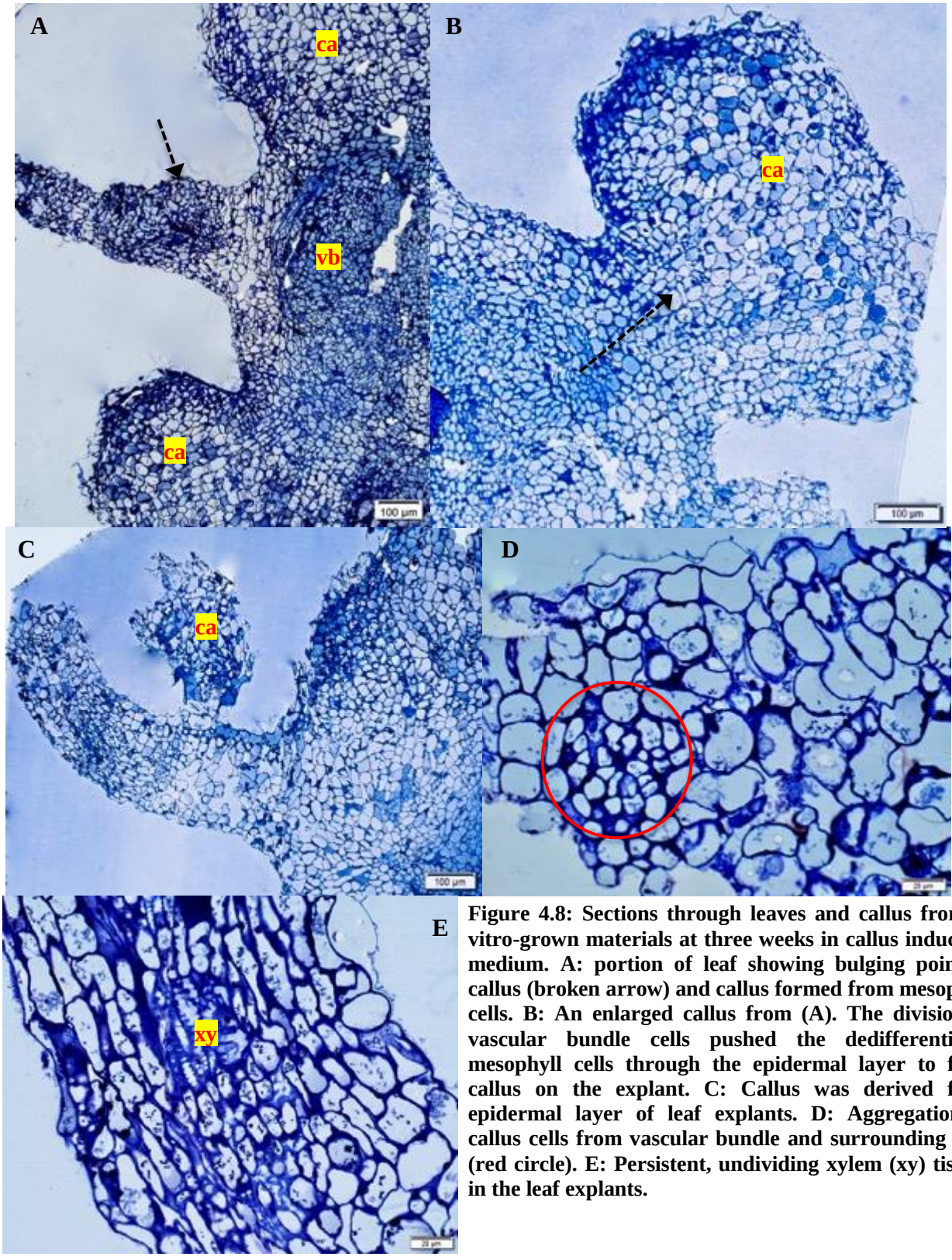


Figure 4.8: Sections through leaves and callus from *in* vitro-grown materials at three weeks in callus induction medium. A: portion of leaf showing bulging point of callus (broken arrow) and callus formed from mesophyll cells. B: An enlarged callus from (A). The division of vascular bundle cells pushed the dedifferentiated mesophyll cells through the epidermal layer to form callus on the explant. C: Callus was derived from epidermal layer of leaf explants. D: Aggregation of callus cells from vascular bundle and surrounding cells (red circle). E: Persistent, undividing xylem (xy) tissues in the leaf explants.

4.3.2.4 Four to six weeks from induction

By the fourth week of induction, the callus had increased in size due to continued cell division and callus masses were readily visible on the leaf explants of both the greenhouse and the *in vitro* materials.

In the callus of greenhouse materials, the accumulation of phenolic compounds and formation of proembryo-like (PEL) structures were observed. The phenolic compounds displayed as deposits on the internal wall of some cells, which accumulated into ring-forms in other cells (Figure 4.9B-E). Some cells, however, had been completely filled with phenolic compounds (Figure 4.9C, E & F). Cell division continued in the callus cells, resulting in asymmetrical daughter cells and newly formed/forming cell walls. Periclinal cell division resulted in 2-celled – apical and basal cells – PEL structures (Figure 4.9C and E). Further division in the periclinal and/or anticlinal plane resulted in multi-celled PEL structures (Figure 4.9B-D). The cells of the PEL were, however, largely vacuolated with few of the cells having a prominent nucleus.

On the other hand, in the callus of the *in vitro* materials, there were no cellular aggregates that could be described as PEL. Instead, callus cells were isodiametric and largely vacuolated with few dividing cells (Figure 4.10B-D). There were a few cytoplasmic dense cells, but the cytomatrix was pulled away from the cell walls (Figure 4.10C).

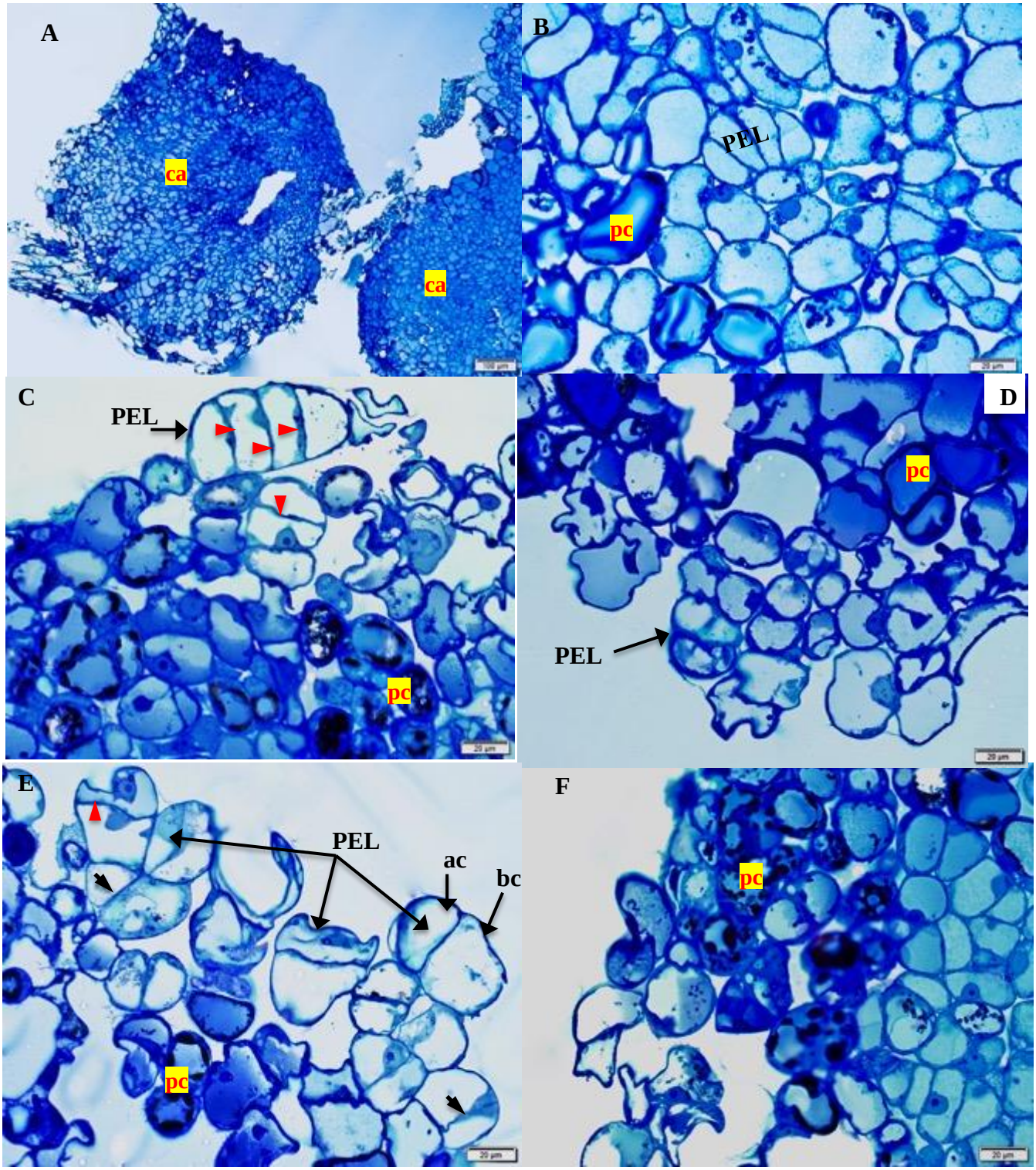


Figure 4.9: Callus from the greenhouse avocado leaf explants that were in induction medium for four weeks. A: callus masses. B: cells within the callus showing phenolic compounds (pc) and dividing cells. C - F: periphery of callus showing at least 2-celled proembryo-like (PEL) structures, arising from periclinal (red arrowhead) and anticlinal (black arrowhead) cell division. Apical (ac) and basal (bc) cells were the components of a two-celled proembryo-like structure (E).

