Occurrence, Anatomy, and Morphology of "Blisters" on Cocoa Seedlings Inoculated with *Crinipellis perniciosa*

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ABSTRACT

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Cocoa seedlings inoculated with *Crinipellis perniciosa* exhibited blisterlike swellings and exudations in addition to the typical symptoms associated with witches'-broom disease. Studies using electron and light microscopy revealed that blisters were formed as a result of abnormal cortical growth induced by the pathogen. The rupturing of blisters resulted in desiccation, necrosis, and brown discoloration of the exposed cells. The exudation was not associated with the disintegration of host tissue and was probably due to the high internal pressure generated by hyperplasia and hypertrophy of infected tissues. Newly formed cells of the infected tissue did not form plasmodesmata in the cell walls.

During a study to devise a methodology for the artificial inoculation of cocoa seedlings for an evaluation of resistance to the witches'-broom pathogen (*Crinipellis perniciosa* (Stahel) Singer) infected seedlings exhibited two additional symptoms besides the characteristic syndrome of witches'-broom disease. One was the appearance of numerous small, lightly colored, blisterlike swellings on the green infected stem. These blisters later enlarged, ruptured, and became discolored. The other symptom was an exudation. Following Stahel's (9) success with seedling inoculation to establish the pathogenicity of *C. perniciosa*, several studies were conducted with seedlings as test material (1,3,5,6,8,10,11). The symptoms reported in these studies on inoculated cocoa seedlings were the characteristic hypertrophy and necrosis of infected tissues, etiolation of leaves, activation and proliferation of axillary buds, and production of broomlike abnormal growth. Evans and Bastos (5) recorded blistering and premature bark formation on the cocoa clones SCA 6 and SCA 12 following inoculation. However, they did not report on the morphology and anatomy of the blisters. The present study was undertaken to obtain information regarding the occurrence, morphology, and anatomy of these blisterlike swellings and the exudation observed on infected cocoa seedlings.

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MATERIALS AND METHODS

Test seedlings were raised from seeds of naturally formed pods of the witches'-broom-susceptible cocoa cultivars Catongo, ICS 43, and ICS 60. The seeds were sown in 400-ml plastic pots containing a potting medium composed of three parts garden topsoil and one part washed sand. Seedlings at the age of 3 wk were inoculated with basidiospores of *C. perniciosa*, which had been collected in petri dishes according to the method described by Evans (4). Initially the basidiospores were suspended in a small quantity of sterile distilled water, and the final dilution was prepared in 0.4% agar in water. The final concentration of basidiospores in the suspension was adjusted to 20,000 spores per milliliter after counting with a hemacytometer. Inoculation was accomplished by placing a drop of the basidiospore suspension on the terminal bud of the test seedling. Because of the viscosity of the agar-basidiospore suspension, the drop of inoculum on the terminal bud slowly seeped down the stem. The inoculated plants were kept in a humid chamber for 24 hr, removed, and placed on benches in the laboratory. Symptoms of witches'-broom disease appeared 8–14 days after inoculation. Some plants showing symptoms were placed in a humid chamber under ambient light and temperature. Control plants were subjected to the same treatment except that the suspension used was basidiospore-free.

Portions of stems of control and infected plants showing various stages of blister development were excised and fixed in a mixture of formaldehyde and acetic acid (1:3, v/v) for light microscopy. Stems were cut into lengths of about 5 mm and dehydrated through an ethanol and tertiary butanol series prior to being embedded in paraffin wax. Sections were cut at about 12 μm on an AO Spencer 820 rotary microtome and stained with Safranin and Fast Green (7).

For transmission electron microscopy, glutaraldehyde-fixed sections were washed in phosphate buffer, postfixed for 1 hr in buffered 2% osmium tetroxide, washed in distilled water, dehydrated in an ethanol series, and embedded in Epon 812. Sections cut at 800 Å with an LKB

Ultratome III were stained with uranyl acetate followed by lead citrate and viewed on a Philips 210 transmission electron microscope operated at 60 kV.

For scanning electron microscopy, fixed stem pieces were freeze-dried, coated with gold-palladium on a Technic-Vote machine, and viewed on a Cambridge Stereoscan SY-10. The electron microscopy was conducted at the University of Nebraska, Lincoln.

