Effects of Corn Plant Age and Cultivar on Resistance to *Cercospora zeae-maydis* and Sensitivity to Cercosporin

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ABSTRACT

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Three cultivars of corn with different degrees of resistance to *Cercospora zeae-maydis* were grown in the greenhouse. Leaf disks from 1-, 2-, and 3-mo-old plants were either treated with 1.2 μM cercosporin or inoculated with a mycelial suspension of *C. zeae-maydis*. Cerosporin treatment caused significantly less ion leakage from disks of older plants. Significantly more stomates of older plant tissue were penetrated by the fungus. No varietal difference was detected for either sensitivity to cercosporin or susceptibility to *C. zeae-maydis*. The data indicate that a real age-dependent resistance to *C. zeae-maydis* exists in corn and suggest that the mechanism of this resistance is different from the varietal resistance seen in the field.

The first symptoms of gray leaf spot of corn caused by *Cercospora zeae-maydis* (Tehon & Daniels) are reddish water-soaked spots. These spots expand to tan lesions that are delineated by the major veins. The grayish cast of the lesion, from which the disease name is derived, occurs only when the fungus sporulates (15). Symptoms of gray leaf spot characteristically appear late in the growing season. Reasons for this are not known, but environmental changes (4,15,16,17) and decreased host resistance (12,17) have been suggested. Likewise, little is known about the varietal resistance that has been developed toward *C. zeae-maydis* (15).

Species of *Cerospora* characteristically produce cercosporin, a red, photodynamic toxin (11). This toxin may play a major role in the ingression of *Cercospora* through host tissue (5,6,19). It is a nonspecific toxin (9), which is consistent with the broad host range of *Cercospora* species (5,6,8,10). Furthermore, light has been shown to be important for the development of several diseases caused by *Cercospora* (5,6,10). In one instance, infection of banana by *Mycosphaerella musicola* (*C. musae*), light was needed for disease development but the initial penetration of the tissue was independent of light (5).

This paper summarizes the findings of a brief laboratory study of the resistance of three corn cultivars to *C. zeae-maydis* and of the sensitivity of those cultivars to cercosporin. Three growth stages of the corn plants were examined to compare age- and cultivar-dependent resistances and toxin sensitivities.

MATERIALS AND METHODS

Plants. Corn cultivars were selected on the basis of differential disease ratings in the field (12; F. Latterell, *personal communication*). A tolerant cultivar (Pioneer Brand 3233), a susceptible cultivar (Pioneer Brand 3184), and a resistant cultivar (Pioneer experimental line) were used. Corn was grown in the greenhouse in 15-cm pots filled with peat-lite with farm fertilizer (1) but without the wetting agent. Plants were fertilized once a month and harvested at the early whorl (1 mo),

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late whorl (2 mo), and silk (3 mo) stages, which corresponded to stages 4, 6, and 8 as described by James (13). Plants grown in short-day seasons took 3 days to 1 wk longer to reach these growth stages.

Fungus. *C. zeae-maydis* isolates were obtained from the USDA-ARS Laboratory at Frederick, MD, and were maintained at 27 C with 12 hr of light (29 $\mu E/m^2/sec$) per day. The fungus was routinely transferred onto V-8 juice medium (200 ml/L V-8 juice, 17 g/L agar, and 3 g/L $CaCO_3$) as agar plugs or as mycelial fragments obtained by flooding the culture with 0.4% (v/v) Tween 20 and loosening the mycelia with a rubber policeman.

Toxin production and isolation. For cercosporin production, *C. zeae-maydis* was grown in 250-ml Erlenmeyer flasks containing 50 ml of GYE medium (15 g/L glucose and 3 g/L yeast extract). Cultures were maintained in a controlled-environment incubator shaker at 26 C with 16 hr of light (13 $\mu E/m^2/sec$) per day at a rotational velocity of 100 rpm. A 5-ml aliquot of a 1-wk liquid culture was used as inoculum.

