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CALLOGENESIS FROM *PERSEA AMERICANA* (MILL.) LEAF TISSUES: A HISTOLOGICAL COMPARISON OF MATERIALS GROWN IN GREENHOUSE AND *IN VITRO*

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Abstract

Phenotypic plasticity has been recorded in cultivars that are grown under different environmental conditions. The present study assessed the morphological differences of callogenic leaf tissues from the greenhouse and the *In vitro* cultured plants of *Persea americana* cv 'edranol' (avocado) using light microscopy. Callus was formed from all tissues of the leaves and morphological observations indicated that *In vitro* materials were less differentiated and more meristematic than greenhouse materials with 80.6% and 72% callus formation, respectively. Further morphological observation of fresh leaves obtained from both sources showed significant differences. Cells of *In vitro*-derived leaves were bigger resulting in thicker leaves (171.19 ± 21.6 µm) than greenhouse-derived leaves (75.98 ± 8.6 µm). Bundle sheath extensions (BSE) were present in the greenhouse leaves but absent in the *In vitro* leaves. *In vitro* leaf cells contained fewer chloroplasts (1 chloroplast/100 µm^2) than the cells of greenhouse leaves (3 chloroplast/100 µm^2). This study established that growth environments could have significant impact on the callogenic competence of explants as well as influence the plant physiology of the parent plants.

Key words: Avocado, Callus formation, Explants, Lauraceae, Light microscopy, Phenotypic plasticity.

Introduction

Plants with the same genotypes can demonstrate differences in traits (i.e. exhibit phenotypic plasticity) if they are grown in different environments (Bradshaw, 1965; Gratani, 2014). The changes in traits can still occur in plants that are raised in controlled growth environments such as in the greenhouse and *In vitro* (Taha & Haron, 2008; Nikolova *et al*., 2013; Mani & Shekhawat, 2017). Consequently, the changes in traits can influence the callogenic competence of the species (Osborn & Taylor, 1990; Pompelli *et al*., 2010; Patay *et al*., 2016). While callus forms naturally in plants as a response to stresses, it can also be induced *In vitro* under appropriate conditions. Various factors such as the source and developmental status of the explant, genotype, the presence of plant growth regulators and the composition of the basal nutrient media influence callus formation (Litz & Gray, 1995; Radojevic *et al*., 1999; Deo *et al*., 2010; Ji *et al*., 2011).

Persea americana Mill. (avocado), which belongs to the Lauraceae, is an economically important but highly recalcitrant tropical tree species. The latter is because the avocado seed has all the characteristics of recalcitrant germplasms such as large seed size, germination of seeds at fruit shed, and sensitivity to drying and chilling (Daws *et al*., 2005). Recalcitrance can be part of the whole physiology of a species (Benson, 2000) as displayed in avocado, not only in its post-harvest seed behaviour (Wolstenholme & Whiley, 1999) but also in its response to *In vitro* manipulations (Hiti-Bandaralage *et al*., 2017). Therefore, by better understanding the underlying developmental processes in avocado it may be possible to improve its response to *In vitro* procedures and perhaps other members of the family.

In avocado, callus has principally been formed from immature zygotic embryos (Mooney & Van Staden, 1987; Pliego-Alfaro & Murashige, 1988; Perán-Quesada *et al*., 2004; Encina *et al*., 2014). The use of zygotic embryos from avocado has been successful in the formation of callus and in the subsequent development of somatic embryos, while the use of other avocado vegetative explants has not been successful in the formation of somatic embryos. There is yet no known record of successful micropropagation through the use of leaf explants neither in avocado nor in the other species of the genus *Persea*. In particular, Young (1983) is the only known author that has documented the use of avocado leaf as an explant. In that work, only callus formation and the factors that favoured more callus formation were highlighted. The use of leaves as explants, in comparison with other plant parts, reduces the impact on the parent plants and their yield, and the leaves can be harvested at any time of the year. In addition, unlike the commonly used zygotic embryos, the use of vegetative tissues retains the characteristics of the parent plant. Therefore, in this study, callus was induced from explanted leaf materials that were obtained from avocado plants, which were grown in the greenhouse and the *In vitro* environment.

The main aim of this study was to compare the morphology of fresh leaves from both sources and to follow the development of callus from the leaves over six weeks. The hypothesis was that since plant cells are believed to have the unique characteristics of totipotence and plasticity, cells of the various leaf tissues of avocado would be meristematic and dedifferentiate to form callus; however, the growth environment and developmental status of the leaves may influence the callogenic competence.

Material and Methods

Parent plant materials: Young *Persea americana* cv. 'edranol' seedlings were obtained from Rietvlei nursery, Tzaneen, Limpopo province of South Africa and were maintained in the greenhouse at 26-28°C, 40% humidity at the University of the Witwatersrand, Johannesburg, South Africa. The seedlings were routinely treated with a pesticide (Malasol, active ingredient: Mercaptothion), fungicides (Previcur®N, active ingredient: Propamocarb – HCl and Odeo, active ingredient: Chlorothalonil) and fertilizers (LAN, 3:1:5 (26) SR and bone meal).

In vitro plantlets were raised from axillary buds of de-leafed shoots obtained from the greenhouse plant materials. The shoots were rinsed under running tap water for 20 minutes, dipped in 70% alcohol for 30 seconds and rinsed again with tap water. Thereafter, the shoots were decontaminated with 1% sodium hypochlorite (NaOCl) containing 20 drops/litre Tween20® for 10 minutes and rinsed three times with sterile, ultra-pure water. The shoots were cut into 1-2 cm long nodal segments (i.e. explants) that contained at least one axillary bud. The nodal segments were cultured on a full-strength MS (Murashige & Skoog) medium, supplemented with 30.0 $g.L^{-1}$ sucrose, 1.0 $mg.L^{-1}$ BAP (6-Benzylaminopurine) and solidified with 3.0 $g.L^{-1}$ gelrite at pH 5.70 ± 0.05. The choice of the culture medium was predetermined in our laboratory. Cultures were incubated at 25 ± 2°C with a photon flux density of 100 $\mu mol.m^{-2}.s^{-1}$ and a 14-hour light/10-hour dark photoperiods.

