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STAINING PROCEDURE FOR SIEVE TUBES IN THE BARK OF *HEVEA BRASILIENSIS* USING O-DIANISIDINE

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A specific stain for the identification of sieve tubes in the bark of *Hevea* particularly in transverse plane is lacking, hence a new staining procedure has been developed. Fresh sections of bark preserved in 4 per cent glutaraldehyde were treated with a mixture containing O-dianisidine 1mg mL^{-1} and 1 per cent hydrogen peroxide in 0.1 *M* phosphate buffer (pH 7), for about 0.5-1 minute. Observations and photomicrographs were taken by using Leica QWin V3 image analysis system attached to Leica DM 1000 microscope. Transverse sections of the soft bark when stained with O-dianisidine gave deep brown coloration for the cell walls of sieve tubes present in the inner soft bark, as well as the recently differentiated ones from the cambium. The cell wall of companion cells, axial and radial parenchyma, and laticifers remained unstained. The study revealed that the staining method using O-dianisidine is suitable for identifying sieve tubes in cross sectional plane of *Hevea* bark.

Keywords: Glutaraldehyde, O-dianisidine, Sieve tubes, Staining procedure

INTRODUCTION

Hevea brasiliensis, the prime source of natural rubber, is exploited commercially by severing the latex vessels present in the bark of the tree trunk (Dijkman, 1951). A number of anatomical and histochemical studies on *Hevea* bark have been carried out earlier using various stains *viz*., Sudan IV (Premakumari *et al*., 1996) and Oil red O (Omman and Reghu, 2003) for laticifers, Tannic acid-ferric chloride - lacmoid (Cheadle *et al*., 1953; Pramod *et al*., 2011) and Aniline blue (Johansen, 1940) for definitive callose, mercuric bromophenol (Mazia *et al*., 1953; Pramod *et al*., 2008) for P-protein, phloroglucinol-HCl (Jensen, 1962; Thomas *et al*., 1995) for lignin, silver nitrite (Johansen, 1940; Thomas *et al*., 2002) for crystals and amido black 10B (Weine, 1957; Thomas *et al*., 2010) for protein storing cells and Iodine-Potassium iodide (Johansen, 1940; Hebant and Fay, 1980; Thomas *et al*., 2002) for starch. Some of the above methods are commonly used for bark characteristic studies in the evaluation process of the newly developed clones of *H. brasiliensis* (Premakumari *et al*., 1996; Pramod *et al*., 2008; Thomas *et al*., 2010).

Sieve tubes of phloem tissues in the soft bark are functionally active for the downward translocation of photo-assimilates for the biosynthesis of latex in the laticiferous system. The survey of

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literature revealed that considerable attention has not been given so far to develop a simple and specific staining method for the identification of sieve tubes in the bark of *Hevea*. The present paper deals with the development of a new staining procedure using O-dianisidine for sieve tubes in the soft bark of *H. brasiliensis*.

MATERIALS AND METHODS

Bark samples of ten mature trees of *H. brasiliensis* (clone RRII 105, 20 year old) were collected from the experimental farm of Rubber Research Institute of India, Kottayam, India during the second week of December 2010 (before defoliation) for the present study.

Fresh bark samples were sectioned in transverse plane at 20-30 µm thickness using Leica SM2000R sledge microtome and fixed in 4 per cent glutaraldehyde prepared in 0.1 *M* phosphate buffer at pH 7.2 at 4 °C refrigeration (Roland, 1978). The procedure for localization of peroxidase enzyme using O-dianisidine (Worthington Enzyme Manual, 1993) was modified as given in Table 1 by increasing the concentration of O-. Sections of the bark were treated with a mixture containing O-dianisidine 1 mg mL^{-1} (Sigma-Aldrich) and 1 per cent hydrogen peroxide in 0.1 *M* phosphate buffer (pH 7), for about 0.5-1 minute.

After treatment, the sections were rinsed 2-3 times with buffer and prepared the micro slides. Sections were also stained with Oil Red-O for laticifers (Omman and Reghu, 2003), Aniline blue (Johansen, 1940) and Tannic acid-Ferric chloride-Lacmoid (Pancean and Parker 1965; Pramod *et al*., 2011) for sieve tubes. Observations and photomicrographs were taken using Leica QWin V3 image analysis system attached to Leica DM 1000 microscope.

RESULTS AND DISCUSSION

For the brevity of description of the results, the bark of *H. brasiliensis* has been demarcated into inner soft and outer hard bark regions based on the structural organisation. The metabolically active tissues including sieve tubes, laticifers, *etc*., are mostly confined to the inner soft bark.

