Research Article

Journal of Plantation Crops, 2014, 42(3):294-300

Localization of peroxidase enzyme in the bark of *Hevea brasiliensis* and its implication in anatomy

Gopika Gopal and Vinoth Thomas*

Rubber Research Institute of India, Kottayam - 686 009, Kerala, India

(Manuscript Received: 25-09-13, Revised: 25-08-14, Accepted: 22-09-14)

Abstract

Localization of peroxidase enzyme with respect to seasons has been carried out in the bark of mature trees of *Hevea brasiliensis* using guaiacol and hydrogen peroxide as the substrate. Cell walls of sieve tubes including sieve plates, and cytoplasm of phloic rays both in the soft and hard region of the bark showed positive indication of peroxidase activity with reddish brown coloration. Sieve tubes differentiated recently from the derivatives of cambium also stained deeply. The cell wall of the phloic rays remained passive for peroxidase activity but the cytoplasm appeared to be granulated and was in a state of streaming motion which was evident in cross sectional view of the bark. Companion cells did not give any indication of peroxidase activity but it was localized in the intercellular spaces of axial parenchyma cells in the soft bark. Phloic rays exhibited seasonal variation for peroxidase enzyme while the activity was localized throughout the year in the sieve tubes. In the samples taken during December- January, phloic rays were unstained throughout the bark. Bark samples collected in the month of March and April showed deep coloration for phloic rays extending from the phellogen to the periphery of the cambial zone. In the month of June, the partially differentiated phloic rays in the cambial zone stained for peroxidase and subsequently differentiated ones were completely unstained. The phloic rays in the months of September and October remain unstained in the soft bark while it gave a brown coloration in the hard bark region.

Keywords: Guaiacol, *Hevea* bark, peroxidase, phloic rays, seasonal activity, sieve tubes

Introduction

Hevea brasiliensis, the prime source of natural rubber, is a deciduous tropical tree exhibiting seasonal activity of cambium that leads to alteration in the anatomy of bark periodically (Thomas *et al*., 1995a; 2002). Structurally, the bark of *Hevea* can be demarcated into inner soft bark and outer hard bark among which functionally active tissues are confined to the soft bark and those tissues in the hard bark region are less functional or shriveled (Bobilioff, 1923). In the bark, the sieve tubes and phloic rays constitute the conductive tissue. Sieve tubes are tubular in structure with sieve plates on either end with pores. The phloic rays in *Hevea* are uniseriate, biseriate or multiseriate of which multiseriate rays were the most abundant (about 95-98%). Sieve tubes serve as the pathway for the downward translocation of photo-assimilates and the phloic rays which extend from the centre to the periphery of the trunk are meant for radial conduction that nourishes various tissues including latex vessels (Hebant and Fay, 1980; Fay and Jacob, 1989). A number of histochemical and enzyme localization studies were carried out in the bark of *Hevea* (Hebant and Fay, 1980; Thomas *et al*., 1995a; 2002; Pramod *et al.,* 2008, 2011) but none of those were focused on the histochemical localization of peroxidase activity in various tissues of the bark with respect to season**.**

Peroxidases are among the most studied plant enzymes, yet its physiological functions are only partially understood. Peroxidases are generally involved in the lignin synthesis (Cottle and Kolattukuddy 1982; Egley *et al.,* 1983), oxidation of IAA (Grambow and Langen-Schwich, 1983), defense mechanisms (Cochrane, 1994; Almagro *et al.,*

* *Corresponding Author: vt@rubberboard.org.in*

2009) and cell differentiation (McDougall, 1992). Biochemical estimation studies of peroxidase enzyme in the bark tissue of *Hevea* revealed that there exists seasonal variation for this enzyme (D'Auzac, 1989; Ambily *et al.,* 2012). The present article describes the histochemical localization of peroxidase activity in the bark tissue of *Hevea* with respect to season.

When metabolic functions in a plant system are altered especially in diseased conditions like TPD, the constant proportion groups of enzymes also change as a whole. To reveal the basic knowledge regarding enzymes and its impact on plant system, enzyme histochemistry and its localization in the bark of the tree has to be performed.

