Nutrition during Spore Production and the Inoculum Potential of Helminthosporium maydis Race T

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ABSTRACT

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Lesion size and lesion number per unit of inoculum were used to measure the inoculum potential of *Helminthosporium maydis* Race T spores produced on various media. These included potato-dextrose agar, V-8 juice agar, and a glucose-mineral salts agar amended with different concentrations of nitrogen-containing compounds or with maize leaves. Inoculum potential was affected by both nitrogen source and concentration. Spores produced on maize leaves and the maize-leaf + mineral salts agar had a higher inoculum potential than spores produced on the other media. Spores grown on media producing high inoculum potential exhibited more rapid spore germination, higher germination percentages, and greater appressorium formation than did spores grown on media of low inoculum potential. Spore size, shape, color, and number of septations were also affected by the medium.

Additional key words: maize, southern corn leaf blight.

Since the outbreak of southern corn leaf blight in 1970 the incitant, *Helminthosporium maydis* race T, Nisikado and Miyake, has been investigated extensively. Inoculum for epidemiological studies was produced on a variety of media, including potato-dextrose agar (8, 9, 12, 13, 15, 17), V-8 juice agar (2), Garraway's medium (6), and corn leaves (5, 16, 24). Determinations were made of the effects of light, temperature, and humidity on the development of the pathogen and the disease. In all these studies, the effect of nutrition on the inoculum potential of the fungus spores was overlooked.

Nutrition, especially nitrogen nutrition, has affected the inoculum potential of several fungi. Banttari and Wilcoxson (1) found that *Phoma herbarum* West var. *medicaginis* Fckl. spores applied to plants in asparagine solution were decreased in germination percentage, number of appressoria formed, and in number of successful penetrations. Weinhold et al. (25) found a direct relationship between the asparagine and glucose concentrations in media used for inoculum culture and the inoculum potential of *Rhizoctonia solani* Kühn. Mycelium of *R. solani* produced on a medium with high concentrations of asparagine caused more disease than did mycelium produced on a low-asparagine medium. Similar relationships were found by Isaac (11) with five species of *Verticillium* Nees, by Maier (14) and Toussoun et al. (22) with *Fusarium solani* (Mart.) Appel. & Wr. f. sp. *phaseoli* (Burk.) Snyd. & Hans., and by Phillips (20) with *Fusarium roseum* f. sp. *cerealis* (Cke.) Snyd. & Hans. In all instances, the medium with a high concentration of carbon or nitrogen produced spores with the greatest inoculum potential.

The objectives of this investigation were: (i) to determine the effect of nutrition on the inoculum potential of *H. maydis* spores. (ii) to develop a medium for producing spores with high inoculum potential, and (iii) to determine the basis for any nutritionally induced change in the inoculum potential of *H. maydis* spores. A brief report of this work has been published (23).

MATERIALS AND METHODS

The isolate used in these experiments was obtained from a single lesion on an ear of maize (*Zea mays* L.) collected near Mitchellville, IA, in 1969. Kernels from this lesion were disinfested in 1.05% NaOCl, and the fungus was allowed to sporulate. Single spores were used to establish cultures.

The basic medium generally contained 1.5 g KH_2PO_4, 0.75 g $MgSO_4 \cdot 7H_2O$, 10.0 g glucose, and 20.0 g Difco Bacto-Agar in one liter of distilled water. Media were developed by adding, to one liter, different nitrogenous compounds: (i) L-asparagine, 0.4 g (LAA = low asparagine added) or 4.0 g (HAA = high asparagine added) (6); (ii) potassium nitrate, 0.64 g (LKA) or 6.4 g (HKA); (iii) ammonium chloride, 0.32 g (LNA) or 3.24 g (HNA); (iv) no nitrogen-containing compound added (NNA); and (v) ground-corn-leaf agar (GCLA), 40-45 g ground (W64A cms-T) fresh maize leaves added in place of nitrogen and glucose. The pH of each medium was adjusted to 6.0 to 6.5 with 1N NaOH or 1N HCl before addition of the agar. Difco potato-dextrose agar (PDA) and V-8 juice agar (19) also were used for spore

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production.

Sterile media, dispensed into sterile plastic petri dishes, were seeded either by mass transfers of mycelium and spores or by washing a sporulating culture with sterile distilled water and then flooding the agar media. Seeded media were placed on a laboratory bench at about 23 C with about 12 hr of light daily, then used for inoculation after two weeks. For production of spores on maize leaves (ML), detached leaves were inoculated with spores with a hand-held atomizer and placed in a moist chamber for several days; spores produced on the resulting lesions were washed from the leaves with distilled water.

