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Histology of *Eurycoma longifolia* seeds as an important explant for future production of hairy root cultures

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Seed morphology and histology analysis of *Eurycoma longifolia* by light microscopes revealed seeds structures of this important medicinal plant at different growing stages. The seed structures of *E. longifolia* consist of main regions such as epidermis, hypodermis, storage parenchyma and procambium. The cotyledon of *E. longifolia* develops into a complex and reticulate vascular system. The seed development phases and the development of the vascular system on the progression of germination provide the insight of the actual and accurate information on the *E. longifolia* cotyledon development period. This information is essential for using the seed as the source of inoculums for the production of the hairy root cultures. Seeds being the storage organ may facilitate the generation of the hairy roots, as it evidently has the essential features like tracheas, which are the main site of infection for *Agrobacterium rhizogenes*.

Key words: *Eurycoma longifolia*, seed, histology, *Agrobacterium rhizogenes*.

INTRODUCTION

Eurycoma longifolia is a medicinally important tropical plant that belongs to the family of Simaroubaceae. It is a popular medicinal plant especially in South East Asia region due to the locals belief, that this plant posses properties to cure many diseases. Various parts of this plant are still being used to treat many diseases by the indigenous people and the knowledge of the healing properties of this plant was passed down from generation to generation, highlights the importance of this plant. The roots of this plant are the most important part of this plant which is used to treat various ailments.

From the roots, several classes of compounds have been identified such as quassinoids, canthin-6-one alkaloids, b-carboline alkaloids, tirucallane-type triterpenes, squalene derivatives and biphenylneolignans. Some of these constituents were shown to possess cytotoxic, antimalarial, antiulcer, antipyretic, and plant growth inhibition activities (Ismail et al., 1999; Jagananth et al., 2000; Jiwajinda et al., 2002; Abd Razak, 2007). In addition, the crude extracts of this plant were reputed to increase male virility and sexual prowess and gained notoriety as a male aphrodisiac (Gimlette et al., 1977) and study carried out by Rosli et al. (2009) was to increase the production of 9-methoxycanthin-6-one, an anti-cancerous properties using callus cultures, indicated the many healing properties of this plant.

Agrobacterium rhizogenes is a Gram-negative, rod-shaped soil bacterium that belongs to the genus *Agrobacterium* and is the etiological agent for hairy-root disease. *A. rhizogenes* causes hairy root disease in

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Abbreviations: MS, Murashige and Skoog; °C, degree Celsius; h, hour; TBA, thiobarbituric acid.

plants by infecting the wound sites of the plant where the transfer of T-DNA segment from the bacteria plasmid to the plant cell happens. The DNA integration and its expression in the plant genome contribute to the development of the hairy root (Grant et al., 1991). Many dicotyledonous plants are susceptible to *A. rhizogenes* and plants have been regenerated from hairy root cultures in a wide range of species (Christey et al., 1997). The infected plants often exhibit an altered phenotype due to the expression of the rol (root locus) loci from the T-DNA (Tepfer et al., 1989). Morphologically, *A. rhizogenes*-induced hairy roots are very similar in structure to wild-type roots with a few observable differences like root hairs are longer, more numerous and root systems are more branched and exhibit an agravitropic phenotype. In addition, an important feature of *A. rhizogenes*-induced roots is their unique ability to grow *in vitro* in the absence of exogenous plant growth regulators (Rao et al., 2002).

Hairy roots production is beneficial because we are able to produce increased roots yields using the same land area as compared to the conventional planting method. Hairy roots can be a promising source for the continuous and standardized production of secondary metabolites (Rosli et al., 2009). This is because hairy roots can produce secondary metabolites over several successive generations without losing genetic or biosynthetic stability. Thus, makes it the best choice for metabolic engineering of the secondary metabolite pathways to enhance the accumulation and secretion of high value metabolites (Kim et al., 2002) and also a better choice compared to propagation through tissue culture method (Jiang et al., 2010), which is a very labour intensive process. Tissue culture processes are usually done under sterile laboratory conditions using sterile media. The hairy root system is more advantageous hairy roots can be induced and grown without the laboratory condition (Christopher et al., 2006), which not only saves the production cost but also increases many fold the yield of the secondary metabolite production in a much simpler way. In addition, the secondary metabolites can be harvested directly with few extraction process compared to tissue culture techniques.

