# Suscept-Pathogen Relationship in Maize Affected by Yellow Leaf Blight

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ABSTRACT

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Seedlings of maize were inoculated with conidia of *Phyllosticta maydis*, the imperfect stage of *Mycosphaerella zeae-maydis*. Over 90% of the conidia germinated within 12 hours, and produced one or two germ tubes. The germ tubes did not branch and were variable in length. Ingress occurred by direct penetration of an epidermal cell by a penetration peg which developed from an appressorium. Earliest penetration was observed 6 hours after inoculation, and 30% of the germinated conidia had given rise to penetration within 24 hours. Ingress was followed by the development of a bulbous to rod-shaped primary hypha. Secondary hyphae grew within epidermal cells and intercellularly in the mesophyll; they did not penetrate vascular tissue. Cytoplasmic granulations and discoloration and thickening of epidermal cell walls of penetrated cells prior to formation of primary hyphae were observed. Alterations in mesophyll cells and tissues in advance of the fungus were also observed. Nuclei in epidermal cells adjacent to the cell penetrated were positioned adjacent to the radial wall closest to the site of penetration.

*Additional key words: Mycosphaerella zeae-maydis*, yellow leaf blight, histopathology.

Yellow leaf blight (YLB) of corn (*Zea mays* L.) is caused by *Mycosphaerella zeae-maydis* Mukunya & Boothroyd (*Phyllosticta maydis* Arny & Nelson) (2, 22). The disease, which was first observed in the United States in 1965 (18), now is present in most of the corn growing areas of this country, and it also is known to occur in Canada, France, and Africa (8, 14, 21). Yellow leaf blight has not caused important losses over large areas, but severe damage has been observed locally under conditions favoring the disease (1, 3, 20).

Several aspects of the etiology of YLB have been studied (2, 6, 9, 10, 19, 20, 22, 30), but little is known of the phenomena leading to infection and colonization of suscept tissue by the pathogen. This report presents results of a histological study undertaken to gain information, by means of light microscopy, of the suscept-pathogen relationship in maize affected by YLB.

## MATERIALS AND METHODS

Seedlings of a commercial cultivar of corn (normal cytoplasm) susceptible to YLB were inoculated at the four-leaf stage (about 14 days old) with *P. maydis* (isolate P24) which was isolated from maize in Cayuga, New York, in 1970. Inoculum was obtained from a 12-day-old culture of isolate P24 grown on potato-dextrose agar (PDA) at 24-26 C and under 12 hours of fluorescent light/day. Inoculum was prepared by adding sterile distilled water to the culture and allowing it to stand without shaking for 15 minutes. Conidia (Fig. 1-A) which oozed from pycnidia were filtered through cheesecloth, agitated, and adjusted to $10^5$ spores/ml. Just before inoculation Tween-20 (polyoxyethylene sorbitan monolaurate) was added to the suspension at the rate of 1 drop/100 ml. The conidial suspension was applied to the foliage with a DeVilbiss atomizer. After inoculation, seedlings were placed in a mist chamber (16) at 20-22 C and 14 hours/day photoperiod of fluorescent light of approximately 10,000 lux. Relative humidity close to 100% was provided for the first 48 hours after inoculation. The misting apparatus then was turned off, but the seedlings were left in the chamber for an additional 174 hours.

Tissue of third and fourth leaves sampled at various intervals after inoculation was examined under a light microscope as whole mounts. Leaf pieces were fixed in a mixture of absolute alcohol and glacial acetic acid (2:1, v/v) for 24 hours, cleared in lactophenol for 24 hours, stained in 0.1% acid fuchsin in lactophenol for 5-6 hours, counter-stained in 0.5% fast green for 20-30 seconds, and mounted in 50% glycerin.

## RESULTS

**Development of symptoms.**—First symptoms appeared 18 hours after inoculation as minute chlorotic specks on the basal and central portions of the fourth leaves. These specks enlarged into small round to elliptical chlorotic areas with a pinpoint yellowish necrotic center in 42-54 hours. Veins interrupted the enlargement of the chlorotic areas in early stages, but later chlorosis extended across the veins. Necrotic spots developed in the fourth leaves 9 days after inoculation;

they appeared as round yellowish zones with a reddish-brown margin and a halo of chlorotic tissue. Irregular dark-brown necrotic lesions appeared in those areas where coalescence of chlorotic lesions occurred.

