# Modes of Penetration of Young Cocoa Leaves by *Crinipellis perniciosa*

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**ABSTRACT**

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Young attached and detached leaves of the cocoa cultivar ICS 43 were inoculated with basidiospores of *Crinipellis perniciosa* to investigate the modes of entry of the pathogen. Light and scanning electron microscopy after 6, 12, and 48 hr of incubation showed that the pathogen enters the leaf by direct penetration and also through stomata. In both modes of penetration either single or multiple germ tubes entered the leaf. Basidiospore germination also resulted in hyphal growth on the lamina.

Extensive cultivation of cocoa (*Theobroma cacao* L.) in the cleared Amazon forest region of Brazil and the subsequent appearance of witches'-broom disease in epidemic proportions renewed interest in this cocoa disease, caused by *Crinipellis perniciosa* (Stahel) Singer. Several scientific studies conducted in the cocoa-growing nations, the United Kingdom, and the United States have resulted in a new understanding of this disease (2–7,13,14,17). However, there is a lack of knowledge regarding the penetration of cocoa tissue by the pathogen. Stahel (15) indicated penetration through the stomatal opening in the case of young pods (cherelles) and published a drawing of the penetration process. No information, however, was provided regarding the penetration of vegetative tissue by the fungus. Baker and Holliday (1) reproduced Stahel's drawing and suggested that penetration other than stomatal entry may be a possibility, on the basis of successful infection by immersion of 4-day-old seedlings in a spore suspension. Suarez-Capello (16), using scanning electron microscopy of inoculated 7-day-old seedlings, was unable to show any particular mode of entry of the fungus into the plant, even though extensive germ tubes and mycelia were produced by the basidiospores. Cronshaw and Evans (3) reported direct penetration of cocoa leaves by a majority of germinated spores and observed only occasional entry through stomata. No photographs have been published to illustrate the modes of entry of the pathogen. This study was therefore initiated to clarify the modes of entry of the pathogen in young leaves of its host.

