A TIME COURSE ANATOMICAL ANALYSIS OF CALLOGENESIS FROM YOUNG LEAF EXPLANTS OF OIL PALM (Elaeis guineensis Jacq.)

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ABSTRACT
Histological and scanning electron microscopy analyses were employed to analyse callus development in oil palm at fortnightly intervals over a period of 28 weeks. The emergence of primary calli on the surface of the oil palm leaf explants was generally observed after 10 weeks of culture. Callus initiation was detected earlier, at six weeks, upon the division of perivascular cells within the leaf explant. This is the first report documenting the presence of a net-like structure termed as an extracellular matrix surface network (ECMSN) on the callus surface and the epidermal layer of the leaf explants of oil palm. We also observed peculiar ‘empty’ epidermal cells and small spherical particles on the surface of the epidermal cells of the leaf explants concomitant with the formation of a callus that have not been reported during callus initiation in other plants. These results may provide basic knowledge and facilitate our understanding of the biological processes involved during oil palm callogenesis. The anatomical changes and the presence of unique features like ECMSN may act as structural markers during callogenesis of oil palm.

INTRODUCTION
Elaeis guineensis (oil palm) is one of the most important crops in the world, and a major source of oils and fats. At present, Malaysia is the second top producer of palm oil in the world (Abdullah et al., 2009). As reviewed by Mohd et al. (2005) and Sumathi et al. (2008), Malaysia produces about 47% of the world’s supply of palm oil. Based on a prediction of the trends in the use of edible vegetable oils with an increasing world population, Corley (2009) postulated that the demand for edible vegetable oils will rise to 250 million tonnes per year. High demand for the edible oils has pressured the oil palm industry in Malaysia to improve the status of palm oil production in order to fulfil this requirement (Corley, 2009). Thus, more than 10 companies in Malaysia have undertaken oil palm micropropagation via somatic embryogenesis (SE) and cell suspension culture to generate a large-scale production of clonal oil palm (Sharifah and Abu, 2007). However, the oil palm industry encounters a number of bottlenecks in the current oil palm tissue culture technique. The processes involved which take up to 15 months to produce somatic embryos, and another one to three years to produce oil palm plantlets, are tedious and laborious. The efficiency of these processes is also dependent on the genotype of the mother plant (Duval et al., 1995; Corley and Tinker, 2003; Mohd et al., 2005).

Clonal propagation of elite oil palms through SE begins with callogenesis which typically generates nodular, fast-growing and granular calli (Duval et al., 1995; Corley and Tinker, 2003). However, only
some of these calli will form friable embryogenic calli (FEC) that are a valuable source of cells with embryogenic capacity. This friable embryogenic tissue is used as the starting material for liquid suspension cultures to regenerate plantlets of oil palm. Tissue is used as the starting material for liquid embryogenic capacity. This friable embryogenic calli (FEC) that are a valuable source of cells with some of these calli will form friable embryogenic laboratories (Schwendiman et al. 1988; Rajanaidu et al., 1997). This has also been reported in other palm species such as coconut, Cocos nucifera (Sáenz et al., 2006), date palm, Phoenix dactylifera (Sané et al., 2006), and macaw palm, Acrocomia aculeatae (Moura et al., 2009). However, polyembryogenic cells or FEC have very low production efficiency during oil palm SE and take six months of culture to reach the peak of FEC production (Corley and Tinker, 2003). More recently, Sharifah and Abu (2007) reported that in some genotypes of oil palm, although the rate of callus production can reach 100%, the rate of embryogenic tissue production in the callus cultures is still low, at a mean of approximately 5%.

In order to overcome these problems, research on this early stage of oil palm tissue culture has to be emphasised because this is the crucial phase in the production of embryogenic tissue. Besides optimisation of the culture medium and tissue culture conditions, understanding the biological changes during oil palm callogenesis may also shed some light towards solving the problems and help in enhancing the efficiency of embryogenic tissue production. The first histological analysis of SE from leaf explants of oil palm was reported by Schwendiman et al. (1988). They focussed mainly on the formation of somatic embryos from calli which were subcultured every two or three weeks. However, no time course details on the morphological and histological changes during callus production of oil palm were reported. Thus, we aimed to analyse the stages of callus formation periodically, as well as to investigate the morphological and anatomical changes during oil palm callogenesis. By doing so, the precise period of callus initiation and development can be determined and manipulated in order to accelerate and enhance callus production, and, thus, improve the production of embryogenic tissue in oil palm. This analysis might also be helpful in identifying putative structural markers during oil palm callogenesis which may have application potential in the oil palm tissue culture industry.

