Anatomical Studies
On Black stripe in Hevea Brasiliensis.

BY

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Abstract

In this paper preliminary observations have been made on the process of infection from the pathogens of black stripe and on the responses of Hevea brasiliensis. It was found that when the black stripe pathogen penetrates the bark, the parenchymous cells around the infected tissue gradually become brownish and finally form a dark brown layer which we call as "browning layer". The formation of the "browning layer", observed in the field, is the preculiar characteristic of the slowly spreading spot. At the same time it was also found that the hyphae exist only on the tissue surrounded by the "browning layer" and not on the "browning layer". It is thus suggested that the "browning layer" can prevent the infection from further spread.

We have already proved that the use of ethylene has higher resistance effect on the bark of Hevea brasiliensis tree towards black stripe[2,4]. This has opened a new path to control black stripe. For development of technical methodology and excellent use of ethylene to control black stripe, it is essential to find clear mechanisms of the use of ethylene to increase the resistance of the bark of Hevea brasiliensis, of course, under normal conditions (i.e. without the use of ethylene), and it is the basis of the study of the resistance of Hevea Brasilensis towards black stripe. Till today people's understanding of the resistance mechanism of Hevea brasilensis is very small[9,10] moreover analytical studies of black strips have still not been reported.

Therefore, starting from the test diagram on the process of infection of black stripe pathogen on Hevea brasiliensis and the anatomical observations of the reaction of the Hevea brasiliensis toward the infection, we investigate the problem of resistance of Hevea brasiliensis.

Materials and Methods

Hevea brasiliensis: The experimental tree is the tree grown in this Institute's experimental farm and clone PB.86

Pathogen: The pathogen used in this experiment is the black stripe (Nada) No.1 (Phytophthora sp.) separated from the diseased Hevea brasiliensis grown in our Institute.

Inoculation: The application of Black stripe material is achieved by artificial inoculation method. Secant inoculation method has been taken from reference (2). Secant inoculation at higher outer portion has been taken from reference (4). Culture is mycelium or zoospores and preparation method is from reference (2).

Method of observing the zoospores entering the section of the bark: By using punching machine take out a piece from the bark and on the microtome cutout sections with thickness of 90 microns. The width of the section is about 0.5 centimeter. Plac it immediately on a glass slide and add 0.1 ml of liquid containing zoospores. Wait for sometime (normally 1-2 hours). The zoospores multiply on the section of the bark, moreover start germinating. At this time add cotton blue, lactic acid phenol to stain and put the cover slide. Observe it under the microscope the condition of the zoospores germinating and penetrating the section of the bark.

Paraffin section: Fixative is formaline glacial acetic acid -50% alcohol (6.2:2.5:50). At this time the infected tissue turns brown (Browning) and it may retain. Stain the section using cotton blue lactic acid phenol or Copperes Halmatoxyline.

Determination of viability (vitality) of Cells: Using 2,3,5-triphenyl tetrazolium chloride reduction reaction (3) determine the viability of infected cells. In the normal living cells one can see quite a few stained bright red mitochondria but in dead or nearly dead cells these are not seen or are very few.

Results

1. Process of pathogen infection:

Germination of zoospores and multiplication on the tree bark: Using the method of bark section, observed the condition of the black stripe zoospores entering the bark tissue. We know that zoospores of black stripe enter through the wound opening in the bark(7), therefore using freshly cut section and inoculating with zoospores one can very easily see how zoospores enter the bark tissue. Soon after the inoculation of the bark section by zoospores, the zoospores immediately stop activity and become round. In 1-2 hours, at 28-32^{o}C temperature spores germinate and form germ tubes (Plate 1.1). The gern tubes of all the spores near the section of the bark grow facing towards the tissue of the bark. After 3-4 hours germ tubes grow 0.15-0.25 mm long and at the same time the whole protoplasm in the spores moves to the germ tubes. The germ tube directly penetrates the outer surface of the injured cells or penetrates the gaps between the cells.

The zoospores have clearly different response to the bark tissue at different positions. This shows that zoospores multiply in large numbers on the sieve tube while multiplication of zoospores on the outer surface of the bark is clearly comparatively less. (Plate 1.).