Culture medium and culture condition: Greenhouse leaf samples were obtained from young avocado leaves from avocado seedlings that were maintained in the greenhouse. The leaves were decontaminated in a solution of 1% NaOCl containing 20 drops/litre tween20© for five minutes. Subsequently, the leaf segments, without the midrib, were cultured in a callus induction medium comprising Gamborg's B5 medium, which was supplemented with 1.0 $mg.L^{-1}$ dichlorophenoxyacetic acid (2,4-D), 0.5 $mg.L^{-1}$ BAP, 30.0 $g.L^{-1}$ sucrose, 4.0 $mg.L^{-1}$ thiamine hydrochloric acid, 100 $mg.L^{-1}$ myo-inositol and solidified with 3.0 $g.L^{-1}$ gelrite at pH 5.70 ± 0.05. *In vitro* leaf materials were obtained from six-weeks-old *In vitro* plantlets and were cultured in the same induction medium for greenhouse leaf material, but without prior decontamination. The chosen culture medium was the optimum out of the 87 media that we previously tried in the laboratory. All cultures were incubated in the dark at 25 ± 2°C for six weeks. We have previously established that dark incubation favoured more callus induction and formation than light incubation (unpublished data).

Light microscopy: Explanted leaves were collected from cultures at 1, 2, 3, 4, 5 and 6 weeks of induction and prepared for light microscopy (LM). Uncultured, fresh leaves from both the greenhouse and the *In vitro* plants were also prepared for LM. Briefly, leaf sections that were 1.5 µm thick were cut using a Reichert-Jung ultracut microtome and fixed following the method of Wilson *et al*., (2017). Sections were mounted, stained and sealed according to those authors. The sections were viewed with an Olympus BX63 OM/FM light microscope and images were captured with an Olympus DP 80 camera attached to the microscope. The dimensions of the cells of the various fresh leaf tissues, and leaves that had been incubated for one week, were measured using the control software of the Olympus BX63 OM/FM microscope. Image J (free downloadable application) was used to analyze the chloroplasts present in the micrographs of fresh greenhouse and *In vitro* leaves. This entailed the counting of the chloroplasts in the sampled leaf micrographs, measurement of the surface area of each chloroplast and the total area occupied by all the chloroplasts in each sample. The latter was expressed as a percentage of the area of the sampled micrograph. Three samples and 20-30 sections from each leaf source were viewed per week.

One-way ANOVA (Analysis of Variance) was used to test significant differences where applicable at *0.05 significant level* using Microsoft Excel data analysis.

Results

Histological analysis of fresh leaf tissues: The arrangement of the avocado leaf tissues from both the greenhouse and *In vitro* followed the same pattern in the order of upper epidermis, palisade mesophyll, spongy mesophyll and lower epidermis. The upper and lower epidermal layers were uniseriate in both leaf sources (Fig. 1). In the greenhouse leaves, vascular bundles were enclosed in an achlorophyllus bundle sheath (Fig. 1A), which was typical of C3 plant materials. The bundle sheath extended to the upper epidermis to form bundle sheath extensions (BSE). The mesophyll layer cells were packed with chloroplasts and these organelles were pushed to the cell periphery by the large central vacuoles. In the *In vitro* materials, lateral sections through the *In vitro* leaves showed elongated and pitted xylem tissues of the vascular bundles (Fig. 1C). The spongy mesophyll cells were more regular-shaped and were more tightly packed (Fig. 1B and C), resulting in smaller air spaces in comparison with the greenhouse leaves. The palisade mesophyll of the greenhouse leaf material was a single layer (Fig. 1A) whereas it was a mixture of single and double layer in the *In vitro* leaf (Fig. 1B and 1C).

There were significantly ($p = 0.0394$) more chloroplasts in the greenhouse leaves (3.0 ± 2.0 chloroplast/100 μm^2) than in the *In vitro* materials (1.0 ± 0.8 chloroplast/100 $\mu m^{2)}$. In addition, chloroplasts significantly ($p = 0.0140$) occupied more areas in the photosynthetic layers (i.e. the palisade and the spongy mesophyll cells) of the greenhouse leaves (8.0 ± 2.8%) than of the *In vitro* leaves (4.4 ± 1.7%). However, there was no significant difference in the surface area of individual chloroplasts obtained in the greenhouse or in the *In vitro* leaves (5.16 ± 0.98 and 4.79 ± 2.33 μm^2, respectively; $p>0.05$).

The cell sizes of the component leaf tissue (widths of epidermis, spongy and palisade mesophyll) were bigger in the leaves obtained *In vitro* than in the leaves from the greenhouse; only the upper and lower epidermal cells were longer in the latter (Fig. 2). Consequently, the avocado leaves obtained *In vitro* were significantly thicker (171.2 ± 21.6 µm) than those obtained from the greenhouse (76.0 ± 8.6 µm).

