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Histochemical localisation of starch in the bark tissue of *Hevea* clones

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Abstract
Histochemical methods are highly useful to understand and locate the various metabolites in the plant tissue system like secondary phloem, bark, of *Hevea*. Phloem serves as the pathway for the transport substances synthesised by photosynthesis and also may provide as a system for storage molecules like starch, lipids, proteins, phenolic substances, tannins etc. In *Hevea brasiliensis*, bark has another important role, in which laticifers are distributed in the form of concentric rows and wounded by the process of tapping to extract latex. Considering the role of starch as a major metabolite in plants, it is significant to understand the occurrence and distribution of it in the bark tissue of *H. brasiliensis*. Samples from 10 clones of *H. brasiliensis* were taken in the present investigation and bark tissues were stained with Iodine-potassium iodide for starch deposition. Soft bark region contiguous to cambium had low level of starch, but the inner and outer hard bark had copious amount of starch occurrence. Among the parenchymatous tissue systems, axial parenchyma cells had recorded intense amount of starch grains compared to ray parenchyma cells. Starch grains were somewhat oval in shape and in many cells they were grouped. The present findings throw light into distribution and pattern of starch grains in *H. brasiliensis*.

Keywords: bark anatomy, *Hevea brasiliensis*, phloem rays, starch granules

Introduction
The rubber tree, *Hevea braziliensis* (Willd. ex A. Juss.) Mull. Arg. is an important plant belonging to the family Euphorbiaceae which is the major source of Natural Rubber. This family is having a significant position among the other taxa, as many of its members possess the most important plant product 'latex'. The milky latex of H. brasiliensis (para rubber) is the sole source of Natural Rubber (NR) which almost satisfies the needs of Rubber Industry. H. brasiliensis stands top, as it possesses very high NR content compared to other rubber yielding plants (Raghavendra, 1991) [25]. Under the genus *Hevea*, 10 species have been recognized so far (Schultes, 1970; 1977; 1987; Wycherley, 1992; Annamma and Abraham, 2005) [32, 34, 35, 44, 1]. Even though bark is the morphologically diverse and conspicuous part of the woody stem, bark remains the less understood part of the stem from the ecological perspective (Paine *et al*., 2010, Rosell *et al*., 2013) [10, 28]. 'Bark' denotes all tissue formed from the secondary cambium towards the outside including the secondary phloem, the secondary cortex, and also the periderm (Evert & Eichhorn, 2006) [5]. The complex internal structure of bark reflects its diverse functions and is significant for the carbon stored in it (Jenkins *et al*., 2003) [12]. The diverse functions include the transport of prepared food to various organs, prevention of the internal structures from fire, damage due to herbivore, pathogen attack (Gill & Ashton, 1968; Pfanz *et al*., 2002; Romero & Bolker, 2008; Romero *et al*., 2009; Lawes *et al.*, 2011) [8, 24, 26, 18]. It also served as main component tissue system for accumulation and storage of water and diverse compounds (Srivastava, 1964; Schmidt and Stewart, 1998; Scholz *et al*., 2007) [38, 30, 31] and provides mechanical support to the main stem (Niklas, 1999) [19]. The significant morphological diversity of the bark suggests that variation in bark may be an important component of variation in plant ecological strategies.

Anatomically *Hevea* bark consists of two distinct zones, the inner soft bark and the outer hard bark (Bryce and Campbell, 1917) [2]. Based on features associated with laticiferous system, the different regions of bark tissue were designated as soft bark, inner hard bark and outer hard bark region (Omman and Reghu, 2008, 2013) [20, 21]. Laticifers are differentiated from the fusiform initials of the cambium, in the form of concentric rings, alternating with other phloic elements such as sieve tubes, companion cells, phloem fibres, axial parenchyma and ray parenchyma. Due to the continued activity of the vascular cambium, new laticifers are differentiated and the older ones are pushed outwards. Outer zone of bark is hard due to the occurrence of copious amount of sclerified stone cells. Total bark thickness comprises the thickness of whole bark tissue that surrounds the wood externally in *Hevea*. The latex vessels running through the bark tissue was wounded by the process of tapping, to exploit the latex from *Hevea* plant. The anatomical feature of laticiferous system in *Hevea* clone has been well studied and taken in account for formulating the tapping system. The bark tissue forming the secondary phloem is mainly responsible for the transport and distribution of sugars synthesized by photosynthesis in the leaves. The present investigation was undertaken for understanding the significance on the distribution of starch metabolite in the phloem tissue system of *Hevea.*

Materials and Methods
Ten Wickham clones (Gl 1, GT1, PB 235, PB28/59, PB 86, RRII 105, RRII 300, RRIM 600, RRIM 703 and Tjir 1) of *Hevea* brasiliensis (Willd. ex Adr. de Juss.) Muell. Arg., were collected from experimental Station of Rubber Research Institute of India, Chethaekal, Ranni, Kerala. The experimental station is situated at 9° 22' N latitude and 76°

50' E longitude with an altitude of 80m above the MSL. These germplasm gardens comprised of 102 Wickham clones, planted in Randomised Block Design (RBD) with three replicates and three trees per plot. The trees were under regular tapping and had an age of 17 years. The samples collected were fixed in formalin-acetic -alcohol (FAA) and were sectioned at 30 – 60 µm thickness at different planes *viz*. cross sectional (CS), tangential longitudinal (TLS) and radial longitudinal (RLS) plane, using Reichert Jung sledge microtome. Sections were treated with Iodine-potassium iodide (Johansen, 1940) for starch and mounted in 50% glycerin and the micro slides were observed under Leitz Aristoplan Research microscope attached to Leica Q 5000 I W Image Analysis System.

