Pak. J. Bot., 46(3): 1039-1045, 2014.

TYLOSOIDS IN LATIFEROUS CANALS OF *TOXICODENDRON VERNICIFLUUM*

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Abstract

This study aims to investigate the ontogeny, anatomy and ultrastructure of the tylosoids in the laticiferous canals of *T. vernicifluum* (lacquer tree), samples were processed for light microscopy and TEM. Results showed that the tylosoids in the lumina of laticiferous canals originated from epithelia through periclinal division. Laticiferous canal invasions varied seasonally, at the beginning of traditional tapping season of raw lacquer, the quinone conglomerations dissolved, laticiferous canal function was renewed and epithelia secreted raw lacquer; at the end of the traditional raw lacquer-tapping season, tylosoids formed and blocked the laticiferous canals by quinone conglomerations until the next raw lacquer-tapping season. The irreversible invasion was mainly distributed in the nonfunctional phloem region near the periderm. These results suggested that the seasonal changes of tylosoids might provide insights into the defense mechanism of laticiferous canals against the invasion of other substances when cavity pressure decreases at the end of the tapping season of raw lacquer.

Introduction

Toxicodendron vernicifluum (Stokes) F. A. Barkley is mainly distributed in East and Southeast Asia and commonly called lacquer tree in China (Webb 2000; ZeLong *et al*., 2009). Raw (oriental) lacquer is the sap obtained by tapping lacquer trees, it has long been used as a coating and painting material because of its excellent waterproofing and antioxidative abilities and corrosion resistance (Niimura *et al*., 1996; Webb, 2000). Raw lacquer is secreted by epithelia and stored in the lumina of laticiferous canals (Zhao & Hu, 1985). In the secondary xylem, both the axial and ray parenchymal cells located next to the vessels may form outgrowths called tyloses inside the pit cavities (Evert & Esau, 2006), tylose formation is often induced by plant pathogens and may serve as a defense mechanism against the spread of pathogens via the vessels (Bonsen & Kucera, 1990); it can also be a response mechanism to frost (Cochard & Tyree, 1990) and wounds (Schmitt *et al*., 1997). Similar structures, called tylosoids (Evert & Esau, 2006), have been observed in the sieve tubes of many species, including *Vitis*, *Grewia tiliaefolia*, *Smilax rotundifolia* (Esau, 1965; Ervin & Evert, 1967; Deshpande & Rajendrababu, 1985), and some other tylose-like structures which originated from epithelia were observed in resin canals of pinus species (Kuroda, 2008). Laticiferous canals are the raw lacquer production and storage structures in *T. vernicifluum,* therefore, the formation of tylosoids in laticiferous canals and their distribution in phloem are highly correlated with raw lacquer production. This study used phloem samples of *T. vernicifluum* to examine the development, secretion features, and seasonal changes of tylosoids and aims to provide basic information on its dynamics and relationship with raw lacquer production.

Materials and Methods

Study site and plant materials: The study was carried out at the central region of Shaanxi Province, which is the main raw lacquer-producing region in China. Stem bark samples were collected monthly from 5 individuals in 2010, which were planted in the Xi'an vivarium (34°2′ N, 108°9′ E, altitude 400 m). Vouchers were deposited in the Herbarium Northwest University under the number 30451.

Light microscopy: For general anatomical characterization, samples were directly fixed with formalin–acetic acid–alcohol fixative solution (90 mL of 70% ethanol, 5 mL of glacial acetic acid, and 5 mL of formalin) for 24 h at room temperature (25°C). The samples then were sequentially dehydrated in 70%, 85%, 95%, and 100% ethanol for 30 min each, vitrified with a gradient from 100% ethanol to 100% xylene, and finally, infiltrated with and embedded in paraffin. The sections were obtained using a microtome (Leica RM 2135) and mounted on microscopic slides. The mounted sections were double-stained with Safranin O and Fast Green FeF, the stained tissues were examined using a light microscope (Nikon 50i) and digitally recorded equipped with a video camera (DS-Fi1).

Unit areas on the sections were chosen for the quantitative analysis of tylosoids and invaded proportions of laticiferous canals. All sections were examined according to the following: laticiferous canals with or without tylosoids, proportion of laticiferous canals completely or partially occluded in different seasons, and seasonal variations of laticiferous canals invaded with diverse distances from the cambium. The data were analyzed using Origin Pro 7.5.

