Potential Biochemical Markers for Somatic Embryos of *Eurycoma longifolia* Jack

Sobri Hussein[1]*, Rusli Ibrahim[1], and Anna Ling Pick Kiong[2]
[1]Malaysian Institute for Nuclear Technology Research, Bangi, 43000 Kajang, Selangor, Malaysia
[2] Department of Bioscience, Faculty of Engineering and Science, Universiti Tunku Abdul Rahman, 53300 Setapak, Kuala Lumpur, Malaysia

Biochemical marker is one of the important tools for the early identification and selection of somatic embryogenesis in plants. Studies in developing the biochemical marker for somatic embryogenesis of *Eurycoma longifolia* disclosed that the regenerated and non-regenerated cotyledons as well as embryogenic and non-embryogenic callus were significantly different in terms of the total protein content as well as the specific activity of peroxidase. The data obtained revealed that embryogenic tissue possess the highest amount of total soluble protein (64.24 mg/g fresh weight) and two protein bands (molecular weight = 25 and 21 kDa) were observed at high intensity. The highest specific activity of peroxidase (578.1 ± 61.6 unit/mg soluble protein) was recorded in embryogenic callus and only 157.1 ± 20.5 unit/mg soluble protein was determined in the non-embryogenic callus. Results obtained also showed that there were variations in the peroxidase banding profiles of the four samples examined. Only two bands were observed in the non-embryogenic callus at the Rf of 0.24 and 0.27, whereas in the embryogenic callus, four bands with high intensity were detected at the Rf of 0.24, 0.41, 0.49 and 0.81.

Keywords: biochemical markers, *Eurycoma longifolia*, micropropagation, plant regeneration, somatic embryos

Eurycoma longifolia has become the targeted medicinal plants by the community in Southeast Asia mainly owing to its aphrodisiac property. It has also been sought as an essential component for the treatments of fevers, aches and as health supplements (Ang and Cheang, 1999). The gradual disappearance of this plant is due to the indiscriminate collection of root tubers as the raw material for the drug preparations. The existence of tissue culture technology especially through somatic embryogenesis can play an important role in this regard. However, somatic embryo often develops asynchronously, and their shapes vary widely with many morphological abnormalities (Ibaraki and Kurata, 2001). As a result, the quality of somatic embryo culture is heterogeneous and is a barrier to the development of a practical somatic embryo production system.

A biochemical marker might be useful for early identification of embryogenic cultures before any morphogenic changes. It will help in optimizing culture conditions for embryogenesis, monitoring the course of somatic embryogenesis, and in discriminating cultures to follow the multiplication process (Blanco et al., 1997). Blanco et al. (1997) further suggested that the proteins, isozymes and ethylene are among the biochemical variables that are able to distinguish between embryogenic and non-embryogenic tissue. Proteins could be useful to identify specific stages in the development of somatic embryos (Yuffa et al., 1994), for an example, Ishizaki et al. (2002) detected a 31 kDa protein that accumulated in the embryogenic calli of *Spinacia oleracea* L.

Bonfill et al. (2003) reported a positive correlation between organogenesis and soluble peroxidase activity in cells coming from orange ovular callus lines as well as *Panax ginseng* callus. In *Phoenix dactylifera* L., somatic embryogenesis is characterized by an increase in peroxidase activity (El Hadrami and Baaziz, 1994). Similarly, in tobacco and *Kabmia latifolia*, it was found that increased peroxidase activity accompanies shoot and root formation from callus (El Hadrami and Baaziz, 1994).

Objective of this study was to identify possible markers for the somatic embryogenesis in *E. longifolia*. Besides, this study also compared biochemical markers for the regenerated and non-regenerated cotyledon with those obtained from the embryogenic and non-embryogenic callus.