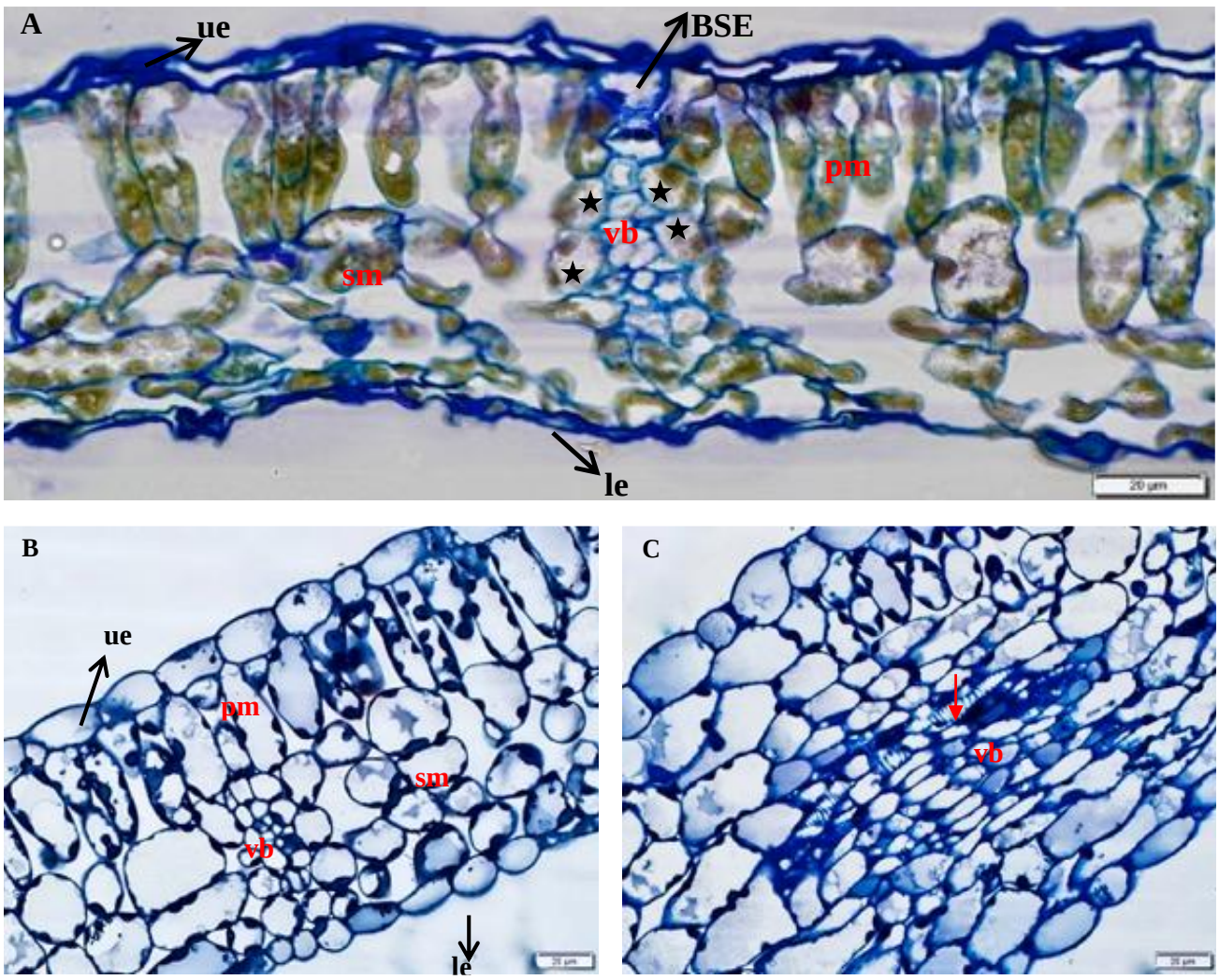


Fig. 1. Transections of fresh and young avocado 'cv' edranol leaves. In the leaves obtained from the greenhouse seedlings (A), vascular bundles (vb) were enclosed with bundle sheath cells (starred cells in A) which extended to from Bundle Sheath Extension (BSE); palisade (pm) and spongy mesophylls (sm) cells were filled with chloroplasts. In leaves obtained from *In vitro* plantlet (B and C), lateral sections of vascular bundles showed pitted xylem (red arrow, C); palisade (pm) and spongy mesophyll (sm) cells had large central vacuoles. Scale bars = 20 µm for all micrographs.

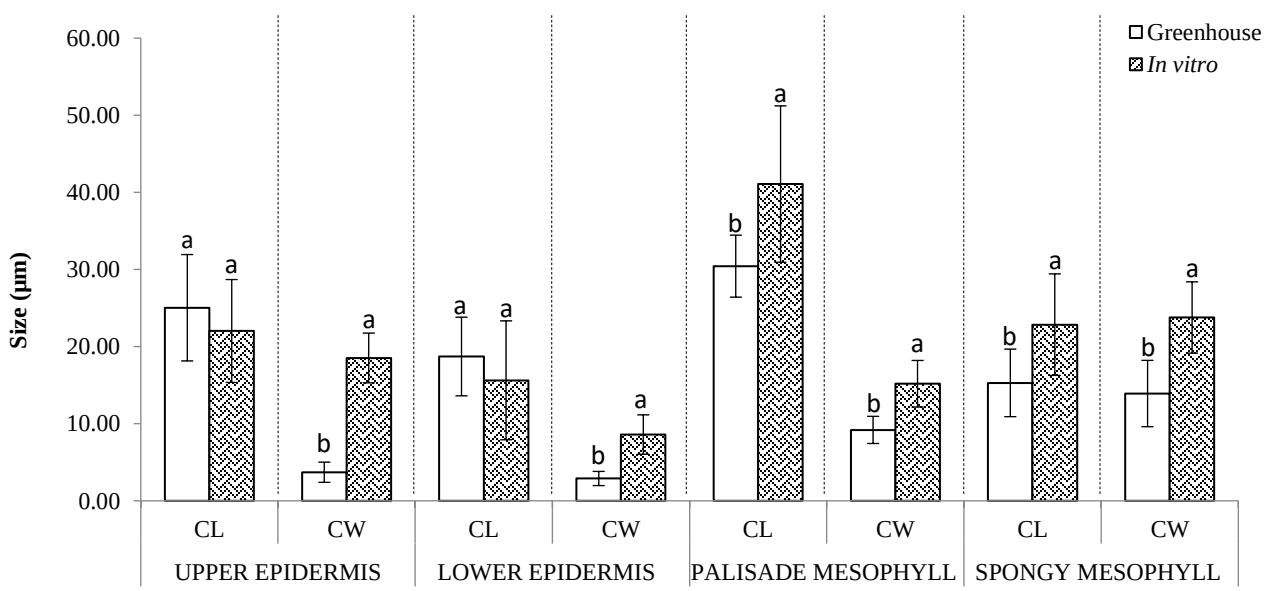


Fig. 2. Cell dimensions (CL – Cell length; CW – Cell width) of the component fresh leaf tissues derived from greenhouse- and *In vitro*-grown *P. americana* cv 'Edranol'. Different superscripts within each cell dimension indicated significant difference.

Table 1. Comparison between the cell sizes of fresh avocado leaves and those incubated for one week in callus induction medium. One way ANOVA was used to ascertain significant difference at *0.05* significant level for each cell type from the two sources. Different superscripts indicated significant difference. n = 20, L – length; W – width.

