Infection of Sugarcane Leaves by *Puccinia melanocephala*

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Research supported in part by the Florida Sugar Cane League, Inc., and by a USDA/ARS Cooperative Agreement for Tropical Agricultural Development 58-7B30-0-218. Journal Series Paper 4131.

Accepted for publication 16 November 1982.

ABSTRACT

Sotomayor, I. A., Purdy, L. H., and Trese, A. T. 1983. Infection of sugarcane leaves by *Puccinia melanocephala*. Phytopathology 73:695-699.

Sequential development of *Puccinia melanocephala* was studied from germination of urediospores to the establishment of the pathogen within sugarcane leaves. The optimal temperature range for germination of urediospores and formation of appressoria was between 15 and 30 C. Penetration occurred only after germ tubes formed appressoria over stomata. Development of infection structures followed the general pattern observed with other rust fungi. Morphology of the appressoria, substomatal vesicles, infection hyphae, and haustoria of *P. melanocephala* were defined. Haustoria of *P. melanocephala* have a central point of attachment and are lobed with fingerlike branches. Plastic leaf replicas and scratched cellophane elicited sequential development of the germ tube and formation of appressorium, penetration peg, substomatal vesicle, infection hypha, and haustorial mother cell. Haustoriumlike structures also were seen on a cellophane membrane. These observations support the hypothesis that a contact stimulus triggers development of appressoria and the subsequent infection structures. Guard cells or stomata on leaves provide the contact stimulus in nature. Penetration of *P. melanocephala* into leaves of the nonhosts, oat and wheat, was similar to penetration of sugarcane leaves. However, the fungus did not develop beyond the infection hypha stage in wheat or the haustorial mother cell stage in oats.

Additional key word: *Saccharum*.

Rust (caused by *Puccinia melanocephala* H. Syd. and P. Syd. (= *P. erianthi* Padw. and Khan)) of sugarcane (interspecific hybrids of *Saccharum*) was reported for the first time in the Americas in 1978 in the Dominican Republic. Subsequently, the disease was reported in northern South America, Central America, the Caribbean region, and in the United States in Florida, Louisiana, Mississippi, Texas, and in 1981, in Hawaii. This sugarcane rust also was reported for the first time in 1978 in Australia (3). *Puccinia melanocephala* is present worldwide, but not in all sugarcane-growing regions (3). In contrast to many other rust fungi, relatively little information is available about the infection processes of *P. melanocephala* from the germination of urediospores to the formation of haustoria.

The sequential development of infection structures of certain rust fungi has been described in association with their respective host plants (2,15,22). Infection structures develop in an orderly manner beginning with the germ tube and its differentiation into an appressorium. Appressoria are essential for *Puccinia* spp. to gain entry into host plants through the stomata. Some exceptions to the typical patterns of stomatal penetration have been reported, especially in rusts of tropical dicotyledonous plants (7). In the absence of the appressorium, penetration and formation of the other specialized structures required for host infection and colonization do not take place. After the appressorium has developed, a penetration peg, substomatal vesicle, infection hypha, haustorial mother cell, and haustorium are produced in sequence and result in the establishment of the rust fungus colony (4,12,14). In addition, analogous structures have been formed by several *Puccinia* species on artificial substrates. Their induction was attributed to a contact stimulus in a specific topography (12,20,21), which apparently substituted for the stimulus provided by guard cells (stomata) on plant leaves. The appearance of some of those fungal structures has been illustrated (12,20).

The objective of the present investigation was to elucidate the infection structures of *P. melanocephala* from germination of urediospores to establishment of the pathogen within the host and to observe colonization and sporulation of the fungus within sugarcane leaves.