Materials and methods

Bark samples from the trunk of six mature trees of *Hevea brasiliensis* (21 year old; clone RRII 105) under tapping were collected from above and below the tapping cut during the months of December-January (2010-2011) (trees without leaves following defoliation), March-April (summer season), June-July (monsoon period), and September-October (post monsoon season). Samples were also collected in the morning and afternoon hours. Fresh sections (TS and TLS) of 30-40 µm thickness were taken using a sliding microtome and stained with 1 per cent guaiacol staining mixture and kept for 10 minutes in dark for incubation. The guaiacol staining mixture was prepared using 0.1 M sodium phosphate buffer, 1 per cent H_2O_2 and 1 per cent guaiacol in the ratio 3:1:1 respectively (Bergmeyer, 1974). Stained sections were mounted in buffer or diluted glycerol and observed under Leica Diaplan attached with Leica QwinV3 Image analysis system. Iodine potassium iodide was used for localizing starch (Johanson, 1940).

Guaiacol is a naturally occurring organic compound readily oxidized by the heme iron of peroxidase enzyme (Bergmeyer, 1974). The rate of decomposition of hydrogen peroxide by peroxidase, with guaiacol as hydrogen donor, is determined by the brown color developed by the formation of certain brown pigments due to browning reactions which are generally catalyzed by peroxidases as the case with o-dianisidine (Higuchi, 1997; Dehon *et al.,* 2002; Gopal and Thomas, 2012). Guaiacol is thus used as a compound for analyzing peroxidase activity in both plants and animals.

$$\text{Guaiacol} + H_2O_2 \xrightarrow{\text{Peroxidase}} \text{Tetraguaiacol} + H_2O$$

Results and discussion

Localization of peroxidase enzymes with respect to different seasons was carried out in the bark tissue of *H. brasiliensis*. Cell walls of sieve tubes and cytoplasm of phloic rays in the soft and hard bark region showed positive indication of peroxidase activity with brown coloration (Figs. 1 and 2). Intercellular spaces of the axial parenchyma cells in the soft bark also stained for peroxidase activity irrespective of the season. Cytoplasm of the phloic rays showed seasonal variation (Figs. 5, 6 and 7) whereas cell walls of the sieve tubes showed peroxidase activity throughout the year. Higher activities of peroxidase were observed in the bark samples collected during the morning hours but the activity was retarded in the afternoon. Staining intensity was found to be more in the cell walls of sieve tubes when compared with the cytosol staining of phloic rays.

Sieve tubes

Sieve tubes in *Hevea* measures an average length of 683 µm and diameter of 40 µm. The peroxidase activity was localized in the cell walls of sieve tubes including those differentiated recently from the derivatives of cambium. Peroxidase activity was detected in both primary and secondary walls of the mature sieve tubes (Fig. 1). Sieve plate occurring on either end of the sieve tubes gave deep staining as an indication for peroxidase enzyme. Companion cells were unstained for peroxidase activity. Sieve tubes in the soft bark region possess a regular outline while those in the hard bark region are uneven in outline. It has been reported that peroxidases are normally associated with cell walls during later stages of cell differentiation (Van Fleet, 1959). From the present study it is obvious that among the different tissues differentiated from the derivatives of fusiform initials, sieve tubes are the first differentiating tissue in the bark as its cell wall showed high level of peroxidase activity even within the cambial zone (Fig. 4).

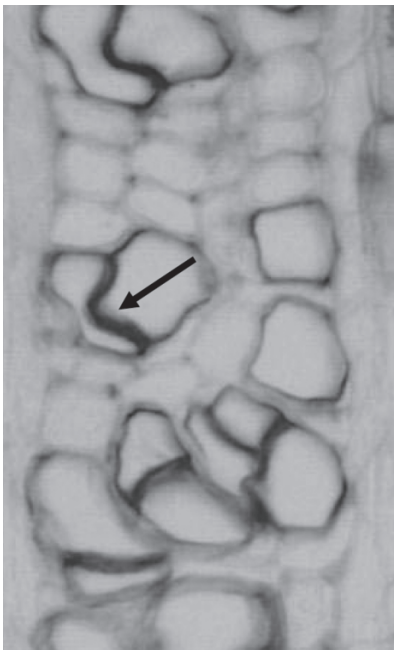


Fig. 1. TS of Sieve tube stained deeply for peroxidase activity with more intense staining for sieve plate (at arrow)