MATERIALS AND METHODS

Plant Material

Cultured non-chlorophyllous young leaf explants of oil palm (Elaeis guineensis Jacq. var. tenera) derived from two different clones of similar genotype (clones 4177 and 4178) were collected every fortnight (day 0 to 28 weeks of culturing) from FELDA Agricultural Services Sdn Bhd, Malaysia. The leaf explants were cultured on Murashige & Skoo (MS) basal culture medium (Murashige and Skoog, 1962) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), and kept in the dark for callus induction.

Histology

Five young leaf explants (0.5 cm²) were sampled and fixed fortnightly in the fixative solution [0.1 M phosphate buffer, 1% (v/v) glutaraldehyde, 2% (w/v) formaldehyde and 1% (w/v) caffeine] for 24 hr at room temperature. The fixed samples were progressively dehydrated in ethanol and butanol at room temperature, and finally embedded in methacrylate resin, Kulzer Technovit 7100 (Wehrheim, Germany). Sections of 5 µm thickness were stained with periodic acid Schiff (PAS) reagent and counterstained with naphthol blue-black (Fisher, 1968). Stained sections were viewed under a Leica DM6000B digital microscope (Wetzlar, Germany).

Scanning Electron Microscopy (SEM)

The leaf explants were cut (approximately 0.5 cm²) and fixed overnight in 0.1 M sodium cacodylate buffer and 4% (v/v) glutaraldehyde. The fixed samples were washed three times using 0.1 M sodium cacodylate buffer for 10 min each time, followed by post-fixation in 1% (v/v) osmium tetroxide for 2 hr at 4°C. The samples were then washed again in 0.1 M sodium cacodylate buffer as described above. The specimens were dehydrated in a series of diluted acetone, and transferred into specimen baskets for drying using a BAL-TEC 030 critical point dryer (Liechtenstein, Switzerland) for 2 hr. The dried specimens were coated with gold (thickness of 300-450 angstroms) using a BAL-TEC SCD005 sputter coater (Liechtenstein, Switzerland), then viewed under a Philips XL30 environment scanning electron microscope (Amsterdam, Netherland).

RESULTS

Callus Initiation

The initial callus called primary callus emerged at the cut edge of the leaf explants after 10 weeks of culture (Figure 1a). The primary callus was observed as a light whitish globular structure. White root-like callus with an elongated structure was also observed on the same leaf explant (Figure 1a). In this study, oil palm leaf explants were cultured on the same
callus induction medium without subculturing throughout the 28 weeks. As a result, the leaf explants produced a cluster of calli that comprised various types of calli. Figure 1b shows the formation of different types of calli in the same cluster after 18 weeks of culture. The frequently produced calli termed as nodular calli (NC) were beige in colour and appeared as compact globular shapes, whereas the white and elongated root-like callus that was observed on primary calli (Figure 1b) was termed as rooty callus. In oil palm callogenesis, NC is the most favoured type of callus because of its capability of producing embryogenic cells. Primary calli and new callus clusters were also found at the later stages of callus culture, demonstrating continuing and asynchronous callus development (Figures 1b-1d). In the 24th week, yellowish friable structures known as FEC were detected on NC (Figure 1d). As expected, the amount of FEC produced in this study was small due to the low rate of FEC production in oil palm tissue culture (Teixeira et al., 1995; Wooi, 1995; Sharifah and Abu, 2007).

Figure 1. Tissue cultured oil palm young leaf explants after 10, 18, 20 and 24 weeks of culture. (a) Callus initiation observed after 10 weeks of culture with the appearance of light yellow primary calli (blue arrows). (b) Various types of calli formed on the same cluster. Whitish primary callus protruded outwards from the leaf surface followed by the emergence of secondary calli comprising light yellow nodular callus and white rooty callus. (c) After 20 weeks of culture, primary callus was also detected typifying continuity in callogenesis. (d) Friable embryogenesis callus was visible after 24 weeks of culture.