MATERIALS AND METHODS

Germination of urediospores. Pieces of sugarcane leaves from plants grown in the greenhouse at 25 ± 5 C were placed on 2% water agar (15 ml) in petri plates that were then put in incubators at 5, 10, 15, 20, 25, 30, and 35 C ± 0.5 C. A small amount of water was sprayed on the surface of the leaf pieces 1 hr before urediospores were placed on the leaf pieces with a dry camel's hair brush. The urediospores used in each experiment were from different lots collected from plants growing in the greenhouse, but each of those lots was established from the same original field collection. The inoculated leaf pieces were removed after 1, 2, 4, 6, 8, and 12 hr at each temperature, fixed, and decolorized by boiling in lactophenol-ethanol (1:2, v/v) for 10 min. Cleared leaf pieces were washed in 50% ethanol and then rinsed in deionized water for 1 min. The water was poured off, and leaf pieces were then immersed in a 0.1% solution of the fluorochrome, Calcofluor (19), in 0.1 M tris-HCl buffer (pH 7.5) for 30 sec. Excess Calcofluor was removed by washing the samples in deionized water with vigorous agitation. Leaf pieces were observed with a Leitz Dialux 20 microscope fitted with a D 13-572 Ploemopak 2.4 epifluorescence attachment with a D 13-413 filter system. Percentages of urediospore germination and percentages of appressoria formed were based on examination of a minimum of 300 randomly selected spores from three different leaf pieces for each time and temperature treatment. Urediospores with a germ tube at least as long as its largest dimension were considered to have germinated. Germ tubes with a terminal swelling over a stoma were considered to have formed an appressorium. The experiment was repeated three times.

Artificial substrates and leaf replicas. Details of in vitro development of infection structures of the sugarcane rust fungus were studied by using synthetic films. Cellophane membranes (1 × 3 cm) were used that were either rubbed with carborundum 0.22-μm (600-mesh) maximum particle size or scratched with fine sandpaper 102–68 μm (grades 150–220) maximum particle size. Urediospores collected from plants grown in the greenhouse were brushed onto the surface of the cellophane membranes with a dry camel's hair

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brush. Inoculated membranes without scratches were used as controls. After application of spores, the films were spread on the surface of 2% water agar in petri dishes and incubated at 25 C for 24–48 hr in darkness. To observe fungal structures, drops of 0.1% Calcofluor in water were applied to cellophane membranes, which were placed on microscope slides.

Comparative observations were also carried out with sugarcane leaf replicas. Positive replicas of polystyrene plastic were prepared according to the procedure of Wynn (20). Leaf replicas were inoculated, incubated, and stained in the same manner as described for cellophane membranes except that replicas dusted with urediospores were placed on moistened filter paper in petri plates.

Sectioned material. The penetration and infection processes of *P. melanocephala* were observed histologically in two sugarcane cultivars. Leaves were excised from cultivars CL 41-223 (rust susceptible) and CP 70-1133 (rust resistant) grown in the greenhouse. For inoculation, portions of the leaf lamina (25–27 cm in length) were laid flat with the abaxial surface exposed in a spore settling tower into which 5 mg of urediospores were introduced by two shots from an air rifle. After inoculation, both cut ends of leaf pieces were placed in deionized water to form an inverted "U" in plastic boxes (25 × 15 × 8.5 cm) and incubated in a dew chamber at 25 C. Leaf samples were taken 12, 18, 24, and 36 hr after inoculation, fixed in formalin-acetic acid-alcohol (FAA), passed through a tertiary-butyl alcohol dehydration series, and embedded in paraffin (9). Serial sections of leaf tissue were cut at 10 μm in a rotary microtome. Paraffin was removed, tissue sections on slides were rehydrated, and then treated with 0.1% Calcofluor in water. Finally, the sections were dehydrated to xylene. Permanent mounts of sections were made in Flo-tex (Lerner Laboratories, New Haven, CT 06513) and examined with a compound microscope fitted with a fluorescence attachment.

Cleared leaves. The morphology and the relationship of infection structures to host cells observed in cleared leaves from plants in the greenhouse and in leaf pieces in growth chambers were similar. Thus, leaf pieces of sugarcane (25–27 cm) were inoculated with urediospores dry-brushed onto the abaxial surface and incubated in a dew chamber at 25 C for 36 hr. They were transferred to a controlled-environment chamber at 25 C, lighted with three 40-W cool-white fluorescent lamps, and programmed for a 12-hr light period. Samples were taken 12, 18, 24, 36, and 48 hr after inoculation and every 24 hr thereafter until 168 hr. By 168 hr, rust pustules had erupted. Sugarcane leaf pieces were fixed and treated with Calcofluor according to the technique described by Rohringer et al (19). Because it was not possible to observe infection structures within the leaf owing to accumulation of the fluorochrome by the cuticle, the cuticle was removed by washing leaf pieces four times in deionized water, and then placing them in warm 1 M KOH for 90 min. Leaf pieces were then soaked in deionized water overnight and placed in lactophenol-ethanol (1:2 v/v) for microscopic observation.