Transmission electron microscopy (TEM): Similar samples for analysis of the ultrastructure of phloem and tylosoid development were fixed in 0.1 mol L^{-1} phosphate buffer (pH 7.0) containing 2% glutaraldehyde for 24 h (4°C). The specimens then postfixed in 1% osmium tetroxide for 4 h at 4°C, dehydrated through an acetone series, and embedded in Epon 812. Semi-thin sections (1μm to 2μm) were cut using Reichert–Jung ultramicrotome and stained with methylene blue, the stained sections were observed under light microscopy. Ultrathin sections were obtained using a Leica EM UC6 ultramicrotome, stained with uranyl acetate (Gibbons & Grimstone, 1960) and lead citrate (Reynolds, 1963), and then observed under a Hitachi H-7650 transmission electron microscope.

Histochemical tests of raw lacquer: For histochemical assays, freshly collected bark specimens (5 mm^3) were embedded in a tissue freezing medium and cut into sections (25μm) using a freezing microtome (Leica CM 1850). The sections were observed under the microscope (Nikon 50i) with one drop of ferric chloride–ethanol solution to detect

urushiol in natural states. For comparison, a parallel study was conducted, where ethanol was used to dissolve the urushiol in the tissue and then the same process were carried out.

Fourier transform infrared (FTIR) microspectroscopy tests: The red components, which were secreted into the invaded laticiferous canals, were detected with FTIR microspectroscopy. Freshly bark specimens were cut using razor blades and dried at 50°C. The sections with invaded laticiferous canals were detected using FTIR microspectroscopy (Nicolet 5700).

Results

Microscopic structures of *T. vernicifluum* phloem and laticiferous canals: The microscopic structure of lacquer tree bark was composed of the cambium, secondary phloem and periderm. The secondary phloem was further divided into functional and nonfunctional phloem, functional phloem was connected to the cambium inside (Fig. 1a), whereas the nonfunctional phloem was located on the outer side of the functional phloem (Fig. 1b). The entire secondary phloem was composed of sieve tubes, companion cells, parenchymal cells, stone cell clusters, laticiferous canals, and phloem rays (Fig. 1c). The sieve tubes and companion and parenchymal cells were dispersed as strips, in which the components were arranged alternately, this arrangement was typical in the functional phloem (Fig. 1a). In the non-functional phloem, sieve tubes were crushed by parenchyma cells, where the strip-arrangement in the non-functional phloem was atypical (Fig. 1b). Laticiferous canals were distributed between the phloem rays in a radial direction. The mature laticiferous canals were mainly distributed in the nonfunctional phloem and the stone cells clusters only existed in the nonfunctional phloem (Fig. 1c).

The *T. vernicifluum* laticiferous canals were produced by the vascular cambium in the functional phloem through schizogenesis. In the first phase, the laticiferous canals were assembled in a rosette shape by the initial secretory cells (Fig. 1d). Then, the intercellular layer dissolved (Fig. 1e), and formed a narrow lumen (Fig. 1f), which then extended into an oval or round shape in the transverse section. The mature lumina were surrounded by a layer of secretory cells, which also had two to three layers of surrounding sheath cells (Fig. 1g).

Development of *T. vernicifluum* laticiferous canals tylosoids: Tylosoid development includes three stages: epithelial proliferation stage, laticiferous canal blockage stage, and tylosoid disaggregation stage. In the epithelial proliferation stage, laticiferous canal epithelia formed tylosoids through periclinal division, the daughter cells had a similar shape to that of the epithelia (Fig. 1h). Tylosoid formation may occur in two ways. In some cases, epithelia from all sides of a lumen may uniformly increase and gradually block the laticiferous canal (Fig. 1i). In other cases, epithelia may increase from one side and block the laticiferous canal (Fig. 1j). According to our results, the latter model best described the tylosoid development.

In the next stage, the tylosoids expanded and squeezed each other within the limited space of the laticiferous canal lumina, hence forming an irregular shape; the expanded tylosoids also squeezed and compressed the sheath cells (Fig. 1k). In the longitudinal section, the laticiferous canals were full of tylosoids, and the sheath cells were compressed and flattened because of the expanded tylosoid cells (Fig. 1l).