Note: FC = friable embryogenic callus; LE = leaf explant; NC = nodular callus; PC = primary callus; RC = rooty callus; WK = week. Scale bars: 0.2 cm (a, c, d); 0.18 cm (b).

Histological Description

Histological analysis revealed that callus initiation began with the division of perivascular cells of six-week-old cultured young leaf explants. Figure 2a shows a section of a leaf explant at day 0 (before culturing), while Figure 2b illustrates cell division of the vascular tissue, marking callus initiation in the six-week-old cultured leaf explants. Perivascular cells were found periclinally divided and were characterised as elongated cells with dense cytoplasm and fewer vacuoles compared to their neighbouring cells. At eight weeks, multi-orientated cell divisions produced a peculiar undifferentiated body structure consisting of cells with large nuclei, dense cytoplasm, small vacuoles and soluble proteins (blue-stained). This structure was surrounded by a thick boundary (dark purple-stained); separating it from adjacent neighbouring cells (Figure 2c).

After 10 weeks of culture, the enclosed cell cluster produced primary calli attached to the
sub-epidermal cells of the leaf explants (Figure 2d). Meristematic cells with large nuclei and dense cytoplasm were present in the innermost part of the primary calli, suggesting that the cells were actively dividing, while highly vacuolated cells were found in the outer layers. This arrangement of cells is similar to cambium tissue. The surrounding cells of the developing callus comprised ‘empty’ parenchyma cells, which putatively underwent programmed cell death (PCD) to allow callus development and dissociation from the leaf explants. The histological results at this time point corresponded to the observation shown in Figure 2a in that callus emerged from the 10-week-old leaf epidermal layer.

Transverse sections of the 12-week-old cultured leaf explants (Figure 2e) revealed that the callus is comprised of two types of cells: dense meristematic cells with large nuclei, and large, vacuolated cells. These zones mirror the arrangement of cambium cells. In addition, secondary calli were possibly derived from the primary calli because a large nucleated meristematic zone was found between these two calli. In a well-developed primary callus, dense meristematic cells were found in the outer part. Meanwhile, a growing secondary callus...
contained a meristematic zone in the centre of the callus similar to the arrangement of cells in the primary callus as observed in Figure 2d. Here, we observed that callus development was asynchronous and continuous, as a newly formed callus (primary callus) was found simultaneously with a secondary callus in the same leaf section in the 12th week (Figure 2e). Interestingly, starch grains were detected (stained pink) in some growing calli (Figure 2f).

Morphological Description

A two-week-old cultured young leaf explant had a smooth epidermis as shown in Figure 3a. After six weeks of culture, small spherical particles were found on the surface of the epidermal cells of the leaf explants (Figure 3b). After 10 weeks of culture, the small spherical particles were also detected on the broken epidermal layer caused by the protuberance of a primary callus (Figure 3e). The width of the small spherical particles ranged from 30-47 µm. After 18 weeks of culture, various types of callus structures were produced on the leaf explants as observed in Figure 3d. Figure 3e shows a newly produced callus emerging from the epidermal layer of a 22-week-old cultured young leaf explant before the production of calli, illustrating continuous and asynchronous callus development. Small spherical particles were also found on the edges of the broken epidermal layer and on the surface of the epidermis close to the newly formed callus (Figure 3e). The appearance of the small spherical particles on the epidermal layer along with the newly formed primary callus suggests that this feature may be associated with the formation of callus. Unlike primary and nodular calli, the root-like calli are filamentous in shape. Also, the arrangement of the calli was unorganised (Figure 3f), and there were tiny hair-like structures covering the surface of the rooty calli (Figure 3g) not found in the nodular and primary calli.

