Pak. J. Bot., 46(3): 1061-1064, 2014.

HISTOLOGY OF SOMATIC EMBRYOS OF *EURYCOMA LONGIFOLIA* (SIMAROUBACEAE): RELEVANCE IN *AGROBACTERIUM RHIZOGENES*-MEDIATED TRANSFORMATION

BHAVANI BALAKRISHNAN[1], SYARIFAH SHAHRUL RABIAH SYED ALWEE[2], CHAN LAI KENG[1] AND SREERAMANAN SUBRAMANIAM[1]*

[1]*School of Biological Sciences, Universiti Sains Malaysia, Minden Heights, 11800 Penang, Malaysia*
[2]*Felda Biotechnology Centre, Felda Agriculture Services Sdn. Bhd.Tingkat 7, Balai Felda, Jalan Gurney 1, 54000 Kuala Lumpur, Malaysia*
[*]*Corresponding author's e-mail: sreeramanan@usm.my / sreeramanan@gmail.com*

Abstract

Histological analysis conducted on somatic embryos of *Eurycoma longifolia* shows the developmental structures that are remarkably similar to seeds found in the wild. The primary components of a growing somatic embryo are its shoot and root apical meristems indicated by dense layers of rapidly growing cells. The increased understanding of *In vitro* culture systems and anatomical changes provide information into cellular processes that govern genetic transformation of *E. longifolia* with *Agrobacterium rhizogenes*. The presence of meristematic regions on cultured somatic embryos suggests that they are suitable for genetic transformation as genetic elements could be transported to these regions where growth and differentiation are centered. This allows the successful integration and expression of transferred DNA in the host organism, leading the way for an efficient *A. rhizogenes*-mediated transformation protocol.

Introduction

Eurycoma longifolia (simaroubaceae), native to Southeast Asia, is a tall slender shrub-tree commonly found as an understory in the lowland forests at up to 500 metres above the sea level. *E. longifolia*, known locally as 'Tongkat Ali' in Malaysia, is popularly sought after in herbal remedies and has been frequently prescribed either as a single ingredient or as a mixture with other herbs and as such, it is sometimes referred to as 'Malaysian ginseng' (Jagananth & Ng, 2000; Kuo *et al*., 2004).

Various parts of this plant are commonly used as folk medicine for the treatment of aches, persistent fever, tertian malaria, sexual insufficiency, dysentery, glandular swelling and as health supplements (Kuo *et al*., 2004). From the roots, several classes of compounds have been identified, of which quassinoids (Ang *et al*., 2000, 2002; Teh *et al*., 2010), canthin-6-one alkaloids (Kardono *et al*., 1991) and β-carboline alkaloids (Kuo *et al*., 2003) form a major portion. Some of these constituents were shown to possess anti-tumour and anti-parasitic (Jiwajinda *et al*., 2002), anti-plasmodial (Ang *et al*., 1995; Chan *et al*., 2004) cytotoxic (Kuo *et al*., 2003), anti-hyperglycemic (Rafidah *et al*., 2004) and plant growth inhibition activities (Jiwajinda *et al*., 2001).

Agrobacterium rhizogenes is a Gram negative, rod shaped, motile soil bacterium responsible for the development of hairy root disease on a range of dicotyledonous plants (Veena & Taylor, 2007). The transfer, integration and expression of T-DNA follow infection of wound sites by *Agrobacterium rhizogenes* from the root-inducing (Ri) plasmid and subsequent development of the hairy root phenotype. Hairy roots can be induced on a wide range of plants and many can be regenerated into whole organisms, often spontaneously. They can be easily distinguished by their rapid, highly branching growth on hormone-free medium and plagiotropic root development. Hairy root cultures have been used extensively in root nodule research, for artificial seed production, for production of plant secondary metabolites, as an experimental system to study biochemical pathways and responses to chemicals and to study interactions with other organisms such as nematodes, mycorrhizal fungi and root pathogens (Christey & Braun, 2004).

*Eurycoma longifoli*a is most commonly propagated using their seeds that are produced once a year. However, they have a low percentage of germination and it takes a long time to germinate due to extremely immature state of zygotic embryo at the time of dispersal. The indiscriminate collection of the taproot from the wild as the raw material for the drug preparations has triggered concerns about diminishing populations, loss of genetic diversity, local extinctions and habitat degradation. Therefore, it needs to be rapidly mass multiplied on a commercial scale to comply with the need of the herbal and pharmaceutical industry (Canter *et al*., 2005; Sobri *et al*., 2005).