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## MATERIALS AND METHODS

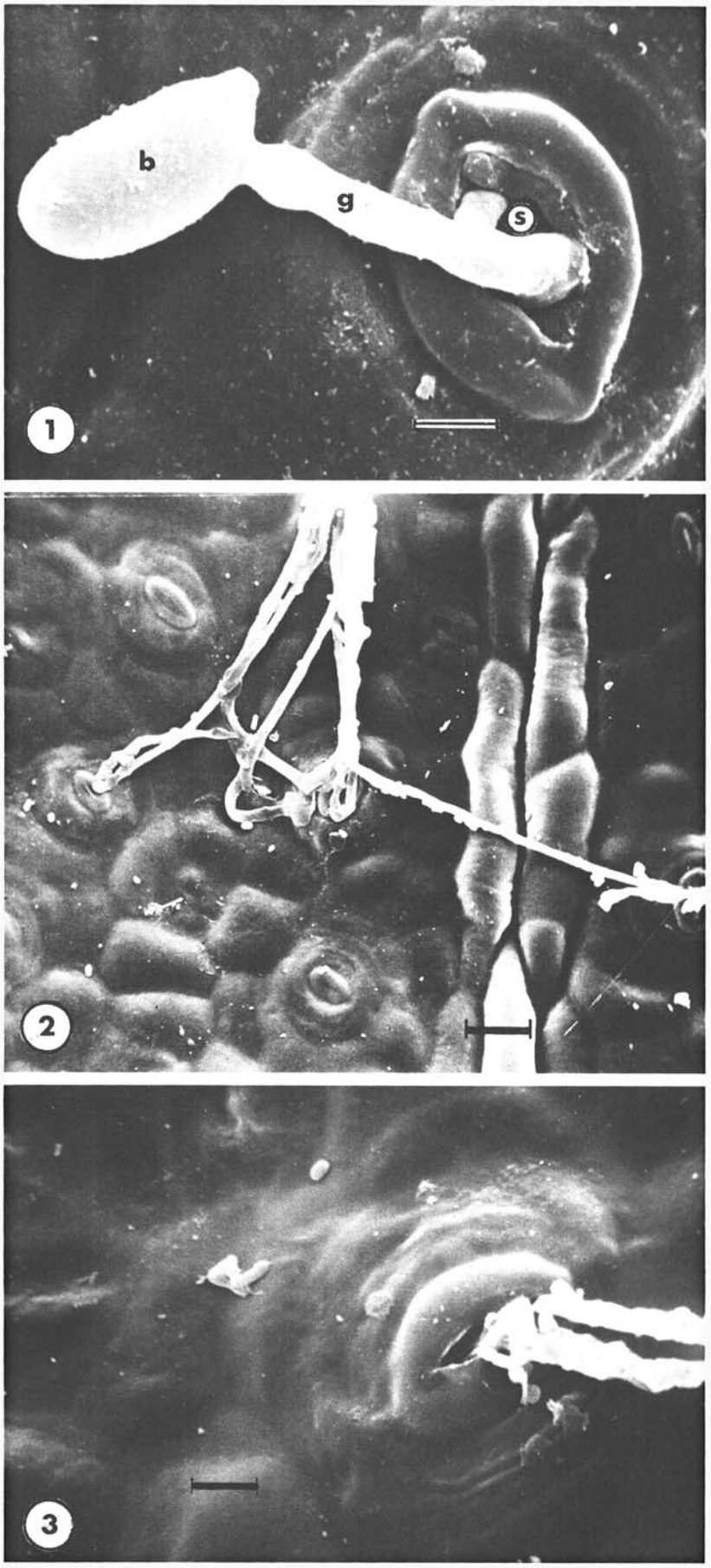
Immature new-flush leaves (5–10 cm in length) were collected from the cocoa clone ICS 43, a highly susceptible cultivar (10). The leaves were surface-sterilized in 0.5% sodium hypochlorite solution and subsequently rinsed in three changes of sterile distilled water. The treated leaves were then placed with the abaxial surface facing upwards in a sterile petri dish lined with moist, sterile filter paper. A polythene sheet with perforations (3 mm in diameter) was placed over the leaves to confine the areas of spore deposition on the lamina. Several newly developed mature pilei of *C. perniciosa* were attached to the underside of the petri dish lid (5), and the lid was then placed over the partially covered leaves. Spore deposition was allowed for 15 min. The petri dish lid with the pilei was then removed and again placed on another petri dish containing

surface-sterilized cocoa leaves covered with a perforated polythene sheet. Following spore deposition the lid with the pilei was removed and replaced by a sterile lid. The petri dishes with the inoculated leaves were incubated for 48 hr in a moist chamber under ambient temperature and light. At different time intervals disks (2 mm in diameter) were cut from areas of the lamina immediately beneath the perforations and fixed in 3% glutaraldehyde. Attached young leaves were also similarly inoculated. The inoculated plants were incubated in a moist chamber for 48 hr. At 6, 12, and 48 hr after inoculation, leaf disks were cut and processed in the same way as those from the detached leaves. The fixed leaf disks were freeze-dried and coated with gold-palladium on a Technic-Vote coating machine and observed on a Cambridge Stereoscan SY-10 at the University of Nebraska, Lincoln.

For light microscopy studies polythene sheets with perforations 7 mm in diameter were used to cover the leaves. After spore deposition the inoculated leaves were incubated for 6 and 12 hr. Disks 5 mm in diameter were cut from leaves and cleared with a saturated solution of chloral hydrate. The cleared leaf disks were stained with cotton blue in lactophenol, mounted on glass slides, and observed for basidiospore germination and penetration.