Figure 3. Morphological changes in leaf explants during oil palm callogenesis. (a) Smooth surface of a two-week-old cultured leaf explant. (b) After six weeks of culture, small spherical particles on the surface of the epidermal cells of a leaf explant (blue arrows) were observed. (c) Primary calli (green arrows) were first found to protrude outward from the leaf explant after 10 weeks of culture. Red arrows point to the small spherical particles found at the sixth week. (d) Various types of calli were formed on an 18-week-old cultured leaf explant. (e) Callus formation was asynchronous as newly developed callus was observed after 22 weeks of culture (green arrow). Small spherical particles were also observed (red arrows). (f) Unorganised, filamentous rooty callus; (g) Magnification of the region marked by the yellow circle in (f). Purple arrows point to tiny hair-like structures covering the surface of rooty calli.
Aberrant Layer and Peculiar ‘Empty’ Epidermal Cells

A vaguely transparent, thick layer was seen on the outer epidermal cell walls of a histo-sectioned leaf explant (Figure 4a) which bore a growing callus (Figures 4a-4d). This aberrant layer was found sporadically on the leaf explants after 10 weeks of culture. Interestingly, a darkly stained thin layer was also observed outside the epidermal cells on another part of the same 10-week-old cultured leaf explant (Figure 4b). Also, a few peculiar structures similar to epidermal cells but without nuclei and cytoplasm were observed on the epidermal layer.

Note: GC = growing callus; NFC = newly formed callus. Scale bars 20 µm (a,b,d,f); 100 µm (c,g); and 150 µm (e,h).
of the leaf explants. The epidermal cells might have been modified to produce ‘empty’ cells coated with bead-like blue patches (Figures 4c-4f). The bead-like blue patches on the cell walls of these ‘empty’ cells might be filled with the same material as that found in the aberrant layer (Figures 4a and 4b). Here, we designated the ‘empty’ cells as peculiar ‘empty’ epidermal cells.

The peculiar ‘empty’ epidermal cells were also observed in the sections of the cultured leaf explants at the 18th (Figure 4g) and 20th weeks (Figure 4h). However, the blue patches could not be detected by light microscopy at other stages of oil palm callogenesis. The width of the peculiar ‘empty’ epidermal cells ranging from 30-45 µm was relatively close to the width of the small spherical particles observed by SEM (Figures 3b, 3c and 3e). The peculiar ‘empty’ epidermal cells were probably analogous to these small spherical particles. However, histological analysis failed to detect the peculiar ‘empty’ epidermal cells in the leaf explant culture at the sixth week although their analogue, the small spherical particles, were detected by SEM on the outer epidermal cells of the leaf explants at this stage (Figure 3b). Nevertheless, the peculiar ‘empty’ epidermal cells observed at the 10th, 18th and 20th weeks (Figures 4c-4h) could correspond to the small spherical particles seen under SEM. Interestingly, the darkly stained aberrant layer and the peculiar ‘empty’ epidermal cells were detected along with the formed callus in the innermost layer of the sectioned leaves (Figures 4c-4h).

Net-like Structure

Scanning electron microscope analysis unveiled a compact net-like structure partially covering the surface of the epidermal walls of six-week-old cultured leaf explants (Figure 5a), but no similar structure was detected on the epidermal surface of the leaf explants. The net-like structure was observed on the outside of the epidermal layer of leaf explants and callus. (a) The surface of a six-week-old cultured leaf explant was covered by a compact network (green arrows). (b) After 10 weeks of culture, the surface of the protruding primary callus was observed to be smooth. (c) The surface of nodular callus at 14 weeks (yellow arrows) was covered by a net-like structure. (d-g) Different forms of the net-like structure were found on the developing callus at 16 weeks. (d) A net-like structure linking adjacent callus cells is indicated by yellow arrows. (e) Tiny bead-like substances were found sporadically (blue arrows). (f-g) Clusters of tiny bead-like substances (blue arrows) with net-like strands (pink arrows) were observed on the surface of callus. (h) A net-like structure was observed on the surface of a nodular callus after 18 weeks of culture (pink arrows). (i) At 20 weeks, the surface of the callus was covered by a smooth compact feature (*), possibly formed by the stretching of the net-like structure during callus development.

Note: CA = callus cell; PC = primary callus cells.