Growth of hyphae in the tissue of the bark:

The growth of hyphae of the pathogen in different positions and tissues of the bark has its own peculiarities.

In the granular bark and yellow bark(5), hyphae clearly develop in between the cells (Plate 1,2-5), only under very few conditions one can see hyphae penetrating the cells. Sometimes one can see hyphae in the cells forming various forms of sucking organs. (Plate 1,3). At the time of infection hyphae in the rays are clearly more as compared to the hyphae in the parenchymous cells in between the rays. At the same time, on the horizontal section of the bark, one can easily see hyphae developing along the rays from outer surface of the bark directly towards inside. (Plate II,1). Therefore we see that rays are comparatively suitable for the hyphae growth. Observations prove that at the begning of the infection, hyphae develop just along the rays swiftly towards inside the bark.

In the water pocketed bark, hyphae growth is very good. Moreover development is comparatively fast. We know that the main structural part of the water pocketed bark(although has conductiv bast) is sieve tube(5). In this the hyphae penetrate the sieve tube: In the sieve tube they form branches (Plate 1,6) or form many sucking organs (Plate 1,7). moreover can penetrate the sieve plate and grow (Plate 1.8). Comparing the plate 1,6-8 with 2, one can see that these hyphae are much more as compared to the

hyphae of the infection, at the begning, in the outside layer of the bark. The development of hyphae in the water pocketed bark is also clearly faster than in the outer layer of the bark. After 3-4 days of inoculation of the bark one can see the formation of a big inside and small outside infection spot. Hyphae in the sieve tube spread fast and the growth is also faster as compared to the lateral spread. Therefore this forms a characteristic clear long shuttle shaped infection spot at the inner layer of the bark. These facts prove that the sieve tube layer is particularly helpful in the growth of hyphae.

Hyphae also pierce through the formed layer and enter the young xylem.

2. Response of the bark towards infection:

Death of the infected cells:

After the black stripe pathogen enters the tissue of the bark, the cells slowly die at the location of hyphae growth. When 2,3,5 triphenyl tetrazolium chloride reagent is added, one can not see the stained red coloured mitochondria in the cells having infection. Following this the protoplast of these cells dissociates and therefore, become light coloured. (Plate II,2). At the time of the begning of the formation of a lump of the infection spot, the infected cells of the bark are already dead. (Plate II,1,2). With this, at the same time, the hyphae continuously spread and surround the living cells. These cells similarly go on dying. As a result the infection spot grows bigger uninteruptedly.

Formation of browning layer:

At the location of hyphae spread the cells of the bark die at the same time. The parenchymous tissue cells near these cells

slowly turn brown (Browning). At the time of hyphae begning to pen trate the bark tissue, the cells that receive infections immediate ly start forming a few brownish cells. (Plate II,1). But following the growth of hyphae, these originally brownish cells die at the locations of hyphae spread and moreover loose the brownish colour. At the same time new brownish cells formation takes place nearby. Following this the spot increases. As the region of disease spot widens more and more all around, more and more dense brownish cells are formed. Also the colour of the brownish cells changes from light brown to dark brown. Finally these brownish cel form a dark brown layer visible to the naked eyes, around the spot and we call this as the "Browning layer". (Plate II,2;Plate III,2,3) and precisely this layer is commonly called as "Chronic development of disease spot". The browning layer is commonly 0.5-1.5 mm wide. Almost all parenchymous cells in this region turn brownish. Cells of the browning layer are live cells and have a large central vacoule, the inner contents have tennin material, nuclei are distributed on the periphery and have disintegrated energy. (Plate III, 4.D).

We have, particularly, paid attention to the relation of th growth of hyphae with the browning of the cells. At the time when browning layer is still not formed, although one can see hyphae, sometime, penetrating on the side of the brownish cells (Plate 1,4) but one can not see the hyphae forming the sucking organs in the brownish cells or piercing the brownish cells. After the brownish layer is formed, one cannot find the growth of hyphae in this. That is to say the hyphae confine their growth in the cells surrounding the browning layer.