	Mean size (µm) ± standard deviation											
	Greenhouse						*In vitro*					
	Palisade		Spongy		Epidermis		Palisade		Spongy		Epidermis	
	L	W	L	W	L	W	L	W	L	W	L	W
Fresh	30.4±4.0^a	9.2 ±1.8^a	15.3±4.4^a	13.9±4.3^a	27.7±6.8^a	3.0±1.1^a	41.1±10.1^a	15.2±3.0^a	22.8±6.6^a	23.8±4.6^a	22.5±2.5^a	19.1±2.4^a
One week	59.5±6.4^b	11.4±1.9^b	23.7±5.0^b	18.4±3.4^b	21.2±4.6^a	17.9±1.3^b	43.7±6.3^a	11.5±1.7^b	26.6±6.1^a	20.1±4.4^a	24.9±6.1^a	16.4±2.0^a

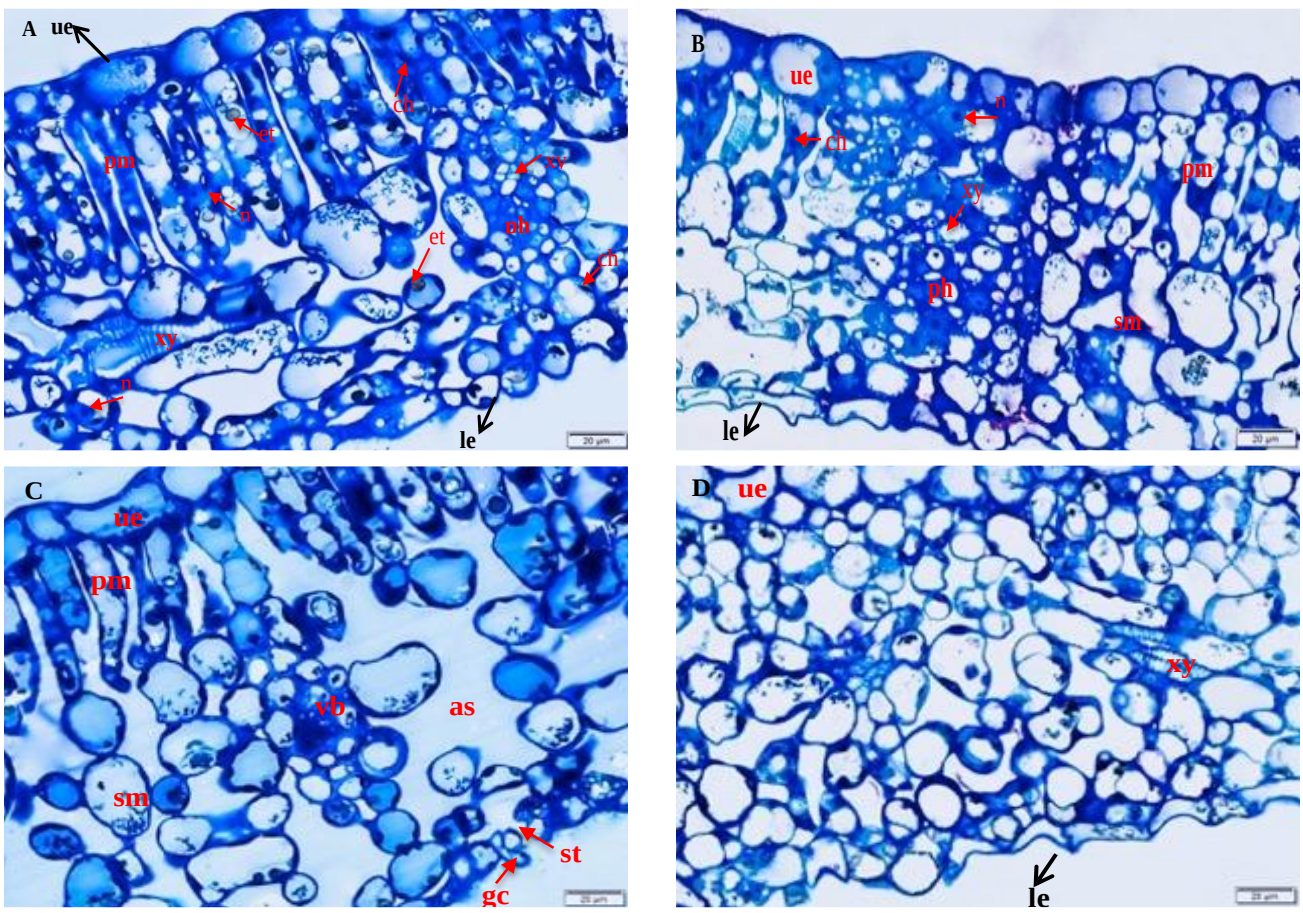


Fig. 3. Avocado 'edranol' leaf explants after one (A and B) and two weeks (C and D) in callus induction medium. Nucleus (n) and chloroplasts (ch) were more prominent and visible in materials from both greenhouse (A) and *In vitro* (B) at one week. Etioplasts (et) were seen in the greenhouse material (A). Cells of the vascular bundles, except for the xylem (xy), were meristematic in both explants. After two weeks, different tissues of the explants from greenhouse (C) materials remained differentiated while palisade and spongy mesophyll cells of the explants from *In vitro* (D) had dedifferentiated. Phloem (ph), nucleus (n), upper epidermal cell (ue), stoma (st), lower epidermis (le), spongy mesophyll (sm), vascular bundles (vb), etioplast (et), guard cell (gc), air space (as). Scale bars = 20 µm for all micrographs.

Formation of callus and histological analysis: From the cultured leaf explants, there was little or no visible callus growth in the first two weeks from both the greenhouse and *In vitro* leaves. However, callus formation was readily visible on the leaf explants from the third week. Leaf explants from the greenhouse and *In vitro* had formed 72.0 and 80.6% callus, respectively, by the sixth week.

After one week in culture, histological analysis of cells revealed a significant increase (p<0.05) in the cell size of the various leaf tissues (Table 1). Vacuolar fragmentation was particularly noticeable in the cells of both the greenhouse and *In vitro* materials (Fig. 3A and 3B). Organelles, including plastids (chloroplasts and etioplasts) and nuclei with prominent nucleoli, were visible. Loss of the green pigment occurred in some of the functional chloroplasts, which led to their transition to etioplasts. Cells of the phloem region of the vascular bundles were apparently meristematic in nature. However, the xylem typically remained unchanged because they comprised dead cells.