Figure 5. Net-like structure observed on the outside of the epidermal layer of leaf explants and callus. (a) The surface of a six-week-old cultured leaf explant was covered by a compact network (green arrows). (b) After 10 weeks of culture, the surface of the protruding primary callus was observed to be smooth. (c) The surface of nodular callus at 14 weeks (yellow arrows) was covered by a net-like structure. (d-g) Different forms of the net-like structure were found on the developing callus at 16 weeks. (d) A net-like structure linking adjacent callus cells is indicated by yellow arrows. (e) Tiny bead-like substances were found sporadically (blue arrows). (f-g) Clusters of tiny bead-like substances (blue arrows) with net-like strands (pink arrows) were observed on the surface of callus. (h) A net-like structure was observed on the surface of a nodular callus after 18 weeks of culture (pink arrows). (i) At 20 weeks, the surface of the callus was covered by a smooth compact feature (*), possibly formed by the stretching of the net-like structure during callus development.
of the leaf explants in the later stages. After the appearance of the primary callus at 10 weeks, it was observed that the callus had a smooth surface as shown in Figure 5b. However, at 14 weeks a net-like structure was observed covering the secondary nodular callus (Figure 5c). At 16 weeks, this feature became more apparent, with the tissue being more varied and present sporadically on the secondary calli (Figures 5d-5g). Higher magnification showed a net-like structure linking the neighbouring cells of the developing callus at 16 weeks (Figure 5d). The net-like structures probably developed sporadically into tiny bead-like structures which were found to be scattered on the surface of the callus cells (Figure 5e). The cluster of the tiny bead-like structures was denser and expanded horizontally with increased density of the net-like strands (Figures 5f-5g). Figure 5f shows the presence of a net-like structure on the surface of NC after 18 weeks of culture. At 20 weeks, the surface of the secondary callus was covered by a smooth compact layer, formed possibly from the degradation or stretching of the net-like structure altering it into a smooth surface (Figure 5i). The presence of the net-like structure was not synchronous and could be seen sporadically at different stages as shown in these figures. After 22 weeks of culture, the frequency of this net-like structure diminished, perhaps due to the reduction in callus formation.

**DISCUSSION**

**Callus Development of Oil Palm**

Oil palm callogenesis from leaf explants was investigated for 28 weeks. During this period, callus was initially observed to emerge after 10 weeks of culture. However, the histological study detected perivascular division in six-week-old cultured leaf explants indicating callus initiation. There are many reports on the cellular origin of callus in different species, such as callus derived from anther connective cells of *Vitis vinifera* L. (grapevine) (Faure et al., 1996), vascular cylinder cells in the hypocotyl explants of *Gentiana cruciata* (Mikula et al., 2005), perivascular zones of root explants of rrottan species (*Calamus merrillii* and *Calamus subinermis*) (Goh et al., 2001), and date palm (*Phoenix dactylifera*) leaf explants (Sané et al., 2006). Auxins were used in the callus initiation from these plants. Auxin is notably involved in the initiation of meristematic activity and has been widely used as a key factor for callus induction and SE (Pasternak et al., 2002; Jiménez and Thomas, 2005; Singla et al., 2007). In this study, the presence of exogenous auxin in the culture medium could be the triggering signal for the dedifferentiation process. The vascular tissue of the leaf explants possibly responded to the exogenous auxin by switching on the dedifferentiation process, leading to callus formation.

According to Paranjothy et al. (1990) and Corley and Tinker (2003), callus initiation in oil palm is dependent on the availability of auxin. Other researchers also documented that the addition of auxin, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthalene acetic acid (NAA), in the culture medium is necessary for oil palm callus induction (Eeuwens et al., 2002; Morcillo et al., 2006). Mattson et al. (1999) revealed that inhibition of auxin flow affects the vascular patterning in leaf explants of *Arabidopsis thaliana* and other dicotyledonous plants, demonstrating the importance of auxin transport in vascular strand development and cell proliferation. In this study, the vascular tissues could have absorbed the exogenous auxin from the culture medium through the cut edges of the leaf explants where there is close proximity to such tissues. Then, a signal was probably generated and transmitted to the perivascular zone to dedifferentiate, as an initial step in callus development.