Cercosporin was extracted from 1-wk liquid cultures by the methods of Kuyama and Tamura (14), except the fungal mat was extracted with acetone rather than ether. The acetone was removed under vacuum at 25 C and the residue was dissolved in chloroform. The culture filtrate was extracted by three chloroform washes, totaling about 10 ml of chloroform per 100 ml of culture filtrate. The combined chloroform solutions were purified on a calcium hydrogen phosphate column (14). Column fractions were examined by thin-layer chromatography on plates prepared by spreading a slurry of 24 g of Silica Gel-G in 48 ml of 2% (v/v) aqueous H_3PO_4. The plates were developed in ethyl acetate:benzene (2:3, v/v) (3) and viewed under ultraviolet and visible light. The fractions in which only cercosporin was detected were combined and the toxin was crystallized (3).

Electrolyte leakage. Ion leakage, an indicator of tissue damage in response to the presence of irradiated cercosporin, was measured with a conductivity bridge (Industrial Instrument, Inc). Leaf disks were cut with a 1-cm cork borer and mixed, then 0.2-g (wet weight) samples were placed in 50-ml beakers with 10 ml of 2% (v/v) methanol in water or 10 ml of 1.2 μM cercosporin in 2% aqueous methanol. The beakers were incubated under constant light (115 $\mu E/m^2/sec$) on a rotating shaker at a rotational velocity of 120 rpm. In preliminary experiments, conductivities of the solutions were monitored every 15 min for the first hour and every hour thereafter. In later experiments, these data were taken at 2, 4, and 6 hr. The relative differences in conductivity of these solutions between 2 and 6 hr were determined by dividing the difference in the 6- and 2-hr conductivity readings of a treated tissue by the analogous difference of a corresponding control tissue. The experiment was repeated three times with duplicate samples for each experiment.

Stomatal penetration. Leaf disks were dipped in a mycelial suspension (0.25 mg dry weight per milliliter) from a 7- to 10-day-old culture grown on solid medium and transferred to plastic petri dishes 100 × 15 mm containing 25 ml of 1% (w/v) water agar. Plates were incubated on wet sponges in glass trays at 27 C under Westinghouse Agro light (108 $\mu E/m^2/sec$) for 12 hr per day. After 6–8 days, the disks were immersed for a few minutes in aniline blue W.S. (18) and the excess stain was removed with a water rinse.

Stained disks were examined with a light microscope, using a double-blind procedure. The percentage of penetration was defined as the ratio of penetrated stomata to the number of stomata that were in direct contact with the fungus. A stoma was counted as penetrated only if a hyphal strand passed completely through the stoma. Twenty stomata were examined randomly from each disk, and five disks were examined per experiment. Each experiment was repeated three times.

Statistics. The GLM procedure of the SAS computer program was used to test for significance (2).