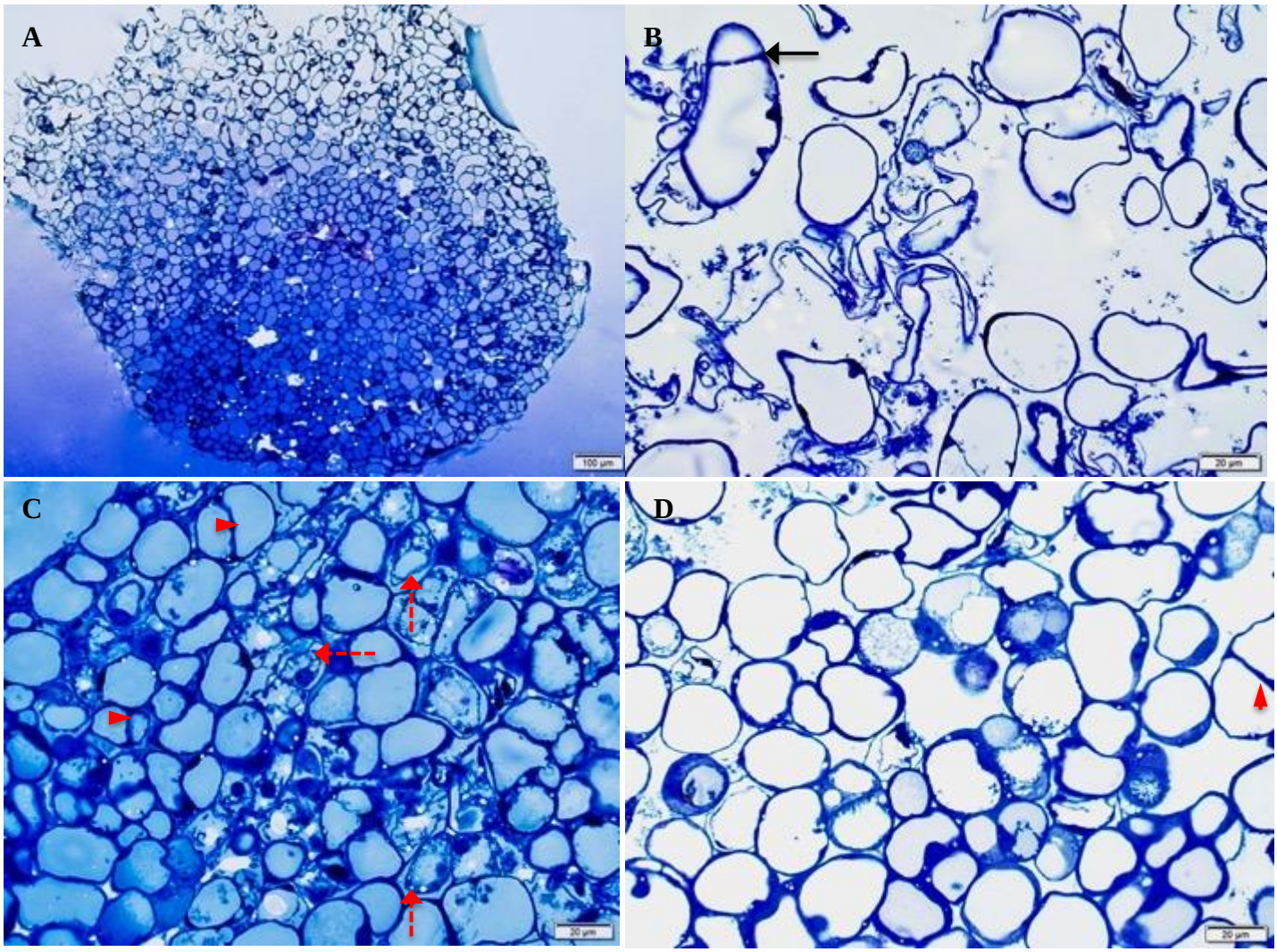


Figure 4.10: Callus developed from four-week-old culture of leaf derived from *in vitro*-grown avocado shoots. A: callus mass. B: Periphery of callus with one asymmetrically-divided cell (black arrow). C: Inner cells of callus comprising dividing cells (red arrowhead) and cytoplasmic-dense cells (broken red arrow). D: Inner cells of callus which were largely vacuolated.

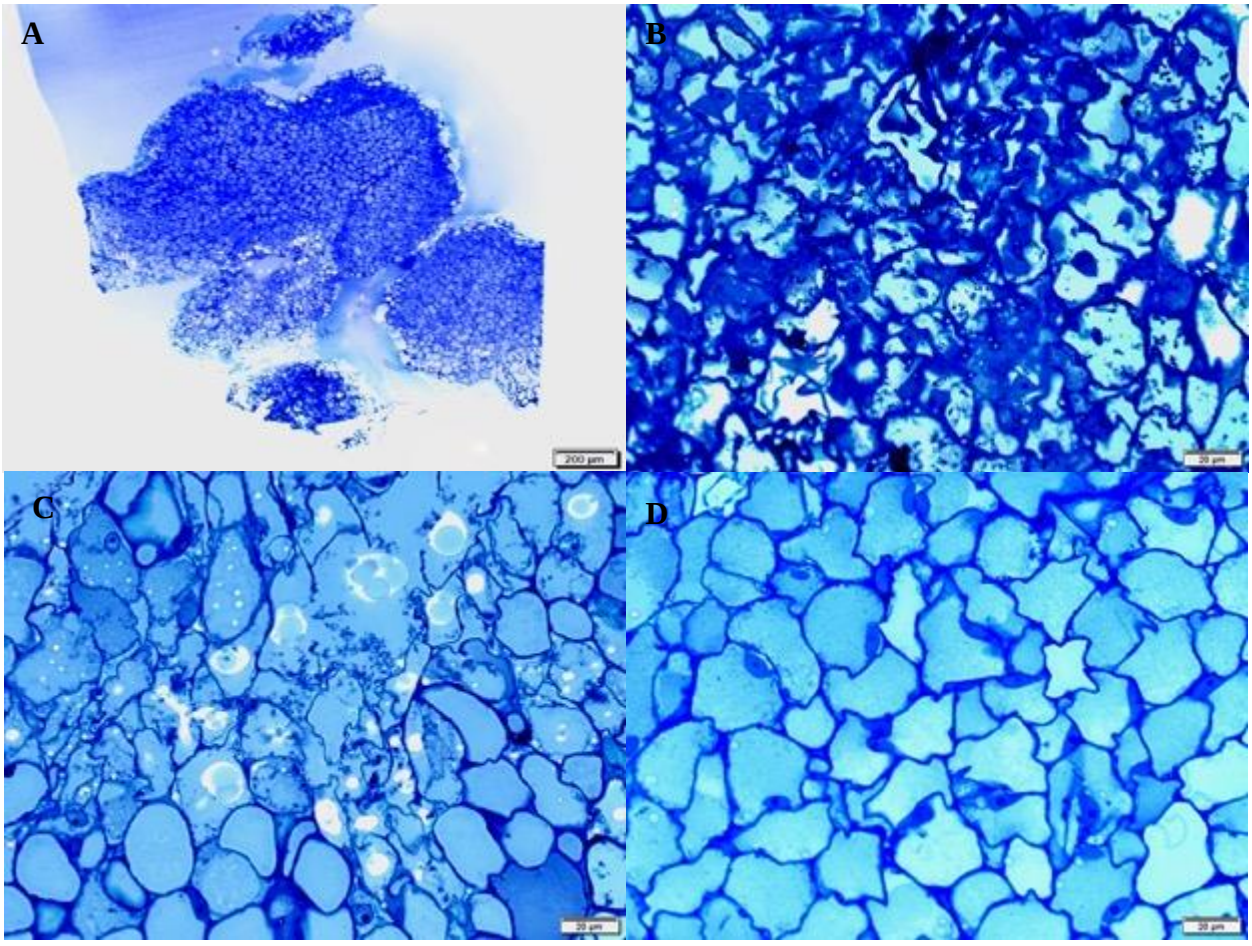


Figure 4.11: Callus from greenhouse-derived leaves that had been cultured for five weeks. A: Fused callus masses. B – D: cells of different regions of the callus. B: callus cells had irregular shapes, appeared plasmolysed, but cells were nucleated. C: Callus cells were highly vacuolated and isodiametric. D: callus cells were polygonal and had relatively small nuclei.