Results and Discussion

In the present investigation, starch grains stained bluish-black with Iodine - Potassium Iodide (I2KI) were mainly localized in axial parenchyma and rarely in ray cells. The frequency of starch bearing cells, as well as the number of grains per cell varied considerably in different zones of bark. Soft bark region contiguous to cambium had low level of starch reserves (Fig. 1 a, arrow head) whereas the outer IHB region (Fig.1 a, arrow), as well as the entire HB region (Fig. 1 b) showed high storage of starch reserves. The storage of starch was more in axial parenchyma cells than in rays (Fig. 1c). In axial parenchyma, the starch grains were mostly accumulated as groups (Fig. 1 d) and in certain cases the grains were randomly distributed within the cells. The starch grains appeared as circular/oval in shape (Fig.1e). In the OHB region, starch grains were distributed in almost all the cells except in stone cells (Fig.1 f). The variation in starch grain size was also noticed in different clones (Fig. 1g and h). Studies on the histochemical status and distribution pattern of reserve metabolites such as starch, lipids, proteins and conversion of reserve metabolites into extraneous materials like phenols and tannin in *Hevea* bark are very limited as revealed by the survey of literature. The situation was the same with respect to cell wall deposits like total polysaccharides and lignin. Starch is the end product of carbon fixation and is the tonoplast of the storage cells, probably from sucrose (Zeigler, 1964; Strafford, 1965; Czaja, 1978) [45, 39, 4]. A large portion of photosynthates is utilized for the growth and development of plants. A considerable fraction is used up in respiration and surplus fraction is deposited as reserve metabolites in the storage tissue which are eventually utilized for growth and respiration (Kramer and Kozlowski, 1960) [17]. Hence in woody species, starch reserves is an important source of various kinds of organic compounds, including sucrose, which is the primary sugar that is transported in plants and regulate vascular differentiation (Shiroya *et al*., 1962; Wetmore and Rier, 1963; Zimmermann, 1971; Giaquinta, 1980; Kozlowski and Pallardy, 1997) [23, 42, 47, 7, 15]. The variation and partitioning of storage granules and lipid droplets have been investigated in several tree species like *Betula pendula* (Harms and Sauter, 1992), *Pinus sylvestris* (Fischer and Holl, 1992), Pinus cembra (Hoch *et al*., 2002), *Quercus petraea* and *Fagus sylvatica* (Barbaroux and Breda, 2002; Barbarous *et al*., 2003). The above studies clearly indicate the level of different reserve metabolites from phloem to xylem, which corelate to growth and development of the trees. In *Hevea*, the present investigation revealed the occurrence of high starch reserves in the axial parenchyma of the secondary phloem. This was similar to earlier reports in various plant systems (Hao and Wu, 1992; Wu and Hao, 1993; Zhang, *et al*., 1994; Courty, *et al* 1999; Thomas *et al*., 2002) [9, 43, 46, 3, 40]. The increased accumulation of starch in the outer hard bark region reflects the storage function. It is interesting to note that the phloic rays were devoid of starch reserves. In this context, it is reasonable to believe that the phloic rays are mainly involved in the conduction and transport of photosynthates, as suggested by Savidge and Wareing, (1982) [29] and the metabolites conducted through them might have been diverted for the biosynthesis of rubber latex in the laticifers by Tupy (1985) [41]**.** Enhanced respiratory and phosphatase activities reported in phloic rays by Hebant and Fay (1980) [10] strongly confirm this view.

It has been reported that the rate of cell differentiation is influenced by quantity of starch reserves in storage tissues (Oribe, 2003) [22].

The present study revealed that copious quantity of starch grains were accumulated in the axial parenchyma especially in the inner hard bark regions. This may also be related to the transport of sucrose from the storage cells to the laticifers as suggested by Jacob *et al*., (1998). This view can be further supported by the presence of numerous plasmodesmatal connections between laticifers and adjacent parenchyma cells in *H. brasiliensis* (Fay *et al*., 1989) [6]. The absence of starch grains in the soft bark region, particularly near the cambial zone, may be due to the utilization of metabolites for cell division and other cellular activities. The meristematic zone is considered as a strong sink for sucrose (Krabel, 2000) [16]. Starch reserves are mobilized to provide carbon for maintenance and growth when photosynthesis is absent or limited (Smith and Zeeman, 2020) [37]. Sucrose is accounted as the major primary photosynthate which is being transported within the source-sink system in plants (Shiroya *et al*., 1962; Zimmermann, 1971; Giaquinta, 1980; Kozlwski and Pallardy, 1997) [23, 41, 7, 15]. Begum *et al*., (2010) postulated that reduced starch granules in phloem and cambium zone may provide required energy during cambial reactivation and xylem differentiation. Hence, the variation in the occurrence and distribution of starch reserve in the secondary phloem may be an indication of the activity of plant tissue system during growth and development.

