Nutritional and Inhibitory Factors in the Resistance of Zea mays to Puccinia graminis

K. T. Leath and J. B. Rowell

Former Research Technician and Research Plant Pathologist, respectively, Crops Research Division, ARS, USDA, Cooperative Rust Laboratory, Department of Plant Pathology, University of Minnesota, St. Paul 55101.

Present address of senior author: U.S. Regional Pasture Research Laboratory, University Park, Pennsylvania 16802.

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ABSTRACT

The nature of fungal inhibition was investigated in the immune host-pathogen combination of *Zea mays* and *Puccinia graminis*. In untreated, mature leaf tissue the fungus was inhibited at the infection-hyphal stage of development and did not form haustoria. In detached tissue, in tissue from plants treated with maleic hydrazide, and in senescent tissue, fungal development was the same as in untreated, mature tissue. Treatment of seedlings with chloroform vapor before or after inoculation delayed fungal inhibition and increased fungal development, including the formation of haustoria. Development of the fungus in immature leaf tissue was similar to that which occurred in the narcotized tissue. The growth of *P. sorghi*, a corn pathogen, was inhibited in leaf tissue infiltrated before or after inoculation with cell-free exudate from germinating uredospores of *P. graminis*. The exudate from *P. graminis* did not inhibit the germination of *P. sorghi* spores in vitro. Thus, the limited growth of *P. graminis* in corn apparently results from the action of a phytoalexin rather than from a nutritional deficiency. Observations of the rust pathogens in corn tissue suggest that the phytoalexin is induced by fungal products released during uredospore germination, reaches inhibitory concentrations within 24 hr after inoculation, and is fungistatic in its action. Attempts to demonstrate a phytoalexin with the drop-diffusion method were unsuccessful. Phytopathology 60:1097-1100.

Additional key words: corn, stem rust, phytoalexin, cross-protection, immunity, narcosis.

Phytoalexins have been postulated as a mechanism of resistance of natural hosts to their rust pathogens (6). However, no evidence has been presented of the existence of this mechanism in the interaction of gramineous hosts and rust pathogens. An easily manipulated combination of a rust pathogen and a grass species having a distinctive phytoalexin response is needed to investigate the stimulation, production, and activity of phytoalexins in such host-parasite interactions.

The immune interaction between corn (*Zea mays* L.) and the stem rust fungus (*Puccinia graminis* Pers.) appears to be such a system. The development of this fungus in corn stops at the infection-hyphal stage within 24 hr after the leaf is penetrated (10). Although walls of mesophyll cells thicken in response to fungal entry of the leaf, resistance of corn to *P. graminis* does not result from structural barriers (11). Most infection hyphae cease growing prior to contact with thickened cell walls, and the fungus is inhibited in the absence of thickened walls in either narcotized or immature tissues. The observed inhibition of fungal development could result either from inadequate nutrition or the presence of inhibitory substances.

The present study examined these possibilities by investigating the effect of altered host nutrition and physiology on fungal development and by testing for the presence of fungitoxic substances with extraction and cross-inoculation methods.

Materials and Methods.—Test host tissue was obtained from dent corn seedlings (*Zea mays* L. Minhybrid 507) grown in soil under alternating 12-hr light and dark periods described previously (10, 11). Uredospores of *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn., race 15B-1, were collected from infected wheat seedlings in the greenhouse as needed for inoculum or extraction. Uredospores of an unidentified isolate of *P. sorghi* Schw. were harvested from infected corn plants in the greenhouse and stored under liquid N until needed. Corn leaf tissue was inoculated and infected by topical or injection techniques used previously (5, 10, 11).

Microscopic examinations of test tissue cleared with chloral hydrate and stained with acid fuchsin were made 48 hr after inoculation, since fungal development had ceased and host responses were evident at this time. Development of *P. graminis* was recorded as the stage at which growth ceased for an individual infection. Given in order of increasing development, these are vesicle, infection hypha, intercellular hypha, and haustorium. "Intercellular hypha" is used here to describe a hypha that grew into the intercellular spaces of mesophyll tissue to a depth of one cell or more. A modified chi sq test for comparing proportions (8) was used to test for significance.