**Penetration phenomena**—Germination of conidia occurred readily; 79% of the spores germinated in 6 hours and over 90% germinated within 12 hours (Table 1). One or two germ tubes per conidium were produced (Fig. 1-B, C), but occasionally three to four germ tubes per spore were observed (Table 1). Emergence of germ tubes was mostly lateral. Of 577 germ tubes from 359 conidia observed in the period 6-24 hours after inoculation, 63% were lateral, 22% were polar, and 15% were subpolar. Germ tubes were 1.2-2.4 $\mu$m wide and highly variable in length, ranging from 2.4-102 $\mu$m at 12 hours to 2.4-140 $\mu$m at 18 hours, with septa 10-25 $\mu$m apart. Branching was rare; occasionally (about 2% of germ tubes examined) a short branch developed from the main germ tube.

Growth of the germ tube ended with the development of an appressorium. Some appressoria were observed 6 hours after inoculation; at 24 hours over 81% of germinated conidia bore appressoria (Table 1). Appressoria were hyaline and thin walled, and were not delimited from the germ tubes by a septum (Fig. 1-B, C); they were mostly round averaging 3 $\mu$m in diameter, although elliptical or clavate appressoria also were observed.

**Penetration.**—Penetration was observed 6 hours after inoculation and the frequency of penetration increased with time (Table 1). Ingress was accomplished by means of a penetration peg (Fig. 1-E) produced from the appressorium, and occurred by direct penetration either into the lumina (about 75% of total penetrations) or at junctions of epidermal walls.

**Postpenetration development.**—Upon gaining ingress into an epidermal cell, the penetration peg gave rise to a bulbous or rod-shaped primary hypha (23) which ranged from 3.6 to 6.0 $\mu$m in width and 6.0 to 8.4 $\mu$m in length (Fig. 1-F). Within 12 hours of inoculation, a short secondary hypha 2.4-3.0 $\mu$m wide developed from the primary hypha and within 24 hours two or three of them were formed. Secondary hyphae grew downward into and through the epidermal cell reaching the upper layer of the mesophyll, or they colonized epidermal tissue by growing intracellularly, primarily parallel to the walls (Fig. 1-G). Growth was intercellular in the mesophyll; when a hyphal tip contacted a cell directly the hypha either grew around the cell without penetration (Fig. 1-I) or it branched with each branch curving around the cell. Secondary hyphae were not seen to penetrate vascular tissue, but instead, when secondary hyphae reached vascular bundles they grew intercellularly in the mesophyll tissue around them.

**Reaction of suscept cells and tissues.**—Detectable deleterious effects were first observed during penetration of epidermal cells before primary hyphae were formed. Anticlinal walls of cells being penetrated or close to the site of penetration showed thickening and a light-brown discoloration, and granular material in the cytoplasm accumulated against the wall (Fig. 1-H). These symptoms were accentuated by the formation of primary hyphae. Nuclei of penetrated epidermal cells were about half of their normal size. With the development of secondary hyphae, the thickening and light-brown discoloration appeared in the wall of a few mesophyll cells below the site of penetration. Concurrently, it was noticed that nuclei of epidermal cells adjacent to the cell penetrated were flattened against their respective wall at a point close to the site of penetration (Fig. 1-D). This phenomenon also was seen in epidermal cells 100 to 150 $\mu$m from the site. Accounts of this phenomenon are presented later in this report.

Secondary hyphae grew slowly in epidermal and mesophyll tissues. Hyphal tips were observed in epidermal cells some 150 $\mu$m and 300 $\mu$m from the cell penetrated at 24 hours and 42 hours, respectively. Conspicuous changes in mesophyll cells appeared in 24 hours; granulation of cytoplasm, disintegration of chloroplasts, and browning and thickening of walls were observed in colonized areas and also in advance of hyphae. After 42 hours, epidermal cells showed marked brown discoloration and partial dissolution of walls. Mesophyll tissue presented a gradation of symptoms from cell collapse and intense darkening in the central area of the lesions to small, round, and loosely arranged chloroplasts and light discoloration and thickening of walls some 600-800 $\mu$m from the site of penetration. These symptoms were restricted to an interveinal area and lesions appeared elongated in shape.

