Influence of Inoculum Composition on the Black Point Disease of Durum Wheat

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ABSTRACT

Helminthosporium sativum and *Alternaria alternata* were repeatedly isolated from black-pointed durum wheat and were found to be pathogenic by greenhouse inoculations and using 5,000 - 10,000 conidia/ml as the inoculum and a 48-hr period of high humidity following inoculation. Both organisms, either alone or in combination, caused decreased kernel weight but the decrease was more pronounced when the ratio of the pathogens in combination favored *H. sativum*. On plants inoculated first with one organism and then 24 hr later with the other, the fungus initially introduced rapidly colonized the wheat heads and restricted the isolation frequency of the second organism. The severity of black point was greatest on the middle to upper part of the spike and on the outer florets of each spikelet. The growth rate of each organism was reduced more than 40% when grown in the presence of the other organism, presumably due to competition for nutrients and not to the production of a diffusible inhibitor.

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Additional key words: epidemiology, multiple infections.

Black point disease, caused by *Helminthosporium sativum* Pammel, King & Bakke, (imperfect stage of *Cochliobolus sativus* Ito & Kurib.) has been economically important in cereal culture for many years (3, 4, 5, 7). Studies of host resistance, isolate virulence, environmental influence, and the importance of associated microorganisms have been limited, or generally restricted to field tests. In order to study the host-parasite relationship, it was first necessary to develop reliable inoculation procedures in the greenhouse. After inoculation procedures were standardized, the study of the combined effect of *H. sativum* and a second black point incitant *Alternaria alternata* (Fries) Keissler (=*A. tenuis* Nees) (10) was undertaken.

The pathological importance of several organisms as black point incitants has been considered (2, 4, 6, 8, 9, 10, 13). However, these studies were limited to observations, field tests, or inoculation tests with a single organism. To our knowledge, double inoculation tests using *H. sativum* and *A. alternata* as black point incitants, have not been reported.

MATERIALS AND METHODS.—The durum (*Triticum durum* Desf.) cultivar 'Golden Ball' (C.I. 6627) was selected because of its resistance to leaf and stem rust and susceptibility to black point. Test plants were inoculated in the greenhouse by dipping heads of the plants into suspensions of conidia of isolates of *H. sativum* or *A. alternata* which were originally obtained from naturally infected durum kernels. The awns were removed prior to inoculation in order to facilitate dipping the heads into the inoculum. Inoculations were made 8 to 14 days after anthesis with *H. sativum* and *A. alternata* tested singly or in combination (dual inoculation). In some tests, the plants were inoculated first with one pathogen and again 24 hr later with the second pathogen (dual-delayed inoculations). After each inoculation the heads were enclosed in plastic bags to maintain high humidity.

Fourteen-day-old cultures of the isolates grown on potato-dextrose agar (PDA) in petri plates were

TABLE 1. The effect of spore concentration and duration of high humidity on the development of black point on 'Golden Ball' durum wheat inoculated with *Helminthosporium sativum*

Hours of high humidity[a]	Inoculum concentration (conidia/ml)	% Black point
0	0	0
6	0	0
24	0	0
48	0	0.1
0	10,000	4
6	10,000	3
24	10,000	23
48	10,000	58
38	0	0
38	590	5
38	3,120	18
38	4,370	19
38	11,250	27
38	17,350	47
48	0	0
48	5,500	56
48	9,000	53
48	13,500	77
48	26,000	85
48	36,000	93
48	90,000	97
48	145,000	91
48	236,000	86

[a] First eight values listed are averages of four tests, 30 heads/dilution; next six values are from one test, 12 heads/dilution; final nine values are from one test, 20 heads/dilution.

flushed carefully with sterile distilled water to obtain the conidial suspensions used as inocula. Spore concentrations were determined with a hemocytometer immediately before inoculation. Since an infection rate of 50% was consistently obtained when the inoculum concentration was held at 5,000 to 10,000 conidia/ml and the inoculated heads were kept at high humidity for 48 hr after inoculation (Table 1), this was the procedure used routinely.

When mature, the heads were harvested and threshed individually in a head thresher. The severity of black point infection was determined by counting the number of discolored kernels per head. Kernel weights were determined individually. The kernels were then surface-sterilized in 0.5% sodium hypochlorite solution for 1 min and transferred to PDA to isolate and identify the causal organism.