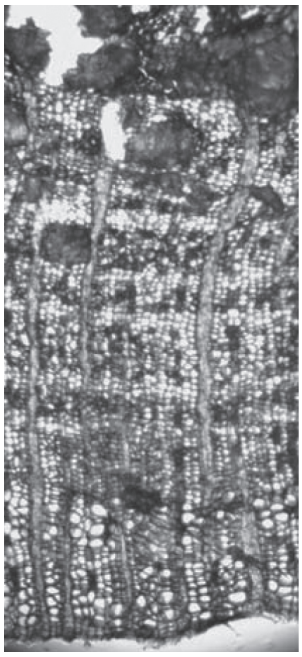


Fig. 2. Full extent of the phloic rays in the bark showing peroxidase activity

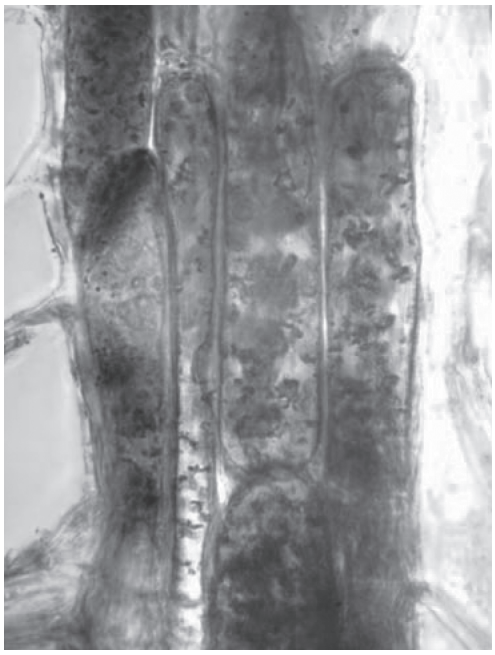


Fig. 3. TS of bark showing stained agglomerations in the ray cells

Phloic rays

The phloic rays in *Hevea* are uniseriate, biseriate or multiseriate of which multiseriate rays are the most abundant (Pramod *et al.,* 2011). Multiseriate measures an average length of 495 µm and 56.81 µm in breadth. The cross sections of the bark when stained for peroxidase were deep brick red color in the cytoplasm of phloic rays while its cell walls remained unstained (Fig. 3). The cytoplasmic peroxidase of phloic rays were localized both in the soft and hard bark region which extended up to the corky layer (Fig. 2). Contrary to sieve tubes, the phloic rays retained its shape and functional ability throughout the bark without showing any symptoms of senescence by the deposition of lignin, as noticed in the case of sieve tubes (Pramod *et al.,* 2011). In the hard bark region the phloic rays appeared to be wavy in nature due to the formation of sclereid groups in a scattered manner (Fig. 2). The cytoplasmic contents of the phloic rays were highly dispersed throughout the cell and these suspended matrices were in a state of streaming movement without developing any spatial pattern as observed in the sieve tubes of *Hevea* (Thomas *et al.,* 2012). The localization of peroxidase in the ray cells were not evident in both tangential and radial longitudinal sections which may be due to the oozing out of the cytoplasmic contents from the ray cells as the thickness of the sections were much lesser than the dimensions of the ray cells. After staining the bark, the colorless staining mixture appeared to be brownish due to

