Etiolated Maize Mesocotyls: A Tool for Investigating Disease Interactions

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ABSTRACT

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Etiolated mesocotyls of maize cultivars with differential resistance and susceptibility to *Helminthosporium maydis* race O and *H. carbonum* race 1, were useful for investigating host-parasite interactions. Mesocotyls and leaves were similarly susceptible or resistant to the pathogens. Pigmentation of the mesocotyl is an important indicator of its response to a pathogen. Uninoculated mesocotyls placed in the light accumulated anthocyanin pigments as a linear function of time. Anthocyanins also accumulated in resistant host-parasite combinations, and eventually their concentration equaled or exceeded that of uninoculated controls. A prominent zone of intense anthocyanin pigmentation often surrounded restricted lesions in resistant interactions. In susceptible host-parasite combinations, however, a significantly lower anthocyanin concentration was evident as early as 24 hr after inoculation and always prior to visible lesion development. In susceptible interactions, less anthocyanin accumulated than in uninoculated controls.

Additional key words: *Colletotrichum graminicola*, *Helminthosporium turcicum*, phenolics, resistance, *Zea mays*.

Our interest in the physiology of corn diseases led to a search for a host tissue with greater uniformity, extractability, and ease of handling than green leaves. Etiolated mesocotyls seemed appropriate because they can be grown rapidly (24) and their biochemical constituents are easily extractable (1,2).

This report presents a method for investigating host-parasite relationships by using etiolated mesocotyls. It describes the susceptible and resistant responses of the maize mesocotyl, which include a unique decrease of anthocyanin accumulation in susceptibility.

MATERIALS AND METHODS

Plant material, pathogens, and inoculation. Seeds of maize (*Zea mays*) hybrids Mo17$_{Ht}$ × B73$_{Ht}$ and B73$_{Ht}$ × Va26$_{Ht}$, and the inbreds Pr, Pr1, B73$_{rhm}$, and B73 were imbibed in aerated water for 7 hr and then incubated between layers of moist germination paper in the dark at 28 C for 84–96 hr depending on the cultivar. Elongation of the mesocotyl occurred in the dark and ceased when the plants were exposed to light (24). Seedlings with mesocotyl lengths of 40 to 50 mm were arranged in plastic boxes so that the mesocotyls were horizontal and above water and the root systems remained submerged.

Spores of the fungi *Helminthosporium maydis* Nisik. and Miy. race O and *H. carbonum* Ullstrup race 1 were obtained from isolates grown on potato-dextrose agar under constant fluorescent light (60 μE·m^{-2}·sec^{-1} across the range 400–700 nm) at 24 C. Spores from 10-day-old cultures were suspended at a concentration of 10^5 spores per milliliter in sterile distilled water containing Tween-20 (one drop per 100 ml of suspension) wetting agent and were applied to mesocotyls from DeVilbis hand atomizers. Boxes of inoculated mesocotyls were initially placed in a growth chamber at 28 C for 4 hr in the light (combined fluorescent and incandescent, 92 μE·m^{-2}·sec^{-1} across the range 400–700 nm). Following the initial exposure to light, seedlings were subjected to a photoperiod of 9 hr dark and 15 hr of light for the duration of each experiment. For the first 24 hr after inoculation relative humidity (RH) was maintained at 100%. Symptom development was observed at 8-hr intervals over a period of 4–7 days.

Plants were also grown in the greenhouse so that leaves could be inoculated with the same fungi used for mesocotyl inoculations. Plants were grown from the same seed lots in a 12-hr photoperiod of supplemental fluorescent light (20) for 2 wk and were then inoculated with suspensions containing 10^4 spores per milliliter. They were incubated in the dark for 18 hr at 100% RH on the greenhouse bench before leaf surfaces were allowed to dry. Symptom development was observed for 8 to 10 days.