At two weeks of callus induction, cells of the greenhouse leaves maintained their organization without any further obvious dedifferentiation (Fig. 3C). On the other hand, there was no distinction between the palisade and spongy mesophyll cells in the *In vitro* materials because the cells had divided and dedifferentiated, thus were isodiametric and meristematic (Fig. 3D). The xylem of the vascular tissues was still visible at two weeks (Fig. 3D).

At the third week of callus induction, there was callus formation in the leaves obtained from both the greenhouse

and the *In vitro*. In the greenhouse leaf materials, cellular aggregates were around both the vascular bundle and the epidermis (Fig. 4A). In the *In vitro*-derived explants, cellular aggregates were associated with the mesophyll cells, which pushed through the epidermis to form visible callus (Fig. 4B). Similar processes occurred directly from the epidermis (Fig. 4C).

The size of the callus from both the greenhouse (Fig. 5A and 5B) and the *In vitro* (Fig. 5C and D) environments started to increase from the fourth week. The accumulation of phenolic compounds was visible in the callus formed from greenhouse-derived materials (Fig. 5B). Some callus cells divided to form an organized group of cells that were proembryo-like, which were found dispersed within and on the periphery of the callus mass (Fig. 5B). In the callus obtained from *In vitro* leaf materials (Fig. 5C), cells were isodiametric and highly vacuolated (Fig. 5D). The cytoplasm of some cells from *In vitro* materials began to pull away from their cell wall (Fig. 5D).

By the fifth week, callus cells from greenhouse materials comprised a mixture of polygonal-shaped cells with small nuclei (Fig. 6A) and isodiametric, highly vacuolated cells (Fig. 6B). In the callus from *In vitro* leaf explants, cell shapes were highly irregular, showing signs of shrivelling and had begun to collapse (Fig. 6C). However, new proembryo-like cell aggregates, suggestive of callus development towards a definite structure, were formed on the periphery of the degenerating callus (Fig. 6D).

Phenolic darkening was prevalent in the callus from the greenhouse explants by the sixth week (Fig. 6E). While phenolics were not seen in the callus from *In vitro* explants, the shrivelled cells of the *In vitro* callus had presumably collapsed, creating a hollow space in the callus mass (Fig. 6F).

In this study, the arrangement of the tissues in the avocado leaves (Fig. 1) followed the general organization of most dicotyledonous leaves, i.e. upper epidermis, palisade and spongy mesophyll layers and lower epidermis (e.g. in Laparra *et al*., 1997). Uniseriate epidermal layer as seen in avocado is also common in other species of the Lauraceae (Zeng *et al*., 2014). The mesophylls layers of avocado leaves from both the greenhouse and the *In vitro* materials are differentiated into palisade and spongy tissues like most dicotyledonous plants (Trigiano & Gray, 2005). The size and the arrangement of the spongy mesophyll allowed for more intercellular space in the greenhouse leaf than *In vitro* tissues. This implied that there would be more gaseous exchange in the greenhouse leaves than the *In vitro* as the proportion of intercellular space has been shown to have a direct relationship with gaseous exchange (Beck, 2010; Pompelli *et al*., 2010). It was also assumed that the cells of the greenhouse leaves would be more saturated with nutrients and plant growth regulators from the induction medium; thus, were expected to form more callus than the *In vitro* materials. However, the leaves from the *In vitro* materials formed more callus than the greenhouse materials. This inferred that there were more influential factors, such as a lesser extent of tissue differentiation and higher meristematic competence (characteristics of the *In vitro* materials), which favoured more callus formation in the *In vitro* leaves.

Persea americana cv. 'edranol' showed phenotypic plasticity both morphologically and physiologically, which was a reflection of the different controlled environments in which they were grown. The plasticity was expressed in the presence or absence of bundle sheath extensions (BSE), number of and the area occupied by chloroplasts, leaf thickness, size of leaf cells and callogenic competence.