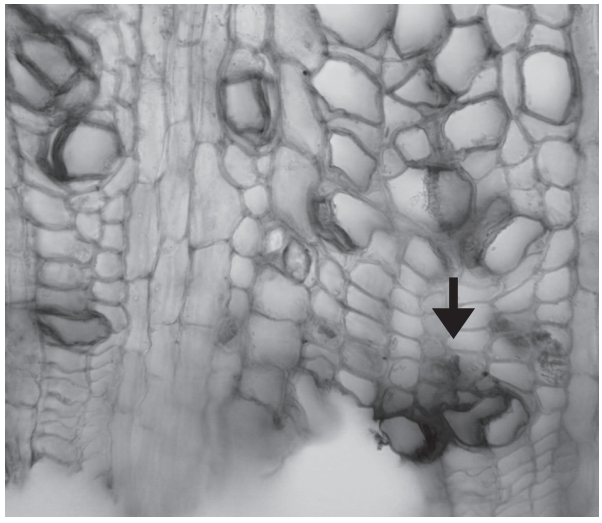
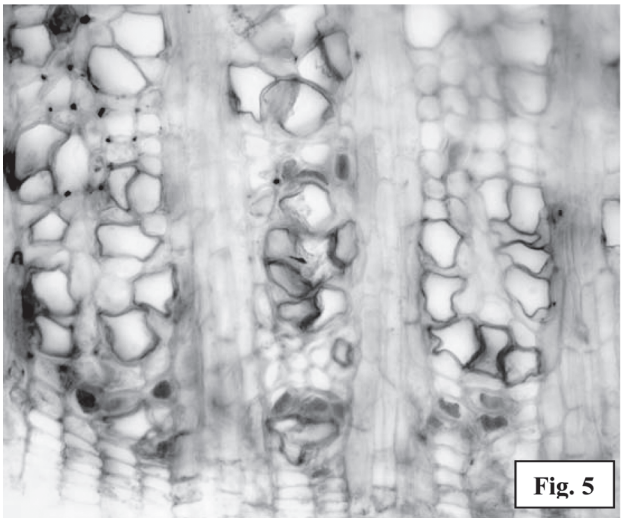
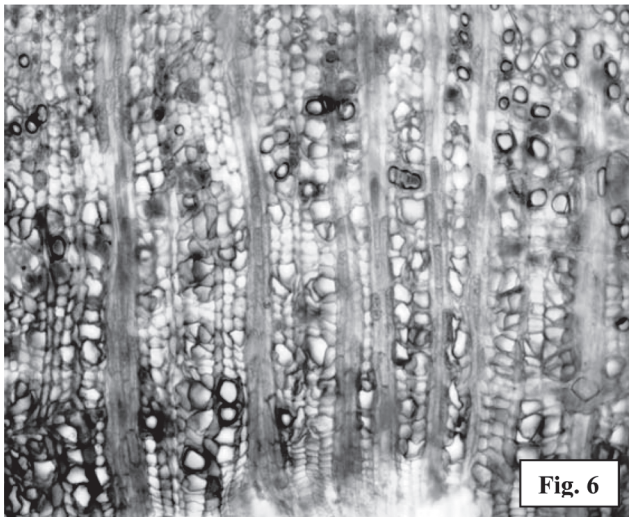


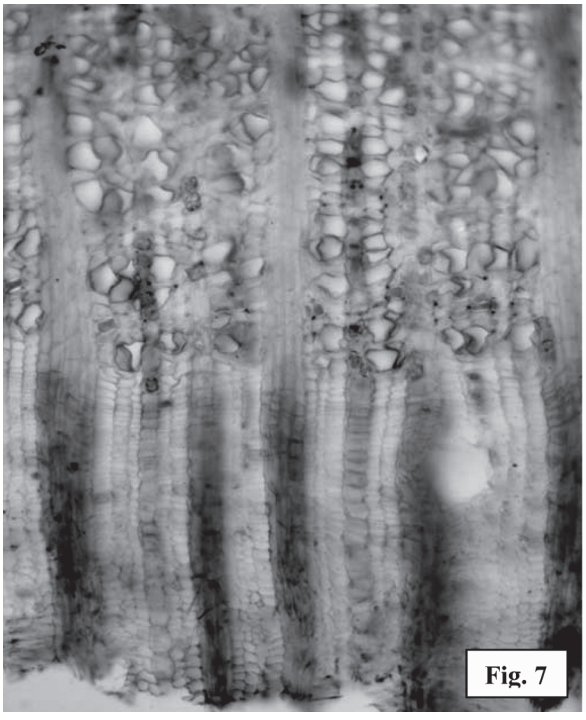
Fig. 4. Recently differentiated sieve tubes showing peroxidase activity (at arrow)