Anthocyanin accumulation as a function of time. The accumulation of anthocyanins was measured in uninoculated mesocotyls and in susceptible and resistant disease interactions. Mesocotyls of the hybrids B73$_{Ht}$ × Va26$_{Ht}$ and Mo17$_{Ht}$ × B73$_{Ht}$ were inoculated with 10^5 spores per milliliter of *H. maydis* race O (susceptible combination) or of *H. carbonum* race 1 (resistant combination). Similarly, the combinations of *H. carbonum* race 1 on the inbreds Pr (susceptible combination) and Pr1 (resistant combination) were investigated. Following inoculation, mesocotyls were incubated as described and anthocyanin content was determined at intervals. Each experiment was repeated three times.

Mesocotyls, selected at random at each sampling time, were excised 4 mm below the coleoptile node and 4 mm above the point of attachment to the kernel, and either were extracted immediately or were frozen. Triplicate samples of three mesocotyls, approximately 0.3 g per sample, were extracted for each treatment. The tissue was weighed and homogenized in 5 ml of 80% methanol acidified with 0.1% HCl. The homogenate was centrifuged at 20,000 *g* for 30 min at 4 C. Anthocyanin content was determined directly by measuring the absorbance of the supernatant at 525 nm (2,9).

That anthocyanins were the compounds being measured was confirmed by acid hydrolysis to anthocyanidins and comparison of the aglycones to commercial standards by thin-layer chromatography (10,17,19).