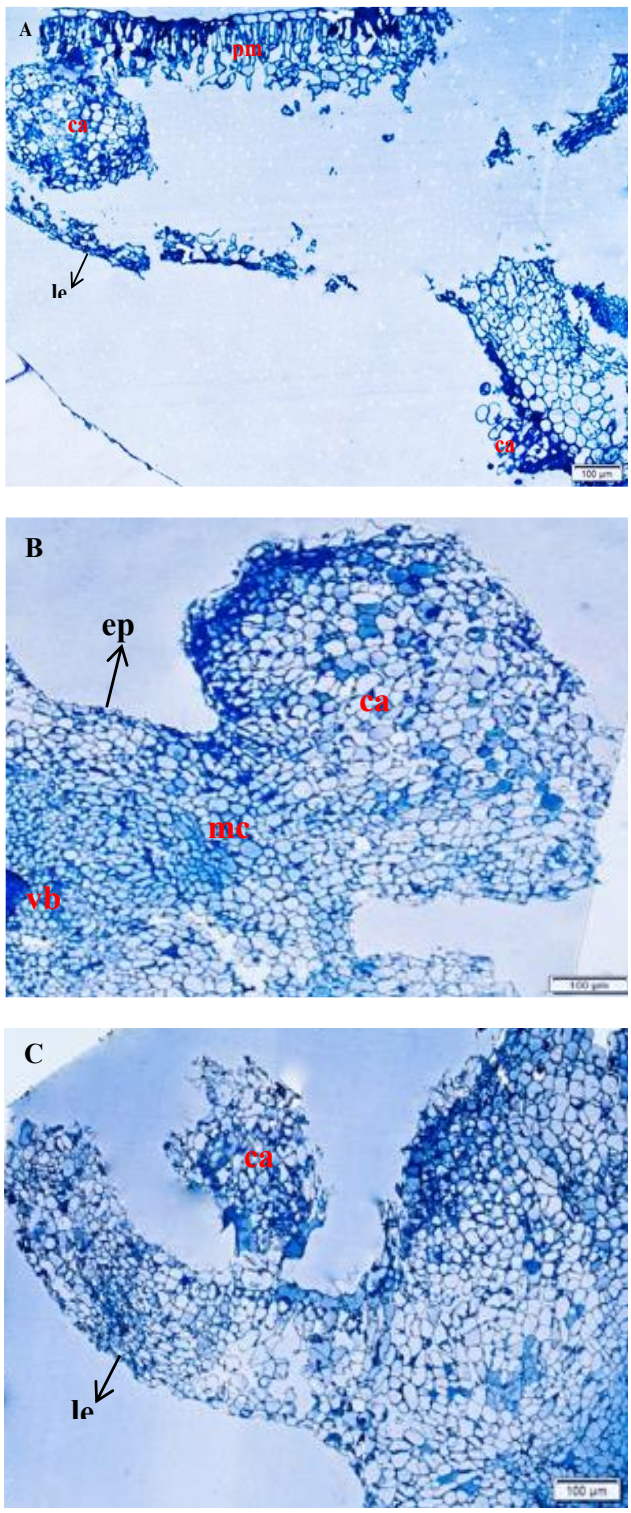


Fig. 4. Cross section through the callus and the leaf explants of 'edranol' avocado after three weeks in callus induction medium. A: cells aggregated around the vascular bundle and epidermal layer to form callus (ca) in greenhouse leaves. Callus formed in the *In vitro* leaves from the mesophylls that surround vascular bundle (B); and on the epidermis (C). ue – upper epidermis; le – lower epidermis; mc – mesophyll cells; vb – vascular bundle; ep – epidermis; pm – palisade mesophyll. Scale bars = 100 µm for all micrographs.

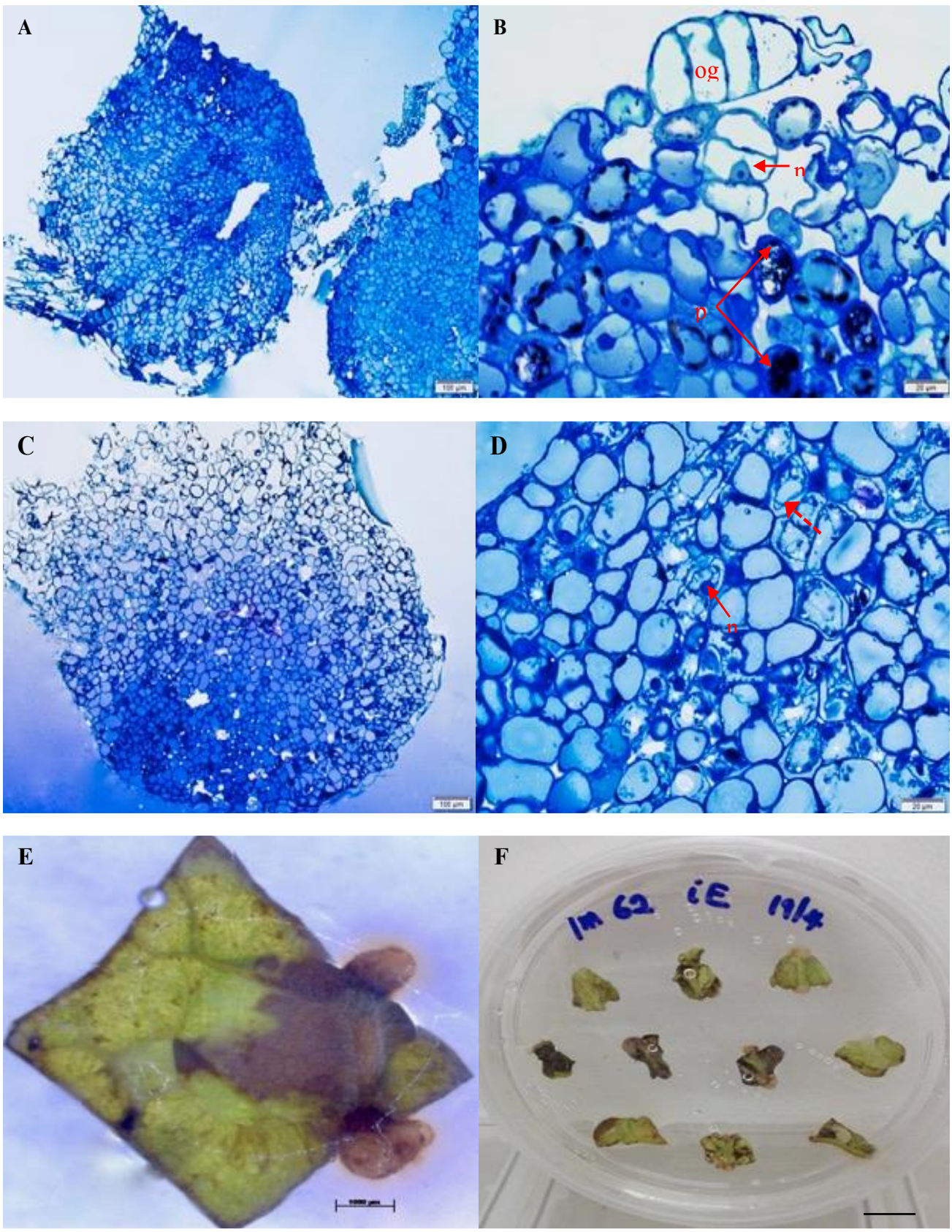


Fig. 5. Light micrographs (A – D) of callus at four weeks of callus induction from the leaf material of avocado cv. 'edranol'. **A**: callus from greenhouse leaf material. **B**: portion of A showing nucleated cells, phenolic compounds (pc) in cells and some organized group of cells (ogc). **C**: callus from *In vitro* leaf material. **D**: portion of C showing largely vacuolated and different sizes of callus cells. Some cells showed retracting cytoplasm from the cell wall (broken arrow line). **E**: explanted greenhouse leaf material showing brown (due to phenolic compound) callus. **F**: explanted *In vitro* leaf material in a Petri dish. n – nucleus. Scale bars: A & C – 100 µm; B & D – 20 µm; E – 1000 µm and F – 1 cm.