RESULTS

About 3 wk after inoculation of the primary stem numerous elongated blisters appeared and gradually increased in size (Fig. 1). Some of these swellings in close proximity to each other eventually coalesced. Subsequently, small cracks appeared on the distended epidermis of the blisters and gradually enlarged, exposing the underlying tissue, which soon became desiccated, necrotic, and discolored under ambient conditions (Fig. 2). However, when inoculated plants were kept at high humidity, the exposed tissue formed a calluslike outgrowth, which remained intact for a comparatively long period of time. The removal of plants from the moist environment resulted in desiccation followed by necrosis and discoloration of the cell mass, which subsequently transformed into brown lesions resembling pockmarks (Fig. 3).

Investigations into the histopathology of the blisters at different stages of development revealed hyperplasia and hypertrophy of cells of the subepidermal region in which blisters were formed (Figs. 4 and 5). These cells did not die until the blisters were ruptured (Fig. 6). In the region of the blisters hyphae were observed in intercellular spaces (Fig. 7). In transections of healthy seedling stems, numerous plasmodesmata were observed in cell walls (Fig. 8). However, in cell walls of hypertrophied tissues, plasmodesmata either were not present or occurred in very low frequency (Fig. 7).

On infected stems, in addition to blisters, small mucilaginous globules exuded on the surface at elevated areas not associated with blister formation (Fig. 9). These globules gradually enlarged and eventually solidified, forming a structure resembling a cabbage head and segmented by numerous deep grooves and fissures (Fig. 10). Histologically the structure was noncellular.

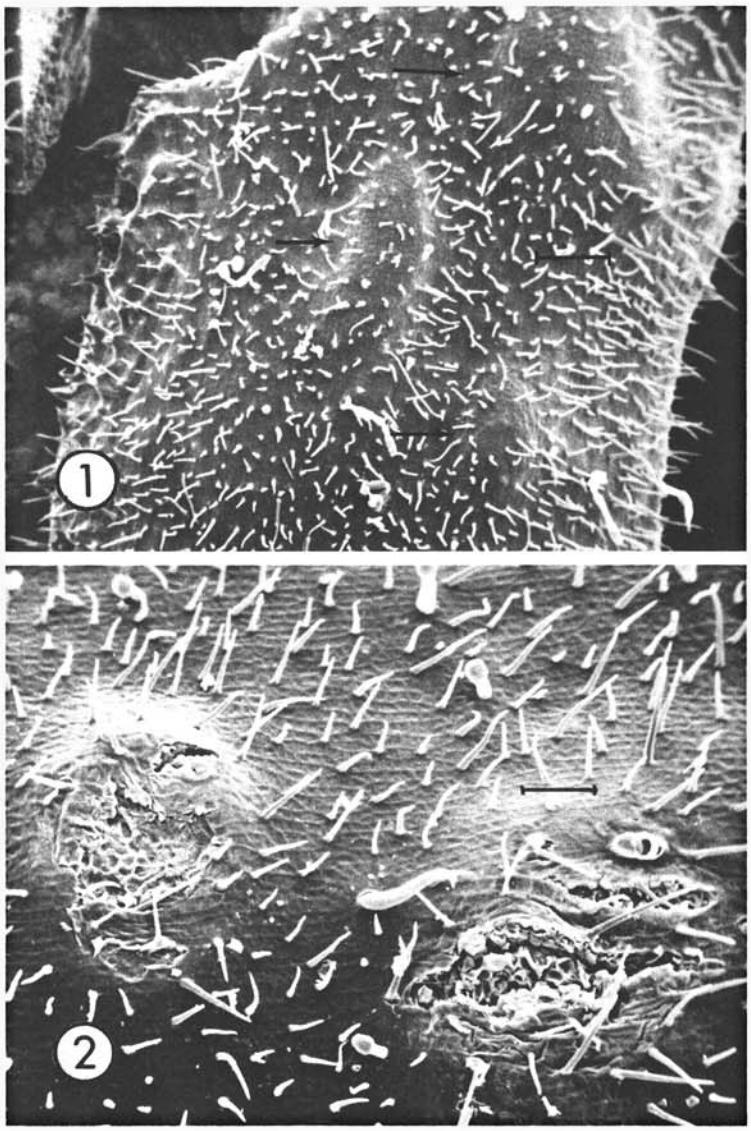
The control plants did not show symptoms of witches'-broom disease, blister formation, or exudation.