Influence of inoculum concentration on anthocyanin accumulation. The hybrid B73$_{Ht}$ × Va26$_{Ht}$ was inoculated with suspensions of *H. maydis* race O spores (susceptible combination) containing from 10^3 to 10^5 spores per milliliter or with suspensions of *H. carbonum* race 1 spores (resistant combination) containing from 10^3 to 10^6 spores per milliliter. Anthocyanins were extracted as described above after 120 hr of incubation.
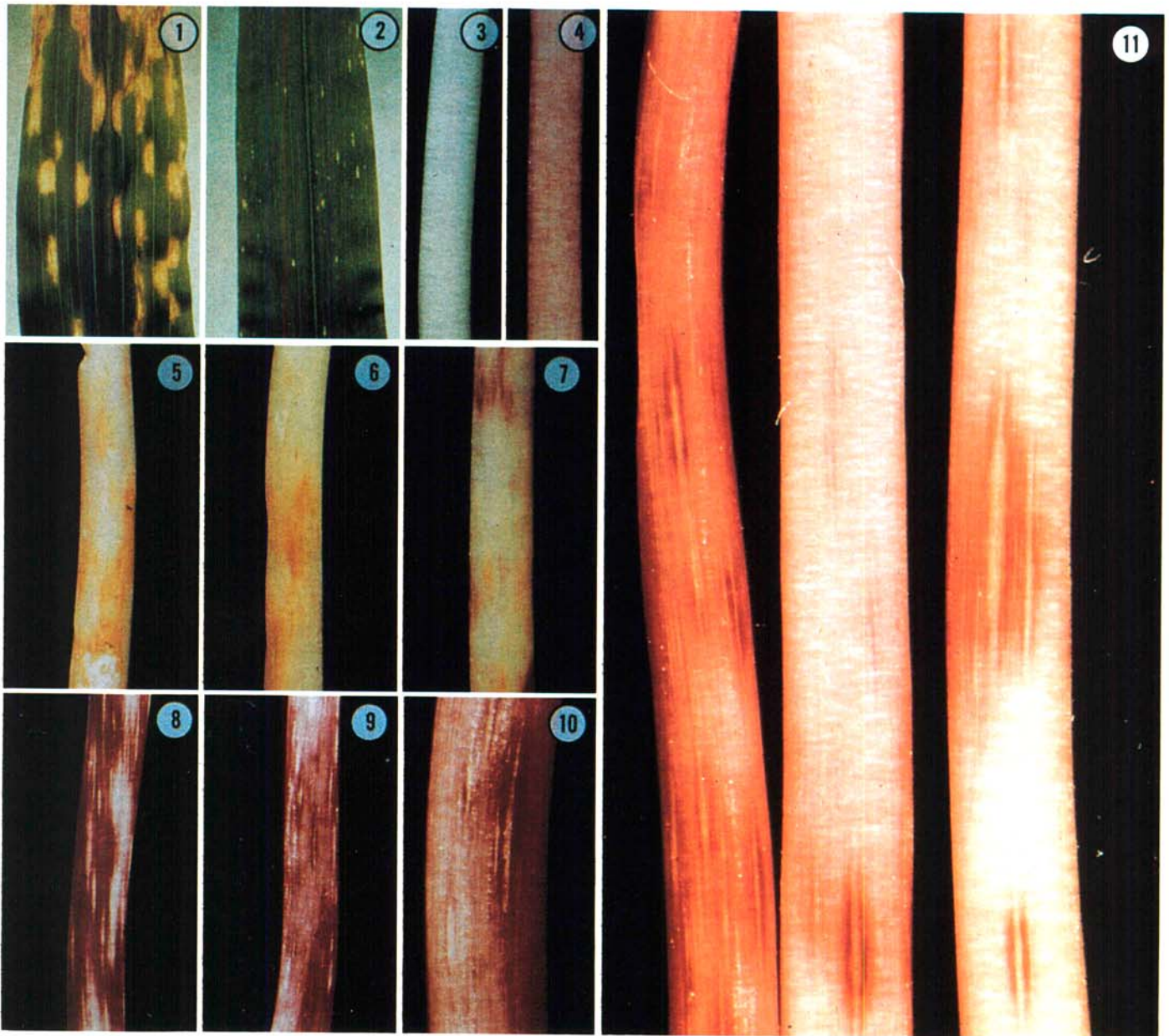
Figs. 1–11. Comparison of symptoms on leaves and etiolated mesocotyls of maize hybrid B73ₕₜ × Va 26ₕₜ following inoculation with *Helminthosporium maydis* race O or *Helminthosporium carbonum* race 1. **1,** The third leaf of the plant showing characteristic lesions of the susceptible interaction with *Helminthosporium maydis* race O (7 days after inoculation). **2,** The third leaf of the plant showing characteristic lesions of the resistant interaction with *Helminthosporium carbonum* race 1 (7 days after inoculation). **3,** Etiolated mesocotyl 96 hr after imbibition of water by the kernel. Note the absence of anthocyanin pigments. The width of a mesocotyl is 2 mm. **4,** Etiolated mesocotyl after 3 days in a 15-hr photoperiod. Note the uniform anthocyanin pigmentation. **5,** Lesion development on a mesocotyl 72 hr after inoculation with *Helminthosporium maydis* race O (susceptible interaction). Note the brown discoloration, areas of sunken tissue, and complete lack of anthocyanin pigmentation. **6,** Lesion development on a mesocotyl 84 hr after inoculation with *Helminthosporium maydis* race O (susceptible interaction). **7,** Lesion development on a mesocotyl 60 hr after inoculation with *Helminthosporium maydis* race O (susceptible interaction). Inoculum coverage was uneven and resulted in the accumulation of anthocyanins around oval areas of tissue. Note the tan discoloration near the center of the oval areas. Necrotic lesions develop from such discolored tissue. **8,** Resistant interaction of the mesocotyl with *Helminthosporium carbonum* race 1 60 hr after inoculation. Note the oval unpigmented areas of tissue which indicate the presence of infection sites. **9,** Resistant interaction of the mesocotyl with *Helminthosporium carbonum* race 1 72 hr after inoculation. Note that the unpigmented areas of tissue have substantially decreased in size (compared to those in Fig. 8) and that a zone of red pigmentation (anthocyanins) surrounds areas of unpigmented tissue. Each of these remaining areas represents an infection site and the areas are characteristic of the restricted lesions of the resistant interaction. **10,** Close-up of the restricted lesion characteristic of the resistant interaction of the mesocotyl with *Helminthosporium carbonum* race 1. Note the accumulation of anthocyanins around the remaining infection sites. **11,** Close-up of restricted lesions characteristic of the resistant interaction of the mesocotyl with *Helminthosporium carbonum* race 1. Note the greater pigmentation in the mesocotyl on the left which received a heavier inoculum load than the pair to its right. Lesion length seldom exceeded 3 mm.