Spores for inoculations were removed from the media by flooding the plates with distilled water and gently rubbing the surface with an L-shaped glass rod. Inoculum was diluted to about 50-60 spores per 0.01 ml of water and refrigerated (1-5 C) until needed (Not more than 1 hr). In several experiments not reported herein, some of the spores produced on various media were washed by centrifugation, resuspended in distilled water and compared in inoculum potential to unwashed spores. No significant difference or trends were detected and this allowed the elimination of the washing procedure.

Two-wk-old plants of the maize inbred, W64A cms-T, were inoculated quantitatively by using an aliquot inoculator attached to the side of a spore-settling, turntable tower (J. A. Browning, M. D. Simons, and G. D. Booth, *unpublished*). The pots of plants were rotated on the turntable at 20 rpm while being atomized with 0.25 ml of the spore suspension during a 9-sec period. The reservoir of inoculum was continuously agitated during inoculation. Inoculated plants were transferred immediately and arranged randomly in a mist chamber. The temperature was adjusted to 26 C, and two Northern Cool Spray Model 100 misters (Northern Electric Co., Chicago, IL 60625) were run until the chamber was moderately humid before the plants were placed inside. Temperature control on the mist chamber was turned off, and the misters were run for 16 hr. Plants were allowed to dry before placement on the greenhouse bench. Temperature rarely decreased more than 4 C during the misting procedure.

Measurement of necrotic and chlorotic lesion lengths and determination of lesion numbers were made 3 to 5 days after inoculation. The necrotic lesion length was measured parallel with the veins. The chlorotic lesion length included the necrotic lesion. Usually, 20 random lesions were measured per treatment.

Germination studies were conducted by using sections of detached W64A cms-T maize leaves, which were placed in moist chambers and inoculated by placing one drop of a spore suspension on each section. Sections were incubated in the dark at 30 C for a defined time period, followed by addition of a small amount of 1% acid fuchsin in lactophenol to each drop. Inoculated leaf sections were viewed with the aid of a compound microscope (×100). Approximately 1,000 spores were observed for germination percentage, germ tubes per spore, and appressoria formation for each treatment and time period. Morphology of the spores produced on various media also was studied by using phase-contrast optics.

Studies on the origin of the observed effects on inoculum potential were made, beginning with mass transfers from a single spore culture to GCLA and HAA. After seven days of growth, mass transfer subcultures were made to both GCLA and HAA from both media. Seedlings were inoculated with spores from the original GCLA and HAA cultures and from each of the four subcultures.

RESULTS

Comparison of spores from leaves and ground corn leaf agar (GCLA).—No significant differences in the inoculum potential were detected between spores produced from sporulating leaf lesions and those produced on GCLA. The necrotic lesion lengths incited by GCLA and maize leaf spores were 4.32 and 5.6 mm, respectively, the chlorotic lesion lengths were 9.50 and 10.38 mm, respectively, and the mean number of lesions per plant per 1,000 spores applied in the aerosol were 1.18 and 1.67, respectively. Spores from GCLA were considered equivalent to those from lesions on maize leaves.

Effect of nitrogen source on inoculum potential.—Comparisons were made in two experiments of the lengths of necrotic and chlorotic lesions incited by spores produced on the various media (Table 1). In each

TABLE 1. Lengths of *Helminthosporium maydis* Race T lesions on maize leaves incited by spores produced on various media

Medium	Nitrogen source	Amount g/liter	Lesion length (mm)[x]			
			Necrotic		Chlorotic	
			I[y]	II[z]	I	II
HAA	Asparagine	4.00	2.4 d	0.7 c	6.9 c	1.2 c
LAA	Asparagine	0.40	6.9 bc	3.1 ab	12.9 ab	7.3 ab
HKA	KNO_3	6.40	5.4 c	...	9.4 bc	...
LKA	KNO_3	0.64	8.9 ab	...	13.3 ab	...
NNA	None	...	7.8 abc	...	11.9 ab	...
HNA	NH_4Cl	3.20	...	1.7 bc	...	7.7 ab
LNA	NH_4Cl	0.32	...	2.6 ab	...	5.9 b
GCLA	Corn leaves	40-45	10.4 a	3.7 a	14.1 a	9.1 a

[x]Values in each column followed by the same letter are not significantly different ($P = 0.05$) as determined by Duncan's multiple range test.

[y]Data taken 5 days after inoculation.

[z]Data taken 3 days after inoculation.

instance in which two levels of nitrogen were compared, as HAA and LAA, spores produced on the medium containing the lesser concentration of nitrogen caused significantly larger necrotic lesions than did spores produced on media with greater nitrogen concentrations. Spores produced on LAA also incited larger chlorotic lesions than those produced on HAA. Spores produced without supplementary nitrogen (NNA) incited lesions with lengths not different from those produced on GCLA or on the lesser nitrogen media. Spores produced on GCLA consistently incited the largest lesions. Larger lesions were incited by spores produced on potassium nitrate and ammonium chloride than on asparagine (Table 1).

