Resistance of Maize to Anthracnose: Changes in Host Phenols and Pigments

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ABSTRACT

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The resistance of corn to *Colletotrichum graminicola* was studied with respect to phenol metabolism and lesion development in susceptible (Mo940), resistant (H91), and hypersensitive-resistant (33-16) inbreds. From chlorophyll content and visual observations, it was impossible to distinguish susceptible and hypersensitive-resistant responses prior to 42 hr after inoculation. These host responses could, however, be differentiated after 62 hr by chlorophyll regeneration in the hypersensitive host-pathogen combination. Host responses could also be differentiated by changes in phenol content. Total phenol content (ethyl acetate- and water-soluble phenols) of all three inbreds increased with time after inoculation. However, phenol content in the resistant and hypersensitive-resistant inbreds increased 48 hr prior to the increase detected in the susceptible inbred. At 42 hr after inoculation the ethyl acetate-soluble phenols in the resistant and hypersensitive-resistant inbreds had increased by approximately 74 and 110%, respectively, as compared to no detectable increase in those phenols in the susceptible inbred. Three phenolic compounds, M1, M2, and M3, accumulated in each of the inbreds after inoculation. These compounds accumulated earlier in the resistant and hypersensitive-resistant interactions than in the susceptible interaction. Compounds M1 and M2 were suggested to be flavonoids of the flavone group based on tests with chromogenic reagents, separation by thin-layer chromatography, and ultraviolet spectral analysis. Both M1 and M2 were fungitoxic to *C. graminicola* in spore germination and chromatographic bioassays. Compound M3, which was not distinguishable from caffeic acid, was not inhibitory to *C. graminicola* at the concentrations tested. Anthocyanin pigments accumulated in the resistant and hypersensitive-resistant hosts, but not in the susceptible host. Anthocyanins accumulated after the initial changes in total phenol content and after the increase in M1, M2, and M3. A role for phenolic metabolism in resistance of corn to *C. graminicola* is suggested, based on a correlation of lesion restriction with the accumulation of total phenolic compounds (compounds M1, M2, and M3) and the appearance of anthocyanins.

Phenolic compounds accumulate in numerous plant species following infection with plant pathogens (13, 14). Many of these compounds, or their oxidation products, are toxic to pathogenic and nonpathogenic fungi and bacteria and have been considered to be factors in disease resistance. Biochemical resistance of corn has been attributed primarily to the presence of cyclic hydroxamic acids (7, 9, 18). The level of cyclic hydroxamates present prior to infection has been correlated with the degree of resistance to *Helminthosporium turcicum* (8). It is doubtful, however, that hydroxamates are primary resistance factors in corn (7, 8). Lim et al. demonstrated the presence of two phenolic compounds in leaf diffusates (17) and water extracts (16) of corn leaves 3-12 days after inoculation with *H. turcicum*. Both compounds inhibited the germination of spores of *H. turcicum*. Similarly, after infection with *H. turcicum,* an inhibitor(s) of spore germination was demonstrated in leaf diffusates from corn lines deficient in hydroxamic acids, but possessing monogenic resistance to *H. turcicum* (7). The nature of the inhibitory factor(s) was not elucidated. Based on cross-protection studies, phytoalexin production has been suggested to account for resistance of corn to *Puccinia graminis* (15), but no specific phenolic phytoalexins have been identified or shown to accumulate in corn in response to infection.

Hammerschmidt and Nicholson (10) demonstrated that the size of anthracnose lesions on lines of corn resistant and hypersensitive-resistant to *Colletotrichum graminicola* were smaller when inoculated plants were grown under high light intensity. An accumulation of anthocyanins in the tissue surrounding lesions was associated consistently with decreased lesion size. The accumulation of anthocyanins, which are flavonoid glycosides, and the associated decrease in lesion size suggested that phenolic metabolism was involved in resistance. In this study, we have investigated the role of phenolic metabolism in the resistance of corn (*Zea mays* L.) to *Colletotrichum graminicola* (Ces.) Wils. and have demonstrated the accumulation of specific phenols associated with differential host responses to infection.

MATERIALS AND METHODS

The corn lines used were the dent inbreds Mo940, H91, and 33-16 (susceptible, resistant, and hypersensitive-resistant to *C. graminicola,* respectively). Seedlings were grown under a light intensity of approximately 37,600 lux at 22.5 C with a 12-hr photoperiod. *Colletotrichum graminicola* isolate 104 (21) was maintained on oatmeal agar in the light (3,228 lux) at 24 C. Spore suspensions were prepared as described (10) and adjusted to 1.0×10^6 spores/ml prior to inoculation. Plants were inoculated when the third leaf above the plumular leaf had fully emerged (16 days after planting). An atomizer pressurized at 0.5 atmosphere was used for inoculation and plants were incubated in a humidity chamber for 18 hr.