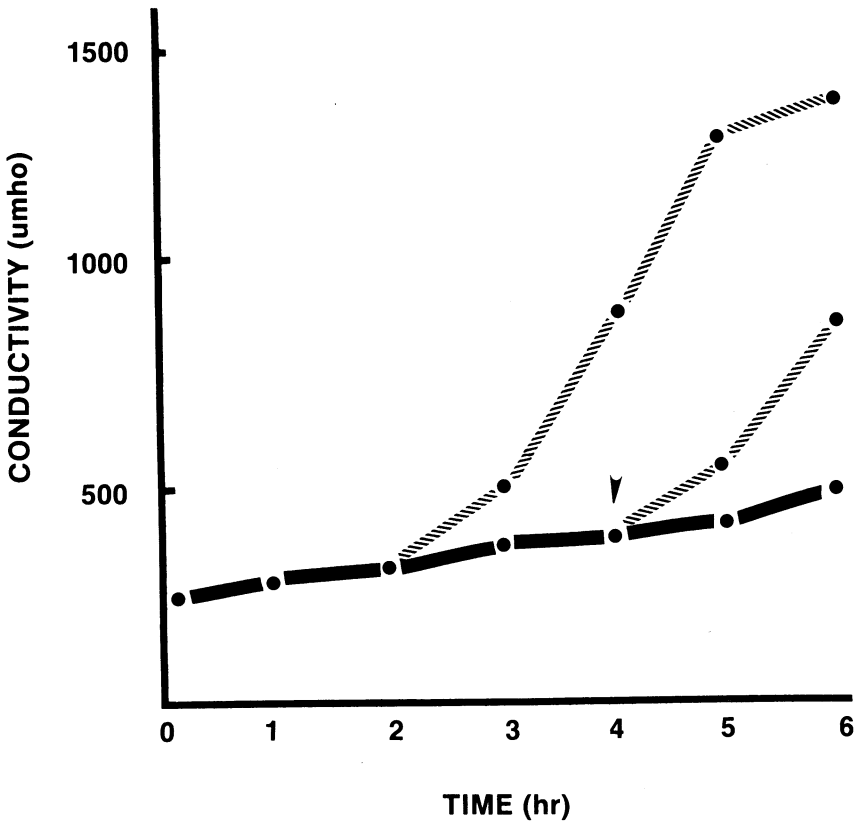

Fig. 1. Conductivity of solutions bathing leaf disks from 1-mo-old corn plants. The bathing solution was either water (solid line) or water containing 1.2 μM cercosporin. The control and one of the cerosporin-treated samples were placed in the light throughout the experiment. The second cercosporin-treated sample was incubated in the dark for the first 4 hr, then placed in the light (arrow).

RESULTS

Toxin production and isolation. Several hundred milligrams of crystalline cercosporin were isolated. This material gave a single spot on TLC when the plates were examined with ultraviolet or visible light. The R_f value of 0.14 was close to the value of 0.13 reported for cercosporin in the same system (14). The visible and ultraviolet spectra of the isolated cercosporin in methanol and in 0.1 N NaOH matched the reported spectra (3). A second red pigment was present in the crude fungal extract but was removed by the column chromatography. In the same TLC system, the R_f for this compound was 0.32 and it was assumed to be isocercosporin (14). This is apparently the first report of the isolation of cercosporin from *C. zeae-maydis*.

Electrolyte leakage. An exposure of 3 hr of light was required before electrolyte leakage of cercosporin-treated tissue increased significantly above the control. However, preincubation in the dark eliminated this lag phase. In the dark, conductivity of the solution containing cercosporin-treated tissue did not differ from the control (Fig. 1).

Statistical analysis showed that there was a significant ($P \leq 0.001$) age-dependent linear decrease in the sensitivity to cercosporin as measured by ion

leakage. No significant differences were found among cultivars of the same age (Fig. 2).

Stomatal penetration. Incubation in the high-moisture conditions used for the study of penetration caused corn leaf disks to become bleached. Stomata in these disks remained closed, but in the older plants, the fungus was able to penetrate nonetheless. The frequency of this penetration increased significantly from less than 5% in the 1-mo tissue to about 10% in the 2-mo tissue and to about 40% in the 3-mo tissue. There was no significant difference in penetration among cultivars (Fig. 3). The fungus formed appressoria when penetrating some stomata but produced no structures when contacting others. The appearance of the fungal germ tubes and appressoria was the same as that reported by Beckman and Payne (4).

DISCUSSION

Cercosporin-induced ion leakage from plants occurs only when the tissue is exposed to light (7). This was true in this study, but a 2-hr lag in ion leakage from the cercosporin-treated corn tissue was also observed. This probably represents the time required for cercosporin to diffuse into the tissue, because it was eliminated by incubating the cercosporin-treated tissue in the dark (Fig. 1). Daub (7) did not observe this lag period, but she was using 5 μM cercosporin, whereas the concentration in this study was only 1.2 μM.

Tissue from older corn was less sensitive to the action of cercosporin, but varietal resistance to this toxin was not detected (Fig. 2). Thus neither age- nor cultivar-dependent resistance to *C. zeae-maydis* can be explained on the basis of differential sensitivity to cercosporin, a finding that is consistent with the literature (9,14).

