Rubber Science, **25**(2): 183-188, 2012

CHANGES IN PROTEIN PROFILE DURING DIFFERENT DEVELOPMENTAL STAGES OF SOMATIC EMBRYOGENESIS IN *HEVEA BRASILIENSIS*

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Received: 12 September 2012 Accepted: 30 September 2012

Jaysree, P.K., Sajeevan, R.S. and Thulaseedharan, A. (2012). Changes in protein profile during different developmental stages of stomatic embryogenesis in *Hevea brasiliensis*. *Rubber Science*, **25**(2): 183-188.

Somatic embryogenesis is a process by which somatic cells are induced to form bipolar embryos through a series of developmental stages. Since proteins directly influence the cellular metabolism, the changes in protein profile provide a suitable tool for examining the biochemical changes associated with somatic embryogenesis. In plant species, total protein levels increased according to the progression in the developmental stages of somatic embryos and *vice versa* which in turn correlated with the regeneration potential. In the present study, total soluble proteins were studied during the course of somatic embryogenesis pathway in *Hevea* as well as at different stages of the development of somatic embryos. SDS-PAGE analysis of total soluble proteins showed marked differences in its profiles. During different stages of somatic embryogenesis pathway, there was more accumulation of proteins in somatic embryos on a tissue fresh weight basis. Three developmental stages of somatic embryos displayed uniform banding pattern for proteins. However, the relative protein content was decreased as the somatic embryos were more advanced in their development, particularly, at the cotyledon stage. This may probably the reason for the low conversion ability of somatic embryos to plantlets and their further establishment.

Keywords: Electrophoresis, *Hevea brasiliensis*, Protein, SDS-PAGE, Somatic embryogenesis

INTRODUCTION

The ability of somatic plant cells to regenerate into whole plants by the process of somatic embryogenesis by *in vitro* culture is a remarkable phenomenon. Since the initial description of somatic embryo induction in carrot (Steward *et al*., 1958), this developmental process has been reported for a variety of plant species. In Indian *Hevea* clones, somatic embryogenesis has been achieved from immature anther (Jayasree *et al*., 1999), immature inflorescence (Sushamakumari *et al*., 2000) and leaf explants (Kala *et al*., 2005). In later years, the efficiency of *Hevea* somatic embryo production has improved considerably, however, the process still exhibit low rates of plant regeneration and survival during hardening process.

Morphological and physiological quality of mature somatic embryos affects their germination and subsequent seedling

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growth and development (Griga *et al*., 2007). Storage proteins are used as sources of amino acids for seed germination (Misra *et al*., 1993) and therefore the proteins are related with the conversion ability of embryos. These storage proteins are also used as markers in comparing the developmental processes of somatic and zygotic embryogenesis (Hakman, 1993). In plant systems, numerous reports are available on proteins associated with somatic embryogenesis. Blanco *et al*. (1997) studied the protein changes associated with plant development from embryogenic callus of sugarcane and discussed the association of soluble protein content and callus regeneration ability. In sandalwood, analysis of total proteins in all the developmental stages of somatic embryos revealed the presence of an array of proteins (Suma and Balasundaran, 2004). Increased levels of storage proteins and free amino acid accumulation were related with increased rate of somatic embryo conversion and increased vigour of seedlings of alfalfa (Horbowioz *et al*., 1995). However, in *Elaeis guineensis,* the reduced vigour of plantlets was attributed to the low levels of storage proteins present in the somatic embryos (Morcillo *et al*., 1999). *Capsicum chinense* somatic embryos also showed low total protein content in cotyledonary staged embryos, which could be correlated with the low rate of conversion to plantlets and high frequency of deformed somatic embryos (Lecona-Guzman *et al*., 2012). In contrast, somatic embryos developed in coffee displayed changes in some proteins that are progressively accumulated throughout the development (Yuff *et al*., 1994). However, there is limited information available on protein pattern associated with somatic embryogenesis in the Indian *Hevea* clones. In the present study, an attempt was made to understand the changes in protein profiles associated with various stages of somatic embryogenesis pathway as well as with different developmental stages of the embryos *viz.* globular, torpedo and cotyledonary stages in *Hevea*.