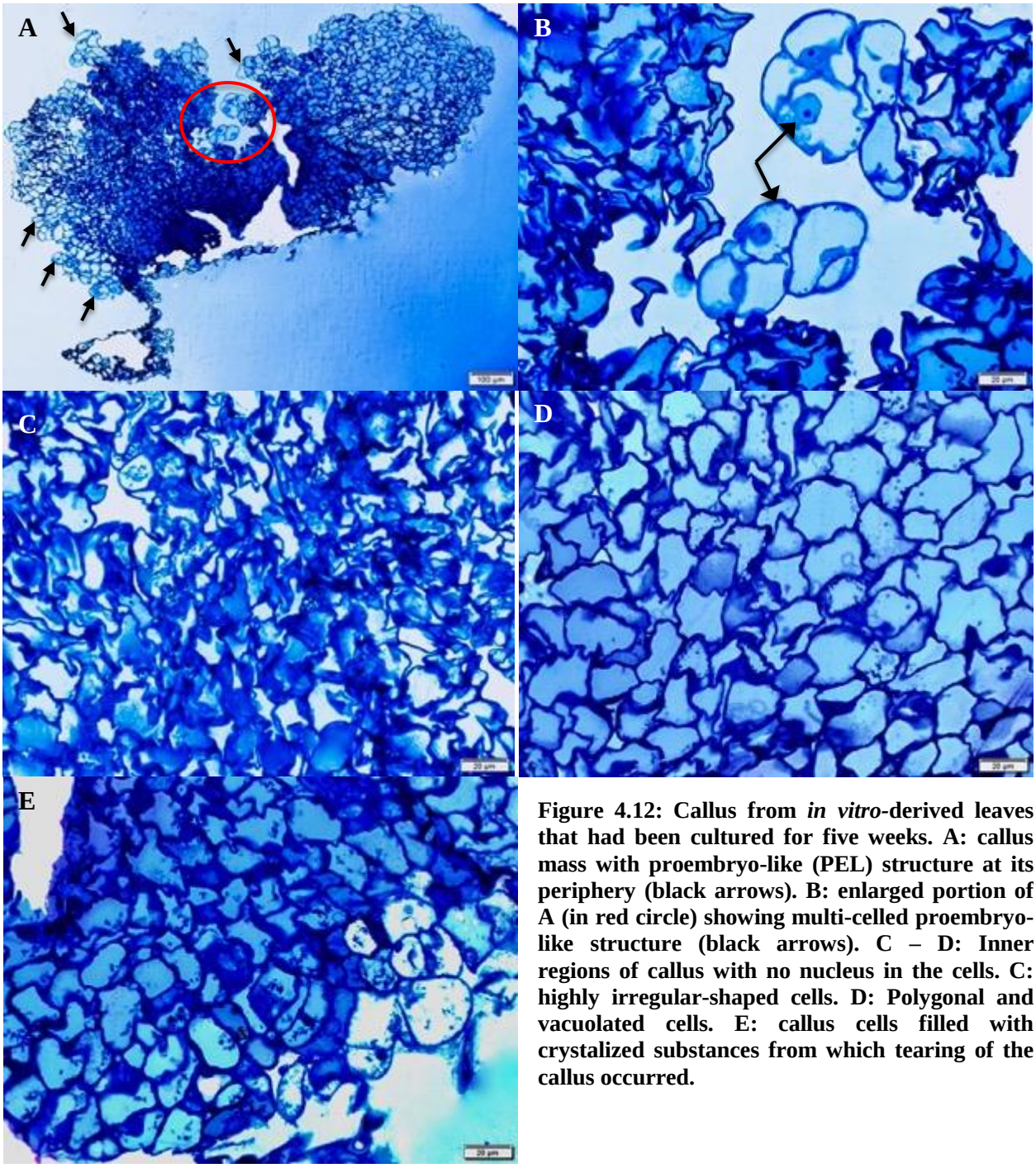


Figure 4.12: Callus from *in vitro*-derived leaves that had been cultured for five weeks. A: callus mass with proembryo-like (PEL) structure at its periphery (black arrows). B: enlarged portion of A (in red circle) showing multi-celled proembryo-like structure (black arrows). C – D: Inner regions of callus with no nucleus in the cells. C: highly irregular-shaped cells. D: Polygonal and vacuolated cells. E: callus cells filled with crystalized substances from which tearing of the callus occurred.

While PEL structures were observed at four weeks in the callus of greenhouse leaf materials, similar structures were only noticed at the fifth week of callus induction from the *in vitro* derived leaf materials. The formation of these structures was limited to the periphery of the callus mass (Figure 4.12A). Cells of the PEL structures had a prominent nucleus (Figure

4.12B). The inner regions of the callus from both the greenhouse- and *in vitro*-derived leaves were made up of different types of cells: (i) highly irregular-shaped, shriveled cells (Figure 4.11B and Figure 4.12C) (ii) isodiametric, apparently anucleated cells (Figure 4.11C and Figure 4.12E) and (iii) polygonal cells with a small or obscure nucleus (Figure 4.11D and Figure 4.12D).

By the sixth week, the cells of the callus from the greenhouse material had been filled with phenolic compounds (Figure 4.13A). The phenolic compounds were not restricted to the peripheral region of the callus but were rather spread throughout the callus (Figure 4.13B). Where phenolic compounds were not found in the callus as seen in the callus from the greenhouse material, the shrivelled cells of the callus from *in vitro* material had presumably collapsed; creating air spaces in the callus mass (Figure 4.13D). However, the cells at the peripheral region of the callus had prominent nuclei and thus could be embryogenic but the cells still appeared plasmolyzed, with irregular shapes (Figure 4.13E).

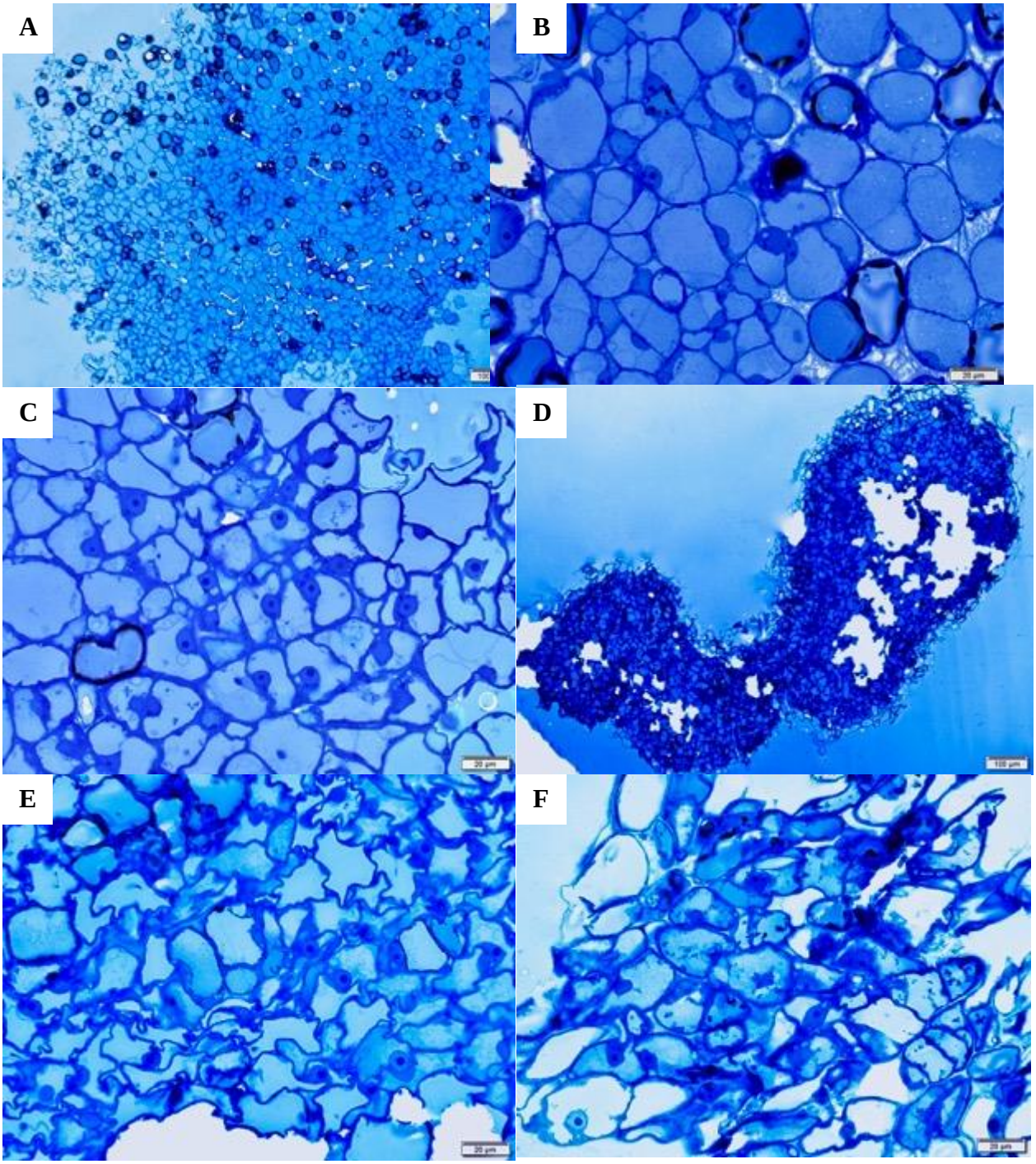


Figure 4.13: Callus cells from greenhouse-derived and *in vitro*-derived leaves that had been in culture for six weeks. (A): showed dispersed phenolics through the callus. The core of the callus consisted of highly vacuolated as well as few nucleated cells (B) while the periphery comprised periclinally-divided cells with prominent nuclei (C). Callus from *in vitro* material at six weeks in culture with hollow spaces created from torn cells (D). Callus cells around the cavity were nucleated but shrivelled with irregular shapes (E) while the cells at the periphery were filled with crystallized substances (F).