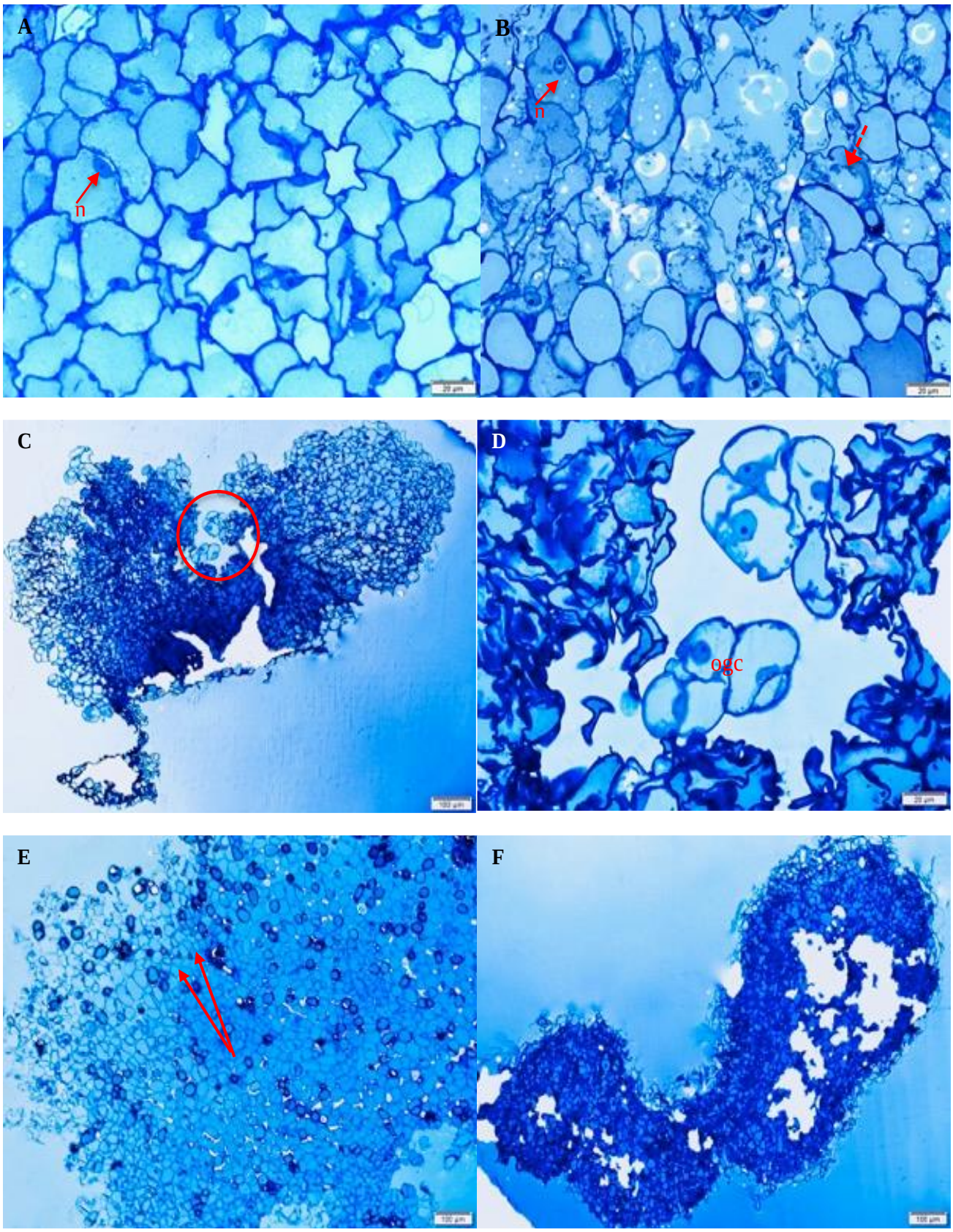


Fig. 6. Light micrographs of callus at five (A – D) and six (E – F) weeks of induction from the leaf material of avocado cv. 'edranol'. Callus from greenhouse material (A & B) comprised both polygonal cells with small nuclei (A) and isodiametric, highly vacuolated cells (B). Callus from *In vitro* leaf materials comprised irregular shaped cells that had begun to shrivel and collapse (C) but there were organized groups of cells (ogc) which formed new clumps on the periphery of the callus mass e.g. encircled portion of C, which was enlarged in D. By the sixth week, phenolic compounds (pc) were dispersed through the callus from the greenhouse leaf material (E). The cells at the center of the callus from *In vitro* leaf material had collapsed, creating hollow spaces in the callus (F). Scale bars: A, B & D – 20 µm; C, E & F – 100 µm.

Discussion

The number of chloroplasts present in leaves, as well as their surface area, has direct relationship with photosynthetic efficiency (Xiong *et al*., 2017). Thus, in this study, while the chloroplasts from both the greenhouse and *In vitro* were relatively similar in terms of their surface area, the higher number of chloroplasts present in the greenhouse leaves suggested a more efficient photosynthetic process than in the *In vitro* materials. Other studies have shown that the photosynthetic rate of *In vitro* plants is generally low due to their growth conditions (Viña *et al*., 1999; Sáez *et al*., 2012).

The presence or absence of BSEs categorizes leaves as heterobaric or homobaric, respectively (Kenzo *et al*., 2007). Bundle sheath extensions are common in the leaves of Lauraceae (Kenzo *et al*., 2007; Boeger *et al*., 2016) and hence, were found in avocado leaves obtained from the greenhouse. One of the functions of BSEs is to improve photosynthetic rates and since *In vitro* materials invest minimally in photosynthesis (due to fewer number of chloroplasts), it is understandable that avocado leaves obtained *In vitro* lacked BSEs in this study. While the classification of leaf forms, based on the presence or absence of BSEs, had been at inter-specific level, this study suggested an intra-specific level of classification.

In accordance with the view of Kessler *et al*., (2002), *In vitro* avocado leaves were significantly thicker than those from the greenhouse. This was directly related to the larger size of the various component tissues of the *In vitro* leaves (Fig. 2), as well as the arrangement of their spongy mesophyll in stacks of three to five cells (Fig. 1B and C). The relative humidity (RH) of *In vitro* culture environment is usually higher than the RH of the greenhouse environment (Chen, 2004). This has a direct relationship on the moisture content of the cells of *In vitro* grown plants, resulting in turgid and bigger cells with higher fresh weight and little dry matter. Similar results were obtained in the component tissues of plantlets of potato grown *In vitro* (Kozai *et al*., 1993).

The process of callus formation from the leaves of avocado 'edranol' was in three stages: the preparatory stage of dedifferentiation and cell division in the first two weeks; aggregation of cells and budding stage by the third week and the growth stage from the fourth week.