The importance of this study is to understand the mechanisms happens during the germination of the seeds as it will be useful information for using the *E. longifolia* seeds as the source of inoculation for the production of hairy roots from this valuable medicinal plant. This is the first paper to report on the histology of *E. longifolia* seeds as an important explant for future production of hairy root cultures using *A. rhizogenes*-mediated transformation system.

MATERIALS AND METHODS

Plant materials

E. longifolia seeds were collected from the nursery at School of

Biological Sciences, Universiti Sains Malaysia. The seeds were surface sterilized using series of washing in Tween-20. Then, the seed coat and the seed outer layer were removed using sterile blades. The embryo was inoculated in Murashige and Skoog (MS) (1962) media and germinates at the $26 \pm 2^\circ\text{C}$ and under 16 h photoperiod (Philips TLD, 36W) at $150 \mu\text{mol.m}^{-2}.\text{s}^{-1}$. The time of inoculation were noted as week 0. The embryos were fixed at each week in fixing solution. This was done till the emergence of the pummel and radical from the inoculated *E. longifolia* seeds.

Histology method

Series of transfer of the seeds were done in alcohol TBA solution with varying concentrations of 50, 70, 85, 98 and 100%. After that, the embryos were treated with TBA solution I and II and continued the treatment with xylene and wax I, II and III. The specimen were then blocked and sliced at 6 micron using Microtome (Leica RM 2135). The specimen slices were then stained using Saffranin (Overnight) and Fast Green (20 min). The slide preparations were then observed using light microscope (SIS Image Analyser, Olympus).

RESULTS AND DISCUSSION

Germination is a complex process during which the seed recover physically from maturation drying, resumes metabolism as to complete the essential cellular events to allow for the embryo to emerge and prepare for subsequent seedling growth. Initially there will be rapid uptake of water by the dry seed until all of the matrices and cell contents are fully hydrated (Nonogaki et al., 2010). This is followed by a period of limited water uptake, which remains unchanged in seeds that do not complete germination, such as in dormant or dead seeds (Nonogaki et al., 2010). The limited water uptake period, will be preceded by a period of increased water uptake, which is related to completion of germination. The steady increase of water reaches the cells of the growing radicle and to the rest of the seedling. This steady increase is due to mitotic divisions and cell expansion (Nonogaki et al., 2010).

The cotyledon of *E. longifolia* is in hemispherical shape (Figures 2a, b and c). The outer most layer of the cotyledon is bounded by epidermis (Figure 1a). Below the epidermis is a single row of cells called hypodermis. The storage parenchyma can be found extensively throughout the cotyledon (Figures 1a, b, c and d). The storage parenchyma appeared to consist of two distinct zones, an outer zone corresponding to the spongy parenchyma and the inner zone corresponding to the palisade tissue (Smith et al., 1966).

The epidermal cells are small, narrow and elongated (Figures 1a and c) and usually will be in the region of $10 \mu\text{l}$ width and $100 \mu\text{l}$ long. The epidermal cells are arranged parallel to one another. The hypodermis region consists of more and less isodiametric cells, which often tend to be slightly flattened in the plane parallel to the surface (Smith et al., 1966). The storage parenchyma consists of two zones. The cells of its two zones differ