## RESULTS AND DISCUSSION

This study showed the germination of basidiospores and the subsequent growth of hyphae on the surface of inoculated leaves incubated for 48 hr. The entry of the pathogen into the leaf was through stomata by primary germ tubes (Fig. 1) and by hyphae from primary mycelium formed externally on the leaf surface (Figs. 2 and 3). Strands of hyphae originating from surface mycelia can effectively enter several stomata simultaneously (Fig. 2), and several hyphae were observed entering a single stoma (Fig. 3). Germ tubes from several basidiospores were also observed to converge and enter a single veinal stoma (Fig. 4). In addition to stomatal entry, direct penetration by germ tubes also occurred. Several germ tubes were seen to enter at the same site of penetration (Fig. 5). There were no morphological modifications in the germ tube tips to indicate the formation of an appressorium or infection cushion (Figs. 1, 4, and 5). Hardwick et al (9) reported that during the ontogeny of the cocoa leaf, stomata on veins develop earlier and become larger than laminar stomata. The phenomenon of the convergence of germ tubes and their subsequent entry into the large veinal stomata was not observed at the smaller laminar stomata. Spore germination and the subsequent entry of the pathogen into the leaf through stomatal pores and by direct penetration




**Figs. 1-3. (1)** Germ tube (g) from a basidiospore (b) entering a laminar stoma (s) after 6 hr of incubation, and branching in the stoma. Bar = 2 $\mu$m. **(2)** Growth of hyphae resulting from the germination of basidiospores and their entry through three stomatal openings. Bar = 10 $\mu$m. **(3)** Stomatal entry by two hyphal branches. Bar = 2 $\mu$m.

took less than 6 hr.

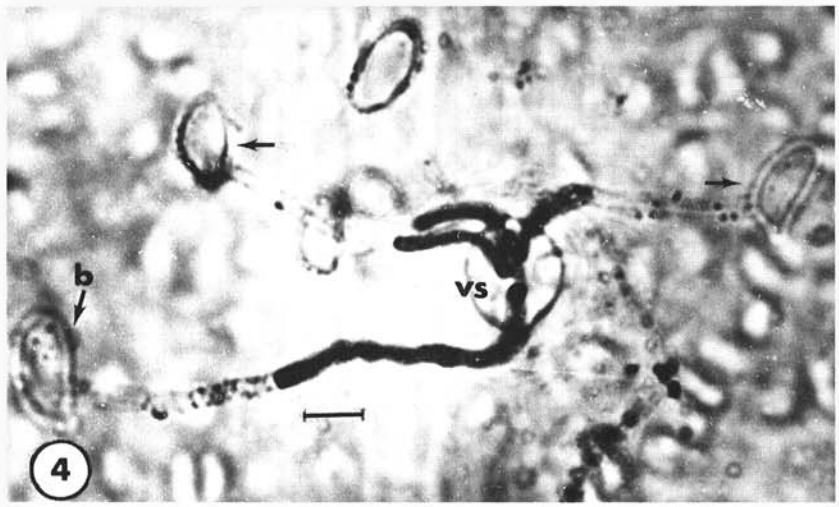
The finding that *C. perniciosa* produces mycelial growth on the leaf surface is in agreement with Suarez-Capello's observations of the growth of the fungus on the epidermal surface of inoculated seedlings (16). The growth of mycelium derived from basidiospores and subsequent multiple penetration of host tissue imply that under natural conditions a single basidiospore may be able to effect multiple entry of the host under prolonged wet conditions. In most of the cocoa-growing areas such conditions often exist. The observation that the incidence of witches'-broom disease tends to be higher in areas of high rainfall than in drier areas may be due, in part, to the occurrence of several multiple infections arising from basidiospores.