4.3.2.5 NAA-induced callus from greenhouse material

At six weeks, callus obtained from NAA-supplemented induction medium comprised isodiametric cells; some of which were still dividing to form new daughter cells. Some cells were nucleated, and few were taken over by phenolic compounds (Figure 4.14). The cells showed no sign of shrivelling.

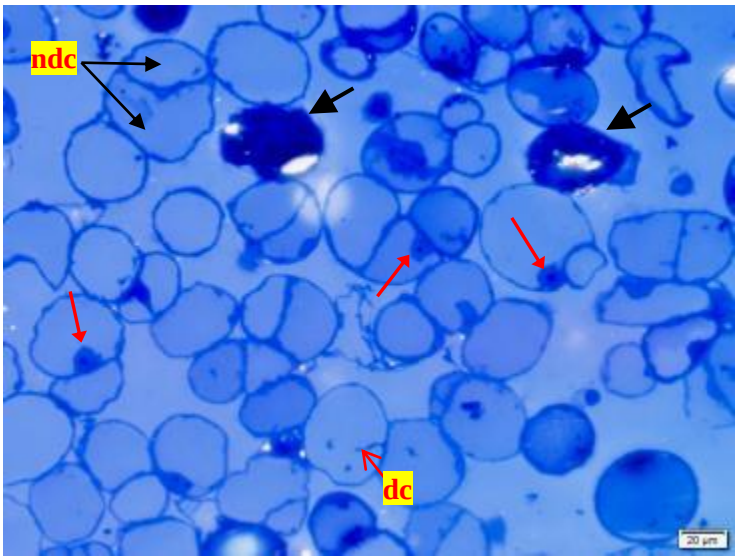


Figure 4.14: Callus cells from greenhouse-derived leaf that was cultured for six weeks on an induction medium that comprised Gamborg's B5 supplemented with 1.0 mg/L NAA and 2.0 mg/L BAP. Phenolic compounds were present in few cells (black arrow), few nucleated cells with visible nucleolus were present in the callus cells (red arrow heads). *ndc* new daughter cells, *dc* dividing cells.

4.3.2.6 Callose deposition in callus cells

Callose was not detected in the cell walls of cells that were derived from callus induced on 2,4-D-supplemented medium (Figure 4.15A & B). However, luminous callose-like substances, whose production may be related to stress, were detected as droplets within the cells. Callus derived from NAA-enriched medium (Gamborg's B5 supplemented with 1.0 mg/L NAA and 2.0 mg/L BAP) showed callose deposition in the cell walls of some cells (Figure 4.15C & E; BF observation and Figure 4.15D &F; FM observation). This was an indication of active cell division in the callus cells.

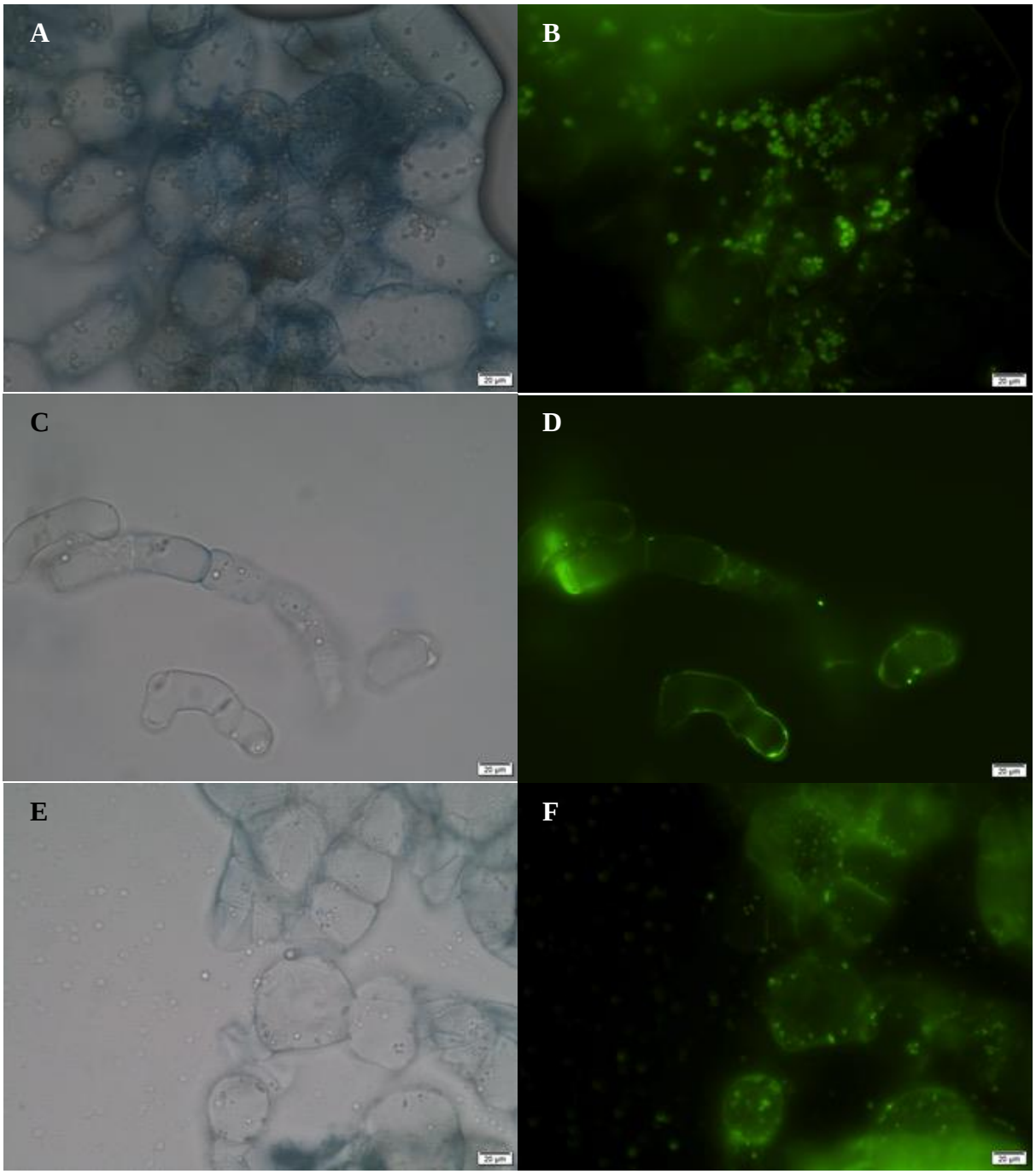


Figure 4.15: Callose deposition in callus cells of avocado. In 2,4-D-enriched induction medium, callose was not detected in the callus cell wall but callose-like substances were detected as droplets within the cells (A & B). Callose (Yellow-green fluorescence) was detected in NAA-derived callus (C & D and E & F). Row I was observed with Bright Field (BF) optics while row II is the equivalent observation with Fluorescence Microscopy (FM).

4.4 Discussion

4.4.1 Fresh leaves obtained from the greenhouse and the *in vitro* materials

The leaf comprises three main tissues: the epidermis (upper and lower), the photosynthetic layer i.e. the mesophyll and the vascular bundle (the vein). Also, different cell types: meristematic, parenchyma and collenchyma cells are usually found in the leaves while sclerenchyma cells will only be seen in leaves with secondary growth (Trigiano and Gray, 2005). The leaves of *Persea americana* cv. 'Edranol' showed the expected arrangements highlighted above. The cellular volume of most cells of asexual plant parts is largely taken up by vacuoles (Esau and Gill, 1991; Taiz, 1992; Marty, 1999). Hence, the cells of fresh leaves from both the greenhouse and the *in vitro* shoots were highly vacuolated thus restricting the intracellular components to the periphery of the cells (Figure 4.2 & Figure 4.3).

4.4.1.1 The epidermis

Epidermal layers play important mechanical supportive and protective roles in plant tissues (Glover, 2000; Takada and Lida, 2014). They are composed of three types of cells: (i) basic pavement cells which make up the majority of the epidermis, (ii) specialized cells which are the guard cells and (iii) the trichomes (Glover, 2000; Takada and Lida, 2014). Leaf epidermis (mainly the pavement cells) is parenchymatous, mostly achlorophyllous and is usually uniseriate, i.e. single layered and transparent. This is to facilitate light transmission from the sun to the chlorophyll-rich mesophyll cells (Beck, 2010). Not only in avocado leaves was the epidermis uniseriate (Figure 4.2 and Figure 4.3) but also the leaves of some other members of the Lauraceae were single-layered (Zeng *et al*., 2014). Examples are *Endlicheria paniculata*, *Nectandra barbellata* and *Ocotea indecora* (Gonçalves *et al*., 2018). However, the leaves of *Caryodaphnopsis spp* in the family have a characteristic double-layered lower epidermis (Zeng *et al*., 2014). Leaf epidermal cells are characterized with different shapes and sizes across species (Rudall, 2007) and some of the shapes can be irregular, polygonal, angular and rounded in the Lauraceae (Zeng *et al*., 2014). In line with these observations, *P. americana* displayed a range of epidermal cell shapes (Figure 4.2 and Figure 4.3). Shape variations within a species can be caused by mutation such as in maize (Kessler *et al*., 2002) or different growth conditions (Pompelli *et al*., 2010) such as seen in the present investigation. According to Elsner *et al*. (2012), heterogeneity of cell sizes, as seen in the epidermal cells of *in vitro*-

derived leaf (Figure 4.3), could be an indication of different differentiation stages in the leaf developmental processes. The stomata were seen on the abaxial side of the avocado leaves, i.e. hypostomatic leaves, which is characteristic of the Lauraceae (Zeng *et al*., 2014).