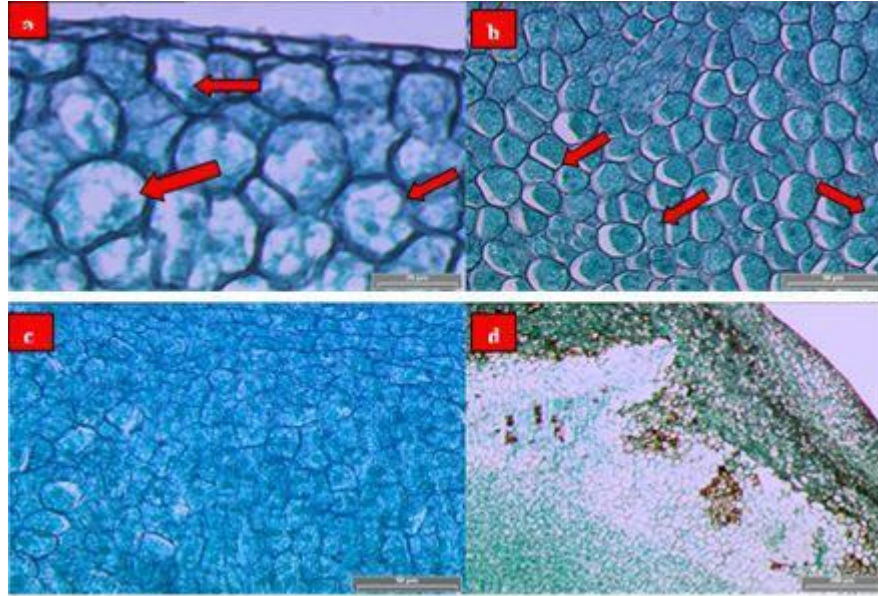


Figure 1. Histological observations of transverse sections of cotyledon of *E. longifolia* (a) Week 1: Small triangular intracellular space confined to the corners of the cells (arrows) (b) Week 2: Small triangular intracellular space observed (arrows) (c) week 3: Lesser intercellular spaces were observed. (d) Week 4: Well defined regions of the cotyledon tissue were observed.

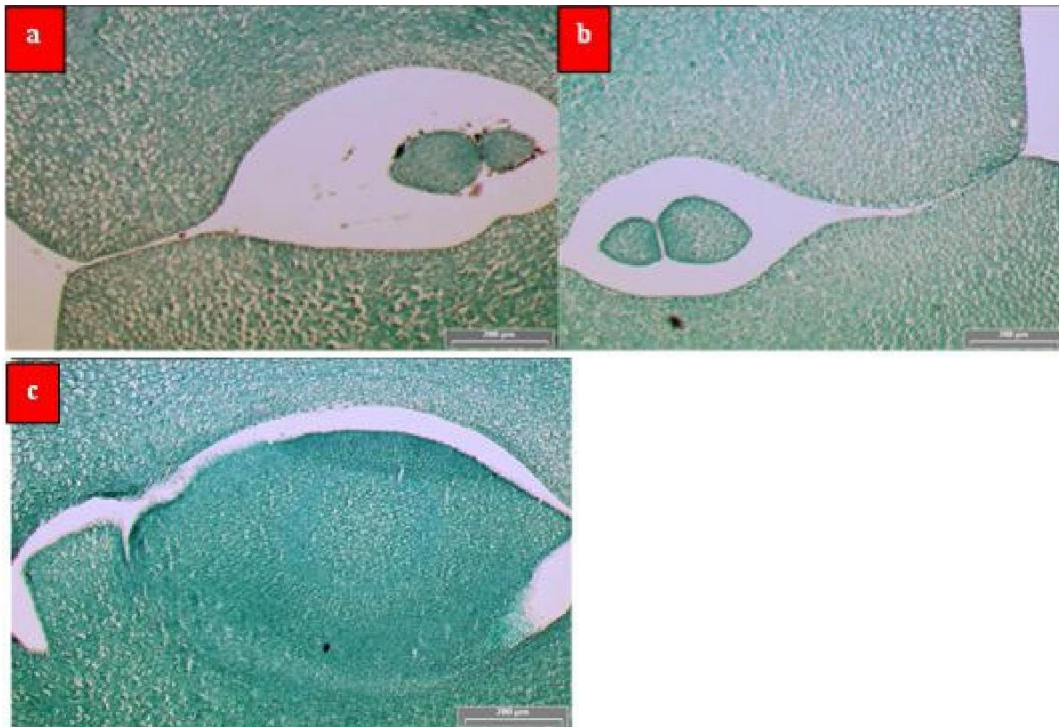


Figure 2. Histological observations of transverse sections of embryo of *E. longifolia* (a) Week 1; (b) Week 2 and (c) Week 3.