Nitrogen source and spore germination.—Rates of germination of spores produced on GCLA, HAA, LAA, and NNA were compared (Table 2). Spores were incubated in V-8 juice broth for the various time periods. Spores produced on GCLA and LAA germinated more rapidly and approached maximum germination sooner than those produced on HAA or NNA. Fifty-percent germination was reached at 55 min for GCLA, 62 min for LAA, and 113 min for HAA, but 50% germination was not reached for NNA during the period of the experiments.

TABLE 2. Germination of *Helminthosporium maydis* Race T spores produced on ground corn leaf agar (GCLA), low asparagine agar (LAA), high asparagine agar (HAA), and no nitrogen agar (NNA)

Time (min)	Medium			
	GCLA	LAA	HAA	NNA
45	40[a]	26	12	0
75	69	71	29	0
105	76	70	36	18
135	98	72	85	59
165	...[b]	92	90	43
195	...	93	89	36
225	...	97	93	37
255	...	...	93	39

[a]Numbers represent the percentages of spores germinated per approximately 1,000 spores in droplets of V-8 juice broth.
[b]No data taken.

TABLE 3. Lengths of lesions on maize leaves incited by *Helminthosporium maydis* Race T spores produced on four laboratory media

Medium[x]	Lesion length (mm)[y]		Number of lesions[z]
	Necrotic	Chlorotic	
PDA	1.2 c	5.1 b	5.8
V-8 agar	1.7 ab	4.6 b	0.2
HAA	1.4 bc	4.3 b	0.2
GCLA	2.0 a	8.5 a	5.9

[x]Abbreviations: PDA = potato dextrose agar; HAA = high asparagine agar; and GCLA = ground-corn-leaf agar.
[y]Data collected three days after inoculation. Values in each column followed by the same letter are not significantly different ($P = 0.05$) as determined by Duncan's multiple range test.
[z]Values represent the mean number of lesions per plant per 1,000 spores applied in the aerosol.

Inoculum potential of spores produced on common culture media.—Lesion lengths and numbers of lesions were measured on leaves that became diseased after being inoculated with spores produced on PDA, V-8 juice agar, GCLA, and HAA (Table 3). As before, spores from GCLA incited a greater number and larger lesions than did spores from HAA. The V-8 juice agar produced spores that incited lesions similar in length to those incited by GCLA spores, but spores produced on V-8 juice agar caused fewer lesions and smaller chlorotic lesions than did GCLA spores. Spores from PDA cultures produced small lesions similar in length to those caused by HAA spores, but incited many more lesions than did HAA spores.

Spore germination and appressoria formation on these media were observed at 6 and 12 hr (Table 4). Spores produced on GCLA had the highest percentage germination, the highest percentage of spores with two polar germ tubes, and the highest percentage of spores that produced appressoria.

Origin of the effect.—The inoculum potential of spores is determined primarily by the medium on which the fungus was last cultured and not prior media (Table 5). The penultimate medium, however, seemed to have a slight carry-over effect when the penultimate medium was GCLA. It appears that the effects of media on inoculum potential were truly nutritional rather than the selection of mutants.

Morphological differences.—Spores obtained from lesions on maize leaves and from GCLA fit the general description of *H. maydis* spores (21): olive-green, 10 to 17 by 30 to 115 μm, curved, tapering toward the ends, 3- to 13-septate, and germinating by polar germ tubes. Spores produced on PDA, V-8 juice agar, and particularly, HAA appeared abnormal, being more darkly pigmented, broader and shorter (GCLA 78.9 μm and HAA 62.6 μm), cigar-shaped rather than curved and tapering, germinating most often by a single germ tube (Table 4), and having significantly fewer septations on the average than did GCLA spores (GCLA 7.8 and HAA 3.9). Although the cell contents of GCLA spores stained well with 0.05% acid fuchsin in lactophenol and the cells appeared full, HAA spores had cells that stained poorly, and cells were incompletely filled or devoid of contents.

TABLE 4. Germination of and appressorium formation by *Helminthosporium maydis* Race T spores produced on various laboratory media at 6 and 12 hr

Medium[a]	Germination (%)		Spores with two germ tubes (%)		Spores that produced appressoria (%)	
	6 hr	12 hr	6 hr	12 hr	6 hr	12 hr
PDA	59[b]	64	37	35	9	17
V-8 agar	47	69	7	33	2	30
HAA	16	23	2	6	0	5
GCLA	98	99	96	98	80	95

[a]Abbreviations: PDA = potato dextrose agar; HAA = high asparagine agar; and GCLA = ground-corn-leaf agar.
[b]Approximately 1,000 spores observed on five leaf sections.