Plant tissue culture provides a suitable technique by which mass multiplication of *E. longifolia* plants could be performed efficiently in a laboratory environment. Usually it is the zygote which develops into an embryo, through embryogenesis and eventually forms a complete plant. However, using careful manipulations of tissue culture medium and incubation conditions, it is possible to reverse the differentiation process and form embryos from other parts of a mature plant. Non-zygotic or somatic embryos are bipolar structures that follow the developmental behaviour similar to zygotic embryos. This pathway is called somatic embryogenesis (Sharma, 2009). Histological analysis of zygotic embryos of *E. longifolia* has been conducted (Monica *et al*., 2011) and they have been described as important explants for future production of hairy root cultures using *A. rhizogenes*-mediated transformation system. The primary objective of this study is to comprehend the mechanisms of somatic embryogenesis which will provide valuable information that can be used as a guide in *Agrobacterium rhizogenes*-mediated transformation of *Eurycoma longifolia*.

Materials and Methods

Plant materials: The source of *E. longifolia* plant used in this study was kindly provided by Felda Biotechnology Centre, Kuala Lumpur, Malaysia. The *In vitro* embryogenic callus cultures provided were induced into somatic embryos and multiplied in full strength Murashige and Skoog medium

(1 MS) (Murashige & Skoog, 1962) containing 1.0mg/L (w/v) 2,4-D (2, 4-dichlorophenoxyacetic acid), 0.5mg/L (w/v) kinetin (6-furfurylaminopurine), 1.0g/L (w/v) activated charcoal and solidified with 2.75g/L (w/v) gelrite, as specified by Sobri *et al*. (2005). Cultures were maintained under a 16 hour photoperiod regime with fluorescent light at 25°C. For histological studies, somatic embryos at various stages of growth were used as samples.

Histology method: Freshly harvested embryo samples were cut diagonally into the sizes of 2-5mm with a sharp knife. The tissue was then fixed in vials containing FAA [70% ethanol: glacial acetic acid: formaldehyde at the ratio of 18:1:1 (v/v/v)] at room temperature for 2 days and thoroughly washed with water every 2 hours, 1 day before beginning the dehydration process. During dehydration, fixed samples were washed using a graded series of alcohol with varying concentrations of 50, 70, 85, 98 and 100% at room temperature. The embryos were then subjected to TBA wash I and II followed by immersion in xylene with wax I, II and III. Wax absorption is followed by embedding using a tissue embedding machine (Leica EG1160) at 61°C. Samples that were incubated in wax overnight were embedded at the center of a mold containing hot paraffin wax and allowed to cool to solidify. Solidified paraffin block were detached from the embedding mold and blocking was performed. Samples were dissected at thickness of 11µM using a microtome (Leica RM2135). The specimen slides were then washed in histoclear solutions I and II continued by a graded series of alcohol at decreasing concentrations (100%, 90%, 70% and 50%) for 2 minutes each. The slides were left immersed for 24 hours in safranin stain (dissolved in 50% ethyl alcohol). To remove the safranin stain, the slides were washed with 70% and 80% alcohol (1 minute each) before immersion in fast green (dissolved in 95% ethyl alcohol) for 20 minutes. The stain was removed by washing the slides twice in 95% alcohol for 1 minute each followed by transfer to 100% alcohol (2 minutes) and histoclear III and IV (3 minutes each). Stained slides are allowed to air dry and mounted with cover slips. Samples were viewed using clinical laboratory microscope (Olympus BX41) and images were captured using Xcam-α.

Results

Somatic embryos were propagated in full strength MS medium supplemented with 2, 4-D, kinetin and activated charcoal. The somatic embryos of *E. longifolia* were confirmed using histological analysis. The supplementation of auxin 2, 4-D with cytokinin kinetin is required for optimum response of the plant and to prevent necrosis of embryogenic callus. Each non-zygotic embryo or embryoid is a bipolar structure, consisting of plumular and radicular ends, thus they closely resemble their zygotic counterpart.

The vegetative shoot apices vary in shape, size and cytohistologic structure, and in their relation to the lateral organs. There is tunica-corpus organization in the shoot apex of angiosperms such as *E. longifolia*. One to five layers tunica have been observed in the dicotyledons (the narrow layer of cells that borders shoot apical meristem in Fig. 1b). To draw a clear cut demarcation line between tunica and corpus (cells that are immediately beneath tunica, darker than the surrounding cells in Fig. 1c) is not a simple matter.