DISCUSSION

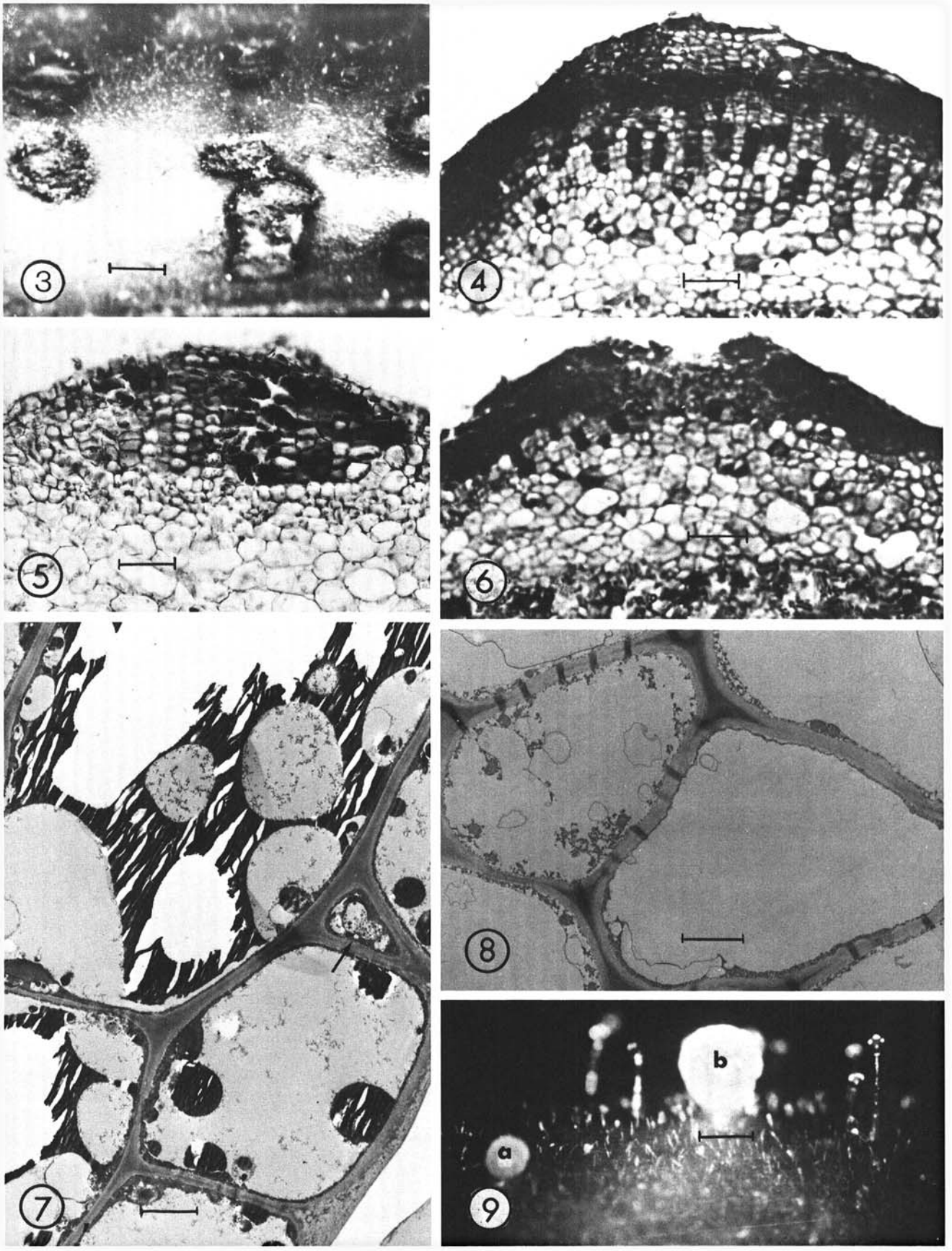
Hypertrophy and hyperplasia are symptomatic of the witches'-broom disease syndrome (8). Blisters observed on inoculated seedlings were formed as a result of infection and postinfection changes induced by the pathogen. The rupturing of blisters was caused by physical pressure exerted on the epidermis by the increased mass of tissues produced by an exaggerated multiplication and enlargement of cortical cells. Exposure of the cell mass to ambient conditions resulted in dehydration, necrosis, and discoloration of cells. Evans (4) reported that within 2–3 wk seedlings of *Theobroma bicolor* L. inoculated with the liana strain of *C. perniciosa* produced premature bark. In this study on seedlings of *T. cacao* L. (cultivars Catongo, ICS 43, and ICS 60) inoculated with the cocoa strain of *C. perniciosa*, no such premature bark formation was observed. However, the brown color of necrotic cortical tissue and remnants of ruptured epidermis gave the appearance of bark tissue.