So far, there is no published report on the morphological changes in developing calli of oil palm using SEM. This study reports the discovery of small spherical particles on the cultured leaf explants using SEM. As pointed out earlier, these unique structures were first detected after six weeks of culture. Unfortunately, similar structures were not seen outside the epidermal cells of six-week-old cultured leaf explant sections. Nevertheless, the histological analysis showed the presence of bead-like blue patches covering peculiar ‘empty’ epidermal cells on the epidermal layer of 10-week-old cultured leaf explant sections. Similar structures were also observed sporadically in the 18th and 20th weeks. Analysis of the width of the peculiar ‘empty’ epidermal cells and small spherical particles showed that these two structures could be related. Although the widths of both structures were not exactly the same, they did fall within a comparable range. The peculiar ‘empty’ epidermal cells observed by light microscopy and the small spherical particles observed under the SEM study could both be of the same structure. However, there are no published reports on the detection of such structures during callus initiation in other plants. The rare detection of these peculiar ‘empty’ epidermal cells on the histological sections could be due to the detachment of these structures during the tissue processing step. The occurrence of the evolved epidermal cells termed as peculiar ‘empty’ epidermal cells coincided with the presence of developing calli; thus, these structures could be linked to the initiation or production of calli. Molecular or cellular signals from the developing calli could possibly be transported via apoplastic
or symplastic pathways to the epidermal cells for the development of the peculiar ‘empty’ epidermal cells.

In most plant species, embryogenic cells are isolated from neighbouring cells by a thickened outer wall (de Touchet et al., 1991; Verdeil et al., 2001; Correidoira et al., 2006; Moura et al., 2008). In oil palm SE, polyembryogenic masses are surrounded by layers of thickened cell walls (de Touchet et al., 1991). Similar results were reported by Correidoira et al. (2006) and Moura et al. (2008), in that thick cell walls were found surrounding embryogenic cells of oak and macaw palm during SE. Moura et al. (2008) postulated that the formation of the thick cell walls was due to the storage of protein and mucilage accumulation, and was associated with the acquisition of embryogenic competency. In this study, the histo-sections of eight-week-old cultured leaf explants showed a group of undifferentiated cells surrounded by dark purple-stained thick boundaries (or walls). The thickened outer cell walls separated the undifferentiated cells from the surrounding degenerative tissues, thus isolating the callus body from the neighbouring leaf cells. The isolation of cells by thickened cell walls could have resulted from middle lamella dissolution which possibly involved the closure of plasmodesmata and callose deposition (Verdeil et al., 2001; Moura et al., 2008) which could then have caused a disruption to symplastic transport and possibly induced the cells to dedifferentiate into new callus cells.

After 10 weeks of culture, the primary callus emerged from the broken epidermal layer of the leaf explant. This was identical to the stage when callus was first observed on the leaf explant. The globular primary callus was whitish in colour and made up of meristematic and highly vacuolated cells, with an arrangement pattern similar to cambium-like tissue. As reviewed by Schwendiman et al. (1988), the division of meristematic cells in the callus leads to the arrangement of a cambium-like zone, ensuring continuous callus growth and proliferation to produce new nodules of callus during oil palm SE. This is in agreement with the observation on the 10- and 12-week-old leaf explants. Continuous cell proliferation gave rise to secondary calli that consisted of various types of calli, including the compact NC that were beige in colour and the undesired white rooting calli. In this study, a low amount of valuable FEC was formed on NC after 24 weeks of culture. Some growing calli were observed to contain starch granules. Schwendiman et al. (1988) also detected the presence of starch in the primary calli and somatic embryos of oil palm. In addition, Sané et al. (2006) and Verdeil et al. (2001) made similar observations on the embryogenic cells of the date palm and coconut, respectively. Meanwhile, Mikula et al. (2005) also revealed that there is an accumulation of starch in the cells produced from vascular cell divisions of Gentiana cruciata hypocotyls that eventually leads to the production of callus tissues.