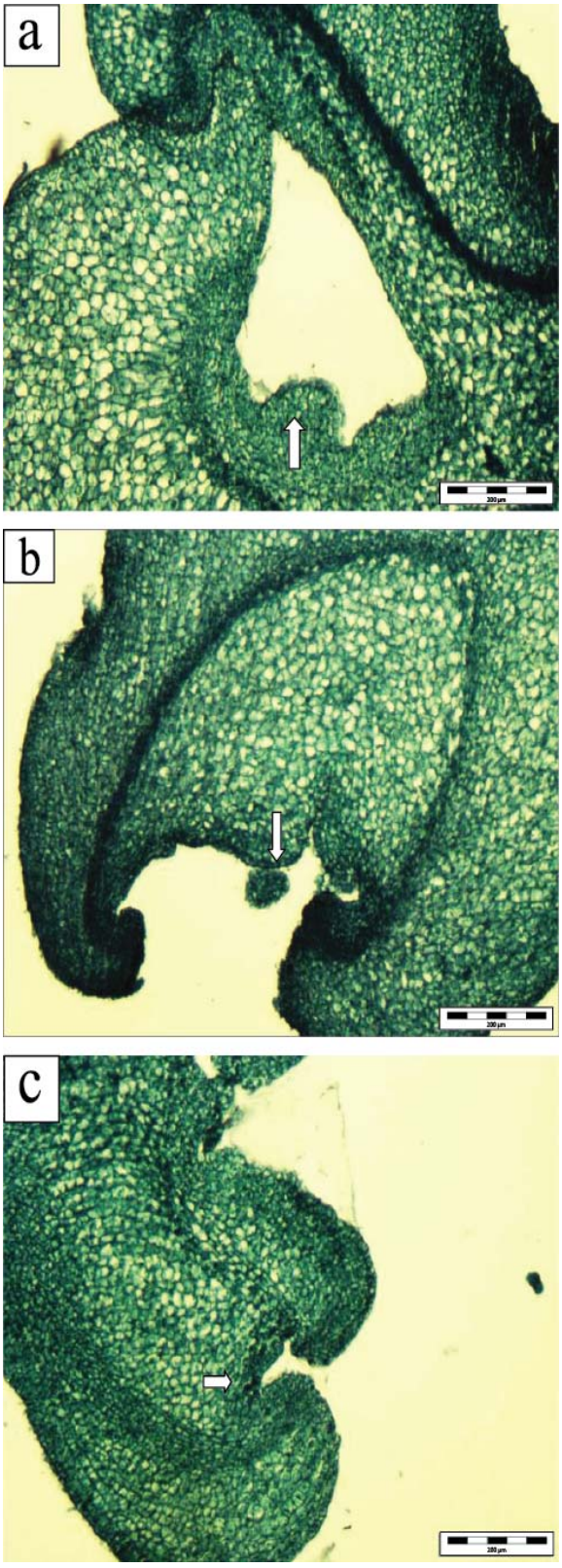

Fig. 1. Histological observations of cross sections of *Eurycoma longifolia* somatic embryos show shoot apex development. (a) Shoot apical meristem (arrow) (b) Tunica (arrow) (c) Corpus (arrow).