Nonhost interactions. To observe the reactions of nonhost plants to *P. melanocephala*, abaxial surfaces of detached oat and wheat leaves, grown in the greenhouse, were dry-brushed with urediospores. After inoculation, the cut edges of the excised leaves were placed in water in plastic boxes as described previously, and the leaves were incubated in a dew chamber at 25 C for 24 hr. Plastic boxes containing the detached leaves were transferred to a growth chamber and maintained at 25 C for 96 hr. Pieces (1 × 2 cm) were cut from inoculated leaves and cleared and stained with "Fluorescent Brightener 24" (Sample A-1383, provided by K. Artz and M. Morf, Ciba-Geigy Ltd., CH-4002 Basel, Switzerland). Kuck et al (10) referred to Fluorescent Brightener 24 as diethanol.

RESULTS

Germination studies. Germination of urediospores and formation of appressoria by *P. melanocephala* occurred between 5 and 30 C (Table 1). Urediospores began to germinate within 1 hr at 25 and 30 C. The optimal temperature range for spore germination was 15–30 C. Germination percentages at 5 and 10 C were lower than those recorded at higher temperatures. Urediospores did not germinate at 35 C.

High percentages of appressoria developed over a range of 15–30 C, but low percentages of appressoria developed at 5 and 10 C.

Artificial substrates and leaf replicas. Infectionlike structures of *P. melanocephala* developed on scratched cellophane membranes at 25 C. Germ tube elongation ceased upon contact with a scratch, and differentiation of an appressorium followed. A septum formed that delimited the appressorium from the germ tube, and from which a projection was produced that corresponded to the penetration peg followed by the development of a structure homologous to the substomatal vesicle (Fig. 1). A septum formed between the penetration peg and the substomatal vesicle. Almost all substomatal vesicles produced two infection hyphae. Haustorial mother cells were separated from infection hyphae by septa, and infection hyphae branched immediately behind the septa. On three occasions a haustoriumlike structure was observed (Fig. 1). Appressoria failed to develop on the surface between scratches, as well as on nonscratched cellophane membranes on which only long germ tubes were observed.

On polystyrene leaf replicas, germ tubes produced appressoria following contact with stomatal impressions followed by development of substomatal vesicles each with two or three infection hyphae. Haustorial mother cells were produced on each infection hypha, but haustoriumlike structures were not observed on leaf replicas.

Sectioned material. Urediospores of *P. melanocephala* germinated and produced globose-to-ellipsoidal appressoria over stomata on leaves of both susceptible and resistant sugarcane cultivars. A short penetration peg passed through the stomatal aperture. Direct penetration through the epidermis was not observed. After passing between the guard cells, the penetration peg enlarged and formed a vesicle (Figs. 2–5) that occupied almost all of the substomatal cavity. Generally two to three (but sometimes four) infection hyphae developed from the substomatal vesicles, usually within 36 hr after inoculation. Infection hyphae colonized the leaf and grew intercellularly. Upon contacting a mesophyll cell, a terminal hyphal cell was cut off by a septum from the rest of the hypha and formed a haustorial mother cell. The haustorial mother cell was always delimited from the infection hypha by a septum. When the haustorial mother cell contacted the host cell wall a haustorium developed within the host cell (Fig. 3). Haustoria developed 36 hr after inoculation. The haustorium appeared to be