During the tylosoid development, the lumen was partially or completely blocked by a bubble-like substance secreted from epithelia in the intercellular space between tylosoids (Fig. 2a). The histochemical (Feigl reaction) test indicated that those bubble-like secretions were quinones (Fig. 2b), and this result was proved by the FTIR microspectroscopy analysis.

During the disaggregation stage, the tylosoids gradually shrank and the nuclei vanished. Eventually, the cell walls disaggregated(Fig. 2c), and cell debris accumulated in the laticiferous canals, moreover, the quinone granules gathered and attached to the tylosoid fragments (Fig. 2d). The quinone granules further melted and aggregated, wrapped the cell debris, formed a compact red quinone conglomeration, and completely blocked the laticiferous canals (Fig. 2e).

Ultrastructure of the *tylosoids*: The mature secretory epithelial cells are characterised by very thin walls with remarkable spherical nucleus, abundant and electron-dense cytoplasm and small vacuoles. The cytoplasm of these cells contains polyribosomes, mitochondria smooth endoplasmic reticulum and plastids (Fig. 2f). In the epithelial proliferation stage of tylosoids development, laticiferous canal epithelia formed the tylosoids by periclinal division. The tylosoids are characterized by very thin walls with plasmodesmata, spherical nucleus, abundant and electron-dense cytoplasm, and small vacuoles (Fig. 2g). In the later tylosoid development stage, the vacuoles became numerous and could coalesce, giving rise to larger vacuoles. Electron-dense accumulations were observed to adhere to the inner surface of the tonoplast (Fig. 2h). Disintegration of the middle lamellae starts from the anticlinal walls and culminates with protoplast fragmentation, and the cell contents are released toward the lumen . Electron microscopy showed that quinone granules of different sizes appeared in the gaps between tylosoid cells during this stage (Fig. 2i).

Seasonal changes of *T. vernicifluum tylosoids*: The tylosoid invasion and quinone conglomerations were reversible, suggesting the seasonal changes of the tylosoids transfixed on the laticiferous canals. During summer, most of the laticiferous canals were unobstructed (Fig. 3b). During autumn, tylosoids formed and blocked the canals (Fig. 3c). Then, during winter and early spring, the tylosoids disaggregated into fragments and formed quinone conglomerations to block the canals. In fact, the laticiferous canals maintained a high proportion of invasion in the phloem (Figs. 3a and 3d). By the end of spring, most of the quinone conglomerations had dissolved. Hence, summer turned out to be the best tapping season with 69.6% yield. The invasion proportions of laticiferous canals in different seasons are shown in Table 1.