RESULTS

Uninoculated mesocotyls. When exposed to light, etiolated mesocotyls gradually turned pink, but failed to develop chlorophyll or related pigments (1,2) (Figs. 3 and 4). Duke and Naylor (2) demonstrated that mesocotyls placed in constant light accumulated anthocyanins as a linear function of time. We also observed an essentially linear increase in anthocyanin content of uninoculated mesocotyls grown under alternating light and dark periods (Figs. 12–15). The rate of accumulation and final pigment concentration were different for each corn cultivar studied.

Resistant interaction. The resistant response of hybrids B73$_{Ht}$ × Va26$_{Ht}$ and Mo17$_{Ht}$ × B73$_{Ht}$ and the inbred Pr1 to *H. carbonum* race 1 developed in a consistent pattern. Unpigmented oval areas surrounded by pigment developed on mesocotyls 36–48 hr after inoculation (Fig. 8). Subsequently, the oval areas decreased in size

and anthocyanins continued to accumulate. An intense zone of red pigmentation, indicating a high concentration of anthocyanins, eventually surrounded areas of unpigmented tissue (Figs. 9–11). These restricted lesions are shown prominently in Fig. 11. The unpigmented tissue of the restricted lesion eventually became necrotic. When viewed under the microscope the necrotic area involved only a few cells. Typically, lesion length did not exceed 3 mm. The restricted lesions on the mesocotyl resembled the small chlorotic flecks typical of the resistant response of green leaves
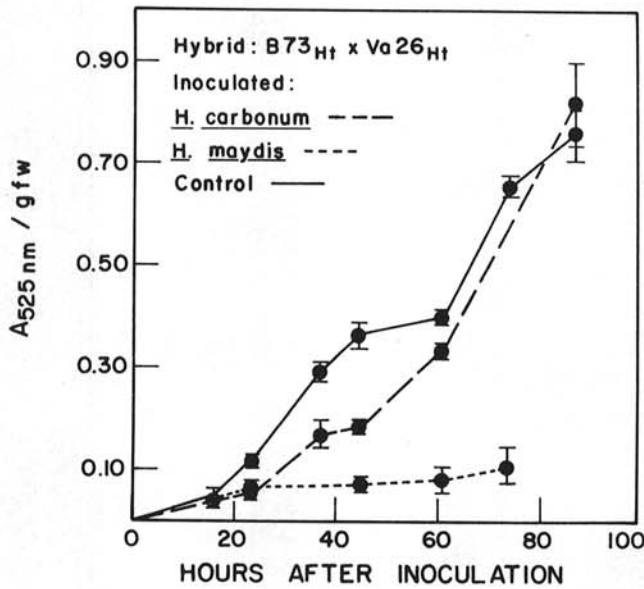

Fig. 12. Anthocyanin accumulation in etiolated maize mesocotyls (B73$_{Ht}$ × Va26$_{Ht}$). Mesocotyls inoculated with *Helminthosporium carbonum* race 1 and *H. maydis* race O represent the resistant and susceptible host-parasite combinations, respectively. Bars indicate the standard error of the mean.