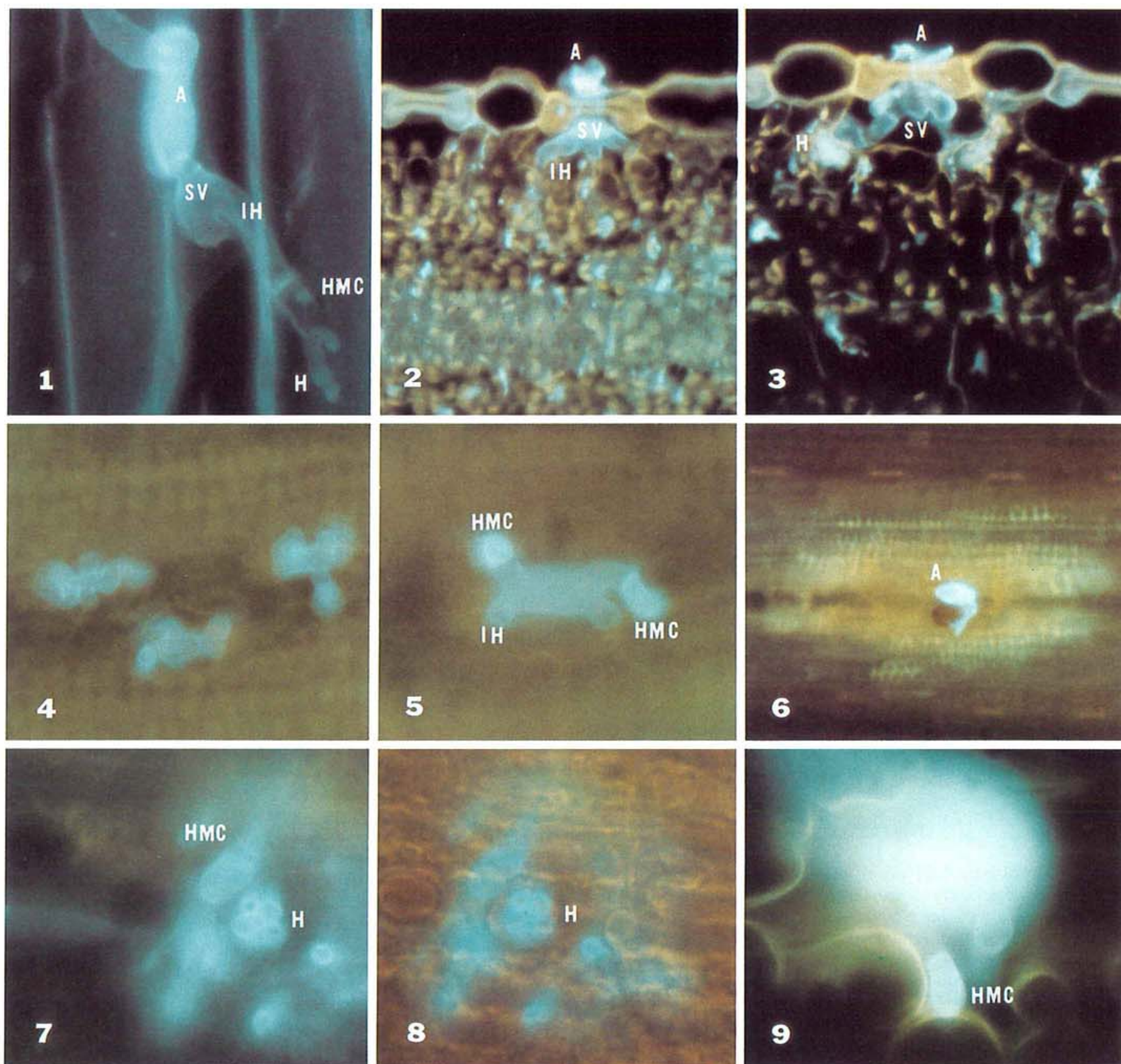
TABLE 1. Effects of temperature and time on percent germination (G) of urediospores and percent formation of appressoria (AP)[a] by *Puccinia melanocephala* on leaf pieces of susceptible sugarcane cultivar CL 41-223

Temperature[b] (C)	1 hr		2 hr		4 hr		6 hr		12 hr	
	G	AP	G	AP	G	AP	G	AP	G	AP
5	0	0	0	0	10	0	60	16	52	11
10	0	0	0	0	12	0	50	10	52	29
15	0	0	0	0	84	26	98	76	97	94
20	0	0	61	9	89	54	97	87	97	97
25	85	12	95	47	94	65	98	86	98	89
30	79	7	95	30	94	53	97	47	99	63

[a] Based on germinated urediospores.
[b] No germination at 35 C.

composed of multiple branches that originated from the point of its entrance into the host cell lumen. The haustorium appeared to have lobes oriented in several optical planes (Fig. 3).