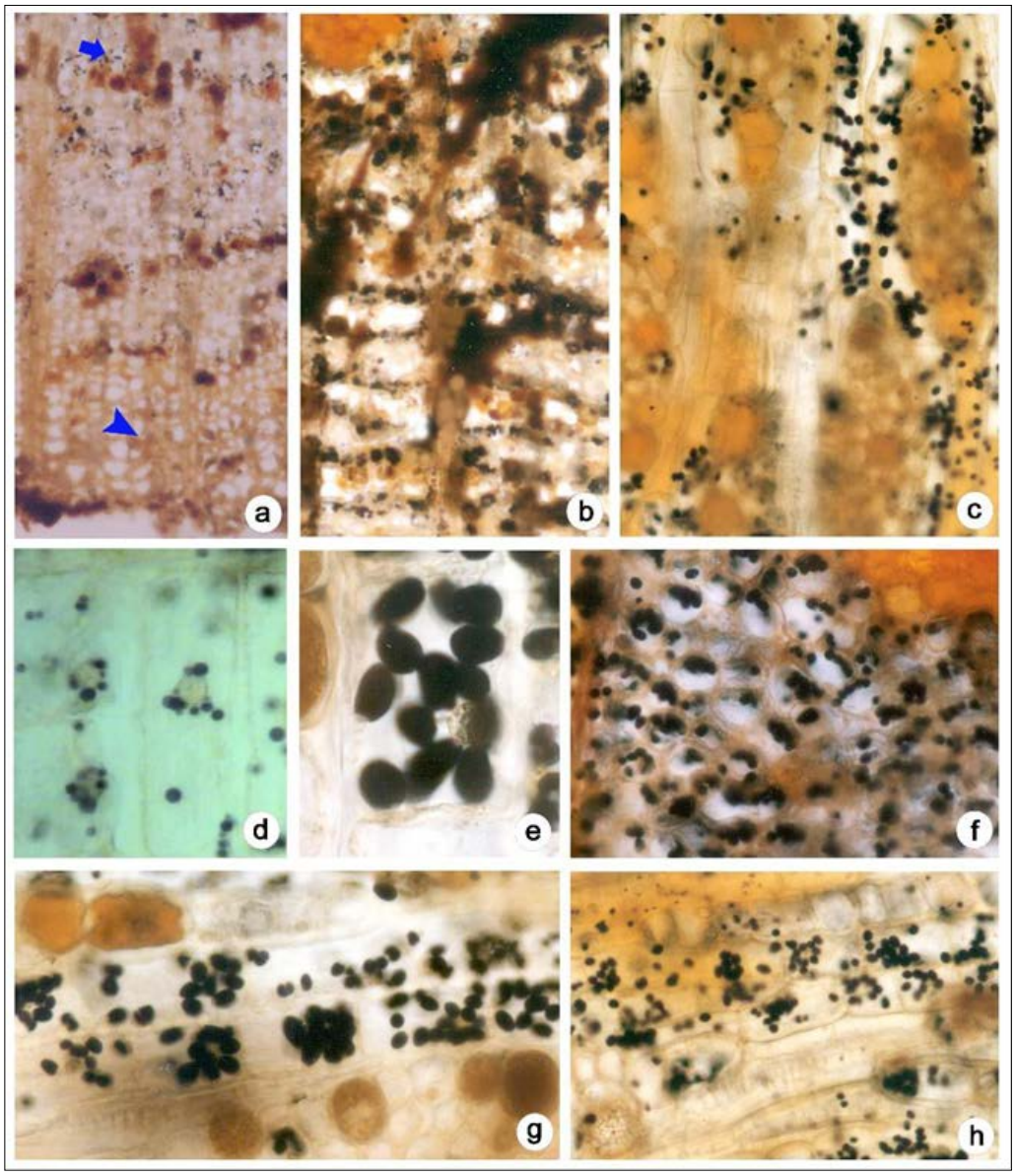

Fig 1: a to h – Histochemical localization of starch in bark tissue. a- less starch grains in soft bark (arrow head) and more starch grains in inner hard bark. b&c – outer hard bark showing high storage of starch in axial parenchyma. d- starch grains grouped. e- oval shape starch grains. f-starch grains surrounding stone cells in outer hard bark. g- PB 28/59 grain size (maximum). h-GT 1 grain size (minimum)

Conclusion

The present investigation throws light in the distribution of starch metabolite in the secondary phloem of *H.brasiliensis.* Starch grains were having round or oval shape. Their distribution was absent in the phloem tissues near the cambial zone. But moving towards the outer region of the bark tissue, the occurrence of starch grains gradually increased. Another most significant specialty was the copious presence of starch grains in the axial parenchyma cells compared to that of ray parenchyma. The distribution and amount of starch grains have great significance in relation to the functional role of plant metabolite in the bark tissue system.

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