Laboratory studies of gray leaf spot of corn are hampered because it is difficult to consistently obtain high levels of *C. zeae-maydis* infection (4,20). This problem was overcome by inoculating small leaf disks and incubating them at high relative humidity. With this method, significant positive correlation was observed between frequency of stomatal penetration by *C. zeae-maydis* and age of the corn tissue inoculated (Fig. 3). In contrast, Beckman and Payne (4) reported that stomatal penetration of greenhouse-grown corn by *C. zeae-maydis* was independent of plant age; however, their definition of percent penetration differed from ours. They compared stomata penetrated with the number of stomata above which an appressorium had formed. We used percent penetration of stomata that had mycelial contact regardless of whether an appressorium was present. Thus, our study, but not that of Beckman and Payne, included observations in which

Fig. 2. Relative conductivity of the 1.2 μM cercosporin solutions bathing leaf disks from three cultivars of corn plants of various ages.

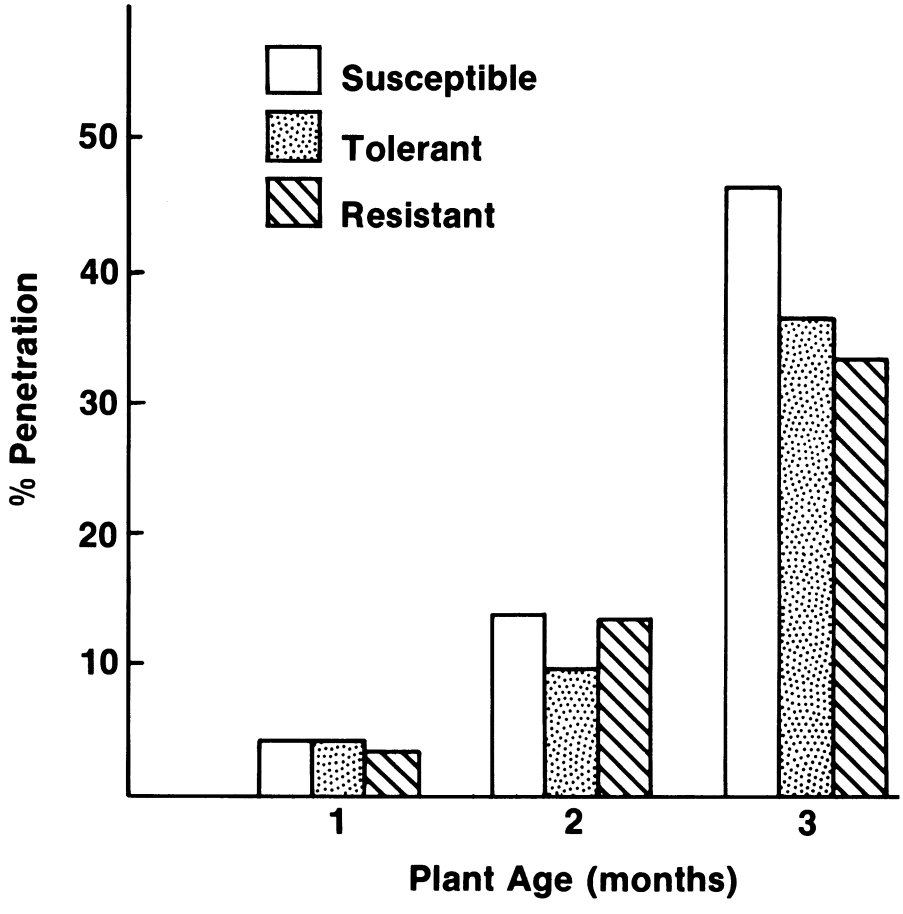

Fig. 3. *Cercospora zeae-maydis* penetration of stomata of leaf disks from three cultivars of corn plants of various ages.

hyphae grew either across or through stomata without stopping to form appressoria. Stomatal contact without penetration was much more common with young corn tissue than with disks from older corn leaves.

The assays used in this study did not detect the cultivar-dependent resistance to *C. zeae-maydis* that is found in the field. The data did, however, correlate well with the known age-dependent responses. This suggests that different factors may be involved in these two types of resistance. It should be noted, however, that the methods of this study only measured the rates of stomatal penetration. It is also possible that age-or cultivar-dependent resistance may depend primarily on differential growth of the pathogen within the tissue.

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