**Effect of penetration and early establishment of P. maydis on the position of nuclei in epidermal cells.**—Whole mounts of leaf tissue sampled 12 hours after inoculation were examined for the position of nuclei in epidermal cells. Ten conidia were randomly chosen for each of the following situations: (i) conidium which had germinated and resulted in subsequent penetration, (ii) conidium which had germinated without appressorium

TABLE 1. Prepenetration and penetration activities of *Phyllosticta maydis* on maize leaves[a]

| Activity | Time after inoculation (hr) 6 (%) | 12 (%) | 18 (%) | 24 (%) |
|---|---|---|---|---|
| Conidia germinating | 79.0 | 92.0 | 90.0 | 98.0 |
| Conidia forming one germ tube | 77.2 | 34.8 | 42.2 | 36.7 |
| Conidia forming two germ tubes | 22.8 | 52.2 | 51.1 | 59.2 |
| Conidia forming appresorium | 13.9 | 43.5 | 60.0 | 81.6 |
| Germ tubes forming appressorium | 13.3 | 24.1 | 40.3 | 60.3 |
| Germinated conidia resulting in penetration | 1.3 | 15.2 | 31.1 | 30.6 |
| Appressoria forming penetration pegs | 8.3 | 35.0 | 48.3 | 30.6 |

[a]Maize seedlings were inoculated with a suspension of $10^5$ conidia/ml of *Phyllosticta maydis*. One hundred spores were observed at each sampling time.

formation and subsequent penetration, and (iii) ungerminated conidium. In those epidermal cells penetrated by the fungus a short secondary hypha was visible inside the cell. For each conidium in each of the three situations, 10 epidermal cells were observed. These cells were distributed roughly in a row across the leaf from the cell bearing the conidium under consideration; i.e., adjacent to the cell upon which the conidium occurred (the first cell proximal to that epidermal cell, the 10th distal to it). The position of the nuclei was recorded as