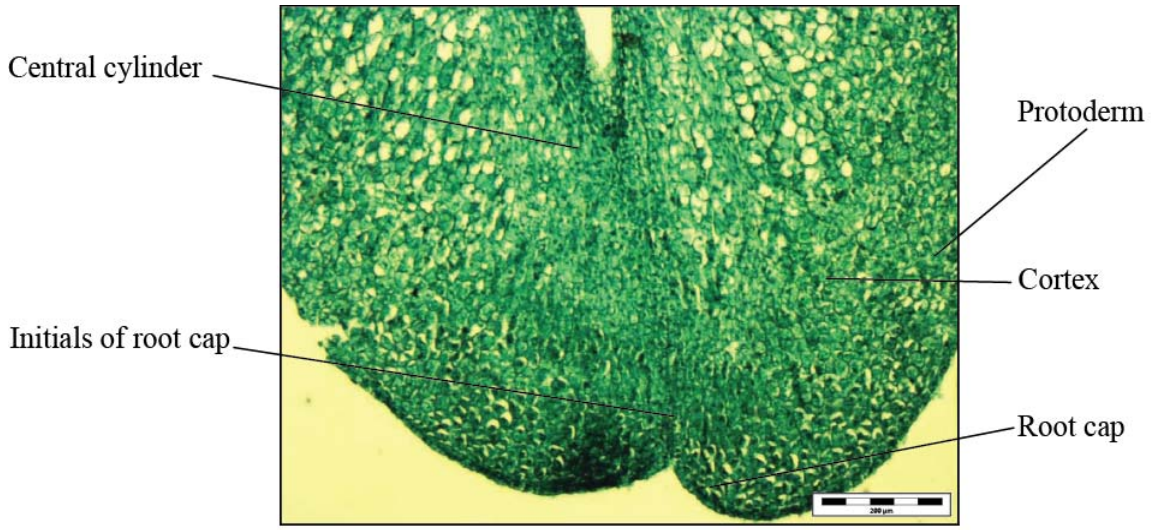

Fig. 2. The root apex of *Eurycoma longifolia.*

During the later stages of embryo development, the cells at the root pole become arranged in a pattern characteristic of the species. This group of cells comprise the apical meristem of the primary root (Fig. 2). The cells of this region are all relatively undifferentiated and meristematic, densely protoplasmic and with large nuclei and they all undergo active cell division. The tissues of the mature root are eventually derived from a number of these cells of the apical meristem, which are termed initials. In contrast to the apical meristem of the shoot, that of the root produces cells not only toward the axis but also away from it, for it initiates the root cap and because of the presence of root cap, the root meristem is not terminal but subterminal in its position, in the sense that it is located beneath the root cap.

Discussion

Usually, the same pattern of growth is observed in cultured somatic embryos, passing through the three sequential stages of embryo formation, such as globular stage, heart-shaped stage and torpedo stage. It is the torpedo stage which is a bipolar structure and gives rise to a complete plant (Sharma, 2009). A common mistake in literature regarding embryogenesis is the failure to demonstrate a well organised shoot meristem. Simply showing a structure with the shape of an embryo is not good enough. Often, many somatic embryos cannot grow into a complete plant due to the absence of a properly organised shoot meristem. It is imperative that through proper sectioning and staining, a well organised shoot meristem should be indeed present. This is a good indication of high quality somatic embryos (Yeung, 1999).

In angiosperms, the number of parallel periclinal layers in the shoot apex may vary during the ontogeny of the plant body and under the influence seasonal growth changes (Pandey, 2007). The root apex also differs from the shoot meristem in that it forms no lateral appendages comparable to the leaves, and no braches. The root branches are usually initiated beyond the region of most active growth, and they arise endogenously. It also produces no nodes and internodes; therefore the root grows more uniformly in length than the shoot, in which the internodes elongate much more than the nodes (Pandey, 2007). Somatic embryos of *E. longifolia* have been shown to resemble the developmental pattern of seeds or zygotic embryos found in the wild as they possess well organized shoot and root apical meristems.

Bringing recalcitrant medicinal plants into cultivation via *In vitro* techniques solves problems inherent in wild or field grown plants primarily by eliminating seasonal and environmental variations. These variations may impede the process of natural product discovery as the levels of bioactive substances generally depend on environmental and biological pressures such as climate and pests. Loss of source caused by extinction could be avoided while providing reliable access and supply to important plant material (Li & Vederas, 2009). Another advantage of the system is that it has been grown in sterile conditions at a laboratory, therefore reducing the chances of contamination that so often plagues *In vivo* explants used in genetic transformation.

Conclusion

Cultured apical meristems of *E. longifolia* are suitable for transformation purposes because these can be easily regenerated into whole plants. Differences in physiological and biochemical attributes of various parts of the same plant render them with different susceptibility to *A. rhizogenes*. Histological studies of somatic embryos of *E. longifolia* suggest that genetic transformation with *A. rhizogenes* is highly possible and could open a whole new path towards molecular tailoring. T-DNA present in disarmed strains of *A. rhizogenes* could be engineered to carry specific genes to increase tolerance or imbue *E. longifolia* with resistance towards diseases, to express foreign proteins and control the mechanisms of secondary metabolite production and to conduct gene analysis using the hairy roots formed by the plant.

Acknowledgements

This study was supported by the Federal Land Development Authority (FELDA), Malaysia and Research University Postgraduate Research Grant Scheme (RUPGRS), Universiti Sains Malaysia, Malaysia.

References

Ang, H.H., K.L. Chan and J.W. Mak. 1995. Effect of 7-day daily replacement of culture medium containing *Eurycoma longifolia* Jack constituents on the Malaysian *Plasmodium falciparum* isolates. *J. Ethnopharmacol.,* 49: 171-175.

Ang, H.H., Y. Hitotsuyanagi and K. Takeya. 2000. Eurycolactones A-C, novel quassinoids from *Eurycoma longifolia*. *Tetrahedron Lett*., 41: 6849-6853.

Ang, H.H., Y. Hitotsuyanagi, H. Fukaya and K. Takeya. 2002. Quassinoids from *Eurycoma longifolia*. *Phytochem*., 59: 833-837.