The treatments compared in Table 1 were made as follows: 1) Maleic hydrazide: plants grown singly in polyethylene bags containing vermiculite and nutrient solution; maleic hydrazide (5 mg in 10 ml water) added to the nutrient solution 9 and 15 days after seeding; and plants inoculated 21 days after seeding. 2) Detachment: leaf segments, 7 mm^2, were maintained in a petri dish on moistened filter paper or water agar in the controlled environment chamber with the intact plants. 3) Chloroform vapor, preinoculation: a 6-hr

exposure of plants to 0.5 ml of liquid chloroform in 11.5 liters of air immediately before inoculation. 4) Chloroform vapor, postinoculation: a 16-hr exposure of plants to 0.2 ml of liquid chloroform in 11.5 liters of air 24 hr after inoculation. 5) Immature tissue: comparison of the mature, green tissue at the tips of the fifth leaf of corn seedlings excised when only 2.5 cm of the leaf was emerged from the whorl to the immature, white, or pale yellow tissue taken approximately 7.5-cm distal from its point of attachment. 6) Senescent tissue: comparison of older, naturally senesced leaves of unfertilized corn plants to mature, vigorous leaves on the same plant.

The drop-diffusate method of Müller (14) was used in attempts to demonstrate a phytoalexin in leaves, leaf sheaths, and ear husks inoculated with *P. graminis*. Diffusates were obtained at 24, 48, and 72 hr after inoculation and at temp from 1-20 C with extraction periods from 2-24 hr. Some diffusates were frozen and reduced under vacuum to one-tenth of their original volume. Fungitoxicity of the diffusates was tested by germination of uredospores of *P. graminis*, growth of hyphal mats of *P. graminis* from exposed, infected wheat tissue, and growth of hyphal fragments of *Fusarium lini* Boll. Germination tests were made by dusting spores directly onto diffusion drops, placing spores on polyethylene against diffusion drops (18), and Müller's agar-block method (15). Hyphal mats of *P. graminis* were prepared by cutting 4-mm^2 pieces from inoculated wheat leaves with the lower epidermis removed. Each leaf piece contained a single infection of *P. graminis* at the fleck stage of development, and was floated with the stripped side up on test droplets; and the development of fluffy, hyphal mats on the exposed surface was observed. Hyphae of *F. lini* were fragmented in a blender and placed with a loop directly into diffusate samples.

RESULTS.—*Effect of tissue condition on fungal development.*—Table 1 shows the extent of development and differentiation of *P. graminis* in paired comparisons between normal, mature leaf tissues and tissues altered physiologically by various treatments. Frequency of stomatal penetration by the fungus was the same in all tissue samples. Fungal development in tissues altered by maleic hydrazide or by detachment did not differ from that in untreated tissue. The physiologic effect of the maleic hydrazide treatment on the host was evident by the darker green leaves and stunted growth of the treated plants.

An increase in the development and differentiation of *P. graminis* did occur in tissues treated with chloroform either before or after inoculation. Haustoria were formed by some of the intercellular hyphae, but the development of most fungal penetrants ceased at earlier stages. The significant increase in development of intercellular hyphae in narcotized tissue indicates that the primary mechanism of fungal inhibition is suppressed by this treatment.

More haustoria and greater mycelial development occurred in immature tissue than in any other tissue tested. Nevertheless, fungal development in the immature tissue was still halted with development of more than half of the penetrations arrested prior to haustorial formation. Thus, the primary mechanism of fungal inhibition appeared weaker in the immature tissue than in mature tissue. Fungal development in senescent tissue did not differ from that in mature, nonsenescent tissue.