*Corresponding author; fax +82-603-89258262
e-mail sobri@mint.gov.my

MATERIALS AND METHODS

Plant Materials

E. longifolia was obtained from Alor Star, Kedah, Malaysia. Somatic embryos of *E. longifolia* were initiated from cotyledon explants cultured in the basal MS medium containing 0.5 mg L^{-1} (w/v) of kinetin and 1.0 mg L^{-1} (w/v) of 2,4-D. Multiplication of the somatic embryos was carried out in the same medium formulation with the addition of 1.0 g L^{-1} (w/v) activated charcoal (Sobri et al., 2006).

Determination of Total Soluble Proteins

Total soluble protein content of the four samples, embryogenic callus, non-embryogenic callus, regenerated cotyledon and non-regenerated cotyledon, was determined using the Bradford method. One g of each sample was ground with a mortar and pestle in an ice bath and extracted with 3 mL of protein extraction buffer (0.1 M Tris-HCl, 1 mM EDTA, 0.1% mercaptoethanol, pH 8.0). The extract was then transferred into an Eppendorf tube and centrifuged at 12,000 rpm at 4°C for 20 min. In order to determine the total soluble protein content, 10 µL of the supernatant was added into 90 µL of protein extraction buffer and 5 µL of protein reagent and was mixed by vortexing. Absorbance at 595 nm was determined after 2 min using a spectrophotometer (Ultraspec III, Pharmacia, UK). The absorbance was compared with the standard curve plotted using bovine serum albumin as a standard at the concentrations of 0, 20, 40, 60, 80 and 100 µg mL^{-1}. The experiment was repeated three times for each sample.

Measurement of Peroxidase Activity

Total peroxidase activities of the four samples, embryogenic callus, non-embryogenic callus, regenerated cotyledon and non-regenerated cotyledon, were also measured. Activity of peroxidase was determined based on the appearance of brown color resulting from guaiacol oxidation in the presence of hydrogen peroxide. Reaction mixture consisted of 0.5 mL sample extract, 2.6 mL of 0.1 M sodium phosphate buffer (pH 6.1), and 0.3 mL of 1% guaiacol. A total of 0.3 mL of 30% H_2O_2 was added prior to reaction. Change in absorbance at 470 nm was followed for three minutes using a spectrophotometer. Peroxidase activity was expressed in unit/mg protein whereby changes in 0.1 absorbance/min/mg protein refer as one unit (Gupta et al., 1990). The experiments were carried out in three replicates for each sample.

Polyacrylamide Gel Electrophoresis (PAGE) Analysis of Protein and Peroxidase Content

A vertical electrophoresis system Mini Protein II (Bio-Rad, USA) was used, and 20 mg of protein was loaded with 6X loading dye for both denaturing and non-denaturing polyacrylamide gel. Sodium dodecyl sulfate-PAGE was performed using a 7.5% stacking gel (0.5 M Tris-Cl, pH 6.8) and 10% separating gel (1.5 M Tris-Cl, pH 7) buffer systems. Gels were run in a buffer of 25 mM Tris-Cl and 192 mM glycine (pH 8.3) at 80 V for 6 h at 4°C. Protein was stained by incubating the gel for 2 h in 0.1% Coomassie Brilliant Blue G-250 (ICN, USA), 45% methanol (Merck, Germany) and 10% acetic acid (Merck) with several changes until the gel background is clear. Discontinuous non-denaturing PAGE was carried out for the determination of peroxidase isozyme pattern under the same condition as mentioned in the SDS-PAGE. The peroxidase activity was visualized by incubating the gel in a solution consists of 100 mL 0.4 M sodium acetate buffer (pH 5.0) with 16 mg o-dianisidine and 40 mL of 30% hydrogen peroxide for 3 min with agitation at 50 rpm. The relative front (R_f) for each band in the gel was calculated using the formula described by Bollag and Edelstein (1991).