Extraction and determination of total phenols, anthocyanins, and chlorophylls.—Leaves were sampled for phenol or chlorophyll content by removing infection sites with a cork borer (5-mm internal diameter) at intervals after inoculation. Infection sites were identified prior to symptom expression by location of appressoria with a microscope and by observation of chlorotic flecks and lesions after symptoms were evident (21). Comparable control (noninoculated) samples were taken at the same time intervals.

Tissue for total phenol determinations was weighed and placed in 10 ml of boiling methanol per gram fresh weight (gfw) for 5 min. The methanol was decanted and the tissue was homogenized in acidified (0.1% HCl) 80% methanol, centrifuged (2,000 *g*, 5 min), and the pellet was extracted twice with acidified 80% methanol (10 ml/gfw). The methanol extracts were combined and concentrated to near dryness by flash evaporation at temperatures not exceeding 32 C. The residue was suspended in glass-distilled water (5 ml/gfw) and extracted twice with hexane (1:1, v/v). The aqueous phase was flash-evaporated to remove residual hexane, adjusted to pH 3.5, and extracted three times (1:1, v/v) with ethyl acetate. Both the ethyl acetate and water-soluble fractions were evaporated to dryness and the residue was dissolved in methanol (1 ml/gfw). Samples were stored at −20 C until assayed.

Phenol determinations were made on samples of both the water-soluble and ethyl acetate-soluble fractions after dilution with water to a concentration ratio of 10 ml/gfw. The phenolic content was determined using the Folin-Ciocalteu reagent (5) and expressed as milligrams of quercetin equivalents per gram fresh weight. A minimum of five samples were taken per time period and three determinations were made from each sample.

For anthocyanin determinations, samples of the water-soluble fractions were diluted to 100 ml/gfw with acidified (0.1% HCl) 80% methanol. Total anthocyanin content was estimated by measuring the absorbance of the diluted aliquots at 530 nm (11, 24).

Chlorophyll content was determined by homogenizing leaf disks containing infection sites in cold (−20 C) 80% acetone (50 ml/gfw). The homogenate was centrifuged at 2,000 *g* for 5 min. Chlorophyll content was calculated and expressed as mg/gfw on the basis of absorbance of the supernatant at 645 and 663 nm by the method of Arnon (2). A minimum of five samples per host were taken per time period and three determinations were made per sample.

Chromatographic analyses.—Both ethyl acetate-soluble and water-soluble extracts were analyzed by thin-layer chromatography (TLC) on silica gel G plates (E. Merck & Co., Darmstadt, Germany) which were pre-washed with chloroform:methanol (1:1, v/v). Samples equivalent to 0.1 gfw of tissue were spotted and separated using the following solvents: *n*-butanol:acetic acid:water (4:1:1, v/v, BAW); the upper phase of ethyl