Attempts to demonstrate a fungitoxic substance.—Drop-diffusate tests for the presence of fungitoxic substances in various, inoculated corn tissues were unsuccessful. No reduction of *P. graminis* uredospore germination, *P. graminis* hyphal mat formation, or *F. lini* hyphal elongation occurred in diffusates from inoculated or noninoculated tissues. Concentration of diffusates to one-tenth their original volume was ineffective. Neither a pre- nor postinoculative antifungal substance was demonstrated by this method.

Attempts were made to demonstrate fungitoxicity within leaf tissue through cross-protection using a rust pathogen of corn, *P. sorghi*, as the test organism. Portions of leaves were inoculated on one surface with uredospores of *P. graminis*, then 20 hr later the same or the opposite leaf surface was inoculated with *P.*

TABLE 1. Extent of development and differentiation of *Puccinia graminis* in mature corn leaf tissue and tissues altered physiologically by various treatments

Paired treatments	No. infections observed	Stage at which fungal development ceased			
		Vesicle	Infection hypha	Intercellular hypha	Haustorium
		%	%	%	%
1) Untreated	135	7	92	2	0
Maleic hydrazide	105	12	86	1	0
2) Attached tissue	199	26	67	7	0
Detached tissue	223	29	62	9	0
3) Room atmosphere	52	12	83	6	0
Chloroform vapor, preinoculation	52	0[a]	32[a]	62[a]	6[a]
4) Room atmosphere	100	12	85	3	0
Chloroform vapor, postinoculation	111	0[a]	49[a]	49[a]	3[a]
5) Mature, green tissue	160	12	79	8	0
Immature, yellow tissue	144	2[a]	25[a]	32[a]	40[a]
6) Mature, green tissue	113	23	73	4	0
Senescent tissue	80	30	68	2	0

[a] Significant from paired treatment at 95% confidence level.

sorghi. Areas of the same leaf not previously inoculated with *P. graminis* served as the control. *P. sorghi* developed little or not at all in leaf tissue inoculated on the same surface 20 hr earlier with *P. graminis*. Physical exclusion, however, was a possibility. When *P. sorghi* was inoculated on a leaf surface opposite an earlier infection of *P. graminis*, no reduction of *P. sorghi* occurred, indicating that either no phytoalexin was produced and physical exclusion caused the observed cross-protection, or that movement of effective concentrations of a phytoalexin across the leaf did not occur.

Upon injection into the leaf whorl, cell-free water on which uredospores of *P. graminis* had germinated causes a chlorosis of emerging leaf tissue (10). Similar spore exudates were prepared and infiltrated under vacuum into 1-cm^2 leaf pieces until the pieces appeared thoroughly soaked. Control segments were infiltrated with distilled H_2O. The leaf pieces remained in either exudate or water for 2 hr at 1 C, then were placed on benzimidazole agar and inoculated with *P. sorghi*. Similar pieces of leaf tissue that were not infiltrated were also inoculated. Results of these tests are given in Table 2, showing that in exudate-infiltrated tissue no haustoria were formed. However, infiltration with distilled water was inhibitory to *P. sorghi* development to a lesser but important degree. Although the exudate might have elicited an antifungal response in the corn, the adverse effects of infiltration with water greatly obscured the results.

When *P. sorghi* colonies were allowed to develop for 24 hr before similar infiltration of the tissue, the effects of infiltration with water alone were eliminated. The effect of this treatment on the development of *P. sorghi* was determined by measuring the maximal dimension of the colony 72 hr after inoculation. The size of *P. sorghi* colonies in exudate-infiltrated tissue was approximately one-fourth that occurring in water-infiltrated or noninfiltrated tissue (Table 3). Toxicity of the spore exudate from *P. graminis* was tested by dusting uredospores of *P. sorghi* onto the exudate. Germination of *P. sorghi* uredospores was 93% on water and 90% on the exudate (800 spores counted/treatment). Germ tube appearance and length were unaffected by the exudate. Thus, the exudate from *P. graminis* was not detectably toxic to *P. sorghi*, but a marked inhibition of this corn pathogen occurred in tissues infiltrated with the exudate.