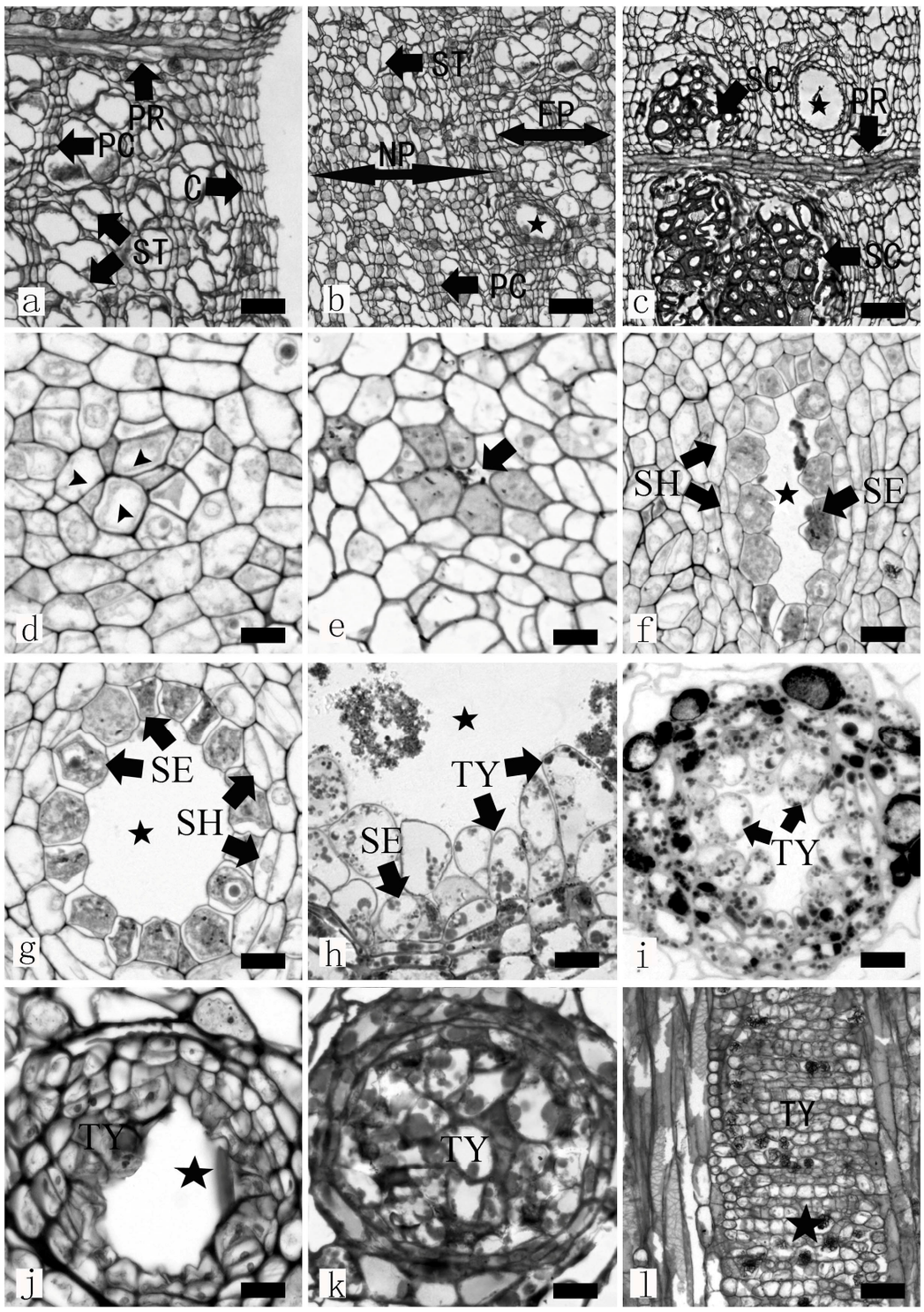

Fig. 1. a-c: Microscopic structure of *T. vernicifluum* phloem, d-l: and Development of laticiferous canals and tylosoids.
a. Transverse section showing sieve tubes and parenchymal cells dispersed as strips in functional phloem, bar=65 μm. b. Transverse section showing the boundary of the functional and nonfunctional phloem, bar=65 μm. c. Transverse section showing the stone cell clusters in the nonfunctional phloem , bar=65 μm. d. The precursor cells of the laticiferous canals, bar=25 μm. e. The precursor of lumen, bar=20 μm. f. Transverse section showing the new narrow laticiferous canals, bar=25 μm. g. Transverse s ection showing the mature laticiferous canals, the arrows indicate the locations of the epithelia, bar=25 μm. h. Epithelia formed the tylosoids via periclinal divisions, bar=15 μm. i. Transverse section showing the epithelia formed tylosoids from all sides of a lumen, bar=20 μm. j. Transverse section showing the epithelia formed tylosoids form one side of the lumen, bar=20 μm. k. Transverse section showing the tylosoids completely blocking the laticiferous canals, bar=25 μm. l. Longitudinal section showing the tylosoids completely blocking the laticiferous canals, bar=30 μm.