from each other in size and shape. The cells of the inner storage tissue, the adaxial surfaces are isodiametric and

rounded in appearance. In contrast, the cells of the storage tissue, underlying the abaxial surface, are larger

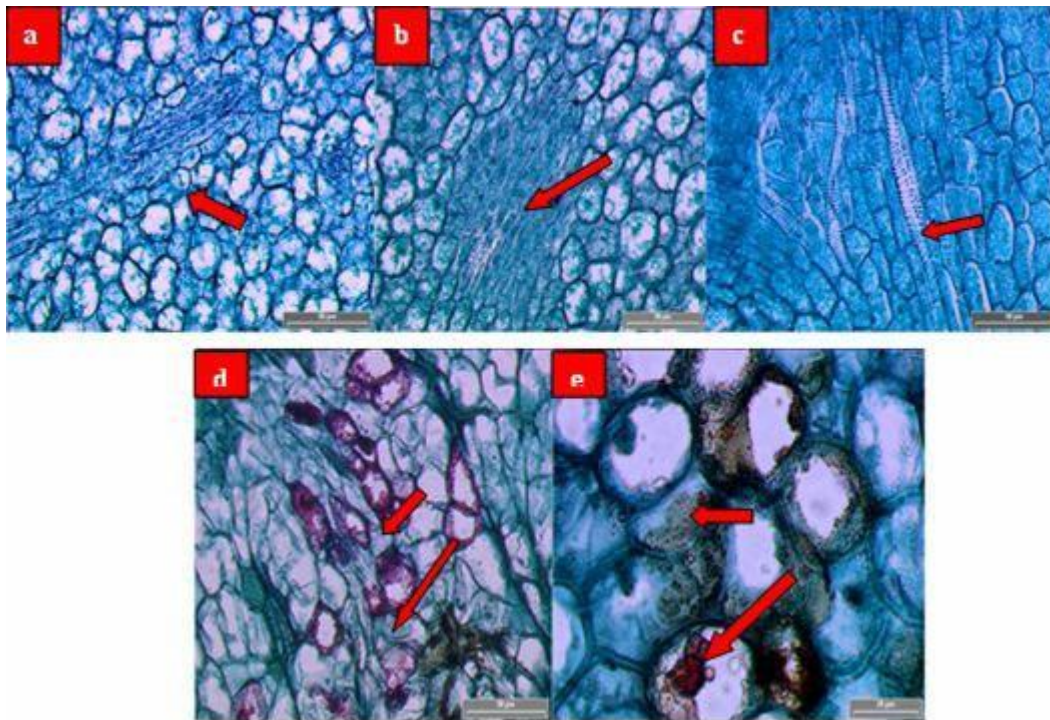


Figure 3. Histological observations of transverse sections of embryo of *E. longifolia* that reveals the vascular system development. (a) Week 1: Vessel elements (arrow) (b) Week 2: Aggregation of vessel elements with primary wall thickening (arrow). (c) Week 3: Annular and helical secondary wall thickening occur in vessel elements. (d and e) Week 4: Mature xylem vessels and the substance stained red with Safranin is lignaceous.

and more irregular in shape (Smith et al., 1966). The cells of both the tissues are compact but irregularly arranged, with small triangular intracellular space confined to the corners of the cells (Figure 1a). As the germination process proceeds, the size of the intercellular spaces increases and larger, more irregular spaces developed by fusion of the small spaces that were initially present (Figures 1b, c and d). By the fourth week, well defined regions of the cotyledon tissue were observed (Figure 1 d).

The embryo of *E. longifolia* is heart shaped as shown in Figures 2a, b and c. As the germination proceeds, the embryo increased in size. Towards the end of the germination process, the embryo fuses with the cotyledon to facilitate the increasing need of nutrient and water uptake for its growth. The cotyledon possesses a well-developed vascular system that runs along the boundary of the inner and the outer storage tissues. In the mature embryo (Figures 3a, b and c) procambium was observed and no mature xylem or phloem elements were detected. Differentiation of vascular tissues from the procambium (Figures 3d and e) was observed to occur during the first two days of germination of *E. longifolia*.