Cleared leaves. Infection structures observed in serial sections were compared with those observed in cleared leaves. Infection structures in leaves were visible only after the cuticle was removed by treatment in 1 M KOH. The integrity of the infection structures was not affected by KOH treatment. After 12–18 hr, vesicles had developed in substomatal cavities. The leaf surface was brushed lightly with a camel's hair brush wetted with lactophenol to remove appressoria and germ tubes; this allowed observation of substomatal vesicles and other infection structures within the leaf. Substomatal vesicles were variable in shape, usually with several armlike projections (Figs. 4 and 5). Infection hyphae developed from the substomatal vesicle, and colonized the leaf intercellularly. There were abundant haustorial mother cells, but haustoriumlike structures could not be identified precisely because of the difficulty in seeing the host cell wall. Use of the method described by Kuck et al (10) allowed observation of many haustorial mother cells from which haustoria developed (Figs. 7 and 8). The walls of cells with



Figs. 1–9. Infection structures of *Puccinia melanocephala* on scratched cellophane and in sugarcane leaf tissue. **1,** Infection structures produced by *P. melanocephala* on scratched cellophane after 48 hr (×2,000). **2** and **3,** Longitudinal sections of sugarcane leaves that illustrate the relationship of *P. melanocephala* infection structures to the host after 36 hr (×1,250). **4,** Substomatal vesicles of *P. melanocephala* with infection hyphae in cleared leaf tissue after 12 hr (×1,250). Germ tubes and appressoria have been removed from the leaf surface. **5,** Substomatal vesicle of *P. melanocephala* in cleared leaf tissue after 18 hr (×2,000). **6,** Germinated urediospore of *P. melanocephala* with an appressorium on the surface of the rust-resistant sugarcane cultivar CP 70-1133 showing yellow or golden fluorescence suggestive of a resistant host response after 120 hr (×500). **7** and **8,** The lobed haustoriumlike structure of *P. melanocephala* in a cleared sugarcane leaf showing the haustorial mother cells from which the haustorium developed after 72 hr (×2,000). **7,** Epifluorescence microscopy only. **8,** Both epifluorescence and transmitted light microscopy. **9,** Haustorial mother cell of *P. melanocephala* in contact with a mesophyll cell of oat after 96 hr (×2,000). Presence of *P. melanocephala* in the substomatal chamber of oat induced the resistant response indicated by the yellow or golden fluorescence of adjacent cells. Legend: A = appressorium, SV = substomatal vesicle, IH = infection hyphae, HMC = haustorial mother cell, and H = haustoriumlike structure.

haustoria exhibited a golden fluorescence when either chloroform-methanol or ethidium bromide was included in the leaf-clearing and staining procedure either with Calcofluor or Fluorescent Brightener 24. Generally, transmitted white light was used as background for better resolution of the host cell wall (Fig. 8).

The first visual evidence of haustoria was detected at 36 hr after inoculation. Haustoria appeared to be lobed or branched with fingerlike projections from a central point of attachment, and were not at all similar to haustoria of other species of *Puccinia* (1,2,15,17,18). Sometimes the haustorial body had two lobes or branches, but many had five or more (Figs. 3, 7, and 8). The haustorial body occupied much of the host cell lumen.

Successive stages of fungal colonization were followed shortly by a period of mycelial growth and hyphal aggregation around the substomatal cavity from which uredia developed. In general, colonization of sugarcane leaves by *P. melanocephala* was similar to that described for other rust fungi (11,21). Mycelial aggregation appeared as a mass of sporogenous hyphae that resembled parenchyma cells. Palisadelike layers of hyphal cells were formed beneath several stomata 168 hr after inoculation, and growth was oriented parallel to leaf venation. Subsequently, rupture of the epidermis occurred after enlargement of paraphyses and the formation of urediospores.

A bright orange-yellow (golden) fluorescence was observed around appressoria on cleared whole leaves of the rust-resistant cultivar CP 70-1133, 5 days after inoculation (Fig. 6). The intercellular mycelial growth was much reduced in comparison with that observed in the susceptible host. However, when fluorescence around appressoria was extensive, fungal structures were not observed within the leaf (Fig. 6).

Nonhost interactions. *Puccinia melanocephala* apparently responded to stomata in the leaves of oat and wheat plants as if they were sugarcane stomata. The prepenetration stage of the infection was similar to that observed in sugarcane cultivars. The rust fungus also penetrated the leaves of these nonhost plants; however, there was no development beyond infection hyphae in wheat or beyond haustorial mother cells in oats (Fig. 9).