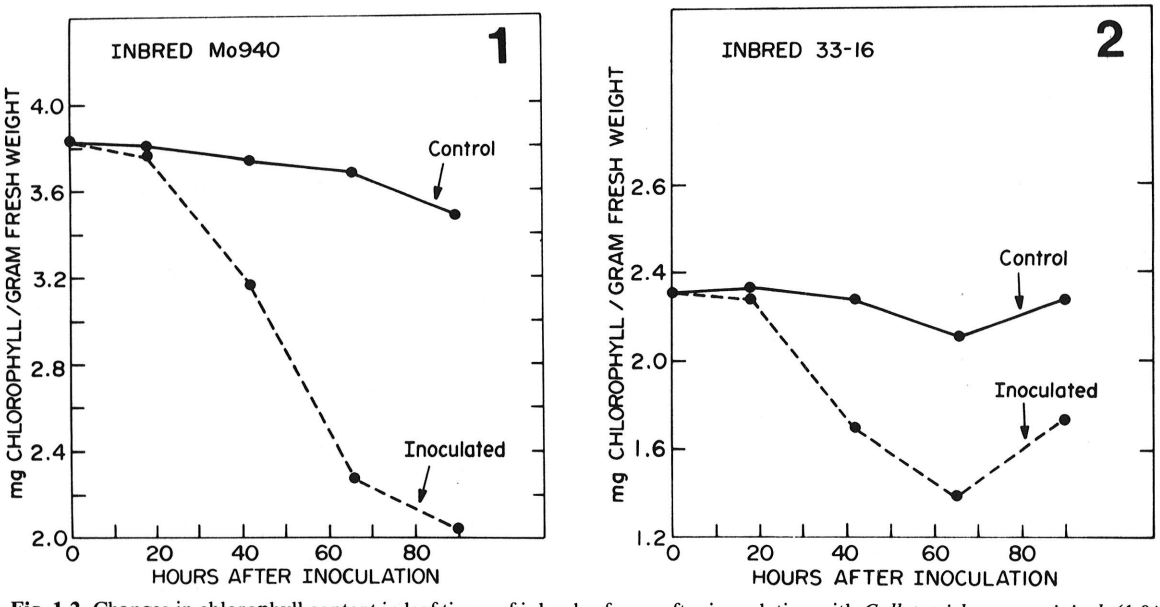
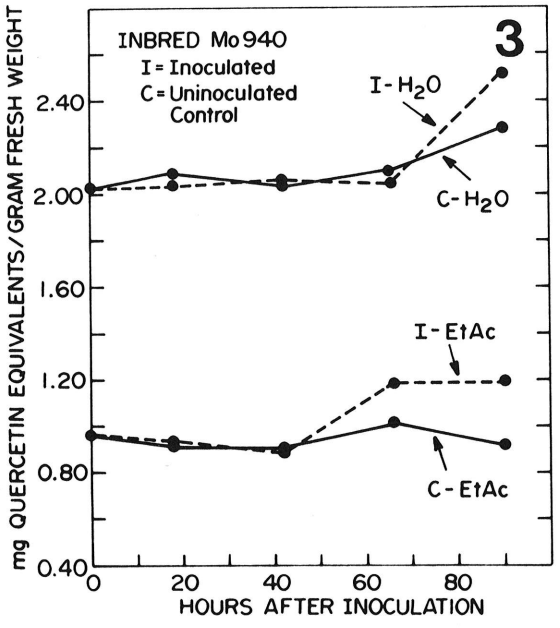


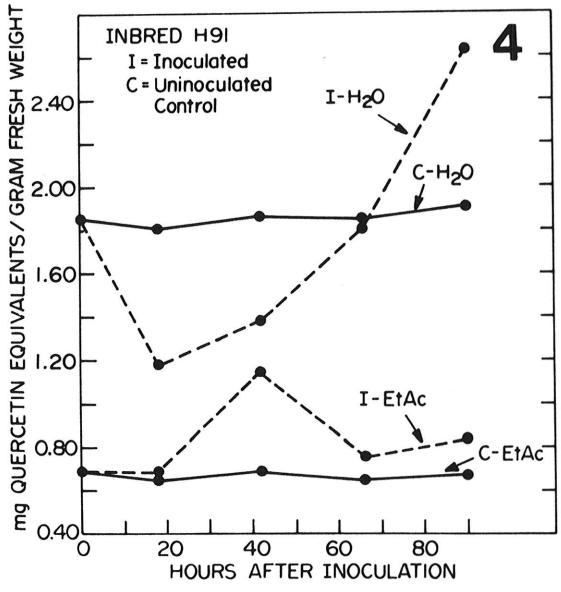
Fig. 1-2. Changes in chlorophyll content in leaf tissue of inbreds of corn after inoculation with *Colletotrichum graminicola* (1.0×10^6 spores/ml). Plants were inoculated when the third leaf above the plumular leaf had fully expanded (16 days after planting). Chlorophyll contents were measured in triplicate for each of five samples. **1)** Inbred Mo940, susceptible to *C. graminicola.* **2)** Inbred 33-16, hypersensitive-resistant to *C. graminicola.*

acetate:formic acid:water (85:6:10, v/v, EFW); the lower phase of chloroform:acetic acid:H_2O (4:1:1, v/v, CAW); benzene:dioxane:acetic acid (90:25:10, v/v, BDA). Phenols were detected under ultraviolet (UV) light (366 nm) before and after fuming the TLC plates with ammonia. After development, the TLC plates were sprayed with the following reagents for qualitative determination of phenolic compounds: (i) 1% aqueous ferric chloride (22); (ii) a freshly prepared mixture of 1% aqueous ferric chloride and 1% aqueous potassium ferricyanide (3); (iii) diazotized *p*-nitroaniline (27); (iv) vanillin-*p*-toluenesulphonic acid (23); and (v) 2,4-dinitrophenylhydrazine in 2N HCl followed by 10% aqueous NaOH (DNPH/NaOH; 6, 22).

Phenolic compounds which had increased after inoculation were located with UV light, and the silica gel containing these phenols was removed from TLC plates with a razor blade. The silica gel then was placed in a microcolumn constructed by plugging the tip end of a disposable pipette with glass wool. Phenols were eluted from the silica gel by passing 2 ml of 95% ethanol through the microcolumn. Following elution of the bands from TLC plates with ethanol, the individual eluants were rechromatographed in 70% acetone on TLC plates and located by UV spectroscopy. The accumulation of each phenol was monitored by measuring the absorption maximum of the eluant at 0, 18, 42, 66, 90, and 160 hr after inoculation.