RESULTS AND DISCUSSION

Total Soluble Protein Content and SDS-PAGE Banding Profiles

Biochemical differentiation based on total soluble protein content revealed that embryogenic callus (EC) contains the highest amount of total soluble protein, 64.24 ± 4.5 mg g^{-1} FW, whereas in non-embryogenic callus (NEC) only 25.5 ± 3.5 mg g^{-1} FW of total soluble protein was detected (Fig. 1). The same pattern was also observed in regenerated cotyledon (RC) and non-regenerated cotyledon (NRC), whereby the highest amount of total soluble protein was observed in regenerated cotyledon (55.41 ± 3.2 mg g^{-1} FW) followed by 41.08 ± 4.3 mg g^{-1} FW in non-regenerated cotyledon. Lakshmanan and Taji (2000) reported that accumulation of protein at maturation is crucial for high vigour and successful conversion of somatic embryo in legumes. Thus, the study of biochemical

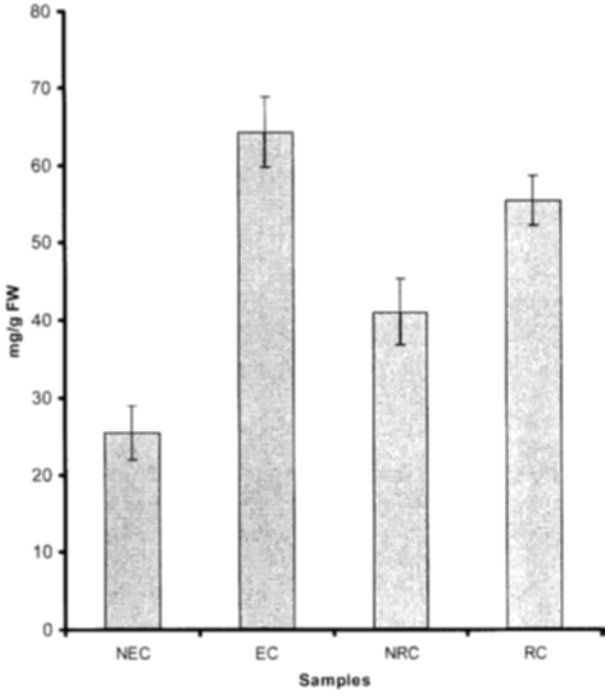

Figure 1. Total soluble protein content in the non-embryogenic callus (NEC), embryogenic callus (EC), non-regenerated cotyledon (NRC) and regenerated cotyledon (RC). Bar indicates SE of means (N=3).

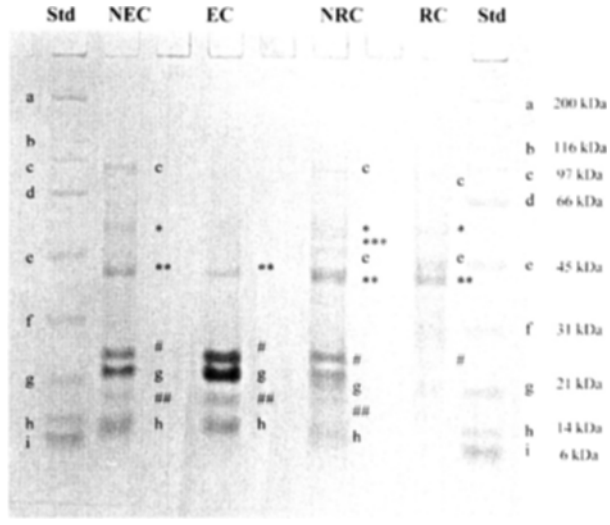

Figure 2. SDS electrophoretogram of protein extracted from different types of *E. longifolia* samples. Std, standard marker; NEC, non-embryogenic callus; EC, embryogenic callus; NRC, non-regenerated cotyledon; RC, regenerated cotyledon; a, myosin; b, β-galactosidase; c, phosphorylase; d, serum albumin; e, ovalbumin; f, carbonic anhydrase; g, soybean trypsin inhibitor; h, lysozyme; I, aprotinin. *, 59 kDa; **, 43 kDa; ***, 56 kDa; #, 25 kDa; ##, 16 kDa.

and physiological aspects of cell growth could lead to a better understanding of somatic embryogenesis (Silveira et al., 2003).