4.4.1.2 The mesophyll

The mesophyll layers are largely responsible for the photosynthetic ability of leaves (Beck, 2010). Leaves of most dicotyledonous plants are characterized by distinct parenchymatous mesophyll types: palisade and spongy layers (Trigiano and Gray, 2005; Beck, 2010); with the palisade mesophyll towards the adaxial surface and the latter towards the abaxial surface. Such distinction characterized the mesophyll of *P. americana* leaf from either the greenhouse or *in vitro* (Figure 4.2 & Figure 4.3). However, there are exceptions to this distinction (Beck, 2010) as seen in the xerophytic *Populus euphratica* tree, whose leaves lack spongy mesophyll cells (Liu *et al*., 2015). The number of layers of the (spongy) mesophylls, as seen in the *in vitro*-derived leaf explants (Figure 4.3) and in *Quercus robur* (Corredoira *et al*., 2006), has a positive correlation with light intensity. Similarly, the proportion of intercellular air spaces has a direct relationship with gaseous exchange (Beck, 2010; Pompelli *et al*., 2010). Thus, there would be more gaseous exchange in the leaves from the greenhouse than in the *in vitro*-derived leaves.

Viña *et al.* (1999) showed that avocado grown *in vitro* had low photosynthetic rate. This could be attributed to the fact that *in vitro* plants develop in culture media that have been exogenously enriched with nutrients; hence, they invest less in the photosynthetic processes. In addition, Xiong *et al.* (2017) made a direct correlation between the number of chloroplasts, their surface areas and photosynthetic rate. Thus, the significantly fewer chloroplasts found in the *in vitro* avocado leaves, when compared with those obtained from the greenhouse (Table 4.1), suggested reduced photosynthetic rate in the former. This result was similar to that of Sáez *et al*. (2012) where photosynthetic rate was compared between *in vitro*- and nursery-grown *Castanea sativa*.

4.4.1.3 Vascular bundles

Leaf vascular bundles are macroscopically seen as midribs and veins. They are made up of xylem and phloem; with the xylem towards the adaxial surface of the leaf and the latter is

towards the lower epidermis (Rose *et al*., 2006). Vascular bundles are morphologically different based on their arrangements and the cellular compositions (Beck, 2010; Sack and Scoffoni, 2013); which in turn results in functional differences (Sack and Scoffoni, 2013). *In P. americana*, a morphological difference between greenhouse- and *in vitro*-derived avocado leaves is the presence of bundle sheath extensions (BSEs) in the former. The presence or absence of bundle sheath extension categorizes leaves as heterobaric or homobaric, respectively; and its occurrence in leaves is irrespective of the growth stage and age of the plants (Kenzo *et al*., 2007). In the study of Kenzo *et al*. (2007), 40% of sampled species of Lauraceae had BSEs while it was over 90% in the study conducted by Boeger *et al*. (2016). Hence, it is not unusual for the leaves of avocado (which is a species from the Lauraceae) to have BSEs. According to Beck (2010) and Boeger *et al*. (2016), BSEs are made up of thick-walled sclerenchyma cells, which were similar to the BSE cells that were observed in the greenhouse-derived avocado leaves in this study (Figure 4.2). Boeger *et al*. (2016) also found that heterobaric leaves were thinner than homobaric leaves; this was in agreement with the comparison of the leaf sizes between greenhouse and *in vitro* leaves (discussed in the next section). The occurrence of BSEs have been associated with the prevailing environmental habitat of the growing plants, in which high light intensity and temperature favour the development of heterobaric leaves while homobaric leaves are usually characteristic of plants that develop under high humidity (Kenzo *et al*., 2007; Boeger *et al*., 2016). Similarly, avocado leaves that were obtained *in vitro* lacked BSEs and their growth environment had a higher humidity than the greenhouse environment. Classification of leaf forms, based on the presence or lack of BSEs, has been recorded at interspecific level (Kenzo *et al*., 2007; Boeger *et al*., 2016) but the current study suggested an intra-specific level of classification. BSEs are believed to offer mechanical support against stress such as drought, facilitate better transportation and improve photosynthetic rates (Kenzo *et al*., 2007; Beck, 2010; Sack and Scoffoni, 2013; Boeger *et al*., 2016). The presence of BSEs in greenhouse leaves further confirmed that they have higher photosynthetic rate in comparison with the *in vitro* materials.

4.4.1.4 Leaf sizes

Kessler *et al*. (2002) stated that large epidermal and mesophyll cells are responsible for increased leaf thickness. Different environmental conditions under which a species grows

usually result in individual plants with some level of morphological differences. Examples of such conditions are sun and shade growth conditions, and as used in this study, the greenhouse and the *in vitro* conditions. Such growth conditions usually result in differences in leaf thickness and area, stomatal area, length, width, density; size of epidermis and mesophylls among others (Osborn and Taylor, 1990; Pompelli *et al*., 2010; Patay *et al*., 2016).

The relative humidity (RH) in air-tight *in vitro* culture vessels is almost 100% which implies that transpiration, if any, is low (Nguyen and Kozai, 1998; Chen, 2004); and always higher than the RH in the greenhouse (Chen, 2004). This has a direct relationship on moisture content of the plant cells. The cells from *in vitro*-grown materials would be turgid, hence bigger, with little airspace, they would also have higher fresh weight and little dry matter. Ultimately the leaves will be thicker than those grown *ex vitro*. Such as was seen in the fresh leaves obtained from the *in vitro*-grown shoot of *P. americana* when compared with those obtained from the greenhouse (Figure 4.4). Similar results were obtained in the plantlets of potato grown *in vitro* (Kozai *et al*., 1993).

4.4.2 Responses of leaf materials cultured on callus induction medium

The process of callus formation from leaves plated into callus induction medium was divided into three stages: the stage of cell division and dedifferentiation in the initial two weeks of culture, the stage of callus cell aggregation and budding of callus by the third week and the stage of callus growth from the fourth week in the callus induction medium.

4.4.2.1 One week from induction

Increase in cell size, such as was recorded at one week of callus induction (Table 4.2), is characteristic of cells triggered into cell division and consequently, the formation of callus (Majda and Robert, 2018). Vacuolar fragmentation was also one of the main changes that occurred in the leaves of *P. americana* at one week of callus induction (Figure 4.5). Morphological changes occur in vacuoles prior to, and during, cell division; one of which is vacuolar fragmentation (Esau and Gill, 1991; Kutsuna and Hasezawa, 2002). A similar event was noticed in the leaf cells of *Helianthus smithii* by the 10th day of induction (Laparra *et al*., 1997). Where it had not occurred, the process could be underway where vacuoles had shrunk and pulled away from the cell membrane (Zieger and Mayer, 2012). As a result of the fragmentation, the vacuoles would occupy less cellular volume than before and cytoplasmic

content would increase (Owens and Poole, 1979; Laparra *et al*., 1997). Small vacuoles would also be less obstructive of cell plate formation during cytokinesis (Esau and Gill, 1991).

In nature, chloroplasts are not only formed from proplastids but sometimes also from the developmental intermediary organelles – the etioplasts (Solymosi and Schoefs, 2010). Etioplasts can be found in enclosed organs such as leaf primordia (Solymosi and Böddi, 2006). Transition often occurs between chloropasts and etioplasts, depending on the availability of light which favours the former or light obstruction which results in the formation of the latter (Wise, 2016). Avocado leaf explants were incubated in the dark, which resulted in the loss of chlorophyll in the functional chloroplasts, thus the chloroplasts were transformed to pale-coloured etioplasts (Figure 4.5A) such as seen in tobacco leaves (Armarego-Marriot *et al*., 2019). However, not all chloroplasts were transformed to etioplasts at one week of incubation in the dark. The persistence of chloroplasts in avocado leaf explants after excision from parent plants and incubated for one week in the dark implied that (i) the transition from chloroplasts to etioplasts took some time and that (ii) the chloroplasts were stable for some times before complete transition and loss of coloration.

4.4.2.2 Two weeks from induction

Subsequent to the changes such as increase in cell size and vacuolar fragmentation observed at one week of callus induction, extensive cellular division was observed in the cells of leaves obtained *in vitro*. Mesophyll cells are believed to be able to acquire meristematic competence because they contain complete protoplasts (Esau, 1977). Such meristematic activity resulted in the dedifferentiation of the mesophyll cells of the *in vitro* materials (Figure 4.6B). The dedifferentiated cells can then be directed towards different developmental pathways under the right conditions. This is because plant cells possess cellular plasticity (Grafi, 2004; Bhojwani and Dantu, 2013). Hence, mesophyll cells, such as observed in this study, are capable of reprogramming through dedifferentiation (Wang *et al*., 2011). Similarly, the photosynthetic layers of the leaves of *Elliottia racemosa* were dedifferentiated after two weeks in culture (Woo and Wetzstein, 2008).