TABLE 5. Lengths of *Helminthosporium maydis* Race T lesions on maize leaves incited by and germination of spores produced on a sequence of media

Sequence of media[x]	Lesion lengths[y]		Germination[z] (%)	Spores with two germ tubes[z] (%)
	Necrotic (mm)	Chlorotic (mm)		
GCLA	4.4 ab	10.1 a	75	66
GCLA to GCLA	6.1 a	11.4 a	78	74
HAA to GCLA	6.3 a	13.5 a	89	88
HAA	1.7 c	3.1 b	21	3
HAA to HAA	1.4 c	2.9 b	20	1
GCLA to HAA	3.8 b	6.3 b	24	4

[x]Abbreviations: GCLA = ground-corn-leaf agar, and HAA = high asparagine agar.
[y]Data were collected three days after inoculation. Values in each column followed by the same letter are not significantly different ($P = 0.05$) as determined by Duncan's multiple range test.
[z]Approximately 1,000 spores observed on five leaf sections at 6 hr.

DISCUSSION

Christensen and DeVay (4) wrote that "virulence of certain pathogenic organisms may be increased or attenuated by growing them on different media." The inoculum potential of *H. maydis* was "increased or attenuated" by the nature of the culture medium. When nitrogen concentration was varied, the inoculum potential of spores produced on agar was inversely related to nitrogen concentration (Table 1). This is contrary to evidence obtained by other investigators with various plant pathogens. Weinhold et al. (25) showed that *R. solani* mycelium produced on high-asparagine medium (2.0 g/liter) was more virulent than that produced on a low-asparagine medium (0.5 g/liter). Isaac's (11) study with *Verticillium,* the findings of Maier (14) and Toussoun et al. (22) on *F. solani* f. sp. *phaseoli,* and Phillips' (20) work with *F. roseum* f. sp. *cerealis* further substantiated the generalization that increased nitrogen concentration in the culture medium increased virulence or resulted in greater inoculum potential of the pathogen. However, Banttari and Wilcoxson (1) observed that spores of *P. herbarum* var. *medicaginis* suspended in 1% L-asparagine solution caused significantly less disease on alfalfa than did spores suspended in a potato-glucose borth. The spores, however, had been produced on wheat seed.

The ground-corn-leaf agar developed for this study consistently produced spores with an inoculum potential equal to that of spores obtained from lesions on maize leaves. The ease with which spores can be obtained through production on this medium would make this method a reasonable substitute for the more involved and time-consuming method of production on maize leaves.

Spores produced on PDA and V-8 juice agar, two media commonly used in epidemiological studies (2, 8, 9, 12, 13, 15, 17), were of an intermediate inoculum potential between spores produced on the ground-corn-leaf agar and spores produced on the high asparagine agar either because they incited fewer lesions or because they caused lesions of reduced length when compared to ground-corn-leaf agar spores (Table 3). The high asparagine agar medium was developed for maximum spore production by *H. maydis* (6), but the spores were abnormal in morphology and germination and were of low inoculum potential. The reduction in percentage germination, the decrease in frequency of polar germination, and the decrease in appressoria formation associated with the high asparagine agar medium and the tested laboratory media are similar to what was reported by Banttari and Wilcoxson (1) working with *P. herbarum* var. *medicaginis*. The reduction in lesion number occurred because of the reduction in the number of successful penetrations. Decrease in lesion size resulted from the reduced rate of germination; penetration rate could be similarly retarded, thus further increasing the time required for lesion initiation.

The observed changes in the morphology of *H. maydis* spores would support the reasoning that high levels of nitrogen in the culture medium interfere with normal spore formation. In other studies, high concentrations of nitrogen decreased the number of septations in *Fusarium* sp. spores (3, 10) and reduced the length and width and changed the shape of spores of *F. roseum* f. sp. *cerealis* (20), *Phomopsis coneglanensis* Trav., and *Cytosporina ludibunda* Sacc. (18). Similarly, in this study, spore size and shape and the number of septations were affected by the culture medium. This would indicate abnormal spore development or retarded spore formation.

Often, when working with plant pathogens, research workers make the false assumption that all spores of one genotype are alike. This study, and those cited above, serve to discredit further this assumption and to reinforce Garrett's (7) concept of inoculum potential—"the energy for growth of a fungal parasite available for infection of a host at the surface of the host . . ." The highest inoculum potential usually is associated with "natural" inoculum. Because the composition of culture media can greatly affect inoculum potential, inoculum for disease studies, and particularly for epidemiological studies, should be produced on a medium known to confer high inoculum potential to the spores. This would reduce the probability that erroneous epidemiological (and etiological) conclusions may be made from inoculum produced in vitro.

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