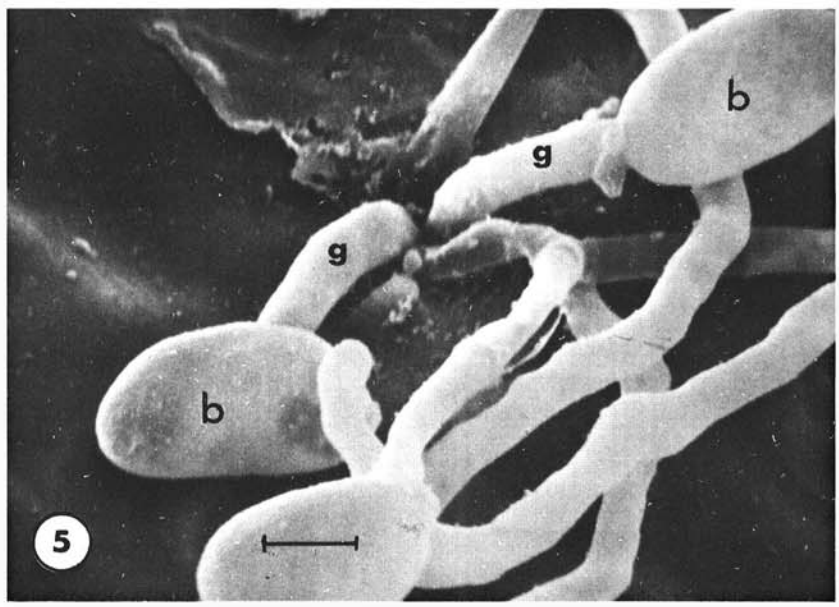
The occurrence of direct penetration was suspected by Baker and Holliday (1) and reported by Cronshaw and Evans (3). The results of this study confirm the occurrence of direct penetration. Cronshaw and Evans (3) did not mention the age of leaf material used in their study but indicated only that the duration of the incubation period was 6 hr. The low frequency of stomatal entry noted by these workers may be related to the stage of development of the leaf.

Tropisms are a critical part of the preinfection phase of disease development (18). In this study, in both stomatal entry and direct penetration, several germ tubes displayed directional growth toward the entry point, and it was speculated that tropism may be involved. Frias (8) implicated tropism as being responsible for the growth of germ tubes of *C. perniciosa* toward stomata and trichome bases of young inoculated cocoa leaves. Hunt (11) reported direct cuticular penetration of germ tubes with appressoria in two rust fungi. In the present study direct penetration by germ tubes without the formation of an appressorium or infection cushion suggests the production at the germ tube tips of a substance or substances that degrade host tissue. Direct penetration and stomatal entry were observed in detached and attached leaves.

Frias (8), using fluorescent microscopy, reported that substomatal vesicles developed after hyphal entry and subsequently gave rise to intercellular hyphae. Occasional branching of the germ tube in the stomatal pore (Fig. 1) and the occurrence of direct penetration (Fig. 5) suggest that vesicle formation is not a prerequisite for hyphal proliferation.

The nuclear state of the pathogenic phase of the fungus is not yet clearly established (4,6,12). Delgado and Cook (4) demonstrated haploidy in basidiospores of *C. perniciosa*. Our results show that germ tubes formed from the haploid basidiospores were capable of entering the host either directly or indirectly within 6 hr of germination. Efforts to maintain the pathogenic phase of the fungus in culture have so far been unsuccessful. Since the pathogen does not remain in the infective phase for long periods, the observation of sustained growth of hyphae on leaves without the loss of the ability to enter host tissues warrants further investigation. The nuclear state of the mycelium on the leaf surface was not determined in this study. Therefore, cytological studies to determine the nuclear status of the hyphae are desirable. Further, the nature of nutrients available on the leaf surface for hyphal growth needs to be investigated.