Primary xylem and phloem were produced from primary meristem cells. At a very early stage of the embryo development, it contains information in the procambium

specifying cyto-differentiation of vascular elements with specialized secondary cell walls (Gahan et al., 1987). The procambium possesses a single cell type, which can be described as small and isodiametric in section. Cell division occurs in any direction, there is no intrusive growth and the cell walls are of uniform thickness. Procambial cells contain proplastids, many small vacuoles and are basophilic (Roberts et al., 1988). Auxin appears to stimulate cambial cell replication and xylogenesis, whereas gibberellins have been implicated in phloem fiber formation and gibberellic acid, could further enhance phloem differentiation (Woodzicki et al., 1982; Roberts et al., 1988). Thus, the presence of the indigenous hormones which are involved in structural differentiation of the seeds, makes the seed an ideal choice for the transformation event.

The initiation of vascular differentiation occurs in progenitor cells as a result of some primary event occurring in the presence of auxin, cytokinin and ethylene (Roberts et al., 1988). Roots do not induce vascular differentiation and roots need not to be present, in order to obtain vascular differentiation in stem tissue (Thompson et al., 1966). Roots, however affect vascular differentiation by orienting the pattern of vascular differentiation from the leaves towards the root tips by acting as a sink for the polar flow of auxin, originating in

the young leaves (Sachs, 1968). The root apices are the source of inductive stimuli that promote vascular development. The major stimulus is cytokinin and it is known to stimulate cell division and to control both the differentiation of tracheary elements (Dalessandro, 1973) and secondary xylem fibers (Aloni, 1982). Both organ wounding and tissue excision induce ethylene biosynthesis which may contribute to cyto-differentiation (Roberts et al., 1988). Organ wounds are based on severing vascular continuity within the organ. This operation disturbs the normal movement of growth regulators and causes the release of endogenous hormones. Leakage of hormones around the wound initiates the re-differentiation of parenchyma into phloem and xylem elements, and, in addition, starts cell division leading to the establishment of a wound cambium (Roberts et al., 1988). Primary wall development, associated with cell enlargement ceases during the initiation of xylem differentiation. Shortly thereafter, secondary wall formation commences. At this time, there is a shift in the metabolic requirement for carbohydrates, that is, there is a greater demand for monomers leading to the production of cellulose and hemicelluloses (Roberts et al., 1988).

The physiological state of the plant material varies with culture conditions, season of the year and the identity of the tissue. Certain organs, e.g., hypocotyls and petioles are generally more reactive towards the infection of *A. rhizogenes* (Tepfer, 1984). This bacterium transfers its transfer-DNA (T-DNA) which is a portion of the large plasmid called the root-inducing plasmid (pRi) to susceptible plant cells where the T-DNA, if integrated into the nuclear genome of the plant cell, will encode genes that direct the synthesis of auxin (indole-3-acetic acid) and also increases the sensitivity of the transformed plant cells to auxin (Hatta et al., 1996). The endogenous production of auxin and the increase in auxin sensitivity by the explants, can lead to the formation of hairy roots at the site of infection (White et al., 1985). It is possible that the hypocotyl segments inoculated with the bacterium were more sensitive to increased auxin supply since *A. rhizogenes* is known to encode genes that increase auxin sensitivity to plant tissue (Hatta et al., 1996).

As conclusion, induction of hairy roots from the cotyledon may be possible in *E. longifolia* due to several reasons. First, the cut area of the cotyledon might induce more attachment of bacterium as it provides more surface area for attachment (Yu et al., 2001). Secondly, because of the differences in physiological conditions, the susceptibility of explants to *A. rhizogenes* is diverse in different tissues of the same plant (Xu et al., 1993) and thirdly due to the fact that the possible correlation of cell division with maximal transformation suggested that cell division and DNA synthesis might be important to the process of transformation (Braun et al., 1975). Hence, this study may contribute to the development of more efficient *E. longifolia* production systems due to the huge

market for the *E. longifolia* derived products. This is possible because the *E. longifolia* seeds structure and its development phase are clearly known and the information can be used to facilitate the hairy root production.

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