Transverse sections of the soft bark when treated with O-dianisidine, gave a deep brown coloration for the cell walls of sieve tubes (Figs. 1-3) in the inner soft bark, including the recently differentiated ones from the derivatives of cambium (Fig. 1). The cell wall of companion cells attached to the sieve tubes, axial and radial parenchyma and laticifers did not show any stainability. Whereas, the latex present in the laticifers also stained black (Fig. 3) with O-dianisidine. When the subsequent sections of the same bark were stained with

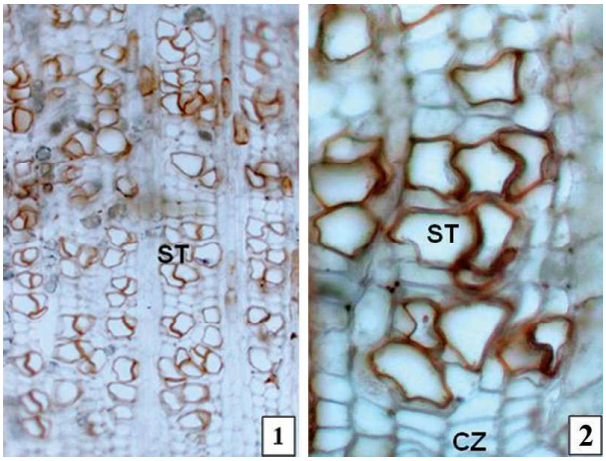
Table 1. **Table showing procedure modification for staining sieve tubes**

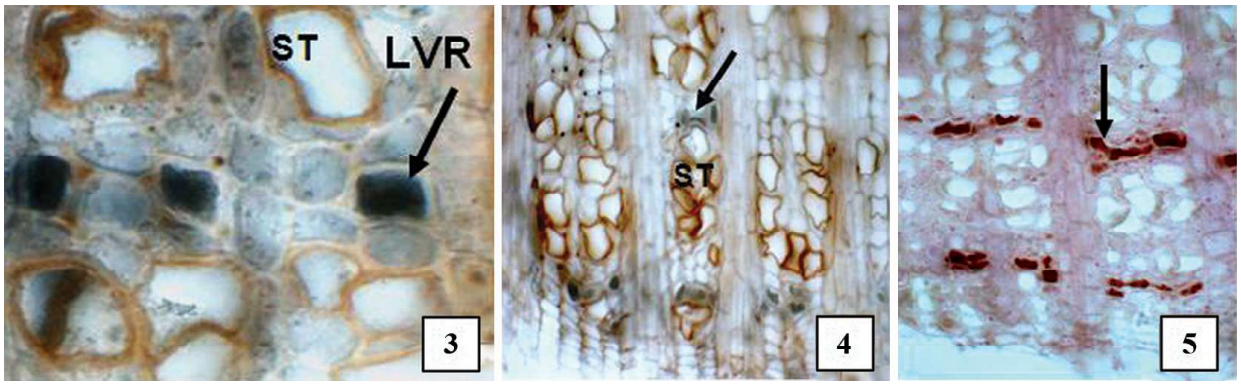
	Procedure (Worthington Enzyme Manual, 1993)	Modified procedure for staining sieve tubes
Concentration of O-dianisidine	0.63*mM*	1mg mL^{-1}
Concentration of hydrogen peroxide	30%	1%
Buffer used	0.2M potassium phosphate buffer pH 7.0	0.1M sodium phosphate buffer pH 7.0
Staining Time	4-5minutes	30 seconds – 1minute

Oil red O the laticifers alone stained deep red and the sieve tubes remained unstained. This result indicated that the tissues stained with O-dianisidine were only sieve tubes (Figs. 4 and 5).

O-dianisidine, also known as 3, 3'-dimethoxybenzidine ($C_{14}H_{16}N_2O_2$), is a colorimetric substrate used in ELISA procedures (Worthington Enzyme Manual, 1993), and for localizing or estimating peroxidase activity in plant tissue (Shannon *et al.*, 1966). In the presence of hydrogen peroxide, O-dianisidine gets oxidised and develop brown to purple colouration to the plant tissue (Stahord and Bravinder-bree 1972). As per the concentration reported in the Worthington Enzyme Manual (1993) for the localization of peroxidase activity in plant tissue, the sieve tubes in *Hevea* was unable to distinguish from the rest of the tissues in the bark due to the lack of proper stainability with the modified procedure, the cell walls of the sieve tubes stained deep reddish brown irrespective of the season (Gopal and Thomas, 2012) whereas the cell walls of the remaining tissues in the bark were unstained. In addition to cell walls of the sieve tubes, the cytoplasm of phloic rays also stained feebly in reddish brown.

A number of histochemical stains have been used to identify sieve tubes in the tangential longitudinal plane. The tangential longitudinal sections stained with Aniline blue or Tannic acid-ferric chloride-Lacmoid and observed under ultra-violet illumination gave fluorescence of callose deposited on the sieve plate. Parker (1965) also reported the stainability of sieve plate and non stainability by using the stain Pancean S. None of these stains are found to be specific for sieve tubes,





Figs. Transverse section of the bark stained with O-dianisidine for sieve tubes.

1. Sieve tubes in the soft bark stained brown keeping the surrounding tissues unstained X 85.
2. Recently differentiated sieve tubes (ST) occurring adjacent to cambial zone (CZ) X 320.
3. Latex in the laticifers stained black with O-dianisidine X 85
4 & 5. For tissue specificity, subsequent sections of the same bark were stained with O-dianisidine for sieve tubes (Fig. 4, at arrow) and Oil red O for laticifers (Fig. 5, at arrow).

particularly in transverse plane as the plane of cutting always need not expose sieve plates.

As the sieve tubes have significant correlation with rubber yield (Gunnery, 1935; Fernando and Tambiah, 1970; Anisio *et al*., 1998), it is considered as an anatomical parameter for the evaluation of *Hevea* clones. The present staining procedure enables easy identification and quantification of sieve tubes in transverse plane of the bark through which, parameters such as diameter, density and grouping pattern of sieve tubes in *Hevea* can be studied.

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