Discussion.—Mechanical, nutritional, and physiologic factors have been examined to establish the nature of the inhibition of *P. graminis* in corn. We found earlier (10, 11) that although corn mesophyll cell walls became thickened in response to invasion of leaf tissue by *P. graminis*, the thickening was not responsible for the rapid cessation of fungal development. Often hyphal growth ceased prior to contact with the thickened walls, and in immature and narcotized tissues fungal inhibition occurred in the absence of thickened walls.

The development of rust fungi is often favored by treatment of resistant hosts with maleic hydrazide (2, 13, 19) and by leaf detachment (3, 4, 19) which increase the content of carbohydrates and free amino compounds (20). Similar treatments did not alter the inhibition of *P. graminis* in corn, suggesting that nutritional factors were not inhibitory.

The mechanism limiting the development of *P. graminis* appears to be an inhibitory substance. No evidence was found of a preformed toxic constituent; instead the delayed inhibition in narcotized and immature tissues as well as the cross-protection results indicate that the inhibitory substance resembles a phytoalexin. The production of a phytoalexin by corn inoculated with *Helminthosporium turcicum* has been demonstrated (12) and Kuć (9) extracted a fungitoxic substance from corn inoculated with *H. carbonum*.

If a phytoalexin is produced in corn in response to *P. graminis*, then the greater fungal development in narcotized and immature tissues is attributable to the slower synthesis of such a material in these tissues. The production rate of other phytoalexins is known to be reduced in narcotized (16, 21) and juvenile (1, 15) tissue. Wall thickening, a synthetic response in corn, is also reduced in narcotized and immature tissues (11). Presumably, fungal growth had ceased at the time of postinoculative treatment with chloroform. Thus, the greater development of the fungus after treatment indicates the fungistatic nature of the in-

Table 3. Size of *Puccinia sorghi* colonies at 72 hr after inoculation in corn leaf tissue infiltrated 24 hr after inoculation with double-distilled water or exudate from germinating uredospores of *Puccinia graminis*

Infiltration treatment	Maximal dimension of *P. sorghi* infections[a]
	μ
None	348
Water	319
Exudate	83

[a] Data represent average of 20 measurements.

Table 2. *Puccinia sorghi* infections reaching various stages of development 48 hr after inoculation in corn leaf tissue infiltrated with double-distilled water or exudate from germinating uredospores of *Puccinia graminis* just prior to inoculation

Infiltration treatment	No. infections observed	Stage at which fungal development ceased			
		Vesicle	Infection hypha	Intercellular hypha	Haustorium
		%	%	%	%
None	137	1	1	0	99
Water	230	78	7	1	15
Exudate	234	92	8	0	0

hibition, which is the general mode of action in phytoalexin inhibition.

Inhibition of *P. sorghi* in tissue previously inoculated with *P. graminis* or infiltrated with exudate from germinating spores of *P. graminis* supports the likelihood of phytoalexin activity. It is known (7, 17) that an organism may be pathogenic to a given host because it does not induce inhibitory concentrations of phytoalexin in that host. Phytoalexins are local in their activity (6) and are readily adsorbed (15), which could account for the lack of inhibition found when opposite leaf surfaces were inoculated with *P. graminis* and *P. sorghi*. A direct toxicity of the spore exudate from *P. graminis* to the germination and germ tube development of *P. sorghi* was not demonstrated, further indicating that the cross-protection effect arose indirectly from effects of the exudate on the host. Spore exudates are known to induce phytoalexin production (6).

The inability to demonstrate phytoalexin activity with the drop-diffusion method is understandable when the requirements of the method are compared with the limitations of the corn-*P. graminis* system. Only a few stomates under each diffusion drop were penetrated by the fungus, and only a few host mesophyll cells responded per penetration. Furthermore, any phytoalexin produced would have to pass through the epidermis in order to be present in the external water drop. Thus, the small volume of source tissue, the difficult diffusion route, and the additional problems of dilution and adsorbability could have contributed to the unsuccessful attempts to detect a phytoalexin with the drop-diffusion method.

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