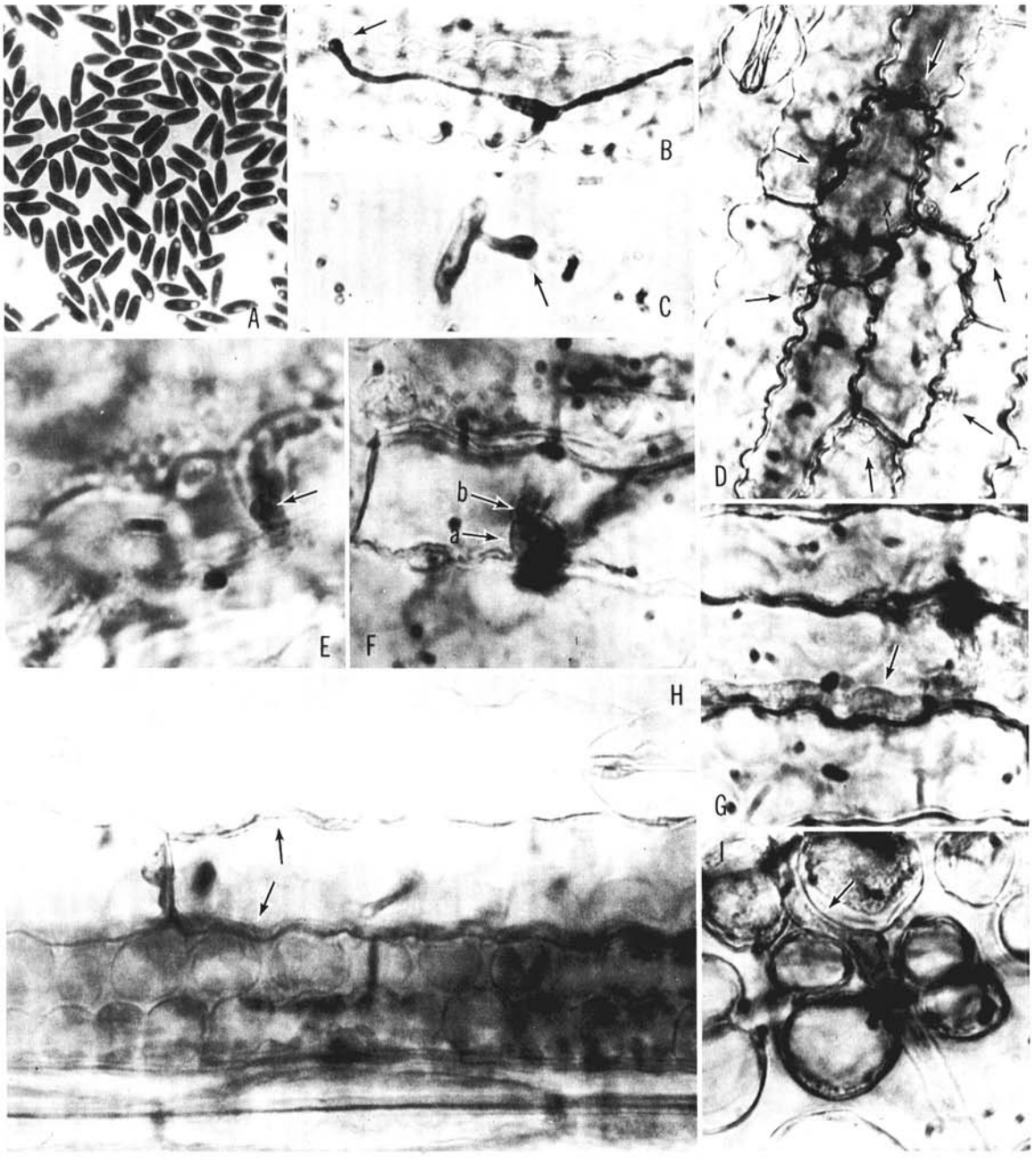


**Fig. 1-(A to I).** Suscept-pathogen relationship in maize affected by yellow leaf blight. **A)** Conidia of *Phyllosticta maydis*. **B)** Germinated conidium of *Phyllosticta maydis* on leaf tissue. Two germ tubes were produced. Arrow points to an appressorium positioned over an anticlinal wall of epidermal cell. **C)** Appressorium (arrow) developed on a short germ tube. **D)** Nuclei (arrows) of epidermal cells adjacent to two cells penetrated by *Phyllosticta maydis* (x). **E)** Round spot (arrow) which indicated the production of a penetration peg from the appressorium. **F)** Primary hypha of *Phyllosticta maydis* (arrow a) and initial development of a secondary hypha (arrow b). **G)** Secondary hypha of *Phyllosticta maydis* (arrow) growing parallel to a wall inside an epidermal cell. **H)** Wall thickening and granular material (arrows) accumulated against anticlinal walls of an epidermal cell penetrated by *Phyllosticta maydis*. **I)** Secondary hypha of *Phyllosticta maydis* (arrow) growing intercellularly in the mesophyll.

outlined by Contreras and Boothroyd (11). Straight-line measurements were recorded for each of the 10 cells, one (x) the distance from the nucleus to the appressorium, germ tube tip, or wall of the conidium, and the other (y) the shortest distance from the epidermal wall of the same cell to the same point of reference. The difference (d) between the two measurements (x-y) provided an indication of the relative position of the nucleus of a given cell to the conidium. Statistical analysis showed that the mean d value in the case of penetration was significantly smaller than the corresponding means for no germination and germination in the absence of penetration (Table 2). This suggested that the position of nuclei in epidermal cells was affected by *P. maydis,* the nuclei becoming oriented toward the site where penetration and early establishment had occurred.

## DISCUSSION

Early events leading to the infection of maize leaves by *P. maydis* were similar to those characteristic of many fungal plant pathogens. Conidia germinated by germ tubes which grew for a short distance on the leaf surface prior to formation of appressoria. A penetration peg developed from the base of the appressorium and allowed the pathogen ingress into the suscept by direct penetration of the cuticle and the wall of the epidermal cell. Germination readily occurred on inoculated maize leaves; about 90% of the conidia germinated within 12 hours. The percent of epidermal cells penetrated by *P. maydis,* however, was low (30.6%), although infection occurred readily, as evidenced by subsequent symptom development.