Healing of the disease spot:

In about 10-15 days of the formation of the browning layer of the disease spot, the cells in the browning layer (mainly rays of parenchymous cells) restore partial activity (Plate III,4), restore injured tissue, and afterwards form new periderm. The diseased dead tissue separates from the healthy tissue and thus the injured surface of the bark is recovered. Afterwards the whole wound is also gradually covered by newly formed bark. This is, at no time, the result of the growth of the recovered tissue produced by the injured layer. This condition and the recovery of the injured bark (6) are similar.

Discussion:

According to the preliminary investigations towards the process of infection by black stripe and the response of the bark towards the pathogen it was found that, the formation of the browning layer is of direct significance, particularly when considering the resistance of Hevea brasiliensis. The two experiments already described are very important: (1). After the formation of the browning layer the growth of hyphae is only restricted to the tissue surrounding the browning layer: (2) The formation of the browning layer is characteristic of the chronic development of ths disease as observed in the field. According to these experiment we assume that after Hevea brasiliensis receives infection the formation of the browning layer can play a role in the retardation of the effect of continued spreading of the infection.

The browning of the tissue in the diseased plants is a general phenomenon. Its main result is the accumulation of the phenolic compounds (8). We already know that phenolic compounds play a role in plant resistance and afterwards again discovered the relationship between metabolism of phenolic compounds with

plant protection (II). In case of Black stripe of Hevea brasilie sis some people also pointout the relationship between phenolic compounds and disease resistance[9,10] and moreover point out tha black stripe pathogen in the tissue of Hevea brasiliensis (fruit or tree bark) can possibly lead to the formation of plant protective material (9). These data are in consonance with the hypothesis that browning layer plays a role in the resistance of the Hevea brassiliensis towards the Black stripe pathogen.

Another important point with the relation of Hevea brasiliensis resistance towards Black stripe is that sieve tube has special advantage for the growth of mycellium. This agrees with the facts investigated in the field that the inner layer of the bark is easily infected[1]. This is the basis of suitably reducing the cutting depths for latex which has definite protective effect[1].

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Foot-Notes

p.71

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*Tan Haiyan, Yun Cuiying and Tang Wenying also participated in this study.

p.73 Fig.1

1. Zoospores. 2. Coarse skin 3. Emergy skin. 4 Yellow skin.

5. Water pocketed skin.

6. Fig.1 Diagram showing the condition of multiplying zoospores of black stripe pathogen on the edge of the tree bark.

7. Using section method observed a number of multiplying zoospores at different locations of the bark tissue. (Average numbers taken from 10 sections of the bark).

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巴西橡胶树条溃疡病的解剖学研究*

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摘　要

本文对条溃疡菌侵染橡胶树皮的过程和橡胶树皮的反应作了初步观察。当条溃疡菌侵入树皮后，受侵染组织周围的薄壁组织细胞逐渐变成褐色，最后形成一个深褐色层，我们称为褐变层。褐变层正是田间观察到的慢性扩展型病斑的特征，同时发现菌丝仅存在于褐变层包围的组织中，而在褐变层中没有菌丝生长。褐变层可能起阻止侵染进一步扩展的作用。

我们已经证明，施用乙烯利有提高巴西橡胶树皮对条溃疡病的抗性的作用[2,4]，这为防治条溃疡病开辟了一个新途径。为了发展和完善使用乙烯利防治条溃疡病的技术措施，有必要弄清楚施用乙烯利提高橡胶树皮抗病性的机理。这种乙烯利作用机理的探讨，当然应以正常情况下（不施用乙烯利）橡胶树对条溃疡病的抗性的研究为基础。迄今为止，人们对橡胶树抗条溃疡病的机理了解甚少[3,5]，而对条溃疡病的解剖学研究还未见报导。为此我们试图从条溃疡病侵染橡胶树的过程和橡胶树对侵染的反应的解剖学观察入手，来探讨橡胶树的抗病性问题。