DECEMBER - JANUARY



MARCH - APRIL



JUNE - JULY

SEPTEMBER - OCTOBER

Figures 5-8: Seasonal variation for peroxidase in the bark of *Hevea*

Fig. 5. Sieve tubes stained for peroxidase keeping the phloic rays unstained (December – January); Fig. 6. Both sieve tubes and phloic rays showed peroxidase activity (March – April); Fig. 7. Phloic rays nearer to the cambial zone alone stained deeply for peroxidase (June –July); Fig. 8. Both sieve tubes and phloic rays showed peroxidase activity (September – October)

the deposition of colored particles that seem to have oozed out from the cells. These particles when observed under microscope revealed that they were identical to these streaming particles present in the cytoplasm of ray cells after staining. When the underlying sections were stained using general histological stains, it did not show such granular particles as with the case of peroxidase staining.

Underlying sections were also treated with I_2KI for the localization of starch grains but these starch grains did not hinder the cytoplasmic streaming of the granulated matrix in phloic rays.

Localization of peroxidase in the cytoplasm of phloic rays showed variation in the staining intensity in the bark samples collected at different seasons. In samples obtained during December - January (trees without foliage following defoliation), peroxidase activity was not observed in the ray cells that extended from the cambial zone to the corky layer (Fig. 5). During the months of March and April (summer season), peroxidase was localized in the entire extend of the phloic rays with more or less same staining intensity, both in the soft and hard bark (Fig. 6). The cytoplasmic contents appeared to be scrambled in nature with deep coloration compared to the cytoplasm in which it was embedded. In the samples during June-July (monsoon period), the phloic rays both in the soft and hard bark remained unstained but the undifferentiated mass of tissues from the cambium, to be designated as phloic rays, gave reddish brown coloration for peroxidase enzyme (Fig. 7). Ray parenchyma possessed granular inclusions, but were less dense compared to the previous season. The bark samples collected during September-October (post monsoon season), demonstrated peroxidase activity throughout with more coloring intensity towards the hard bark region (Fig. 8). During this period the recently differentiated phloic rays stained feebly for peroxidase activity. Another set of samples were collected during December-January of the succeeding year and it was found that the phloic rays were devoid of peroxidase activity. Thus, it was observed that the peroxidase activity in the phloic rays varies with respect to season, but throughout the year the sieve tubes showed cell wall peroxidase activity**.**

It was reported that phloic rays in the hard bark were functionally less active or dead (Bobilioff, 1923; Thomas *et al.,* 1995a). From the present study it is obvious that the phloic rays maintained its continuity up to the corky layer of the bark and exhibited seasonal variation for peroxidase activity. Thus, the phloic rays seem to have a strong role in maintaining the vitality of the tissue in the hard bark too. Also, the phloic rays have connectivity with the phellogen and phelloderm tissues of the cork. Photo assimilation in the phelloderm cells and transpiration by lenticels are some of the major functions of corky layer (Thomas *et al.,* 1995b) which can be accomplished by means of vital rays.

The seasonality for the occurrence of peroxidase has been already established in many plant species (Polle and Glavac, 1993; Zahra *et al.,* 2009). Peroxidase activity in the bark tissue of *Hevea* was estimated and found that there exists seasonal variation (Ambily *et al.,* 2012). While localizing peroxidase, seasonal occurrence for this enzyme was noticed in the phloic rays while the activity was present throughout the year in the sieve tubes. It has been reported by Van Fleet (1959) that the tissue that differentiates at a later stage showed peroxidase activity compared to that of early differentiated ones. But in the present study, it has been observed that the sieve tubes which differentiated immediately from the derivatives of cambium also showed intense brown coloration as the ones that differentiated at an earlier period. This indicates that the peroxidase has some unknown functions other than the designated ones (Meudt and Stecher, 1972; Chibbar and Huystee, 1984; Marjamaa *et al.,* 2009).