Bootsma (6) and Castor et al. (9) have studied factors affecting the germination of conidia of *P. maydis*. Although these authors agree on the importance of free moisture for germination, they differ as to the optimum temperature and the time at which maximum germination occurs. Our observations on the growth of the germ tubes contrast with the report of extensive elongation and branching that occurred when conidia of *P. maydis* were suspended in 0.1% sucrose solution (6). In our study, no attempt was made to elucidate the effect of exogenous nutrients on prepenetration activities of *P. maydis*.

The penetration of epidermal cells was followed by the development of primary and secondary hyphae, which indicated a successful establishment of the pathogen in the suscept tissue. Initial stages of pathogenesis, as shown by the granulation of the cytoplasm and the discoloration of the walls in epidermal cells, were observed in the course of penetration prior to establishment. Such cell alterations could be the result of mechanical injury or the action of a metabolite(s) released by the pathogen, or both. The location of epidermal nuclei near the anticlinal walls most close to the site of penetration was associated with the initial stages of infection. Migration of nuclei of suscept cells toward the infecting hyphae has been reported both in susceptible (5, 7, 24, 25) and in resistant (11, 28, 29) types of interactions; and has also been associated with artificial (4, 24, 25) or natural (25) wounding of plant tissues. In incompatible interactions (11, 29), migration of the nuclei toward the site of penetration was interpreted as the earliest reaction of resistant cells to invasion by the pathogen. We did not attempt to ascertain the time course over which nuclear migration occurred in uninfected epidermal cells of maize leaves, or to establish all stages of infection with which it was associated. Orientation of the nuclei of epidermal cells removed from the invaded one was observed when a secondary hypha had grown a few micrometers within the cell. This phenomenon may represent a reaction of epidermal cells of maize to a chemical wound; i.e., a wound caused by a metabolite(s) which might be

TABLE 2. The effect of penetration by *Phyllosticta maydis* on the position of nuclei of epidermal cells of maize leaves

| | Position of nuclei in contiguous epidermal cells relative to germinated and nongerminated conidium on an epidermal cell[a] | | |
|---|---|---|---|
| Epidermal cell | 1. Conidium germinated, with appressorium and penetration peg formed | 2. Conidium germinated, without appressorium, and no penetration | 3. Ungerminated conidium |
| 1 (proximal) | 14.5[b] | 14.6 | 19.1 |
| 2 | 11.6 | 20.5 | 18.2 |
| 3 | 10.3 | 23.4 | 12.8 |
| 4 | 12.7 | 19.7 | 15.2 |
| 5 | 7.0 | 14.1 | 17.4 |
| 6 | 3.7 | 9.6 | 14.0 |
| 7 | 7.5 | 12.2 | 17.8 |
| 8 | 10.8 | 21.6 | 26.6 |
| 9 | 9.7 | 14.7 | 18.7 |
| 10 (distal) | 9.2 | 21.1 | 21.9 |
| Means[c] | 9.70 A | 17.15 B | 18.70 B |

[a]The difference (d) in $\mu$m between straight-line measurements from a specific origin to the nucleus of a given cell (x) and the point in the wall of the same cell closest to the origin considered (y). Origins for measurements were: 1) appressorium with penetration peg; 2) the tip of the germ tube, without appressorium, and in the absence of penetration; and 3) the wall of the ungerminated conidium.

[b]Each figure is the mean of measurements of d values for 10 epidermal cells adjacent to the origin considered (the first cell proximal to the origin, the tenth distal to it).

[c]Means followed by the same letter do not differ significantly, $P = 0.01$, according to Tukey's honestly significant difference procedure.

produced by the pathogen and diffuses through the suscept tissue. The host-specific toxin produced by *P. maydis* induces chlorosis and leakage of electrolytes in leaves of Texas male-sterile maize (10), and might be involved in such a chemical wound.

Secondary hyphae of *P. maydis* colonized epidermal and mesophyll tissues of maize leaves. Although mycelial growth was intracellular in the epidermis, it followed the configurations of the intercellular spaces in the mesophyll. Moreover, the hyphae branched when they contacted a mesophyll cell, which seems to indicate inability to effect penetration. The vascular tissue appeared to be quite resistant to penetration and damage; the fungus is restricted primarily to the mesophyll cells of the leaf. In this regard, *Phyllosticta maydis* seems to behave the same as many leaf-spotting fungi which either do not colonize vascular bundles (12, 13, 15, 17, 26) or colonize them only after the susceptible tissue has undergone a physiological change (27).

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