Fungitoxicity assay.—Eluants from chromatographic analyses were tested for fungitoxicity by a modified "On-the-Chromatogram Inhibition Assay" (OCIA). The eluants were concentrated to dryness, dissolved in 95% ethanol (1 ml/gfw) and 100 μliter aliquots were applied as 0.5-0.7 cm diameter spots to Whatman 3 MM filter paper. The papers were sprayed with a spore suspension (7.5×10^5 spores/ml) of *C. graminicola* in the nutrient solution described by Allen and Kuć (1), except that sucrose was substituted for glucose. The papers were incubated on a rack in a moisture chamber for 48 hr. Fungitoxicity was ascertained by the presence of zones of inhibition which appeared as white spots surrounded by a gray background of mycelium. Eluants obtained by the same





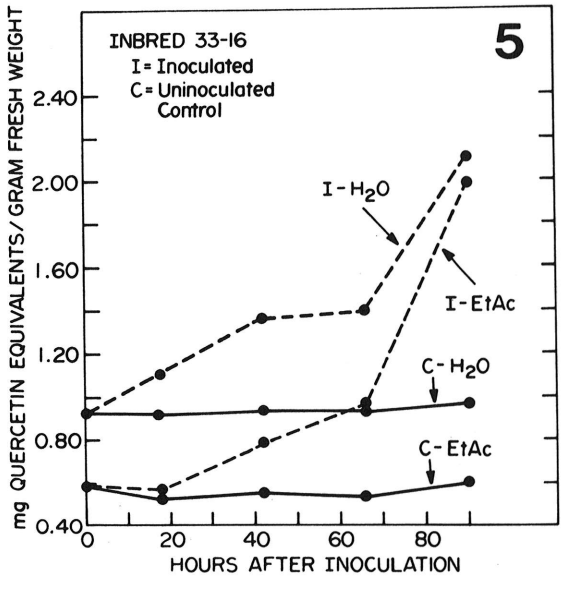


Fig. 3-5. Changes in total water-soluble and ethyl acetate-soluble phenols in leaf tissue of corn inbreds after inoculation with *Colletotrichum graminicola* (1.0×10^6 spores/ml). Plants were inoculated when the third leaf above the plumular leaf had fully expanded (16 days after planting). Phenolic content of infection sites on this leaf was measured in triplicate for each of five samples at 18, 42, 66, and 90 hours after inoculation. **3)** Inbred Mo940, susceptible to *C. graminicola*. **4)** Inbred H91, resistant to *C. graminicola*. **5)** Inbred 33-16, hypersensitive-resistant to *C. graminicola*.

method, but from noninoculated tissue, served as controls.

Fungitoxicity also was tested by spore germination assays (1×10^5 spores/ml) using eluants from TLC plates which had been concentrated to dryness and redissolved in 0.02 M (pH 6.0) phosphate buffer. Spores suspended in phosphate buffer served as controls.

RESULTS

The reaction of each corn inbred to *C. graminicola* first appeared as chlorotic flecks between 24 and 48 hr after inoculation (21). During this time, it was impossible to distinguish between resistance and susceptibility. Development of lesions beyond the chlorotic fleck stage on Mo940 (susceptible) became apparent between 50 and 66 hr after inoculation and lesions were well established by 90 hr. These susceptible-type lesions (21) continued to enlarge for 7 to 8 days after inoculation. Lesion development on inbred H91 (resistant) was similar to that on Mo940 (susceptible) except for a slower development and cessation of enlargement by 6 days after inoculation (21). Unlike Mo940, a red pigment accumulated in the healthy tissue surrounding lesions on H91 between 70 and 90 hr after inoculation (10). Lesion development on 33-16 (hypersensitive-resistant) ceased after formation of necrotic lesions between 66 and 90 hr after inoculation. These necrotic lesions were approximately the same size as the initial chlorotic flecks which formed on all three inbreds within 24 to 48 hr after inoculation. The healthy tissue surrounding lesions on 33-16 also accumulated a red pigment, but to a lesser extent than in H91.

Chlorophyll content was determined in Mo940 and 33-16 to detect differences in host response to infection. From 18 to 42 hr after inoculation, which corresponded to the appearance of chlorotic flecks, chlorophyll content decreased rapidly in both inbreds (Fig. 1, 2). Between 66 and 90 hr after inoculation the chlorophyll content in 33-16 increased, indicating the cessation of lesion development and re-establishment of chlorophyll in previously chlorotic tissue (Fig. 2). However, chlorophyll content in Mo940 (Fig. 1) had continued to decrease during this period. These changes and times correspond with the times of cessation of lesion development in 33-16 and continued lesion development in Mo940.