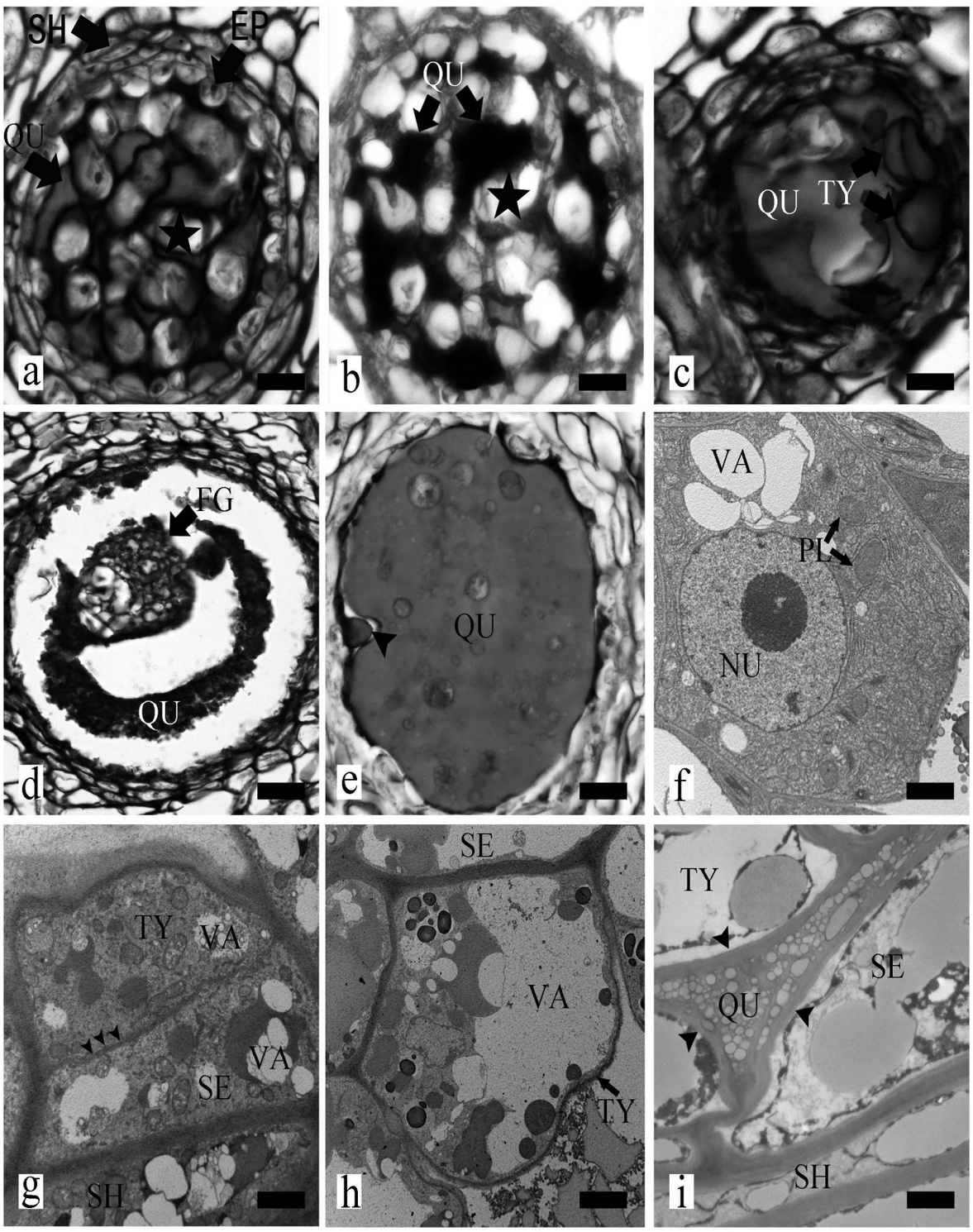

Fig. 2. Microscopic structure and histochemical tests of quinones graunules (a-e), Ultrastructure of epithclium and tylosoids (f-i).
a. Transverse section showing the quinone granules in the spaces between the tylosoid cells, bar=20 µm. b. Histochemical analysis results of quinone granules, bar=22 µm. c. Transverse section showing the cracked tylosoid cells, bar=20 µm. d Transverse section showing the quinone granules adsorbed on the tylosoid fragments, bar=30 µm; e. Transverse section showing the quinone conglomerations in irreversible laticiferous canals, new formed quinone vesicles attached on the conglomeration which formed in the previous year, bar=30 µm. f. Ultrastructure of the mature epithclium, bar=0.5 µm; g. tylosoids in the lumina of laticiferous canals originated from epithelia through periclinal division, arrow heads indicated the new formed cell wall, bar=0.35µm; h. Electron-dense accumulations were observed to adhere to the inner surface of the tonoplast, bar=0.23µm; i. Details of the quinone granules and cell walls of the tylosoids, bar=0.2µm.