There are numerous reports of ultrastructural changes induced in hosts by pathogens (2). However, to our knowledge there are no reports concerning plasmodesmata in infected plant cell walls. The absence of plasmodesmata in walls of newly differentiated cells of hypertrophied cocoa tissue and their presence in the walls of the healthy cells indicate that the formation of plasmodesmata is affected by infection. In host tissue infected by biotrophs growing intercellularly, disintegration of the middle lamella and degradation of the cell wall occurred (2). No such phenomena were observed in cocoa tissue infected by *C. perniciosa*.

Exudation is sometimes a pathological reaction of the host to infection and in many cases is associated with the disintegration of host tissues (citrus gummosis, induced by *Phytophthora citrophthora*, is an example). In infected cocoa tissue the process of exudation was probably initiated by high internal pressure generated by the substantial increase in the volume of the cortex. Tissues in the infected area did not show any signs of disintegration during the process of exudation.



Figs. 1 and 2. (1) Blisters (arrows) in three stages of development on a cocoa seedling stem. Bar = 130 μm. **(2)** Blisters at different stages of rupturing, exposing the underlying tissues. Bar = 260 μm.



Figs. 3–9. **(3)** Seedling stem with lesions formed from ruptured blisters. Bar = 130 μm. **(4)** Transection of a stem with a blister in an early stage of formation and hyperplasia and hypertrophy of subepidermal tissue. Bar = 75 μm. **(5)** Transection of a stem with a blister prior to rupture. Bar = 75 μm. **(6)** Transection of a stem with a ruptured blister. Bar = 75 μm. **(7)** Transmission electron microscopy of infected tissue of a blister, showing intercellular hyphae (arrow) and the absence of plasmodesmata in the cell walls. Bar = 2.5 μm. **(8)** Transmission electron microscopy of healthy cortical stem tissue, showing numerous plasmodesmata in the cell walls. Bar = 2.5 μm. **(9)** Early (a) and late (b) stages of globule formation resulting from exudation. Bar = 130 μm.

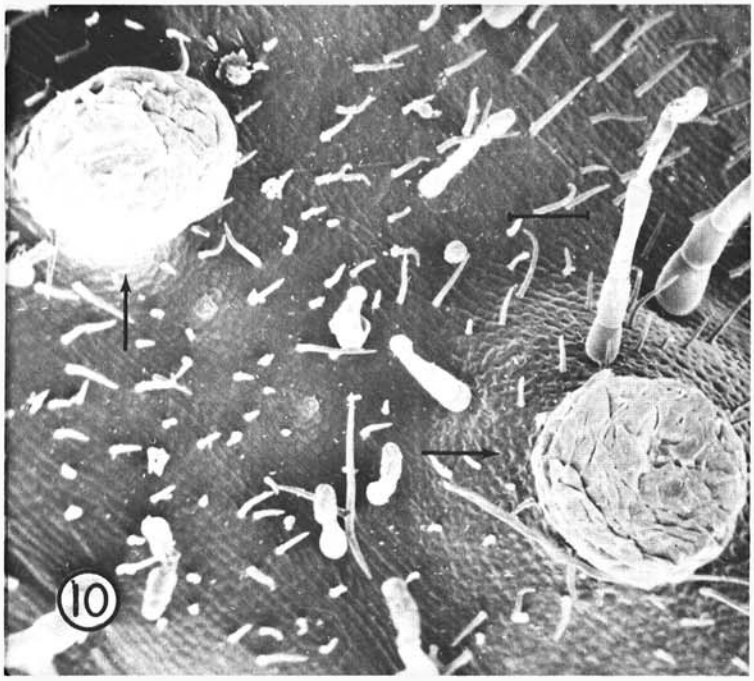


Fig. 10. Scanning electron microscopy of solidified exudate (arrows) forming cabbage-head-like structures. Bar = 250 μm.

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