Fig. 13. Anthocyanin accumulation in etiolated maize mesocotyls (Mo17$_{Ht}$ × B73$_{Ht}$). Mesocotyls inoculated with *Helminthosporium carbonum* race 1 and *H. maydis* race O represent the resistant and susceptible host-parasite combinations, respectively. Bars indicate the standard error of the mean.
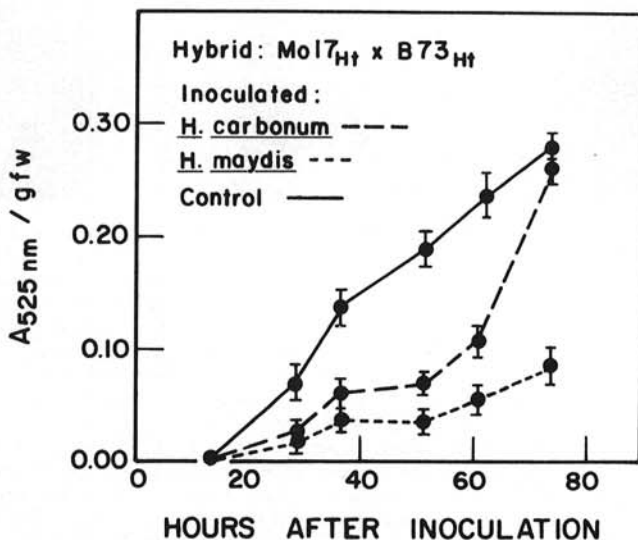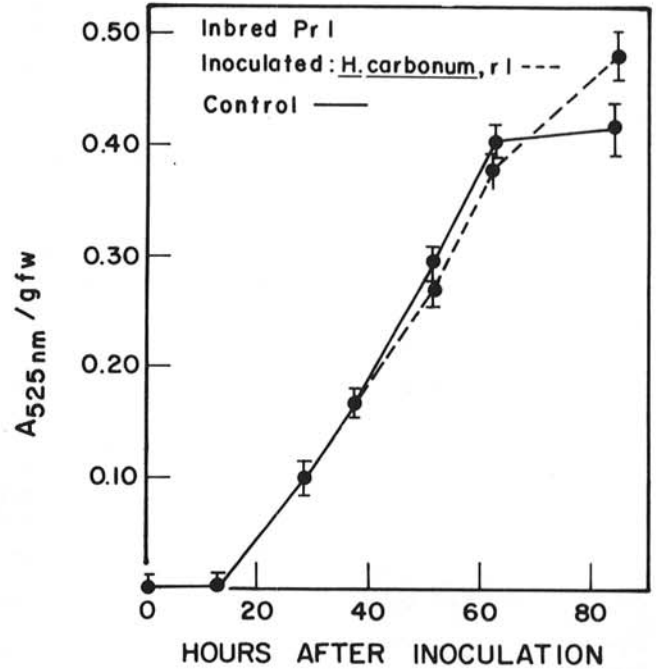

Fig. 14. Anthocyanin accumulation in the resistant interaction of etiolated maize mesocotyls of inbred Pr1 with *Helminthosporium carbonum* race 1. Bars indicate the standard error of the mean.
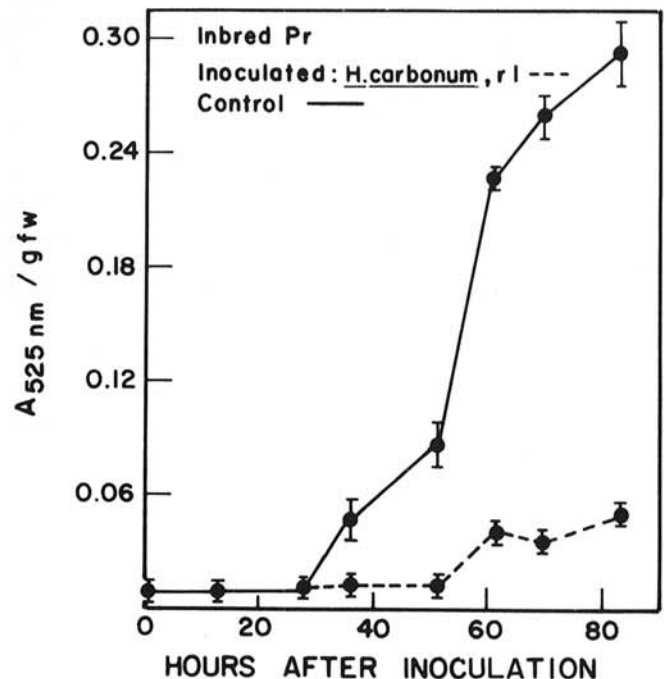

Fig. 15. Anthocyanin accumulation in the susceptible interaction of etiolated maize mesocotyls of inbred Pr with *Helminthosporium carbonum* race 1. Bars indicate the standard error of the mean.