An obvious change to avocado leaf explant cells, in the preparatory stage of callus formation, was the significant enlargement of the cells after one week in culture (Table 1). An increase in cell size, among other things, usually precedes cell division (Majda & Robert, 2018) towards callus formation. Also, the vacuoles in the cells of different tissues had fragmented to form smaller vacuoles (Fig. 3A and 3B). Vacuolar fragmentation, as here seen in avocado leaf cells, often occurs before and during cell division (Esau & Gill, 1991; Kutsuna & Hasezawa, 2002). These dedifferentiation processes have also been recorded in the leaf cells of *Helianthus smithii* 10 days after induction of callus (Laparra *et al*., 1997). In addition, cell division marks the ontogeny of callus formation, which was seen in avocado leaf tissues such as the vascular bundles of both greenhouse and *In vitro* materials (Fig. 3A – C), and in the photosynthetic layers of the leaves obtained *In vitro* (Fig. 3D). The interaction of the plant growth regulators, auxin and cytokinin (2,4-D: 1.0 $mg.L^{-1}$ and BAP: 0.5 $mg.L^{-1}$, as used in this study) triggered such cell division, which resulted in dedifferentiation (Wang *et al*., 2011). Hence, the palisade and spongy mesophyll layers of the *In vitro* leaves dedifferentiated; consequently, there was loss of distinction between the two layers (Fig. 3D). Similarly, mesophyll layers of *Elliottia racemosa* were undifferentiated after two weeks on culture medium (Woo & Wetzstein, 2008).

Unlike the *In vitro* leaf material, the palisade and spongy mesophyll layers of the greenhouse leaf materials did not dedifferentiate during the process of callus formation (see Figs. 3C, 4A). This suggested that these tissues had differentiated further down their developmental pathway and they could not revert to a state of increased developmental competence – dedifferentiation. The inability to dedifferentiate implied that not all plant cells are totipotent; rather totipotency is dependent on many factors, one of which is the developmental state of the cells (Fehér, 2019). This view is contrary to popular belief.

Leaf tissues such as the vascular bundle (e.g. in *Bruguiera sexangula*, Mimura *et al*., 1997) and epidermis (e.g. in bay laurel, Canhoto & Cruz, 1996) have been shown to be the source of callus formation because they are meristematic and sometimes, embryogenic. Similarly, the vascular bundle (Fig. 4A), the mesophyll cells (Fig. 4B) and the epidermal cells (Fig. 4A and Fig. 4C) were callogenic in this study. While it is shown that all the leaf tissues were capable of callus formation, the developmental stages of the leaves from both sources influenced their totipotency. Thus, the avocado *In vitro* tissues demonstrated that they are not as fully differentiated as the greenhouse tissues; hence they could regain and increase developmental potency.

The formation of the proembryo-like, organized group of cells that developed from the greenhouse- and *In vitro*- callus at fourth and fifth week respectively, were similar to those described by Santos *et al*., (2006) and seen in the callus of *Dieffenbachia* (Shen *et al*., 2016). The growth of the proembryo-like structures showed the potential of the callus to develop into definite structures such as somatic embryos, although that could not be achieved in this study.

Accumulation of phenolic compounds accompanied callus growth from the fourth week in the callus obtained from greenhouse leaves and this had increased by the sixth week. Phenolic compounds accumulate in highly vacuolated cells (Alemannno *et al*., 1996) and often lead to deleterious browning of callus. Naturally, different organs of the avocado plants, such as the fruits, are prone to accumulation of phenolic compounds, which leads to browning (Kosińska *et al*., 2012; Toledo & Aguirre, 2017; Castro-Lopéz *et al*., 2019). Apart from the plant species, growth medium and environments contribute to the accumulation of phenolic compounds (Jones & Saxena, 2013). In addition, 2,4-D, the auxin used to induce callus in this study, has been shown to trigger more browning than other auxins (Nikolaeva *et al*., 2009). On the other hand, the accumulation of phenolics was not prevalent in the callus from the *In vitro* derived explants, however,

callus growth comprised some cells with retracting cytoplasm. These cells with retracting cytoplasm had shrivelled by the fifth week and eventually collapsed by the sixth week. It is possible that the collapse occurred because the cells were rapidly expanding under the influence of the auxin and were mostly vacuolated with little or no cytoplasmic contents. This, with progressive culture days, led to the loss of cellular integrity. Another probability for the shrivelling of the cells would be that the induction medium was hypertonic, which would lead to the plasmolysis of the cells (Alemannno *et al*., 1996). Similarly, Sparapano and Bruno (2004) and Ramulifho *et al*., (2019) recorded plasmolyzed cells due to hypertonic callus growth medium.

Conclusion

This study has shown that morphological and physiological differences are related to growth environments. It further shows the varied responses of this recalcitrant species to callogenic triggers. The leaf explants derived from the *In vitro* materials were not as fully differentiated as those from the greenhouse. However, they were more meristematic and thus more capable of callus formation than the fully differentiated greenhouse-derived leaf tissues. The growth of proembryo-like structures from the callus from both explant sources provides ground for further investigation into morphogenic/embryogenic competence of the callus from this truly recalcitrant species.

The less differentiated leaf tissues of *In vitro* plants may be attributed to one or both of the following possibilities. Either (i) there is a difference in their developmental timeline when compared with plants grown under greenhouse conditions and/or (ii) *In vitro* tissues invest minimally in growth and developmental processes (e.g. photosynthesis). While this study did not address acclimatization and hardening of *In vitro*-grown materials to a natural environment, it did show that *In vitro* materials are less invested in photosynthesis. This contributes to the acclimatization problem, which is ubiquitous in plant propagation and particularly in avocado. Collectively, this may be related to the recalcitrant nature of avocado and probably, the other members of the Lauraceae. These factors may reduce the micropropagation of avocado for commercial purposes.

The development of somatic embryos from the explants of this truly recalcitrant species is still largely elusive, however, understanding the role of developmental status in the callogenic competence of the explants is a step towards a greater goal of somatic embryo formation and ultimately plant regeneration. For further research, the effect of other induction media could be attempted. The manipulation of the callus towards further development can be monitored at histological level to further understand the underlying mechanisms of callus development.

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