MATERIALS AND METHODS

Somatic embryogenesis and plant regeneration from immature anther has been carried out by employing the reported protocol (Jayasree and Thulaseedharan, 2005). In brief, after surface sterilization, immature anther of *Hevea*, clone RRII 105, were cultured in callus induction medium containing 2.0 mg/L 2, 4-dichlorophenoxyacetic acid and 0.5 mg/L kinetin. Callus induced from explants were transferred to embryo induction medium consisted of 0.2 mg/L naphthaleneacetic acid, 0.7 mg/L kinetin and 2.0 mg/L GA_3. Embryogenic callus produced were further differentiated into somatic embryos. For embryo germination, mature somatic embryos were cultured on plant regeneration medium containing 0.5-1.0 mg/L benzyl adenine and 2.0 mg/L GA_3. Tissues at four stages of somatic embryogenesis pathway were selected for protein study. The four different stages are (1) fresh callus induced from explant (2) embryogenic callus (3) embryos and (4) plantlets. Somatic embryos at three stages of development such as globular, torpedo and cotyledon stages were also used for the study.

For total soluble protein extraction, all samples (500 mg) were crushed into a fine powder in liquid nitrogen and thawed in 2 per cent ice cold dithiothrietol (DTT). Crude extracts were centrifuged at 10,000 rpm for 20 min at 4 °C. Supernatant fractions were

mixed with 8 volume of ice cold acetone and kept in ice for 2h to precipitate the protein. The precipitate was centrifuged at 5000 rpm and gently washed twice with ice cold 100 per cent acetone. The final pellet was air dried and solubilised in 1.5 ml of buffer (8M Urea, 4 per cent CHAPS, 50 mM DTT). The protein sample (30 µl) mixed with 10 µl loading buffer was boiled for 4 minutes. Proteins from each sample was analysed by SDS-PAGE according to the method of Laemmli (1970) using a 12 per cent linear gel. The gel was stained with coomassie brilliant blue for studying the protein profiles. To ascertain the reproducibility, the experiment was repeated thrice.

Fig. 1. Induction and types of somatic embryos in *H. brasiliensis*
a. Induction of somatic embryos
b. Somatic embryos at globular stage
c. Somatic embryos at torpedo stage
d. Somatic embryos at cotyledon stage

RESULTS AND DISCUSSION

Immature anthers cultured on callus induction medium induced yellow callus after 35 days of culture. Upon transfer to embryo induction medium, yellow colour of callus was changed to brownish. After 2 months of culture, yellow mucilaginous embryogenic callus was produced from the older brown callus. Somatic embryos were induced from these embryogenic mass after 4 month culture. The embryogenic mass displayed active morphogenic growth and then successfully developed into embryos (Fig. 1a). Different stages of embryos such as globular (Fig. 1b), torpedo (Fig. 1c) and cotyledon (Fig. 1d) were observed in the same medium. Mature cotyledonary embryos were subsequently converted into plantlets.

Somatic embryogenesis pathway showed marked difference in their protein profiles at four different stages of development (Fig. 2a). During callus induction, only one prominent and broad protein band with molecular weight higher than 66 kDa was appeared which may contain more than one protein. There may be very low abundant proteins in the other regions of the SDS-PAGE which could not be detected by coomasie brilliant blue staining. The protein loaded was on a tissue fresh weight basis. The low protein content may be attributed to the fact that the callus induction phase represents the dedifferentiation stage during which minimal proteins were required or synthesised. During embryogenic calli formation, more proteins were produced with molecular weight ranging from 14 to 97 kDa. During this stage many proteins are required for various biochemical pathways leading to differentiation. However, the intensity was more for low molecular weight proteins particularly below 20 kDa. In the embryogenic callus of *Santalum album* also Suma and Balasundaran (2004) reported a relative abundance of low molecular weight proteins (14 to 43 kDa). In the present study, when embryogenic callus was differentiated into embryos, a relative abundance of both