(Fig. 2). Similar lesions and pigment accumulation were observed in mesocotyls of inbred B73$_{rhm}$ inoculated with *H. maydis* race O, a resistant response controlled by a single gene (21). Pigment accumulation around infection sites was not as intense as that observed following inoculation with *H. carbonum*.

When mesocotyls of the hybrids B73$_{Ht}$ × Va26$_{Ht}$ or Mo17$_{Ht}$ × B73$_{Ht}$ were inoculated with *H. carbonum* race 1 anthocyanins did not accumulate appreciably during the first 48 hr (Figs. 12 and 13). This initial lag was followed by a marked increase in the rate of anthocyanin development, and by 72–86 hr after inoculation the quantity of anthocyanins in the tissue had reached or exceeded the quantity in uninoculated mesocotyls (Figs. 12 and 13). The lag in anthocyanin accumulation which was evident in the above interactions was not observed in the resistant interaction of inbred Pr1 with *H. carbonum* race 1 (Fig. 14). However, the accumulation of anthocyanins after inoculation eventually exceeded that of uninoculated controls (Fig. 14).

Susceptible interaction. When mesocotyls of the hybrids B73$_{Ht}$ × Va26$_{Ht}$ and Mo17$_{Ht}$ × B73$_{Ht}$ or the inbred B73 were inoculated with *H. maydis* race O (susceptible host-pathogen interactions) lesions first appeared after about 36 hr. The slightly sunken areas enlarged and turned yellow to tan (Figs. 5 and 6). Within 72–84 hr, lesions had become necrotic and had coalesced. Eventually the entire mesocotyl shriveled. Anthocyanins did not accumulate in the susceptible interaction (Figs. 5, 6, 12, and 13). Lesion enlargement, coalescence, and necrosis resembled the lesion development that occurred in green leaves (Fig. 1). Similar symptoms, including the lack of anthocyanin pigmentation were observed in the susceptible interaction of inbred Pr with *H. carbonum* race 1 (Fig. 15).

If spores were unevenly distributed over the tissue in susceptible host-pathogen combinations, small quantities of anthocyanins sometimes accumulated. In such cases anthocyanins surrounded large, oval areas of tissue (Fig. 7) from which necrotic lesions subsequently developed.

Other resistant and susceptible interactions. Other host-pathogen combinations illustrating various genetic relationships between host and parasite were tested using the mesocotyl system. These included *H. turcicum* Pass. races 1 and 2 against the Ht1 gene for resistance to race 1 (22) and *Colletotrichum graminicola* (Ces.) Wils. against corn cultivars of differential resistance and susceptibility (15,16,20). In all cases the lesions formed on the mesocotyl demonstrated that resistance or susceptibility expressed by leaves is also expressed by mesocotyls. In resistant interactions, anthocyanin accumulation in the mesocotyl was equal to or greater than that in uninoculated tissue. In susceptible host-pathogen combinations anthocyanins did not accumulate in the mesocotyl.

Anthocyanin accumulation as a function of inoculum concentration. Inoculum concentration influenced disease development and anthocyanin accumulation in resistant and susceptible host-parasite combinations (Fig. 16). In the resistant interaction of the hybrid B73$_{Ht}$ × Va26$_{Ht}$ with *H. carbonum* race 1, inoculation with concentrations as low as 10^4 spores per milliliter resulted in an increase in anthocyanin content. But anthocyanin content was lower in tissue inoculated with a high concentration (10^6 spores per milliliter) than in uninoculated tissue. Microscopic observation of the mesocotyl tissue indicated that increasing the inoculum concentration increased the proportion of host cells killed. The same trend also was observed in green leaves inoculated with increasing spore concentrations (Fig. 17). Thus, anthocyanin accumulation decreased at high inoculum concentrations, possibly because fewer host cells were alive.