Change in total phenolic content.—At different times after inoculation, the pattern of changes in total phenolic content in the three corn inbreds differed quantitively for both water-soluble and ethyl acetate-soluble phenols (Fig. 3, 4, 5). The level of phenolic compounds in healthy (noninfected) tissue also differed among inbreds. The differences suggested that the initial level of phenolic compounds in healthy tissue was not related to the degree of disease resistance or susceptibility because Mo940 (susceptible) had the greatest amount and 33-16 (hypersensitive-resistant) had the least. The phenolic content did not increase in the inoculated susceptible inbred, Mo940, compared to control tissue, either in the water-soluble or ethyl acetate-soluble fractions during 42 hr after inoculation. Slight increases in ethyl acetate-soluble and water-soluble phenols were detected at 66 and 90 hr after inoculation, respectively (Fig. 3). Water-soluble phenols in the resistant inbred H91 (Fig. 4) decreased initially and then increased between 18 and 66 hr after inoculation and continued to increase through 90 hr after inoculation. Ethyl acetate-soluble phenols from H91 increased between 18 and 42 hr after inoculation and then declined and leveled off through 90 hr after inoculation. Both the water-soluble and ethyl acetate-soluble phenols in the hypersensitive-resistant inbred, 33-16 (Fig. 5), had begun to increase by 18 hr after

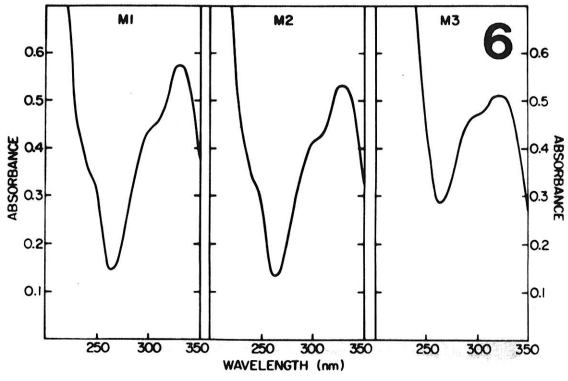


Fig. 6. Ultraviolet absorption spectra of compounds M1, M2, and M3 in 95% ethanol.

TABLE 1. Fluorescence, chromogenic spray reactions, and spectral properties of compounds M1, M2, and M3 which accumulate in corn inbreds 33-16, H91, and Mo940 after inoculation with *Colletotrichum graminicola*

Compound	UV[a]	UV/NH_3[a]	$FeCl_3$[b]	$FeCl_3$-[c] $K_3Fe(CN)_6$	DPNA[d]	VAN-PTS[e]	DPNH[f]	Absorbance[g] maxima
M1	Blue	Blue-green	Gray-green	Blue	Tan	NR[h]	Brown	328, 305s, 242s
M2	Blue	Blue-green	Gray-green	Blue	Tan	NR	Brown	327, 307s, 242s
M3	Blue	Bright-blue	Green	Blue	Tan	NR	NR	320, 300s, 242s

[a]Plates observed under ultraviolet radiation (366 nm) before and after fuming with ammonia.
[b]1% aqueous $FeCl_3$.
[c]Fresh 1:1 mixture of 1% aqueous $FeCl_3$ + 1% aqueous $K_3Fe(CN)_6$.
[d]Diazotized *p*-nitroaniline.
[e]Vanillin-*p*-toluenesulphonic acid.
[f]2,4-dinitrophenylhydrazine in 2N HCl followed by 10% NaOH.
[g]Absorbance maxima in 95% ethanol and s = shoulder or inflection.
[h]NR = No reaction.

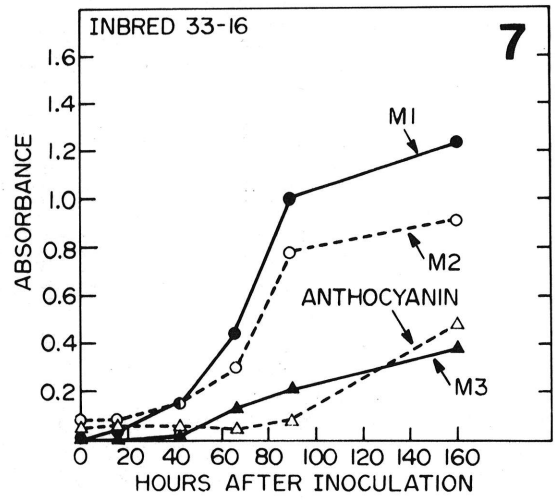
inoculation. The water-soluble and ethyl acetate-soluble phenols in 33-16 continued to increase, and the greatest increase occurred between 66 and 90 hr.