The severity of black point associated with spikelet and floret position in a head was rated as follows: 0 = sterile floret (i.e., no kernels); 1 = healthy kernel with no discoloration; 2 = tip or crease of kernel discolored; 3 = 1/2 of kernel discolored; 4 = 3/4 of kernel discolored; 5 = kernel totally discolored and generally shriveled. The severity value was then calculated according to the formula (11): sum of all readings × 100/total number of observations (i.e., 60) multiplied by the maximum rating (i.e., 5). Spikelets were numbered from the base to the tip of each head; florets of each spikelet were labeled A, B, or C, with B as the central floret, and A and C the left and right florets, respectively. Generally, only three florets made up a spikelet; if more formed, the central floret was labeled B and data were collected from one floret on each side.

Inhibition of growth of one organism by the other was tested on Czapek's medium or PDA in petri dishes. The organisms were transferred to the dishes either simultaneously or one organism was transferred 3 days in advance of the other organism. The growth of each organism was determined by measuring for each colony the distance from the site of inoculation to the point of maximum growth in the direction of the other organism. Two tests were made; six and 10 replications per treatment were used in the first and second tests, respectively.

RESULTS.—*Isolation.*—For 166 North Dakota grain samples obtained between August 1965 and January 1966, 173 isolations were attempted from some of the discolored kernels in each sample and these yielded 46 isolates of *H. sativum*, 17 isolates of *A. alternata*, six isolates of *Fusarium* sp., 39 isolates of nonsporulating fungi, and two bacterial isolates. Sixty-three samples produced no growth. No attempt was made to identify nonsporulating isolates.

All identified and some nonidentified isolates were used to inoculate Golden Ball heads. Symptoms developed only from *H. sativum* and *A. alternata*

Fig. 1. 'Golden Ball' wheat grain: upper row infected with *Helminthosporium sativum*, center row infected with *Alternaria alternata*, and bottom row healthy.

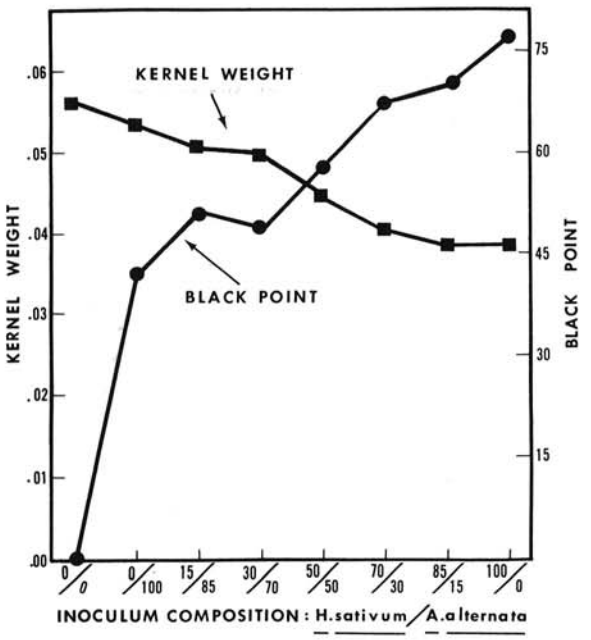

Fig. 2. Individual kernel weight and percentage black point of 'Golden Ball' durum wheat inoculated with *Helminthosporium sativum* and *Alternaria alternata* alone or in various (v/v) combinations of 10,000 conidia/ml suspensions of the two organisms.

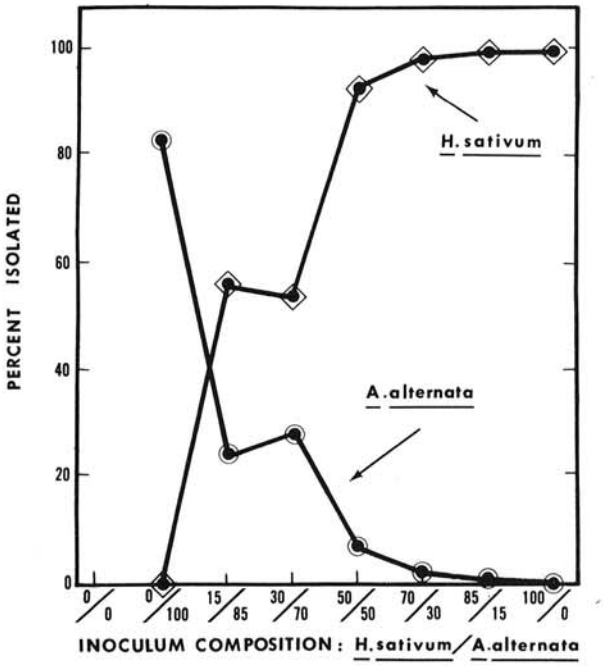

Fig. 3. Percentage of *Helminthosporium sativum* and *Alternaria alternata* isolated from 'Golden Ball' durum wheat inoculated with various (v/v) combinations of 10,000 conidia/ml suspensions of the two organisms.

inoculations. The severity of black point, as well as the color and effect on the grain, varied with many isolates. *H. sativum* isolate 15 and *A. alternata* isolate 37 were selected as standard isolates for future tests. Grain infected with isolate 15 was dark in color and shriveled, whereas grain infected with isolate 37 was less dark in color and only slightly shriveled (Fig. 1).