In the susceptible interaction of hybrid B73$_{Ht}$ × Va26$_{Ht}$ with *H. maydis* race O all levels of inoculum resulted in a decrease in anthocyanin content (Fig. 16). At the lowest level tested (10^3 spores per milliliter) fewer lesions developed and consequently some anthocyanins accumulated in the tissue.

DISCUSSION

The results reported here show etiolated maize mesocotyls to be a convenient tissue for the study of host-pathogen interactions. As pointed out by Keen and Horsch (13), extra caution must be

exercised if the artificially inoculated host organs are not those naturally affected in the field. Results obtained by studying such tissue can, however, be useful; for example, etiolated hypocotyls have been used successfully to investigate induced resistance and the time and site of phaseollin accumulation in the green bean (3,4,18). A criterion of our investigation was to establish that mesocotyls exhibit the same susceptibility or resistance to a pathogen found in leaf tissue. This criterion has been satisfied by demonstrating that resistant and susceptible lesion types occur on mesocotyls when leaves exhibit resistance or susceptibility, respectively (Figs. 1,2,5–11). Moreover, the expected resistance or susceptibility of mesocotyls was demonstrated in corn cultivars
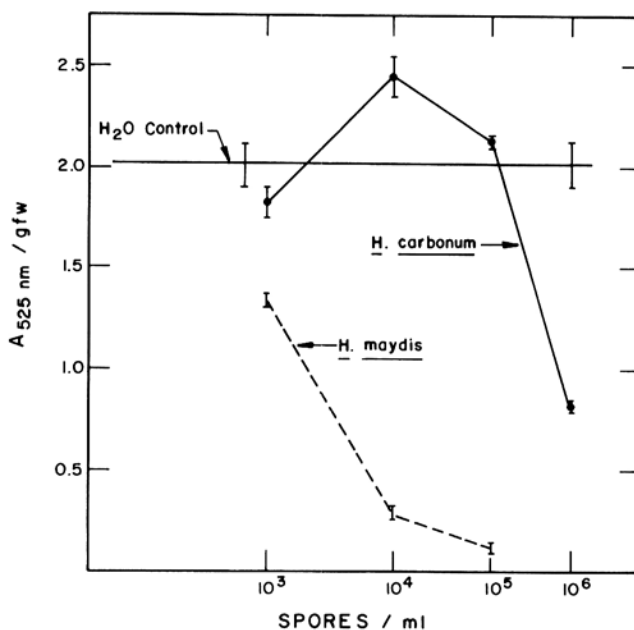

Fig. 16. Influence of inoculum concentration on anthocyanin accumulation in etiolated maize mesocotyls of the hybrid B73$_{Ht}$ × Va26$_{Ht}$. Mesocotyls were incubated for 120 hr (after inoculation) prior to extraction of anthocyanins. Mesocotyls inoculated with *Helminthosporium maydis* race O represent a susceptible interaction and those inoculated with *H. carbonum* race 1 represent a resistant interaction.
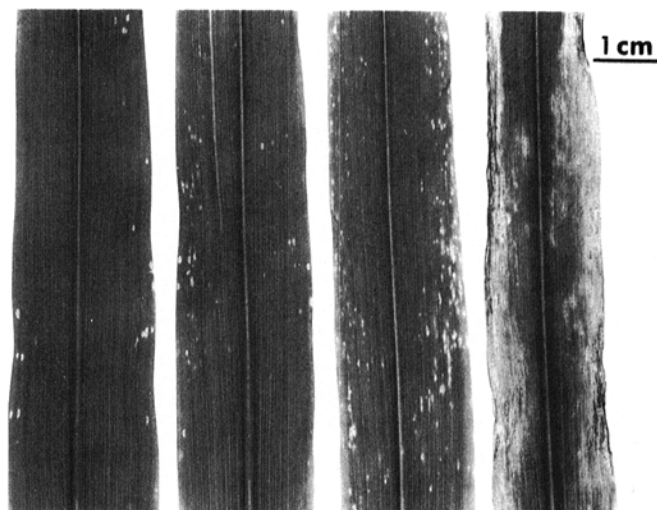