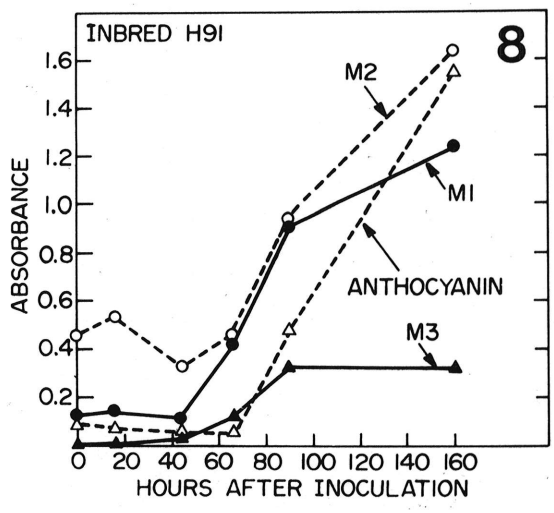
At 42 hr after inoculation, the ethyl acetate-soluble phenols in the resistant and hypersensitive-resistant inbreds had increased by approximately 74 and 110% of the noninoculated controls, respectively (Fig. 4, 5). No increase in ethyl acetate-soluble phenols was detected in the inoculated susceptible inbred at 42 hr (Fig. 3). At 90 hr after inoculation ethyl acetate- and water-soluble phenols in the susceptible inbred (Fig. 3) had increased by approximately 26 and 11%, respectively, as compared to increases of approximately 242 and 132% in the hypersensitive-resistant inbred (Fig. 5).

Accumulation of individual phenolic compounds.—The use of TLC demonstrated that three phenolic compounds in the ethyl acetate fraction had accumulated after inoculation in each of the inbreds studied. The compounds were separated using the EFW solvent. The BAW solvent did not differentiate ethyl acetate fractions from the control and inoculated samples. The compounds, referred to here as M1 (R_f 0.81), M2 (R_f 0.56), and M3 (R_f 0.99), were shown to be phenols by their reaction with several indicator reagents. The difference in colors produced with the reagents (Table 1) and in UV absorption spectra (Fig. 6) indicated that M1 and M2 may be similar in structure. None of the compounds gave a positive test with vanillin-*p*-toluenesulphonic acid which indicated that they were not catechins (23). Compounds M1 and M2 gave a positive test for a carbonyl with the DNPH/NaOH reagent (6, 22).

The only phenols in the water-soluble fractions which had accumulated in 33-16 and H91 were anthocyanins. No other phenolic compounds, including anthocyanins, had accumulated in the water-soluble fraction of Mo940.

The accumulation of M1, M2, and M3 was monitored spectrophotometrically by measuring absorption of rechromatographed eluates at the absorption maximum for each compound (Fig. 6, Table 1). Compound M1 began to accumulate in inbred 33-16 (Fig. 7) between 18 and 42 hr after inoculation, and then rapidly accumulated between 42 and 90 hr. Inbred H91 (Fig. 8) accumulated M1 rapidly between 42 and 90 hr after inoculation. Both 33-16 and H91 exhibited a further increase in M1 after 90 hr, but the rate of accumulation was less. Inbred Mo940 (Fig. 9) exhibited a slight accumulation of M1 by 66 hr,





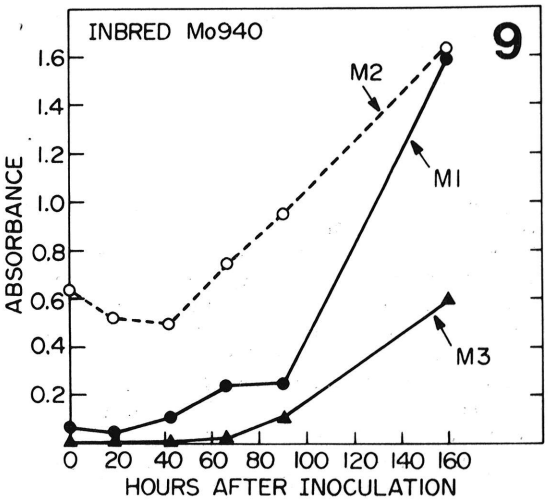


Fig. 7-9. Accumulation of compounds M1, M2, and M3 and anthocyanins in leaves of corn inbreds after inoculation with *Colletotrichum graminicola*. Relative levels of M1, M2, and M3 were determined by measuring absorbance of rechromatographed eluates from TLC plates at the absorption maximum for each compound. Compounds M1, M2, and M3 measured after separation of 0.1 gram fresh weight (gfw) equivalent of ethyl acetate-soluble fractions by thin-layer chromatography and elution of bands into 95% ethanol (2.0 ml/gfw). Anthocyanins were measured directly from water-soluble fractions after dilution with 80% acidified (0.01% HCl) methanol to a concentration ratio of 100 ml/gfw. **7)** Inbred 33-16, hypersensitive-resistant to *C. graminicola*. **8)** Inbred H91, resistant to *C. graminicola*. **9)** Inbred Mo940, susceptible to *C. graminicola*. Note the lack of accumulation of anthocyanins.

but there was no significant accumulation until after 90 hr.