It is generally accepted that plant cells retain their plasticity during development and are totipotent. However, the mesophyll cells of the leaves from the greenhouse did not divide and dedifferentiate during the induction process for callus formation from avocado leaves (Figure

4.6A). This suggested that the mesophyll cells of that material had differentiated to the extent that genetic plasticity had been lost and the cells could not revert to a state of meristematic competence. This agreed with George (1993) that totipotency excludes terminally, and sometimes fully, differentiated tissues. According to Fehér (2019), this generally accepted competence in plants is rather dependent on many factors, one of which was the developmental status of plant cells.

4.4.2.3 Three weeks from induction

The vascular bundle and epidermal cells formed callus in the greenhouse leaves (Figure 4.7). In addition to these tissues, callus also developed from the mesophyll cells of the *in vitro* leaf materials (Figure 4.8). The dedifferentiation of all tissues in the *in vitro* leaves to form callus, while the palisade mesophylls of the greenhouse leaves persisted in their fully differentiated state, suggested that the *in vitro* leaves were not as fully differentiated as the greenhouse leaves. Hence, the tissues of the *in vitro* leaves could easily revert to an undifferentiated state with increased developmental capability. According to Gueye *et al*. (2009), the callogenic competence of plant cells is dependent on the degree of their differentiation.

Callus formation from the leaf of *Bruguiera sexangula* (Mimura *et al*., 1997) and oil palm (Yusoff *et al*., 2012; Gomes *et al*., 2017) was initiated from the vascular tissue, similar to the callus formation from the leaf of avocado derived from the greenhouse (Figure 4.7). Yusoff *et al*. (2012) observed perivascular division and dedifferentiation in cultured leaves prior to callus formation. In this regard, Rose *et al*. (2006) proposed that the uptake of exogenous auxin by the phloem and bundle sheath cells of the vein made the cells pluripotent from which callus cells were formed.

Epidermal cells have been shown to have meristematic capability in pineapple guava (Canhoto and Cruz, 1996) and embryogenic competence in *Helianthus smithii* (Laparra *et al*., 1997) and *Limonium sinensis* (Dam *et al*., 2010). Normally, epidermal cells undergo anticlinal cell division; but for callus formation to arise from the epidermal layer was a consequence of periclinal cell division (Kurczynska *et al*., 2012), such as seen in avocado leaves of greenhouse materials (Figure 4.7C). Whatever the origin of the callus, the proportion of auxin (2,4-D) and cytokinin (BAP) used to induce the callus from both sources was optimal.

Different regions – peripheral, middle and centre – of the callus of oil palm were characterized by different cell types. Vacuolated and elongated cells with large air spaces were found at the periphery; large vacuolated cells with less airspace at the middle and isodiametric, small, cytoplasmic-dense nucleated cells characterized the core of the callus (Gomes *et al*., 2017). The callus formed in this study, both from greenhouse and *in vitro* materials, had heterogeneous cell types at the different regions (Figure 4.7, & Figure 4.8B).

4.4.2.4 Four to six weeks from induction

It was expected that the callus mass would increase in size every week (4^{th} to 6^{th} week) from the time of its formation in the third week. This was due to cell proliferation resulting from continuous cell division as seen in the works of Laparra *et al*. (1997) with *Helianthus smithii* and Meira *et al*. (2019) with macaw palm.

Santos *et al*. (2006) presented a schematic that described the various cell divisions that resulted in the development of proembryonic masses. A callus cell would divide periclinally to give a 2-celled proembryo. The top and bottom cells are typically named apical and basal cells, respectively. Further cell division could be anticlinal and/or periclinal to produce a multi-celled proembryos (Santos *et al*., 2006). Such proembryo-like masses were found within, and on the periphery of, the callus derived from the greenhouse avocado leaves (Figure 4.9B-E) and the pattern of formation was similar to those described by Santos *et al*. (2006). Proembryos that developed from a similar pattern of cell division were also obtained in the callus of *Dieffenbachia* (Shen *et al*., 2016).

There were organized cell clusters in the callus of coconut (Verdeil *et al*., 2001), which was similar to those on the callus from the *in vitro* material at five weeks (Figure 4.12B). However, the cells of the clusters from coconut were densely cytoplasmic with prominent nuclei and nucleoli; hence they were termed proembryos (Verdeil *et al*., 2001). The clusters from *in vitro* callus (Figure 4.12A & B) had no nucleus or they were obscure where present. Such were the clusters found in the callus of rubber and thus were described as degenerating embryos (Michaux-Ferriere *et al*., 1992). These clusters may develop into secondary, non-embryogenic callus after the collapse of the primary callus (as seen in Figure 4.13D) or degenerate altogether.

It is possible for callus to degenerate shortly after formation (e.g in Laparra *et al*., 1997). It is presumed, in this study, that the shriveled cells at the centre of the callus (Figure 4.11B & Figure 4.12C) are evidence of such degeneration (Figure 4.13) because the cells were mostly vacuolated with little or no cytoplasmic contents. This, with progressive culture days, led to loss of turgor pressure in the cells. Another probability for the shriveling of the cells would be that the induction medium was hypertonic, which would lead to the plasmolysis of the cells. Sparapano and Bruno (2004) and Ramulifho *et al*. (2019) recorded plasmolyzed cells due to hypertonic callus growth medium.

Another degenerative event during the growth of callus is the accumulation of phenolic compounds. This often happens in highly vacuolated cells with little or no accumulation of storage materials (Alemanno *et al*., 1996). The accumulation of phenolic compounds started within a week of callus formation (Figure 4.9) in 2,4-D derived callus and spread extensively within three weeks (Figure 4.13). In contrast, only a few cells of NAA-derived callus accumulated phenolic compounds (Figure 4.14). Accumulations of phenolic compounds, which lead to browning, have been discussed in the previous chapter (section 3.4.1.1.8.2). Every tissue of the study species, *P. americana*, is prone to browning by phenolics, and this characteristic is carried through to its callus. The phenolic compounds in callus can sometimes be higher than those found in the parent plants; such was the callus of *Byrsonima verbascifolia* (Castro *et al*., 2016). In addition to the study species, the growth medium and environments also contribute to the accumulation of phenolic compounds. The results in this study, both in the previous- and this chapter, showed that 2,4-D triggered more phenolic compound accumulations than NAA. 2,4-D with kinetin led to the production of highest levels of polyphenols in *Isodonn japonicus* (Choi *et al*., 1995).

4.4.2.5 NAA-induced callus from greenhouse material

Callus derived by NAA had been shown to be morphologically different from those obtained via 2,4-D. The general consensus tends towards associating anomalies with the callus and somatic embryos derived from 2,4-D. (Rodriguez and Wetzstein, 1994; 1998). By the sixth week, NAA-derived calli were still meristematic (Figure 4.14) contrary to 2,4-D over the same period (Figure 4.13). These observations were similar to those of Rodriguez and Wetzstein, (1998).

4.4.2.6 Callose deposition in callus cells

Callose is synthesized in the cell membranes and deposited particularly on newly formed/forming cell walls and cell plates (Bhojwani and Dantu, 2013; Piršelová and Matušiková, 2013). It is also often found deposited in various locations in the plant cells depending on its required functionality. Some of these other locations include plasmodesmata, phragmoplast and sometimes, across an entire tissue (Chen and Kim, 2009; Piršelová and Matušiková, 2013; Nedukha, 2015).

Callose plays important roles in intercellular communication, support, protection and transportation, among others (Piršelová and Matušiková, 2013). Thus, the deposition of callose, seen as luminous yellow-green, in the cell wall of NAA-derived callus (Figure 4.15D & F) was an indication of (i) such roles being carried out and (ii) the availability of enzymes within the callus among which were callose synthases (iii) ongoing cell division and new cell wall formation. Callose deposition in callus cell walls is one of the markers of embryogenic competence (Kurczynska *et al*., 2012). Thus, it was noticed in the embryogenic callus of Moroccan olive (Mazri *et al*., 2013) and coconut (Verdeil *et al*., 2001). It has also been shown to share a commonality with sexual reproduction events in plants (Verdeil *et al*., 2001; Üna *et al*., 2013; Shi *et al*., 2016a).

In 2,4-D-derived callus, the luminous yellow-green substance was found across the cytoplasm of the cells but not in the cell walls (Figure 4.15B). The absence of the callose in the cell walls implied that cell division and callus growth had ceased and thus, no new cell walls were formed. This corroborated the results presented in the previous chapter (section 3.3.1.1.8.3 and Figure 3.4). The identification of the substance across the cytoplasm was not known but there are various possibilities for its identification. One, the substance could be callose-like compounds such as glycoproteins and other polysaccharides. This is because aniline blue is not only suitable for the detection of callose but can also detect these compounds (Śnieżko, 2000). Two, the substance could be Golgi body vesicles bearing callose-like compounds such as polysaccharides (Bhojwani and Dantu, 2013). Three, the substance could be callose because according to Piršelová and Matušiková (2013), callose can also be found across the tissue, particularly in response to stress. In this regard, 2,4-D, though an auxin, is a stress-inducing chemical (Zavattieri *et al*., 2010) and thus could be associated with the production of the substance across the callus cells.

Several authors had shown that callose deposition in callus cell walls is one of the markers of embryogenic competence (e.g. Kurczynska *et al*., 2012; Mazri *et al*., 2013). However, the fact that it was detected in the cell wall of NAA-derived callus cells did not confer embryogenic competence on the callus; considering there are other factors that qualify a cell as embryogenically-competent. These factors, as highlighted earlier, were lacking in the NAA-derived cells.