Mesophyll cells of wheat around the substomatal cavity showed a golden fluorescence, and the substomatal vesicle appeared to have deteriorated. In contrast, fungal structures in oat leaves appeared as if they developed in sugarcane leaves with bone-shaped substomatal vesicles, from which usually three or four infection hyphae developed. Commonly, the tip of one or two infection hyphae in oat was delimited by a septum to form a haustorial mother cell (Fig. 9). Cells adjacent to the haustorial mother cells appeared to be necrotic, but sometimes only a thickening of the cell wall was observed that was characterized by an orange-yellow fluorescence (Fig. 9).

DISCUSSION

The results of our observations of plant leaves inoculated with urediospores of *P. melanocephala* support published results that germination occurs over a wide range of temperatures from a minimum of 5 C to a maximum of more than 30 C. However, the failure of urediospores to germinate at 35 C may be the primary cause of reduced amounts of disease when temperatures exceed 30 C. Because appressoria are apparently essential for penetration of the sugarcane leaves by *P. melanocephala*, any factor (eg, temperatures below 5 C or above 30 C, or lack of the correct topographical stimulus) that interferes with formation of appressoria will prevent infection. Our results demonstrate that temperature affects not only urediospore germination but also the formation of appressoria, especially below 10 C and above 30 C.

Structures that resemble appressoria, penetration pegs, substomatal vesicles, infection hyphae, haustorial mother cells, and haustorialike structures developed in vitro from urediospores following germination on artificial membranes and, except for haustoriumlike structures, on polystyrene replicas of sugarcane leaves. All structures were produced without additional nutrients, supporting the hypothesis that they are initiated by topographical stimuli (12,20,21). Several authors (12,20,21) have reported formation of appressoria by other rust fungi in response to topographical stimuli, such as wrinkles, craters, and scratches, on the surface of artificial membranes, as well as on plastic leaf replicas. The stimulus for appressorium formation on leaves is provided by contact with stomata (20). This stimulus is the trigger in the sequential formation of infection structures from urediospore germ tubes. The germ tube of *P. melanocephala* can distinguish the stomata from almost all other surface features, such as leaf hairs, venation, and protuberances, and Purdy (16) reported that 99% of appressoria of *P. melanocephala* were formed over stomata. The contact stimulus provided by stomata also appears to be the stimulus for development of subsequent infection structures, and this concept is supported by our observations.

The infection processes of sugarcane rust followed the general pattern of the uredio-stage of other rust fungi that penetrate the host through stomata (12,21).

The appressorium was essential for penetration as well as for the development of subsequent infection structures, because these structures were never observed in the absence of an appressorium. Substomatal vesicle development, and the number of infection hyphae arising from it, differed in relation to other rust fungi (2,15). Generally, the form of the infection structures is characteristic of a particular rust pathogen (8,13,15).

Formation of haustoria resulted from contact between the haustorial mother cells and the walls of the host mesophyll cells. The sugarcane rust haustorium consisted of fingerlike projections or branches that occupied much of the host cell lumen. Consequently, the orientation of those projections in different optical planes caused the haustorium to appear to be lobed. Several authors have shown variation in haustorial morphology for temperate and tropical rust fungi (2,17,18), but none have illustrated haustoria similar to those produced by *P. melanocephala*.

Penetration and formation of infection structures in susceptible and resistant sugarcane cultivars appeared to be similar up to the point where the interaction between the host and pathogen resulted in no further fungal development. Heath (5) mentioned that rust resistance is a complex phenomenon, and that the hypersensitive response, whether or not it has a primary role in the restriction of fungal growth, might be only the visible response of a series of interactions between the plant and its potential pathogen. The so-called hypersensitive collapse of cells around the fungal structures suggested by the orange-yellow fluorescence developed by the resistant cultivar CP 70-113 and limitation of rust in this and resistant cultivars of sugarcane will now be investigated.

Rust fungi that penetrate nonhost plants may develop differently than in their specific host (12). In both wheat and oat leaves, *P. melanocephala* developed as it does in sugarcane. Infection processes were stopped in wheat at the point of infection hypha development, whereas in oats infection stopped at the haustorial mother cell formation stage. Host cells adjacent to fungal structures in both wheat and oats showed a golden fluorescence. Perhaps, a fungal inhibitor is produced by wheat and oat leaves in response to the sugarcane rust fungus, as was reported in corn inoculated with *P. graminis* Pers. f. sp. *tritici* (11). However, other possible determinants of nonhost resistance to rust fungi have been mentioned (6).

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