There was a slight accumulation of compound M2 in 33-16 by 42 hr (Fig. 7), but the greatest rate of accumulation occurred between 66 and 90 hr. Some M2 appeared to be present in inbreds H91 and Mo940 at the time of inoculation (Fig. 8 and 9), and both inbreds exhibited an accumulation of M2 between 42 and 160 hr after inoculation.

Compound M3 began to accumulate in inbreds 33-16 and H91 (Fig. 7 and 8) between 42 and 66 hr after inoculation, but did not accumulate in Mo940 until more than 66 hr after inoculation (Fig. 9). Compound M3 was indistinguishable from a commercial preparation of caffeic acid (Sigma Chemical Co., St. Louis, MO 63178), by UV spectra, chromatography with CAW and BDA solvents, and reaction with chromogenic spray reagents (Table 1).

Anthocyanins (10) began to accumulate in both 33-16 and H91 between 66 and 90 hr after inoculation (Fig. 7 and 8), which was after the initial increases in M1, M2, and M3. Anthocyanins were not detected in Mo940 following inoculation (Fig. 9).

Fungitoxicity assays.—Both compounds M1 and M2 were fungitoxic as determined by the OCIA technique. A sample of compound M1, dissolved in 2 ml of 95% ethanol, which exhibited 0.2 absorbance units at 327 nm, completely inhibited growth of *C. graminicola* in OCIA bioassays. Compound M2 was only slightly inhibitory at 0.4 absorbance units (328 nm). The zones of inhibition were surrounded by a red-brown border after 20 hr of incubation. Compound M3 was not inhibitory at a concentration equal to that represented by 0.6 absorbance units (Fig. 9) and the fungus eventually grew over the area where the compound had been applied. The entire spot had turned a red-brown color after a 24-hr incubation period. Caffeic acid was not inhibitory to *C. graminicola* at a concentration less than 0.005 M. In spore germination assays, M1 and M2 (at the same concentrations used in the OCIA assay) completely inhibited germination whereas M3 had no effect on germination over a period of 30 hr. Approximately 50% germination and appressorium formation occurred in phosphate buffer controls over the same period. The fact that sucrose was present in the OCIA assay medium, but not in the spore germination medium, may account for the complete inhibition of germination in the presence of M2.

DISCUSSION

Haspel-Horvatovic and Horickova (12) demonstrated that the loss and subsequent regeneration of chlorophylls during disease development in powdery mildew of barley and *Monilinia laxa* on apricot could be used to distinguish resistant from susceptible host responses. In our experience (10, 21) it is impossible visually to distinguish resistant and susceptible host responses during the chlorotic fleck stage of lesion development of corn anthracnose (through 42 hr after inoculation). The results herein also demonstrated that throughout this stage of disease development, responses of the inbreds Mo940 and 33-16 could not be distinguished on the basis of chlorophyll loss (Fig. 1, 2). However, in the hypersensitive-resistant inbred, 33-16, the loss of chlorophyll ceased after 66 hr and then began to increase through 90 hr. A regeneration of chlorophylls in previously chlorotic tissue surrounding lesions suggested that cessation of lesion development in the hypersensitive reaction had begun. The chlorophyll content in Mo940 continued to decrease throughout this period. Thus, chlorophyll determinations monitored lesion development and suggested a time for cessation of lesion development in the hypersensitive response by the regeneration of chlorophyll in previously chlorotic tissue.