The sieve elements serve as a pathway for the transport of peroxidases to the endodermal cell walls for the production of suberin (Van Fleet, 1942). Thomas *et al.,* (1995b) reported that following wounding of tissues, deposition of suberin and lignin occurs for sealing the wound in *Hevea*. After conducting the ring barking experiment on the tree trunk of *Hevea*, Dijkman (1951) concluded that the peroxidase enzyme is transported through the sieve tube system in the bark to the site of action. This may be one of the reasons for observing peroxidase activity throughout the seasons in the sieve tubes of *Hevea.* It has been reported that the peroxidase activity is resistant to various chemical and physical agents, especially acids and heat which presumes that the enzyme is stable at least in certain instances. From the result of this study pertaining to the localization of peroxidase, it is concluded that it is ideal to collect the bark samples during early morning hours rather than later collection.

The high yielding clones of *Hevea* are vulnerable to a disorder termed tapping panel dryness (TPD) which is evident by the cessation of latex flow from the tapping panel (Thomas *et al.*, 2006). A number of morphological, anatomical and biochemical changes were encountered with TPD (D'Auzac *et al.,* 1989; Thomas *et al.,* 2006), among which high amount of lignification of the tissues were observed in the affected part of the tree. Peroxidase has a major role in lignin biosynthesis.

References

Almagro l., Gomez Ros, L.V., Belchi-Navarro, S., Bru, R., Ros Barcelo, A. and Pedreno, M.A. 2009. Class III Peroxidases in plant defense reactions. *Journal of Experimental Botany* **60**(2): 377-390.

Ambily, P.K., Krishnakumar, R. and Reghu, C.P. 2012. Physiological and anatomical changes in the laticiferous tissues of *Hevea* under ethylene stimulation. *Proceedings of 24th Kerala Science Congress,* 29-31 January 2012, RRII, Kottayam, pp.87-89.

Bergmeyer, H.U. 1974. *Methods of Enzymatic Analysis 1*, Academic Press, New York. 2nd edition 495p.

Bobilioff, W. 1923. *Anatomy and Physiology of Hevea brasiliensis. Part 1. Anatomy of Hevea brasiliensis*. Art. Institute Orell Fussil, Zurich.

Chibbar, R.N. and Huystee, R.B. 1984. Characterisation of peroxidase in plant cells. *Plant Physiology* **75**: 956-958.

Cochrane, M.P. 1994. Observations on the germ aleurone of barley. Phenol oxidase and peroxidase activity. *Journal of Experimental Botany* **51**: 507-520.

Cottle, N. and Kolattukuddy, P.E. 1982. Absicisic acid stimulation of suberization. Induction of enzymes and deposition of polymeric components and associated waxes in tissue cultures of potato tuber. *Plant Physiology* **70**: 775-780.

D'Auzac, J., Jacob, J.L. and Chrestin, H. 1989. *Physiology of Rubber Tree Latex*. CRC Press, Florida.

Dehon, L., Macheix, J.J. and Durand, M. 2002. Involvement of peroxidases in the formation of the brown coloration of heartwood in *Juglans nigra. Journal of Experimental Biology* **53**: 303-311.

Dijkman, M.J. 1951. *Hevea*: *Thirty Years of Research in the Far East*. University of Miami Press, Florida.

Egley, G.H., Paul Jr, R.N.Vaughn, K.C. and Duke, S.O. 1983. Role of peroxidase in the development of water-impermeable seed coats in *Sida spinosa* L. *Planta* **157**: 224-232.

Fay de, E. and Jacob, J.L. 1989. Anatomical organization of laticiferous system in the bark. In: *Physiology of Rubber Tree Latex*. (Eds.) d' Auzac, J., Jacob J. L. and Chrestin H., CRC press, Inc, Boca Raton, Florida, U.S.A.

Grambow, H.J. and Langen-Schwich, B. 1983. The relationship between oxidase activity, peroxidase activity, hydrogen peroxide and phenolic compounds in the degradation of indole-3-acetic acid *in vitro. Planta* **157**: 131-137.

Gopal, G. and Thomas, V. 2012. A method for staining sieve tubes in the bark of *Hevea brasiliensis. Proceedings of 24th Kerala Science Congress,* 29-31 January 2012, RRII, Kottayam, pp. 615-616.