In comparison with other callus described in the literature, the callus obtained from this study could be largely categorized as non-embryogenic although there were intances of embryogenic cells that formed PEL. However, the formation of embryogenic callus with the sole aim of further development into somatic embryos is only one out of the many applications of callus. These applications have been highlighted by Efferth (2019). Similarly, while the production of phenolic compounds might not have been favourable in the callus meant for further development, these compounds are still beneficial too as they are part of the human diet, among other benefits (Balasundram *et al*., 2006). Thus, the non-embryogenic callus of *Persea americana* can be channeled towards profitable applications such as the production of secondary metabolites.

CHAPTER FIVE: GENERAL DISCUSSION AND FUTURE RECOMMENDATIONS

The focus species in this research, *Persea americana*, Mill., is versatile in its use and application, as not only the fruit but other parts are in high demand. Demand is fast outweighing the supply and hence alternative means of propagation, such as somatic embryogenesis, have been reported, first by Mooney and Van Staden (1987) and by other researchers (Table 3.2). In most cases, however, the zygotic embryos were the explant of choice. The use of zygotic embryos implies that potential fruits would be destroyed, could be impacted by seasonal availability and variability, and its products could be affected by somaclonal variations. These issues can be mitigated with the use of alternative vegetative explants such as the leaf as in the research reported here.

A crucial step in the indirect somatic embryogenesis pathway is callus formation. However, the use and application of callus have gone beyond the primary goal of developing somatic embryos for regeneration of whole plants (Franklin and Dixon, 1994), of which only embryogenic callus is required. The undesired type of callus (non-embryogenic) that is not suitable for plant regeneration has been shown to have potential for a range of applications from curative, pharmaceutical to beauty (reviewed by Efferth, 2019).

There are factors that impact the callogenic ability of explants for callus formation. These include the cultivars and growth habitats of the parent plants; basal nutrient medium; types, concentrations and combinations of PGRs and culture room incubation methods (Franklin and Dixon, 1994). Each of these factors impacted the ability of avocado leaf explants to form callus in a consistent manner.

Plants raised in the greenhouse and in the *in vitro* environments often have physiological and morphological differences. Physiologically, the sealed *in vitro* culture environment does not allow for efficient exchange of important gases (such as CO_2) for the plants in cultures when compared with plants that are growing in the open. This, in turn, affects physiological functionalities such as photosynthesis and respiration (Jackson *et al*., 1994). Morphologically, the lack of a proper stomatal closure system, in *in vitro*-derived leaf materials, results in an increase in stomatal malfunctioning. The resultant effect would be poor water regulation by the leaves of the *in vitro* plants (Brainerd and Fuchigami, 1982; Chen, 2004). Also, leaves of *in vitro* plants usually lack, or have a poorly developed, cuticular waxy layer (Brainerd and Fuchigami, 1982; Chen, 2004; Isah, 2015) and thin epidermal cell wall as seen in avocado leaf

(Figure 4.3). In addition, the relative humidity of culture vessels is usually high (Chen, 2004) and this is associated with a range of anomalies (Sciutti and Morini, 1993; Isah, 2015) e.g. low cellular contents of the cells of the growing materials of *Phalaeonopsis* (Cha-um *et al*., 2010).

In the work reported here, there were physiological differences between the fresh avocado leaves, sourced from plants that were grown under greenhouse and *in vitro* conditions (Figure 4.6), which presumably were responsible for the differences in quantitative and qualitative callus formation. Higher callogenic competence recorded for *in vitro* leaves (Chapter Three) could be largely attributed to the less differentiated developmental status of the *in vitro* materials (Chapter Four). The less differentiated developmental status was reflected in the dedifferentiation of the mesophyll tissues of the *in vitro* leaves, which was not observed in the greenhouse-derived leaves. Morphologically, thinner cell walls of epidermal cells, without a cuticular waxy layer of the leaf of the *in vitro*-derived plant (Figure 4.3), and the fact that the tissues of the *in vitro* leaf had more robust cells (Figure 4.4), could have also contributed to the higher percentage of callus formation in the *in vitro* leaf explants than in the greenhouse materials (Figure 3.2 and 3.3). However, qualitatively, the callus consisted of mostly non-viable cells that eventually shriveled and collapsed (Figure 4.12). Callus formation is a reflection of the health, the cellular components and competence of the explanted organ (George, 1993); which in the case of the *in vitro*-derived leaf explants, would have high water content, highly vacuolated cells with little cellular components. These showed that the calli of the *in vitro* materials were not embryogenically competent.

The not-fully-differentiated developmental state of *in vitro* materials was both beneficial and disadvantageous for avocado micropropagation manipulations. In this regard, the use of axillary buds derived from *in vitro* shoots, to generate second-generation shoots, was not favourable (Chapter Two). According to Nhut *et al*. (2007), immature, less differentiated tissues have not yet acquired the level of physiological competence that would support differentiation towards organogenesis i.e. shoots. Thus, the use of under-developed tissues of the *in vitro*-derived nodal explants was not favourable for shoot development. On the other hand, leaf explants from the immature *in vitro* shoots favoured more callus formation than their greenhouse counterparts (Chapter Three). This was because less differentiated materials are more meristematic than fully differentiated materials and hence can more readily revert to

a dedifferentiated state (Zhang *et al*., 2001). Therefore, the selective effectiveness of *in vitro* materials towards organogenesis or callogenesis is an important point of consideration for future work.

It is proposed that one or both of the following possibilities were responsible for the less differentiated developmental status of the *in vitro* materials. One, the developmental timelines were different for plants raised under the greenhouse and the *in vitro* growth conditions. Two, the functions of some mechanisms (e.g. photosynthesis) were not, or minimally, required under the *in vitro* growth conditions and hence resulted in the lower developmental status of the *in vitro* tissues.

Additional differences, which were consequences of the growth conditions of the parent plants, were the presence of bundle sheath extensions and the increase in the number of chloroplasts in the greenhouse materials. These two features are associated with the efficiency of photosynthetic mechanisms in the leaves. Conversely, the *in vitro* materials would not be adequately equipped to carry out the process of photosynthesis – a process that is not as necessary in a sucrose-enriched medium, under the *in vitro* growth conditions. However, considering that successful acclimatization of regenerated plantlets is the climax of micropropagation procedures (Duan *et al*., 2020), under-developed photosynthetic mechanisms could be a major contribution to the low success rate associated with acclimatization (Leite *et al*., 2017).

Murashige & Skoog medium (MS) (1962) followed by Gamborg's B5 (Gamborg *et al*., 1968) were the two basal nutrient media that were mostly, and successfully, used in the establishment of embryogenic cultures in the somatic embryogenesis protocols for avocado (Table 3.2) with the use of immature zygotic embryos as explants. In the current research however, only Gamborg's B5 was found to be more suited for callus formation from leaf explants than MS (Figure 3.4 and 3.6). These two basal nutrient media are the mostly commonly used in plant tissue culture (Greenway *et al*., 2012). It is probable that different explants from a species would require different basal nutrients (George, 1993; Bhojwani and Razdan, 1996) such as the immature zygotic embryos in previous works (Table 3.2) and the leaf explants in the current research. This would be possible because each basal nutrient medium has a predetermined chemical composition with specific concentrations (Al-Khayri,

2011); just as different plant parts consist of different and specific cellular compositions (George, 1993). It should be noted however, that the avocado cultivar 'Edranol' used in the present research had not been previously reported in literature for callus formation or somatic embryogenesis. Hence, there is no record of the requirements for induction of callogenic competence in its different tissues and organs. Thus considering that avocado is a woody species, WPM, other than MS and the Gamborg's B5 used in this study, can be tried for better callus formation in the future.

One of the most important requisites for callus formation is the use of appropriate types and concentrations of PGRs. Callus forms as a result of dedifferentiation through cell division (Bhojwana and Dantu, 2013); and auxins are known to stimulate such cell division and cell growth (Bhojwani and Razdan, 1996; Srivastava, 2002; Bhojwana and Dantu, 2013). The induction of callus formation from avocado leaf explants required the combination of auxin and cytokinin, and not auxin alone (Chapter Three). Those results showed that there was no visible callus formation in the first two week of induction. However, Chapter Four showed the underlying processes of cell enlargement and cell division that eventually culminated in callus formation. These processes were stimulated by the synergistic interaction of the PGRs, auxin and cytokinin, which were 2,4-D and BAP, respectively (Chapter Four). While auxin stimulates cell enlargement and elongation, cytokinin stimulates cell division and proliferation and thus the combined effect of both PGRs on leaf cells resulted in callus formation. In nature, such interactions exist in the naturally-occurring phytohormones and are responsible for directing the growth and development of plant tissues (Reyes-Olalde *et al*., 2017) among other processes. While these interactions can sometimes be antagonistic in nature as part of the normal requirements for functional plant processes, intentional *in vitro* manipulations, especially for callogenesis, require a balance in the concentrations of both the auxin and cytokinin for optimal results (Fehér, 2019). Unbalanced ratios between the auxin and the cytokinin usually result in either shoot or root formation. Light Microscopy (LM) showed that, not only the vascular bundles and the epidermal tissues were capable of forming callus as seen in the greenhouse materials, but also the mesophyll tissues as seen in the *in vitro* materials (Chapter Four). This showed that the cells of these leaf tissues were responsive to the cross signaling and stimulations received from the PGRs in the induction medium.