The pattern and time of phenol accumulation in each inbred differed. However, changes in the total phenols of the susceptible inbred Mo940 clearly were different from the changes observed in the resistant and hypersensitive-resistant inbreds H91 and 33-16. Total phenol content of inbreds 33-16 and H91, but not Mo940, changed prior to necrotic lesion formation (Fig. 3, 4, 5). Total phenols increased most rapidly in 33-16, and the increase corresponded with the formation of the necrotic hypersensitive lesion and termination of lesion development as estimated by chlorophyll determination. Inbred H91 showed an initial decrease in water-soluble phenols followed by an increase in both ethyl acetate and water-soluble phenols. Since corn contains water-soluble glycosides of several phenolic acids (4), the decrease in water-soluble phenols could represent conversion to ethyl acetate-soluble aglycones or their utilization as intermediates in subsequent phenol biosynthesis. Evidence for this is suggested by the increase in ethyl acetate-soluble phenols which immediately followed the decrease in water-soluble phenols (Fig. 4). Also, the nine-carbon phenolic acid pool (cinnamic acids) in corn apparently is utilized readily in the presence of light to form flavonoid phenols without activation of phenylalanine ammonia lyase (28). Total phenol content in the susceptible inbred Mo940 did not increase until after the beginning of lesion expansion and visible lesion necrosis. The increase in total phenols was observed first at 66 hr after inoculation–approximately 48 hr after the first changes were detected in H91 and 33-16.

Macri et al. (20) reported no differences in total phenol accumulation in the interaction of *Helminthosporium maydis* race T with N and T cytoplasms of a single corn genotype. However, their criteria for resistance and susceptibility were based on cytoplasmically inherited toxin sensitivity rather than on resistance controlled by nuclear genes. Our data demonstrate a clear difference between hypersensitive-resistant, resistant, and susceptible responses of corn to *C. graminicola* based on phenol accumulation. These differences suggest a role for phenolic metabolism in the resistance mechanism. Total phenols, however, cannot be used alone to implicate phenolic metabolism since shifts in individual components may occur that are only detectable by chromatographic analysis.

Three different compounds, designated M1, M2, and M3, accumulated in infected tissue of each of the inbreds. Comparison of TLC analyses, UV spectra, and chromogenic spray reagent tests indicated that the same compounds accumulated in each corn inbred (Table 1, Fig. 6). However, the time of accumulation of M1, M2, and M3 differed for each inbred. The compounds rapidly accumulated in 33-16 (hypersensitive-resistant) and H91

(resistant), but not in Mo940 prior to 90 hr after inoculation. Thus, the pattern of accumulation of M1, M2, and M3 coincided with the time of lesion restriction for each host.

The use of chromogenic spray reagents (Table 1) suggested the following chemical nature for compounds M1 and M2: (i) $FeCl_3$-$K_3Fe(CN)_6$ and DPNA tests gave positive phenol reactions (3, 27); (ii) the green color resulting from treatment with $FeCl_3$ suggested orthodihydroxylation (22); (iii) failure to react with vanillin-*p*-toluenesulphonic acid suggested that M1 and M2 were not catechin (flavan 3-ols) or leucoanthocyanidin (flavan 3,4-diols derivatives (23); (iv) reaction with DNPH/NaOH indicated the presence of a carbonyl group (6, 22); (v) the change from blue to blue-green fluorescence and an increase in visible yellow coloration after ammonia fuming suggested a flavonoid structure (19). These tests in conjunction with ultraviolet spectra for M1 and M2 (Fig. 6) suggest that M1 and M2 may be flavonoids of the flavone type (19). Additional evidence that M1 and M2 may be flavonoids is the rapid accumulation of anthocyanin pigments in the resistant and hypersensitive-resistant responses (Fig. 7 and 8). Anthocyanins are the end products of flavonoid biosynthesis and accumulate in tissue surrounding lesions that become more restricted under high light intensity (10, 12). Since biosynthesis of anthocyanins and other flavonoids is light-controlled (24, 25, 26, 29), flavonoid phenols may be involved in resistance to *C. graminicola*. If M1 and M2 are flavones, the accumulation of anthocyanins in resistant tissue is understandable, since an excess of toxic flavonoid precursors which accumulated during the resistance response could be diverted to such nontoxic flavonoid glycosides as anthocyanins.

The fungitoxicity of M1 and M2, and accumulation of these compounds preceding lesion restriction, suggests that M1 and M2 are involved in the resistance of corn to *C. graminicola*. The OCIA indicated that the compounds themselves are toxic to the fungus. Compound M3, which was indistinguishable from caffeic acid, did not inhibit the fungus at the concentration tested. It is doubtful that caffeic acid plays a primary role in the resistance mechanism as a fungitoxic compound since the levels present are too low. However, caffeic acid accumulation may reflect an over production of phenolic precursors used in the biosynthesis of other phenolic compounds or a replenishment of the cinnamic acid pool depleted by such metabolism. This hypothesis would appear to be supported by the data which show that total ethyl acetate-soluble phenols in 33-16 and H91 increased by approximately 242 and 132% compared to a 26% increase in the susceptible host Mo940. However, the importance of M1 and M2 as specific resistance factors relative to other phenols which accumulate can not be evaluated at this time.

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