**Figs. 4 and 5. (4)** Directional growth of several germ tubes from basidiospores (arrows) toward a veinal stoma (vs), resulting in multiple entry. Protoplasts (darkly stained) migrate from the basidiospore (b) toward the terminal part of the germ tube. Bar = 5 μm. **(5)** Direct penetration of germ tubes (g) from basidiospores (b) at the same site on a leaf surface. Bar = 2 μm.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## LITERATURE CITED


1. Baker, R. E. D., and Holliday, P. 1957. Witches' broom disease of cacao (*Marasmius perniciosus* Stahel). Phytopathol. Pap. 2. Commonwealth Mycological Institute, Kew, Surrey, England. 42 pp.
2. Calle, H., Cook, A. A., and Fernando, S. Y. 1982. Histology of witches'-broom caused in cacao by *Crinipellis perniciosa*. Phytopathology 72:1479-1480.
3. Cronshaw, D. K., and Evans, H. C. 1978. Witches' broom disease of cocoa (*Crinipellis perniciosa*) in Ecuador. 2. Methods of infection. Ann. Appl. Biol. 89:193-200.
4. Delgado, J. C., and Cook, A. A. 1976. Nuclear condition of the basidia, basidiospores and mycelium of *Marasmius perniciosus*. Can. J. Bot. 54:66-72.
5. Evans, H. C. 1978. Witches' broom disease of cocoa (*Crinipellis perniciosa*) in Ecuador. 1. The fungus. Ann. Appl. Biol. 89:185-192.
6. Evans, H. C. 1980. Pleomorphism in *Crinipellis perniciosa*, causal agent of witches' broom disease of cocoa. Trans. Br. Mycol. Soc. 74:515-523.
7. Evans, H. C., and Bastos, C. N. 1980. Basidiospore germination as a means of assessing resistance to *Crinipellis perniciosa* (witches' broom disease) in cocoa cultivars. Trans. Br. Mycol. Soc. 74:525-536.
8. Frias, G. A. 1987. An inoculation method to evaluate resistance to witches' broom disease of cacao. Ph.D. thesis, University of Florida,

Gainesville. 110 pp.
9. Hardwick, K., Baker, N. R., and Bird, K. J. 1979. Control of chloroplast formation and photosynthetic performance in developing cocoa (var. Amelonado and Amazon) leaves. Pages 135-142 in: Proc. Int. Cocoa Res. Conf. 7th.
10. Holliday, P. 1957. Further observations on the susceptibility of Imperial College selections to witches' broom disease. Pages 48-53 in: Report on Cocoa Research, 1955–1956. Imperial College of Tropical Agriculture, St. Augustine, Trinidad.
11. Hunt, P. 1968. Cuticular penetration by germinating urediospores. Trans. Br. Mycol. Soc. 51:103-112.
12. Pegus, J. E. 1972. Aspects of the host-parasite relationship in the *Theobroma cacao* L./*Marasmius perniciosus* Stahel disease complex. M.Sc. thesis, University of the West Indies, St. Augustine, Trinidad. 140 pp.
13. Purdy, L. H., Trese, A. T., and Aragundi, J. A. 1983. Proof of pathogenicity of *Crinipellis perniciosa* to *Theobroma cacao* by using basidiospores produced in in vitro culture. Rev. Theobroma 13:157-163.
14. Rocha, H. M., and Wheeler, B. E. J. 1985. Factors influencing the production and the deposition and germination of basidiospores of *Crinipellis perniciosa*, the causal fungus of witches' broom on cocoa (*Theobroma cacao*). Plant Pathol. 34:319-328.
15. Stahel, G. 1919. Bijdrage tot de kennis der Krullotenziekte. Bull. Dep. Landbouw Suriname 33:1-26.
16. Suarez-Capello, C. 1977. Growth of *Crinipellis perniciosa* (Stahel) Singer in vivo and in vitro. Ph.D. thesis, University of London. 134 pp.
17. Wheeler, B. E. J., and Mepsted, R. 1981. Pathogenic races of *Crinipellis perniciosa*? Pages 365-370 in: Proc. Int. Cocoa Res. Conf. 8th.
18. Wynn, W. K., and Staples, R. C. 1979. Tropisms of fungi in host recognition. Pages 45-69 in: Plant Disease Control. R. C. Staples and G. H. Toenniessen, eds. John Wiley & Sons, New York.