材 料 和 方 法

橡胶树：实验树是本院实验农场的实生树和无性系PB86。

病原菌：本实验使用的病原菌是我院从感病橡胶树上分离出来的条溃疡菌那大1号（Phytophthora sp.）。

接种：条溃疡病材料用人工接种方法获得。割线接种方法按文献〔2〕，割线外高部位接种按文献〔4〕。菌种用菌丝体或游动孢子，制备方法按文献〔2〕。

观察游动孢子侵入树皮的切片方法：用打孔器从树皮上取下树皮块，在切片机上切成厚90微米的切片，切片宽约0.5厘米。立即将树皮切片置于载玻片上，加含有游动孢子的溶液0.1毫升。经一定时间后（通常1～2小时），游动孢子已在树皮切片上定殖，并开始萌发，

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*参加本项试验工作的还有谭海燕、云翠英和唐文英同志。

这时加棉兰乳酸酚染色，并加盖玻片在显微镜下观察孢子萌发和侵入树皮切片的情况。

石蜡切片：固定液用福尔马林一冰醋酸一50％乙醇（6.2:2.5:50），这时受侵染的组织变成褐色（褐变），可以保存下来。切片用棉兰乳酸酚或铁矾苏木精染色。

细胞生活力的测定：用2，3，5一氯化三苯基四氮唑盐还原反应[8]测定受侵染细胞的生活力。在正常生活的细胞中可以看到许多染成鲜红色的线粒体，而在死亡和濒于死亡的细胞中则看不到或很少看到它们。

结 果

一、病菌侵染过程

游动孢子的萌发和在树皮上的定殖：

用树皮切片的方法观察了条溃疡菌游动孢子侵入树皮组织的情况。我们知道，条溃疡菌的游动孢子是从树皮的伤口侵入的[7]，因此用新鲜树皮切片接种游动孢子，可以很容易看到游动孢子是怎样侵入树皮组织的。当树皮切片接种游动孢子后不久，游动孢子即停止活动，变为圆形。在28～32℃条件下经1～2小时，孢子萌发形成芽管（图版Ⅰ，1）。在树皮切片附近的所有的孢子形成芽管都朝向树皮组织生长。3～4小时后，芽管长达0.15～0.25毫米，同时孢子中全部原生质均进入芽管。芽管直接穿入切片表面受损伤的细胞或穿入细胞间隙。