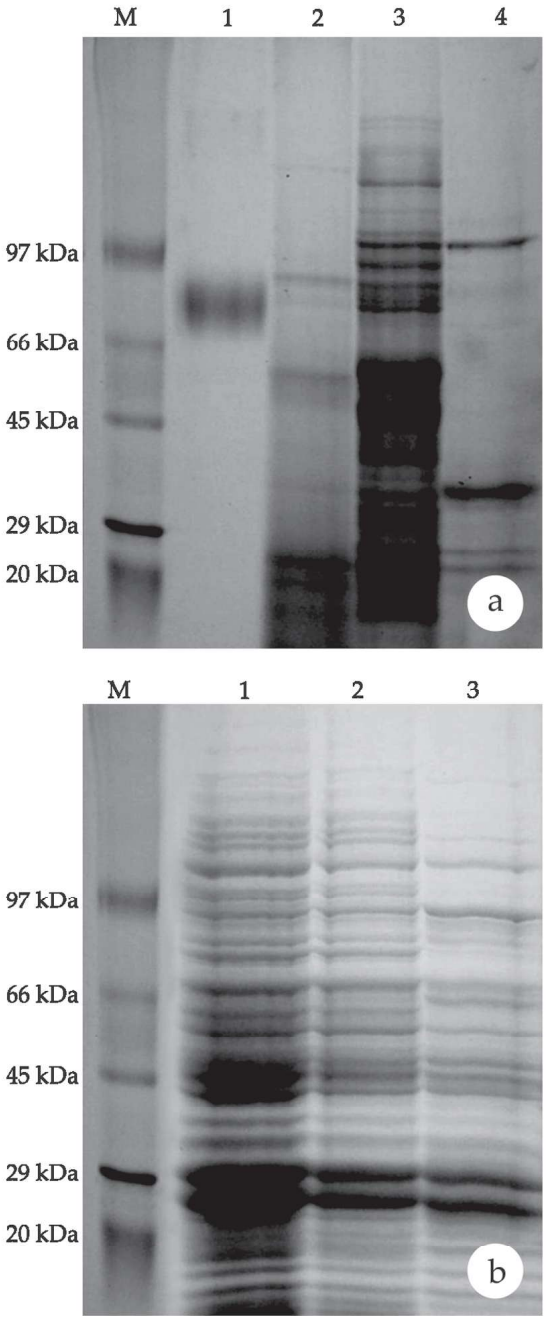

Fig. 2. Protein profiles at various stages of a. Somatic embryogenesis b. Somatic embroys

2a. Lane M - Marker
Lane 1 - Callus
Lane 2 - Embryogenic callus
Lane 3 - Somatic embryos
Lane 4 - Plantlets

2b. Lane M - Marker
Lane 1 - Globular embryos
Lane 2 - Torpedo embryos
Lane 3 - Cotyledon embryos

low and high molecular weight proteins were observed. More than 15 bands were clearly detected at this stage. The increased protein accumulation could be attributed to the fact that these polypeptides may be synthesised during the embryo development and it correlates with embryogenic potential. When somatic embryos were converted into plantlets, there was an apparent reduction in protein content (on tissue fresh weight basis). This observation may probably due to the synthesis of more structural proteins during this stage of development and also the protein loaded in the SDS-PAGE was on a fresh weight basis of the tissue.

A detailed analysis of the protein profiles at different stages of somatic embryo development such as globular, torpedo and cotyledon stages were also carried out (Fig. 2b, lanes 1-3). A large number of low and high molecular weight proteins could be resolved. At all developmental stages, somatic embryos showed similarity in their protein profile. However, the intensity of the protein bands slightly decreased as the somatic embryos advanced their development. Two bands that could be distinguished in a molecular weight range between 20 and 30 kDa showed much higher intensity than other proteins. Another group of proteins corresponding to molecular weight within the range of 45 kDa were also expressed in much higher intensity. Compared to globular stage, the intensity of these proteins was less in torpedo and cotyledon embryos. In *Ocotea catharinensis* Mez. (Lauraceae), a slow growing tree, it was reported that the globular stage embryos significantly accumulated higher level of total proteins. However, these proteins were gradually decreased with embryo maturation (Catarina *et al*., 2003). Soybean

somatic embryos also showed a reduction in protein content by 25% during embryo maturation (Chanprame *et al*., 1998). In *Capsicum chinense,* somatic embryos accumulated high proteins at globular stage which decreased in content as the embryo progressed to cotyledon stage and the low protein content at the cotyledonary stage could be correlated with low rate of plant conversion and high frequency of abnormal embryos (Lecona-Guzman *et al*., 2012). However, coffee somatic embryos at globular, heart and torpedo stages, showed change in some proteins that are progressively accumulated throughout the development (Yuff *et al.,* 1994). According to Horbowioz *et al*. (1995), the increased level of storage proteins and free amino acid accumulation increased the rate of somatic embryo germination and also increased the vigour of seedlings. In the current study more protein accumulation was observed in the early globular stage somatic embryos and the content apparently decreased as the embryos matured into late stages on a fresh weight basis of the tissues. The low level proteins in cotyledon staged embryos may affect the conversion rate of somatic embryos and the vigour of plants.

CONCLUSION

SDS-PAGE analysis of total soluble proteins during four stages of somatic embryogenesis pathway in *H. brasiliensis* revealed significant variation in their protein profiles on a tissue fresh weight basis. Among the four sequential stages, more accumulation of total proteins was observed in somatic embryos. Protein profiles of somatic embryos at three developmental stages displayed uniform banding pattern, however, the intensity of bands slightly decreased as the somatic embryos progressed in their development from globular to cotyledon stage. These results lead to the conclusion that low level proteins in cotyledon staged embryos may affect the conversion rate of somatic embryos and the vigour of plants and therefore, refinement at embryo induction stage will be needed for the accumulation of more proteins during later stages and to improve the conversion ability of *Hevea* somatic embryos.

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