Extracellular Matrix Surface Network

Both light and electron microscopy investigations revealed the presence of a net-like structure on the outer cell walls of the epidermal layer of the oil palm leaf explants. A similar structure has also been reported in other monocotyledonous and dicotyledonous plants such as Zea mays (Šamaj et al., 1999), Cocos nucifera (Verdeil et al., 2001), Brassica napus (Namasivayam et al., 2006), Triticum aestivum (Konieczny et al., 2005; Pilarska et al., 2007), Actinidia deliciosa (Popielsarska-Konieczna et al., 2008) and Drosera spatulata (Blehová et al., 2010). In these studies, this structure has been designated as the extracellular matrix (ECM) or extracellular matrix surface network (ECMSN). In our SEM study, the detection of a darkly stained aberrant layer on the outer epidermal wall of a developing callus in the inner part of the leaf explant might correspond to ECMSN observed on growing calli. The first morphological detection of the net-like structure on the surface of the leaf epidermis was after six weeks of culture (Figure 5a) and the appearance of ECMSN became more obvious on the developing calli after 14 weeks, when the number of leaf explants producing calli was at the highest in this study. However, this ECMSN was only detected on some growing calli. In oil palm SE, the embryogenic tissue is typically derived from the primary callus or/and NC (Schwendiman et al., 1988; Rajanaidu et al., 1997; Konan et al., 2010). In this study, no microscopic sequence of embryogenic cell formation from NC was recorded. However, the presence of ECMSN in the developing callus could be an indicator that the cells were acquiring the ability to form embryogenic tissue later.

The presence of ECMSN during SE has been reported previously by many researchers on many different plants, and the presence of this structure is also associated with the acquisition of embryogenic competence (Namasivayam, 2007). Konieczny et al. (2005) reported that ECMSN only appears on the cells of Triticum aestivum with regenerative potential. Namasivayam et al. (2006) reported that the compact fibrillar layer designated as ECM is found covering the pre-embryogenic tissue of Brassica napus. They also reported that the ECM layer contains small osmiophilic granules attached to fibres extending from the outer cell walls of the epidermal layer. In 2001, Verdeil et al. disclosed the existence of a fibrillar matrix with pectin epitope coating the embryogenic cells of Cocos nucifera. Dubois et al. (1991) also reported that the surface of proembryoid tissue is linked by fibrillar strands during direct SE of Cichorium. Interestingly, the
observations in our study are similar to those of a study by Popielarska-Konieczna et al. (2008) that reported a heterogenous layer of ECMSN covering the endosperm-derived calli of *Actinidia deliciosa*. They also proposed that the presence of ECMSN is linked to the acquisition of morphogenic competence.

Previous histochemical analyses have revealed that ECMSN might comprise arabinogalactan proteins, pectins and lipids. These components indicate that ECMSN may play a role in cellular proliferation, cell-cell adhesion and cell-cell communication, as well as embryogenic acquisition and embryo development (Konieczny et al., 2005; Namasivayam et al., 2006; Pilarska et al., 2007; Šamaj et al., 2008; Blehová et al., 2010). Šamaj et al. (2005) also reported that ECMSN is composed of arabinogalactan proteins and pectins, which are involved in cellular growth and apoptosis during SE. However, the composition of ECMSN may differ depending on genotype (Pilarska et al., 2007). Future studies should focus on determining the composition of oil palm ECMSN in order to have a better understanding of the role of this structure in SE of oil palm.

**CONCLUSION**

Perivascular cell division could be an indicator of callus initiation in oil palm callogenesis derived from leaf explants. Cell division at the sixth week was concomitant with the appearance of ECMSN that was detected on the epidermal surface of the oil palm leaf explants also at six weeks, and then on the surface of growing calli at 14 weeks and in the later stages. The novelty of this study comprises the finding of ECMSN, peculiar ‘empty’ epidermal cells and small spherical particles on the surface of epidermal cells of the leaf explants during callus formation which had not been reported previously in oil palm. These structural features may be linked to callus initiation and the acquisition of embryogenic competency which have the potential to be manipulated as structural markers for oil palm callogenesis. In addition, the outcomes of this time course study are useful for gene expression analyses at different stages of callogenesis.

**ACKNOWLEDGEMENT**

The authors thank Yayasan FELDA for their financial support, FELDA Agricultural Services Sdn Bhd for providing tissue culture materials, MPOB for their histology facility, and Ms Rosna Angsor for her assistance in the histological analyses.