Table 1. Invaded proportion of laticiferous canals in different seasons (%).

Season	Invaded proportion		Run-through
	Partly invaded	Completely invaded	
Spring	30.0 ± 1.73	65.0 ± 2.34	5.0 ± 0.28
Summer	17.9 ± 1.41	12.5 ± 0.30	69.6 ± 3.11
Autumn	36.1 ± 0.84	31.1 ± 0.47	32.8 ± 1.15
Winter	38.2 ± 2.71	36.8 ± 3.49	25.0 ± 1.73

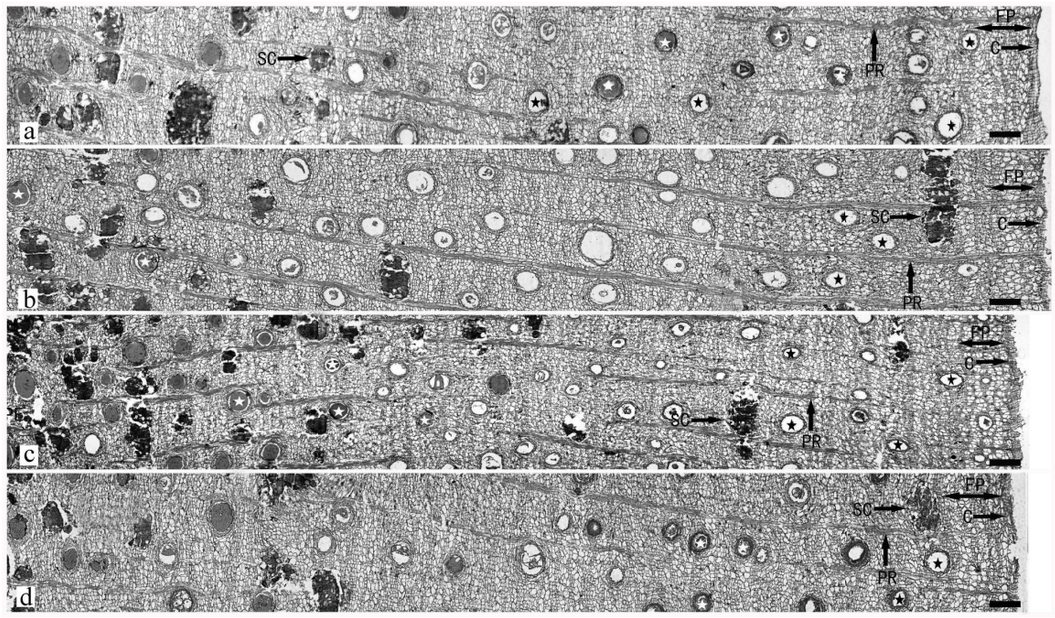

Fig. 3. Transverse sections of phloem during different seasons (a-d, bar=500 μm).
a. Transverse sections of phloem collected during spring showing a high proportion of laticiferous canal invasions. b. Transverse sections of phloem collected during summer showing the unobstructed laticiferous canals. c. Transverse sections of phloem collected during autumn showing the tylosoids blocking the laticiferous canals. d. Transverse sections of phloem collected during winter showing a high proportion of laticiferous canal invasions again.