As would be expected, the choice of PGRs for plant tissue culture is usually based on (i) previous related works that have been carried out on the species and (ii) the most widely used PGRs. For avocado, the auxin, picloram, was often used to generate embryogenic cultures (Table 3.2) and 2,4-D is the most widely used auxin for callus induction (Bhojwana and Dantu, 2013). Hence both auxins were tested in the current research to generate callus. The use of picloram for callus formation was not optimal (Table 3.5), which was contrary to expectation. 2,4-D within 0.5 – 1.0 mg/L (similar to the concentration range suggested by Bhojwana and Dantu, 2013), on the other hand, resulted in ca. 70% and 90% callus formation from the greenhouse and the *in vitro* materials, respectively (Table 3.4 and Figure 3.2). Picloram seemed to be a very potent auxin and was lethal to the leaf explants even at low concentration. Conversely in previously reported work (Table 3.2), picloram was effectively and successfully used for explanted immature zygotic embryos. Understandably, the immature zygotic embryo is highly meristematic and embryogenic when compared with leaf explants. Probably, the use of picloram on the highly metabolic immature zygotic embryos further stimulated their meristematic and embryogenic competence, hence the success recorded in previous research (Table 3.2).

While the use of 2,4-D resulted in substantial callus formation, a major setback was the browning of the callus formed, which ultimately led to loss of callus culture. Hence, NAA was tested to induce callus. Materials treated with NAA took longer to initiate callus formation and did not form as much callus as 2,4-D, but the extent of browning was reduced (Table 3.10(iii); Figure 3.5; Figure 4.14). It is safe to reason that, in a procedure where the same concentrations of both of these auxins are exogenously supplied, cells will accumulate more 2,4-D intracellularly than NAA. This is because the mechanism of uptake of 2,4-D by cells is different and more efficient than that of NAA (Delbarre *et al*., 1996). Consequently, 2,4-D will result in more callus formation than NAA (detailed in Chapter Three) such as seen in pecan (Rodriguez and Wetzstein, 1998). However, for callus that will be directed towards plant regeneration, NAA-derived callus is associated with fewer anomalies and seemed to be more embryogenic than 2,4-D-derived callus (Rodriguez and Wetzstein, 1994; 1998). According to Campanoni and Nick (2005), 2,4-D is ten times more potent than NAA at stimulating cell division but does not stimulate cell elongation. On the other hand, NAA is efficient at stimulating cell elongation but 2,4-D is inhibitory. Hence, in a complimentary

manner, both auxins can be used to stimulate cells to form more calli (because of 2,4-D) that are less prone to browning and have embryogenic characteristics (because of NAA). Also, higher concentrations of NAA, than used in this study, can also be used in the future to achieve higher response to callus formation.

The production of phenolic compounds was prevalent in 2,4-D-derived callus from the greenhouse material. In this study, it is believed that the presence of phenolics (which when oxidized) led to deleterious browning and ultimately the loss of callus cultures. In other reports, however, there is evidence that phenolic compounds contribute to the embryogenic competence of callus (e.g. Kim *et al*., 2006; Reis *et al*., 2008). In fact, accumulation of phenolic compounds has been associated with the acquisition of desiccation tolerance (Risenga, 2014). Acquisition of desiccation tolerance is one of the requisites for the maturation of somatic embryos. Thus, the presence and action of phenolic compounds in a callus is more diverse and complex than hypothesized in this study.

Subculturing of callus to fresh medium is meant to sustain the growth of callus because of the freshly supplied nutrients (Nakasha *et al*., 2016). However, in the current research, rather than stimulating the expansion and formation of fresh callus cells, subculturing had an adverse effect on callus growth, most of which resulted in browning and death.

It was expected that the callus mass would comprise localized embryogenic clusters, which would have differentiated to embryos upon transfer to appropriate differentiating medium (Bhojwana and Dantu, 2013). Indeed, there were cell clusters that were proembryo-like in this research (Figure 4.9 and 4.12). However, when these clusters were compared with the descriptions given in the literature (e.g. Kurczynska *et al*., 2012) and other images such as those shown by Verdeil *et al*. (2001), the morphology of these structures did not conform to the general description of embryogenic materials. Thus, when callus was transferred to the various developmental media, the only change noticed was that of callus colour (Chapter Three) and the callus did not survive further subculture. Perhaps, cells of the proembryo-like structures did not develop sufficient cytoplasmic components that would sustain them in a metabolically active and embryogenic state. This emphasized the importance of the physiological state of the callus to be manipulated for further development. Thus for a callus

which is not embryogenic, a differentiating/developmental medium will not confer embryogenic competence on such callus.

Light microscopy (Chapter Four) substantiated the results obtained through tissue culture (Chapter Three) in the following ways: (i) avocado leaves obtained *in vitro* can form more callus and in shorter time than those from the greenhouse, (ii) callus obtained from *in vitro* leaf tissues had a short life span due to little or no cytoplasmic cellular components, (iii) there was more browning in 2,4-D-derived callus than the NAA counterpart and (iv) callus stimulated by NAA possessed more characteristics (e.g. deposition of callose on the cell walls) of embryogenic callus than that triggered by 2,4-D.

The study opened up, for the first time, the response of avocado cv. 'edronol' leaf to callus formation. It showed that (a) the requirements for initiating callus in the leaf were different from the commonly used immature zygotic embryos of avocado; (b) there were striking differences in avocado leaves sourced from the greenhouse and the *in vitro*-grown shoots and subsequently, the callus derived from them; (c) callus obtained from avocado leaf was not amenable to subculture; (d) the response of the plant tissues to 2,4-D and NAA was different and likewise so was the callus formed from using the two auxins and (e) the callus obtained has potential for further manipulation due to the development of proembryo-like structures. Such manipulation towards further development can be attempted in the future. Where non-embryogenic callus is formed, they can be explored for their usefulness in other applications other than plant regeneration.

APPENDIX

A. Sample fixation for light microscopy

a. Sodium (Na) phosphate buffer solution

Solution X: 0.2M NaOH (8.8 g/L of sodium hydroxide)

Solution Y: 0.2M NaH_2PO_4 (24.0g/L of sodium dihydrogen phosphate)

To make 0.1M Na phosphate buffer, add 35ml of solution X to 50ml solution Y and make the solution up to 100ml by adding 15 ml of ultra-pure water, pH 7.2.

b. 2% glutaraldehyde fixative

Dilute 25% glutaraldehyde to 4% (stock solution) with ultra-pure water; and keep it refrigerated.

Mix equal volumes of 4% glutaraldehyde and 0.1M Na phosphate buffer together to make 2% glutaraldehyde.

To fix samples, submerge tissues in the 2% glutaraldehyde solution for 8 hours (up to overnight) at 20 – 25^{o}C.

c. Rinsing:

Remove 2% glutaraldehyde and samples and rinse 4 times with 0.05M Na phosphate buffer for one hour. (Leave samples in rinsing buffer for 15 min each time).

NB: mix equal volumes of 0.1M Na phosphate buffer and ultra-pure water to give 0.05M Na phosphate buffer.

d. Post fixation in 2% osmium tetra oxide (OsO_4)

Mix one vial (2ml) of 4% OsO_4 solution with 2ml of 0.1M Na phosphate buffer to make 2%.

Post fix samples in 2% OsO_4 for 2 hours at room temperature. Remove OsO_4 and rinse samples with 0.05M buffer for one hour.

e. Primary dehydration

Dehydrate samples serially through 10, 25 and 50% ethanol. (Leave samples in each ethanol concentration for 15 minutes).

NB: to obtain various concentrations of ethanol series, serially dilute absolute ethanol (≈ 100%) to 75, 50, 25 and 10% v/v with ultra-pure water.

f. Staining with uranyl acetate

Stain samples with uranyl acetate for one hour in the dark. Uranyl acetate solution is made by dissolving 0.1g uranyl acetate in 10ml 75% ethanol. The solution is stored in a brown bottle and wrapped with foil paper as solution is light sensitive.

g. Secondary dehydration

Remove uranyl acetate and rinse samples with 75% ethanol for 15 minutes. Dehydrate samples four times sequentially: two times in 100% ethanol and two times in propylene oxide. Each dehydration step is for 15 minutes.

h. Embedding

Mix epoxy resin in 1:1 with propylene oxide and submerge samples in the mixture for 4 – 5 hours. Replace the mixture with pure resin and allowed to infiltrate overnight.

NB: Epoxy resin comprises:

Resin – 5g vinylclohexene dioxide VCD)

Hardener – 13g nonelyl succinic acid (NSA)

Plasticizer – 3g DER

0.2g SI

Add components gravimetrically and use at room temperature.

i. Polymerization

Polymerize samples with fresh resin in resin trays at 70°C for 8 – 12 hours.

B. Toluidine blue stain

The stain consists of a mixture of

60ml of 1% (w/v) sodium bicarbonate

40ml glycerol and

1g toluidine blue.

C. Aniline blue stain

a. Sørensen's phosphate buffer

Solution 1: 0.2 M NaH_2PO_4

Solution 2: 0.2M Na_2HPO_4

To make 100ml 0.1M (pH = 8.0), mix 2.7ml of solution 1 with 47.3 ml of solution 2. Make the volume to 100ml with ultra-pure water

b. make aniline blue stain

Add 0.5% (0.5g) water soluble aniline blue (w/v) to 100ml freshly made Sørensen's phosphate buffer.

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