In embryogenic callus, two protein bands with high intensity were observed at the molecular weight of 25 and 21 kDa, and another three bands, which were not clearly visible, were found at 45, 16 and 14 kDa (Fig. 2). On the other hand, in the non-embryogenic callus, seven bands were successfully separated at 97, 59, 43, 25, 21, 16 and 14 kDa, three bands with high intensity were observed in non-embryogenic callus. The process of somatic embryogenesis involves the commitment of specific cells to a sequential pattern of selective gene expression (Kairong et al., 1999). Thus, differences in the protein pattern between embryogenic and non-embryogenic callus would be applicable in the early identification of embryogenic callus (Ramanayake and Wanniarchch, 2003). Proteins closely associated with embryogenic cells have also been identified and postulated as markers of embryogenic potential in *Spinacia oleracea* L. (Ishizaki et al., 2002), *Daucus carota*, *Brassica napus*, *Cichorium*, *Hordeum vulgare*, *Triticum aestivum* (Islas-Flores et al., 2000) and *Lycium barbarum* L. (Kairong et al., 1999).

Comparison between the regenerated cotyledon and the non-regenerated cotyledon disclosed that three intense bands (molecular weights of 43, 25 or 21 kDa) were clearly visible in non-regenerated cotyledon. Another six bands, which were not clearly visible, were found at 97, 59, 56, 45, 16 and 14 kDa. In the meantime, only two intense bands (45 and 43 kDa) were observed in regenerated cotyledon.

Specific Activity of Peroxidase and Peroxidase Banding Profile

The specific activities of peroxidase in the four samples indicated that peroxidase activity was generally higher in the embryogenic callus and the regenerated cotyledon compared to that in the non-embryogenic callus and the non-regenerated cotyledon. According to Laukkanen et al. (1999), the regeneration capability is a complicated phenomenon that is regulated by several factors. Enzymes such as peroxidase and polyphenol oxidase might also have indirect effects on it. The peroxidase activity obtained in these samples was ranged from 157.1 to 578.1 unit mg^{-1} soluble protein (Fig. 3). The highest specific activity of peroxidase (578.1 ± 61.6 unit mg^{-1} soluble protein) was recorded in the embryogenic callus followed by 428.4 ± 36.1 unit mg^{-1} soluble protein in the regenerated cotyledon. The non-embryogenic callus and the

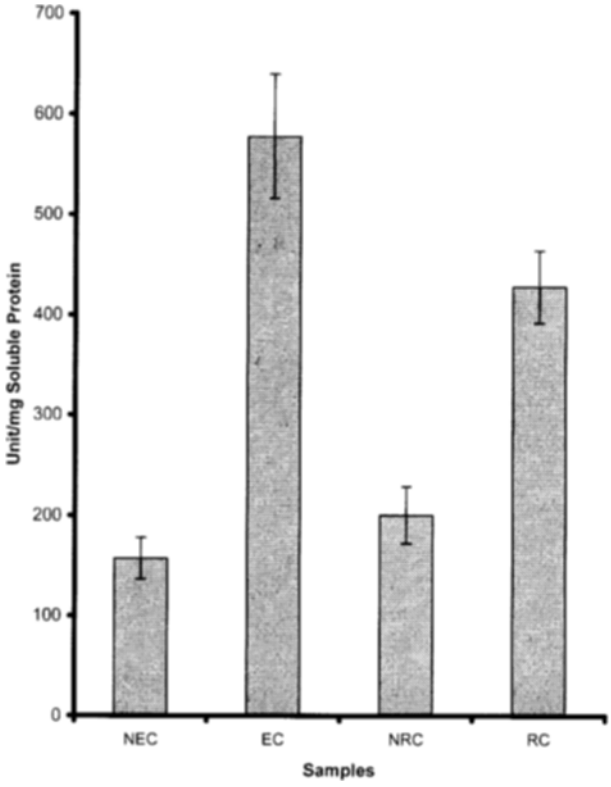

Figure 3. Specific enzyme activity of peroxidase in the non-embryogenic callus (NEC), embryogenic callus (EC), non-regenerated cotyledon (NRC) and regenerated cotyledon (RC). Bar indicates SE of mean (N=3).

non-regenerated cotyledon showed lower peroxidase activities, 157.1 ± 20.5 and 200.6 ± 28.2 unit mg^{-1} soluble protein, respectively. This result is comparable to Laukkanen et al. (1999) in which the changes in peroxidase activity of the embryogenic and non-embryogenic tissues of *Pinus sylvestris* L were determined.