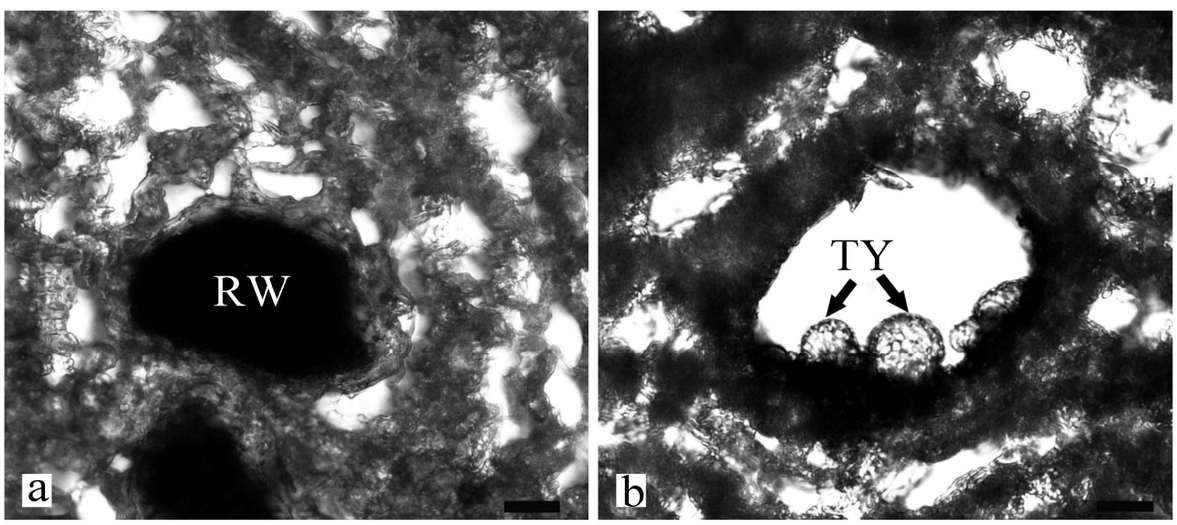

Fig. 4. Histochemical tests of raw lacquer and tylosoids.
a. Histochemical analysis results of raw lacquer, bar=35 μm. b. Histochemical analysis results of raw lacquer after the tylosoid formation, almost no raw lacquer was found, bar=30 μm.
The stars indicate the locations of the laticiferous canals. C: cambium; CW: cell wall; FG: fragments of tylosoids; FP: functional phloem; NP: nonfunctional phloem; NU: nucleus; PL: plastid;PR: phloem ray; QU: quinone coagulations; RW: raw lacquer; SC: stone cell; SE: secretory cell; SH: sheath cell; ST: sieve tube; TY: tylosoid; VA: vacuole.

The urushiol histochemical results suggested that the lumina of laticiferous canals were filled with raw lacquer during the traditional raw-lacquer-trapping season (June to September) (Fig. 4a). At the end of the trapping season, tylosoids were formed, and very little amounts or no raw lacquer was found in the lumina (Fig. 4b).

Tylosoid distribution in *T. vernicifluum phloem*: Tylosoids were mainly formed in the laticiferous canals of nonfunctional phloem. Most irreversible invasions of laticiferous canals are distributed in the nonfunctional phloem region near the periderm.

The quinone conglomerations within the 0 mm to 1.5 mm space from the periderm to the nonfunctional phloem were not completely dissolved, causing irreversible invasion to laticiferous canals. Histochemical analysis showed that this irreversible invasion led to the loss of storage functions during the tapping season, as the few secreted quinone vesicles continued to attach to the conglomerations formed in the previous years (Fig. 2e). The proportional and seasonal changes of the tylosoids in the laticiferous canals of the unit areas at different distances from the cambium are shown in Fig. 5.

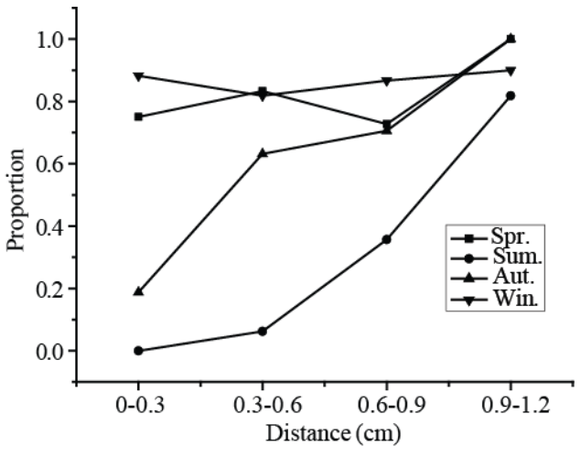

Fig. 5 Seasonal changes of laticiferous canals invaded in unit area of different distances from the cambium.