Fig. 17. Symptoms on the fourth leaf of the maize hybrid B73$_{Ht}$ × Va26$_{Ht}$ 7 days after inoculation with *Helminthosporium carbonum* race 1. From left to right inoculum concentration was 10^3, 10^4, 10^5, and 10^6 spores per milliliter, respectively. Note the extensive tissue necrosis that occurred in the leaf inoculated with 10^6 spores per milliliter.

with differential resistance or susceptibility to *H. maydis* race O, *H. carbonum* race 1, *H. turcicum* races 1 and 2, and *C. graminicola*.

The uninoculated maize mesocotyl accumulates anthocyanin pigments following exposure to light (2). In resistant host-parasite combinations anthocyanins accumulate around restricted infection sites (Fig. 11) and total pigment accumulation may be greater than in uninoculated tissues. In susceptible host-parasite combinations anthocyanin accumulation is markedly decreased (Figs. 5 and 6). *We do not suggest that anthocyanins are in any way responsible for resistance nor that they are stress metabolites*, but we have found that anthocyanin accumulation can be used as a conspicuous indicator of resistance or susceptibility.

Variables affecting growth and pigment accumulation of uninoculated mesocotyls must be considered if the anthocyanin response is to be used for the investigation of resistance and susceptibility. Appropriate periods of imbibition and seedling growth in the dark must be established for each host cultivar and maintained so that mesocotyls of consistent length and age are obtained. The rate and extent of pigment accumulation is dependent on the cultivar. For example, uninoculated mesocotyls of inbreds Pr and Pr1 accumulated less anthocyanin over the same time period than did uninoculated mesocotyls of hybrid B73$_{Ht}$ × Va26$_{Ht}$. Therefore, it is essential to establish the amount of pigment accumulated in uninoculated tissue so that in inoculated tissue resistant and susceptible reactions can be correctly evaluated.

Parameters which affect disease development in green leaves alter disease development and the anthocyanin response in mesocotyls. Environmental conditions such as photoperiod, temperature and relative humidity affect disease development. When using mesocotyls these factors can be easily regulated in a controlled environment chamber, since seedlings require only a small growth area. Disease development in both leaves and mesocotyls is also influenced by inoculum concentrations. Thus, pathogens should be tested at several inoculum concentrations to verify disease reaction type.

Some pathogens, like *H. turcicum* and *C. graminicola*, require a longer time to elicit full symptom expression than *H. maydis* or *H. carbonum*. In the susceptible interaction with such fungi more anthocyanin accumulates in the mesocotyl than in interactions with more rapid disease development, but anthocyanin accumulation is always less than that found in uninoculated controls (*unpublished*). Thus, it is necessary to allow adequate time for symptom expression before evaluating an interaction on the basis of anthocyanin accumulation.

Evidence is presented here which suggests that characteristic and predictable changes in anthocyanin accumulation accompany infection in the maize mesocotyl. The accumulation of anthocyanins around lesions in the resistant interaction in the mesocotyl is consistent with the previous observations that anthocyanins accumulate after lesion restriction in maize leaves resistant to *C. graminicola* (7,8). Similarly, anthocyanin content of the host tissue changes in diseases of apple (12), tulips (25), and snapdragon (11). Inhibition of anthocyanin accumulation in susceptible reactions has not previously been reported.

We propose that changes in pigment accumulation may be a useful tool for evaluating susceptibility and resistance in maize. Anthocyanins are phenolic pigments (6), and phenolics have often been implicated in disease resistance (5,14,23). Further studies of anthocyanin metabolism and of the specific anthocyanin response might elucidate the role of phenolics in diseases of maize.

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