This result based on spectrophotometry indicates that high activity of isozyme particularly peroxidase is closely associated with the regeneration capability of *E. longifolia* Jack. These results agreed with those of Bonfill et al. (2003) on *Panax ginseng* calli which showed that the change from reductant to oxidant state is closely related with the formation of embryogenic structures. Therefore, it was suggested that peroxidase might play an important role in plant regeneration and it could be one of the possible markers for the determination of somatic embryo in *E. longifolia*. Bonfill et al., (2003) also reported that changes in isoperoxidases precede the morphological appearance of the organs and thus can be considered as markers of the differentiation processes.

As shown in Figure 4, there were variations in the

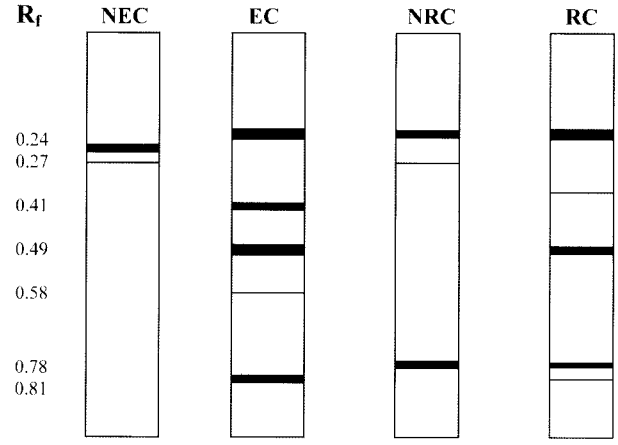

Figure 4. Typical zymogram of peroxidase extracted from the different types of *E. longifolia* samples under the electrophoresis conditions of 7.5% stacking gel, 10% separating gel. NEC, non-embryogenic callus; EC, embryogenic callus; NRC, non-regenerated cotyledon; RC, regenerated cotyledon.

peroxidase banding profiles in the four samples examined. Only two bands were observed from the non-embryogenic callus at the R_f 0.24 and 0.27 whereas in the embryogenic callus four bands with high intensity were observed at the R_f 0.24, 0.41, 0.49 and 0.81. Meanwhile, comparison between the regenerated and the non-regenerated cotyledon also showed that there was significant difference in the band intensity and the number of bands. The data revealed that only two bands were clearly shown in the non-regenerated cotyledon at the R_f of 0.24 and 0.78. As for the regenerated cotyledon, three intense bands were observed at the R_f of 0.24, 0.49 and 0.78. Different number of bands and their intensities among the samples indicate the involvement of peroxidase in plant embryogenesis. Dramatic changes in isoenzyme expression pattern during growth and development is well known in plants (Coppens and Dewitte, 1990). Duncan et al. (2003) reported that embryogenic maize callus contained isoperoxidase, esterase and malate dehydrogenase isoenzymes that were not present in non-embryogenic callus. Differentiation and development of embryogenic cells in the somatic embryogenesis of *Lycium barbarum* L. are regulated by three antioxidant enzymes, superoxide dismutase, peroxidase and catalase (Kairong et al., 1999).

Often the reason for inadequate regeneration from somatic embryos in a given genotype is the failure to recognize the limiting factor, which in most cases is the lack of an appropriate inductive signal (Murthy et al., 1999). Therefore, further research on the pro-

teomic profiling can be carried out in order to distinguish between the embryogenic callus and the non-embryogenic callus at the early stage.

ACKNOWLEDGEMENT

This research was supported by the Ministry of Science, Technology and Innovation of Malaysia.

Received July 8, 2005; accepted October 5, 2005.

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