Canter, P.H., H. Thomas and E. Ernst. 2005. Bringing medicinal plants into cultivation: opportunities and challenges for biotechnology. *Trends Biotechnol*., 23 (4): 180-185.

Chan, K.L., C.Y. Choo, A. Noor Rain and I. Zakiah. 2004. Antiplasmodial studies of *Eurycoma longifolia* Jack using the lactate dehydrogenase assay of *Plasmodium falciparum*. *J. Ethnopharmacol*., 92: 223-227.

Christey, M.C. and R.H. Braun. 2004. Production of hairy root cultures and transgenic plants by *Agrobacterium rhizogenes*–mediated transformation. In: Methods in Molecular Biology 286: Transgenic Plants: Methods and Protocols. (Ed.): Peňa L. Humana Press Inc., New Jersey, USA, pp. 47-60.

Jagananth, J.B. and L.T. Ng. 2000. *Eurycoma longifolia*. In: Herbs-The Green Pharmacy of Malaysia. Vinpress Sdn. Bhd. and Malaysian Research and Development Institute, Kuala Lumpur, Malaysia, pp. 45-46.

Jiwajinda, S., V. Santisopasri, A. Murakami, M. Kawanaka, H. Kawanaka, M. Gasquet, R. Eilas, G. Balansard and H. Ohigashi. 2002. *In vitro* anti-tumor promoting and anti-parasitic activities of the quassinoids from *Eurycoma longifolia*, a medicinal plant in Southeast Asia. *J. Ethnopharmacol.,* 82: 55-58.

Jiwajinda, S., V. Santisopasri., A. Murakami, N. Hirai and H. Ohigashi. 2001. Quassinoids from *Eurycoma longifolia* as plant growth inhibitors. *Phytochem*.,58: 959-962.

Kardono, L.B.S., C.K. Angerhofer, S. Tsauri, K. Padmawinata, J.M. Pezzuto and A.D. Kinghorn. 1991. Cytotoxic and antimalarial constituents of the roots of *Eurycoma longifolia. J. Nat. Prod*., 54(5): 1360-1367.

Kuo, P.C., A.G. Damu, K.H. Lee, and T.S. Wu. 2004. Cytotoxic and antimalarial constituents from the roots of *Eurycoma longifolia*. Bioorg. *Med. Chem*., 12: 537-544.

Kuo, P.C., L.S. Shi, A.G. Damu, C.R. Su, C.H. Huang, C.H. Ke, J.B. Wu, A.J. Lin, K.F. Bastow, K.H. Lee and T.S. Wu. 2003. Cytotoxic and antimalarial β-carboline alkaloids from the roots of *Eurycoma longifolia. J. Nat. Prod*., 66: 1324-1327.

Li, J.W.H. and J.C. Vederas. 2009. Drug discovery and natural products: End of an era or an endless frontier? *Sci*., 325: 161-165.

Monica, D., L.K. Chan, S.R.S.A. Syarifah and S. Sreeramanan. 2011. Seed histology of recalcitrant *Eurycoma longifolia* plants during germination and its beneficial attribute for hairy root production. *J. Med. Plants. Res.*, 5(1): 93-98.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plantarum.,*15(3): 473-497.

Pandey, B.P. 2007. Apical meristems. In: Plant Anatomy. S. Chand and Company Limited, New Delhi, India, pp. 117-133.

Rafidah, H., H.L.P. Azimahtol and N. Meenakshii. 2004. Screening for antihyperglycaemic activity in several local herbs of Malaysia. *J. Ethnopharmacol.,* 95: 205-208.

Sharma, H.P. 2009. Somatic embryogenesis. In: Plant embryology- classical and experimental. Narosa Publishing House, New Delhi, India, pp. 16.3-17.6.

Sobri, H., I. Rusli, A.L.P. Kiong, M.F. Nor'aini and D. Siti Khalijah. 2005. Micropropagation of *Eurycoma longifolia* Jack via formation of somatic embryogenesis. *Plant Biotechnol.,* 22: 349-351.

Teh, C.H., H. Morita, O. Shirota and K.L. Chan. 2010. 2, 3-Dehydro-4α-hydroxylongilactone, a novel quassinoid and two known phenyl propanoids from *Eurycoma longifolia* Jack. *Food Chem.,* 120: 794-798.

Veena, V. and C.G. Taylor. 2007. *Agrobacterium rhizogenes*: recent developments and promising applications. *In Vitro Cell. Dev. Biol.–Plant*., 43: 383-403.

Yeung, E.C. 1999. The use of histology in the study of plant tissue culture systems- some practical comments. *In Vitro Cell. Dev. Biol.–Plant*., 35: 137-143.

(Received for publication 7 June 2013)