Hebant, C. and Fay, E. 1980. Functional organization of the bark of *Hevea brasiliensis* (Rubber trees): A structural and histoenzymological study. *Z. Pflanzenphysiol.* **97**: 391-398.

Higuchi, T. 1997. *Biochemistry and Molecular Biology of Wood.* (Ed.) Timell, T.E. Springer Verlag, Berlin.

Johansen, D.A. 1940. *Plant Microtechnique*. Mc Graw - Hill Book Company, Inc. New York.

Marjamaa, K., Kukkola, E.M. and Fagerstedt, K.V. 2009. The role of xylem class III peroxidases in lignification. *Journal of Experimental Botany* **60**(2): 367-376.

McDougall, G.J. 1992. Plant Peroxidases and cell differentiation. In: *Plant Peroxidases 1980–1990, Topics and Detailed Literature on Molecular, Biochemical, and Physiological Aspects*. (Eds.) Penel, C., Gaspar, T.H. and Greppin, H. University of Geneva, pp. 101-115.

Meudt, W.J. and Stecher, K.J. 1972. Promotion of peroxidase activity in the cell wall of *Nicotiana. Plant Physiology* **50**: 157-160.

Polle, A. and Glavac, V. 1993. seasonal changes in the axial distribution of peroxidase activity in the xylem sap of beech (*Fagus sylvatica* L.) trees. *Tree Physiology* **13**: 409-413.

Pramod, S., Thomas, V. and Rao, K.S. 2008. P-protein in the sieve elements of *Hevea brasiliensis* trees affected by tapping panel dryness. *Natural Rubber Research* **21**: 139-144.

Pramod, S., Thomas, V., Rao, K.S. and Krishnakumar, R. 2011. Definitive callose deposition in the tapping panel dryness affected bark of *Hevea brasiliensis*. *Journal of Sustainable Forestry*, **30**: 329-342.

Thomas, V., George, E.S. and Saraswathyamma, C.K. 2006. Grafting and debarking techniques to manage tapping panel dryness in *Hevea brasiliensis.* In: *Tapping Panel Dryness of Rubber Trees*. (Eds.) James Jacob, R. Krishnakumar and N.M. Mathew Rubber Research Institute of India, Kottayam, India. pp. 225-230.

Thomas, V., Premakumari, D., Reghu, C.P., Panikkar, A.O.N. and Saraswathyamma, C.K. 1995a. Anatomical and

histochemical aspects of bark regeneration in *Hevea brasiliensis*. *Annals of Botany* **75**: 421-426.

Thomas, V., Sailajadevi, T., Nair, R.B., Sankar, S. and Saraswathyamma, C.K. 2002. Seasonal activity of cambium and changes in bark structure of *Hevea brasiliensis*. *Indian Journal of Natural Rubber Research* **15**: 55-65.

Thomas, V., Shankar, S., Gopal, G., Pramod, S. and Rao, K.S. 2012. Chaotic and linear spatial patterns in the sieve tubes of *Hevea brasiliensis* Book of Abstracts, 5th Chaotic modelling and simulation International Conference, 12th -15th June 2012, Athens, Greece. pp. 163-164.

Thomas, V., Vinod, K.K., Pothen, J., Saraswathyamma, C.K. and Potty, S.N. 1995b. Occurrence of bark chlorophyll on mature rubber trees of *Hevea brasiliensis*. *Proceedings of the 82nd Indian Science Congress,* January 3-8, Calcutta.

Van Fleet, D.S. 1942. The significance of oxidation in the endodermis. *American Journal of Botany*. **29**: 747-55.

Van Fleet, D.S. 1959. Analysis of the histochemical localization of peroxidase related to differentiation of plant tissues. *Canadian Journal of Botany* **37**: 449-458

Zahra, P., Majid, R. and Amin, B. 2009. Seasonal changes of peroxidase, polyphenol oxidase enzyme activity and phenol content during and after rest in pistachio (*Pistacia vera* L.) flower buds. *World Applied Sciences Journal* **6**(9): 1193-1199.