游动孢子对不同部位的树皮组织的反应有明显差别，这表现在筛管层定殖的游动孢子数量多，而在树皮外层定殖的游动孢子显著地较少（图1）。

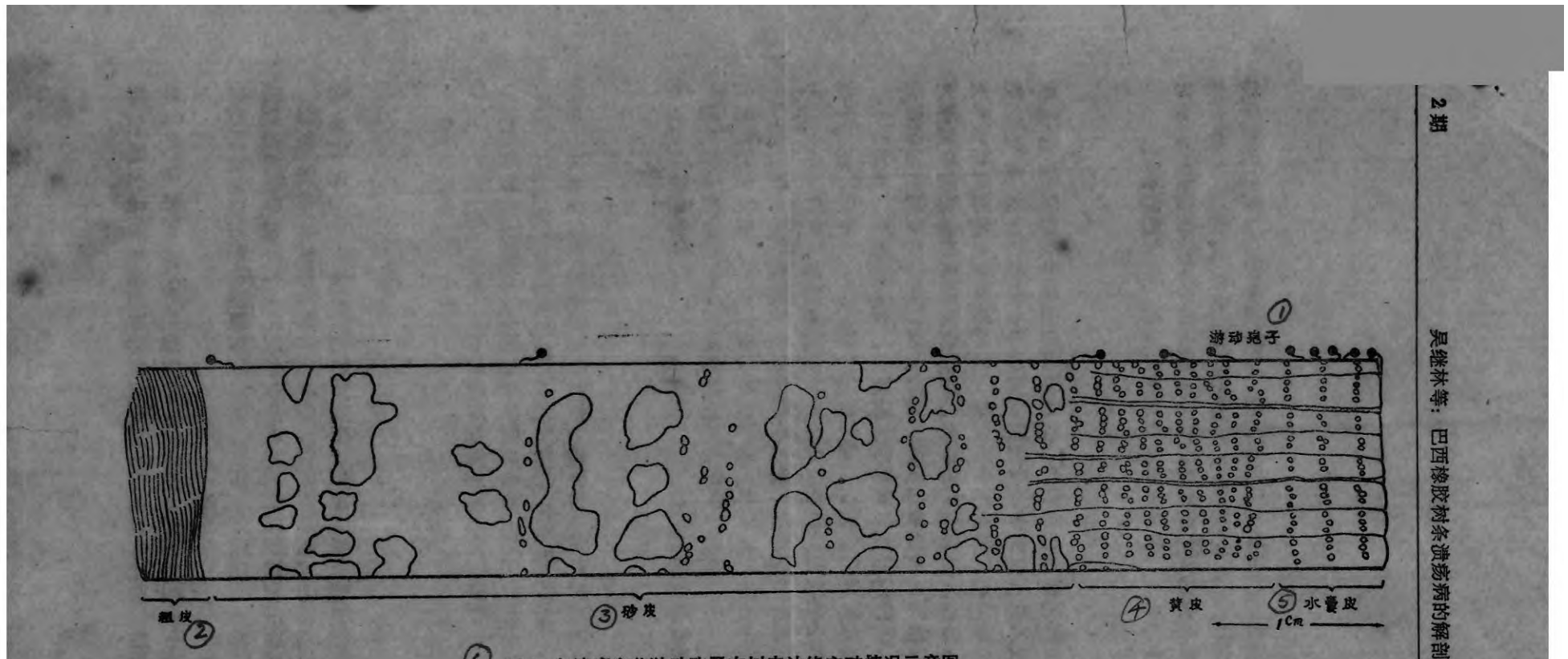
菌丝在橡胶树皮组织中的扩展：

条溃疡菌菌丝在橡胶树皮的不同部位和不同组织中扩展的情况都有它各自的特点。

在砂皮和黄皮[5]，菌丝明显地沿着细胞间隙伸展（图版Ⅰ，2—5），仅在极少数情况下看到菌丝穿过细胞。有时可以看到菌丝在细胞中形成各种形状的吸器（图版Ⅰ，3）。在侵染开始时，射线中的菌丝明显地比射线之间的薄壁组织细胞中的菌丝多，同时在树皮的横切片上很容易看到菌丝沿着射线从树皮外层一直向里伸展（图版Ⅱ，1）。由此看来，射线是比较适于菌丝生长的。观察表明，在侵染初期，菌丝正是沿着射线迅速向树皮里层扩展的。

在水囊皮，菌丝生长好，而且扩展较迅速。我们知道水囊皮（即有输导功能的韧皮部）的主要结构成分是筛管[6]。在这里菌丝侵入筛管中：它们在筛管里形成分枝（图版Ⅰ，6）或形成很大的吸器（图版Ⅰ，7），并能穿过筛板而扩展（图版Ⅰ，8）。比较图版Ⅰ，6—8与2，可以看到这里的菌丝比树皮外层初期侵染的菌丝粗壮得多。在水囊皮中菌丝的扩展也明显地比在树皮外层快，在树皮接种3～4天后，即可看到形成一个里大外小的病斑。菌丝在筛管中蔓延的速度也比横向蔓延的速度快，因而在树皮内层形成了一个特别明显的长梭形病斑。这些事实表明筛管层是特别有利于菌丝生长的。

菌丝也穿过形成层进入幼嫩木质部中。



⑥ 图1．条溃疡病菌游动孢子在树皮边缘定殖情况示意图

⑦ 用切片方法看到的在树皮组织的不同部位定殖的游动孢子数量（10个树皮切片的平均数）。树皮的分层参考文献[5]，各层宽度按实际比例，但孢子已放大。

二。橡胶树皮对侵染的反应

受侵染细胞的死亡：

当条溃疡菌侵入橡胶树皮组织后，在菌丝伸展所到之处，细胞逐渐死亡。死亡的细胞加2，3，5-氯化三苯基四唑盐试剂时，看不到细胞中有染成红色的线粒体。随后这些细胞的原生质体分解，因此在切片中成为浅色的（图版Ⅱ，2）。在开始形成块状病斑时，病斑的树皮细胞已经死亡（图版Ⅱ，1，2）。与此同时菌丝继续向周围生活的细胞伸展，这些细胞亦相继死亡，结果病斑不断扩大。