Discussion

Functions of–tylosoids: The seasonal variations in the secondary phloem of some forest trees in Nigeria showed that tylosoids formed on sieve tubes at particular time every year, and the formation of tylosoids was a result of the pressure drop in the sieve tube as the tree ages (Lawton & Lawton, 1971). Histochemical analysis in present study indicated that at the end of the tapping season, the lumina would contain little or almost no raw lacquer, which might cause the reduced pressure in the lumina. The seasonal changes of tylosoids may be taken as a mechanism for preventing other substances from filling the laticiferous canals when the lumina pressure drops. Hence, the irreversible blockage of the cavities is prevented. During spring, the quinone conglomerations in most laticiferous canals disappeared. This mechanism allowed the laticiferous canals to clear up and resynthesize raw lacquer for the tapping season in summer. The seasonal changes were defined as blockage and run-through in laticiferous canals. Tylose is a protuberant structure formed by the penetration of a parenchymal cell into a treachery cell, which causes the partial or complete blockage of the vessel lumen (Evert & Esau, 2006). The main function of tylose is to provide effective protection against fungi and bacteria (Stevenson *et al*., 2004). Aside from pathogens, wounds also induce tylose formation (Sun *et al*., 2006; Delvaux *et al*., 2010). A study on wound-induced vascular occlusions in *Vitis vinifera* (Vitaceae) found that pruning caused prodigious tylosis formation in vessels of grape stems (Sun *et al*., 2006, 2008). On the other hand, a tylosoid is an outgrowth of parenchymal cells into sieve elements or intercellular resin ducts (Evert & Esau, 2006). This study showed that tylosoids were formed only in nonfunctional phloem laticiferous canals and proliferated from epithelial cells to block the lumina. The development process was similar to the tylosoid formation in radial canals.

Previous studies showed that quinone has antibacterial effects (Daum *et al*., 2009) and can inhibit insect feeding (Ferkovich & Norris, 1972). This study showed that the quinone conglomerations were mainly located near the periderm, which is the first protective layer of plants. A few irreversible laticiferous canal invasions were observed outside the periderm. Hence, we believe that quinone conglomerations also have the same defensive functions against microorganism infection and animal feeding.

Varying secretions in T. vernicifluum laticiferous canals: Both *T. vernicifluum* laticiferous canals and *Hevea brasiliensis* laticifers exist in the phloem, the trapping methods of raw lacquer and rubber are also similar, tylosoids can also form in *H. brasiliensis* laticifers and gradually lignify, leading to the cessation of latex production (Nicole *et al*., 1991). A study on *H. brasiliensis* phloem showed that necrosis in rubber trees can be characterized by cell wall degradation, alteration of the middle lamella, vesiculation of the endomembrane, formation of tylosoids, and internal coagulation of rubber. Moreover, the tylosoids of rubber trees were observed in the trunk instead of roots. The laticifers contained coagulations, and the fusion of rubber particles led to the cessation of latex yield (De Faÿ & Jacob, 1989). A study on *H. brasiliensis* roots infected by 2 fungi, *Rigidoporus lignosus* and *Phellims noxiu*, also indicated latex coagulation inside the laticifers, which usually occurs at a very low rate in healthy rubber trees (Nicole *et al*., 1986).

The results of present study showed that laticiferous canal tylosoids could lead to the cessation of raw lacquer production. Histochemical analysis indicated the absence of urushiol in the lumina during this time. Moreover, the histochemical results showed that the cavity is filled with raw lacquer from June to September. However, at the end of the tapping season, the epithelium inside the lumina gradually stopped secreting raw lacquer, hence the small amount or even absence in the cavity. Previous studies indicated that latex coagulation could lead to the cessation of latex yield (Nicole *et al*., 1986; Nicole *et al*., 1991). Thus, quinone coagulations might play the same role in raw lacquer production.

The lacquer consists of urushiol derivatives (60%~70%), water (~20%), oil-soluble proteins, laccase, and gums (~10%) (Hatada *et al*., 1994). Moreover, urushiol derivatives are easily oxidized and aggregated to form stable quinones. The anatomical analysis of the phloem in this study showed that the irreversible invasion of laticiferous canals was mainly distributed in the nonfunctional phloem region near the periderm. However, a few irreversible invasions of laticiferous canals were observed outside the periderm, which were eventually detached from the said layer.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31300157).

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(Received for publication 7 October 2012)