褐变层的形成：

在菌丝伸展所到之处，树皮细胞逐渐死亡的同时，这些细胞附近的薄壁组织细胞逐渐变成褐色（褐变）。在菌丝开始侵入树皮组织时，受侵染的细胞即开始形成少数褐变细胞（图版Ⅲ，1）。但随着菌丝的扩展，在菌丝伸展所到之处，这些原来褐变的细胞死亡并失去褐色，同时附近有新的褐变细胞形成。随着病斑的扩大，在病斑周围越来越变宽的范围内形成越来越密的褐变细胞，而褐变细胞也从浅褐色变成深褐色。最后这些褐变细胞在病斑周围形成一个肉眼可见的深褐色层，我们把它称为褐变层（图版Ⅱ，2；图版Ⅲ，2，3），而这时的病斑正是通常所称的“慢性扩展型病斑”。褐变层通常宽0.5～1.5毫米，在这个范围内的几乎所有的薄壁组织细胞都发生褐变。褐变层的细胞是生活的细胞，具有一个大的中央液泡，内含有单宁类物质，细胞核分布在周缘，并有分裂能力（图版Ⅲ，4．D）。

我们特别注意到组织的褐变与菌丝生长的关系。当褐变层还未形成时，虽然看到菌丝有时在褐变细胞旁边穿过（图版Ⅰ，4），但从未看到菌丝在褐变细胞中形成吸器或穿过褐变细胞。在褐变层形成以后，未发现其中有菌丝生长。即是说菌丝限于在褐变层包围的组织中生长。

病斑的愈合：

在病斑的褐变层形成约10～15天，褐变层中的细胞（主要是射线薄壁细胞）恢复分生活动（图版Ⅲ，4），形成愈伤组织，然后形成新的周皮，将感病死亡的组织与健康组织隔离，即树皮伤口的表面愈合，后来，整个伤口也逐渐被新形成的树皮覆盖；这是从未受损伤的形成层产生的愈伤组织生长的结果。这种情况与一般树皮伤口的愈合[6]是相同的。

讨 论

根据对条溃疡菌侵染橡胶树皮的过程和橡胶树皮对侵染的反应的初步研究，我们认为在橡胶树的抗性方面，特别值得注意的是褐变层的形成。已叙述的两个事实是很重要的：（1）在褐变层形成以后，菌丝仅限于在褐变层包围的组织中生长；（2）褐变层的形成正是田间观察到的慢性扩展型病斑的特征。根据这些事实，我们假定橡胶树受侵染后形成褐变层可能起阻止侵染进一步扩展的作用。

组织发生褐变在植物病害中是个普遍的现象，它主要是酚类物质积累的结果[8]。早已知道酚类物质在植物抗病中起作用，而后来又发现了酚类的代谢与植保素形成之间的联

系[11]。在橡胶树条溃疡病方面，也有人提出酚类物质与抗病性有关[9,10]，还提出条溃疡病菌在橡胶树组织中（是果实而不是树皮）也可能诱导形成植保素[9]。这些资料与褐变层在橡胶树对条溃疡病抗性中起作用的假设是一致的。

与橡胶树对条溃疡病的抗性有关的另一重要之点是筛管层特别有利于菌丝的生长。这与田间观察到的树皮里层易于感病的事实相符合[1]。这是割胶深度适当减浅具有一定防病作用[1]的根据。

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ANATOMICAL STUDIES ON BLACK STRIPE IN HEVEA BRASILIENSIS

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Abstract

A preliminary observation was made on the process of infection from the pathogen of black stripe and on the responses of Hevea brasiliensis. It was found that when the pathogen penetrates the bark, the parenchymous cells around the infected tissues gradually become brownish and finally form a dark brown layer called "browning layer". The formation of browning layer is considered the very characteristic of the slowly spreading spot observed in the field. It was also found that hyphae exist only on the tissues surrounded by the browning layer but not on the browning layer. It is therefore suggested that the browning layer may serve to prevent the infection from further spread.