Dual inoculation.—Suspensions of *H. sativum* and *A. alternata* were prepared at concentrations of 10,000 conidia per ml. Durum heads were inoculated with each organism alone or with mixtures of inocula of both organisms obtained by combining different amounts of the suspensions. Ratings are expressed in ml of each suspension. Three tests using 20, 20, and 50 heads per treatment were made. The severity of black point was determined for all kernels produced, and isolations were made from 100 infected kernels selected randomly from each treatment.

The results were similar for all three tests. Kernel weight decreased and percentage black point increased as the ratio of *H. sativum* to *A. alternata* conidia was increased (Fig. 2). *A. alternata* was less frequently reisolated as the inoculum ratio of *H. sativum*/*A. alternata* rose to one (Fig. 3).

Dual-delayed inoculation.—When inoculations with the two organisms were separated by 24 hr, *A. alternata* was recovered less frequently than *H. sativum* (Fig. 4). The severity of black point was lowest and kernel weight was close to that of the noninoculated control whenever *A. alternata* was either the only inoculum, or the initial inoculum (Fig. 4, 5). As the period of high humidity was increased

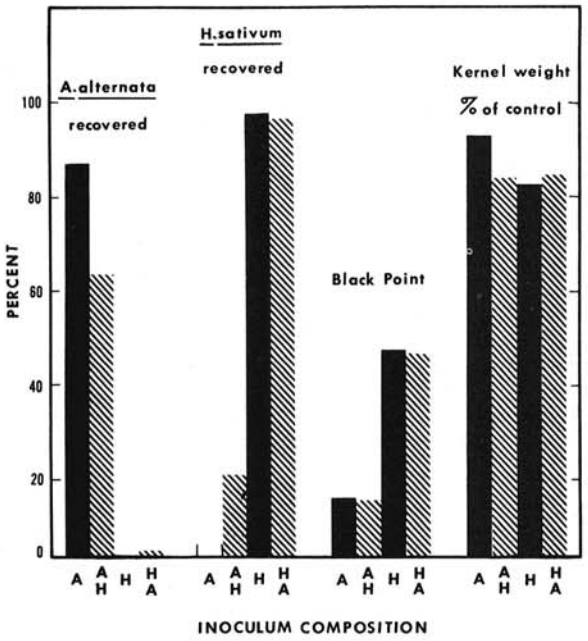

Fig. 4. Recovery of *Helminthosporium sativum* and *Alternaria alternata* from 'Golden Ball' durum and the degree of black point and concomitant kernel weight of durum inoculated with each organism individually or both organisms with a 24-hr delay between inoculations: H = *H. sativum*, A = *A. alternata*; A over H or H over A indicate a 24-hr delay between inoculations, with the top organism used as inoculum initially.

from 48 to 72 hr after inoculation, the severity of black point due to *H. sativum* increased, and kernel weight decreased (Fig. 5). The effect of humidity on black point severity and kernel weight was not as pronounced for heads inoculated singly with *A. alternata* as for those inoculated singly with *H. sativum*. When *A. alternata* was the initial inoculum, black point severity was low and kernel weight was nearer that of the water-inoculated control.

Spikelet-floret position and black point susceptibility.—Heads of Golden Ball durum inoculated with *H. sativum* were dissected 2 weeks after inoculation. Severity of black point was highest for kernels recovered from spikelets four through twelve (Table 2). The central floret of each spikelet, especially those near the base and the tip of the spike, had the lowest severity rating.

Cultural growth rate.—Each organism influenced the growth of the other in vitro (Table 3). Simultaneous inoculation with both organisms, reduced growth of either fungus from 62-65%. However, when *A. alternata* was the initial inoculum, *H. sativum* growth was reduced 44% and *A. alternata* growth was reduced 20%. When *H. sativum* was the initial inoculum *A. alternata* growth was reduced 42% and *H. sativum* growth was reduced 26%. The average colony radius of a 10-day-old culture of *A. alternata* was 28.4 mm, and that of *H. sativum* was 50.7 mm. Thus, *H. sativum* had the most rapid growth rate of the two organisms. No clear zones of inhibition developed when the organisms were cultured together. Neither organism was inhibited when agar blocks were aseptically removed 1-2 cm from the outer edge of the colony on culture plates of one organism, and placed on freshly prepared agar inoculated with the other organism.

DISCUSSION.—Although various microorganisms were isolated from black-pointed durum, *H. sativum*

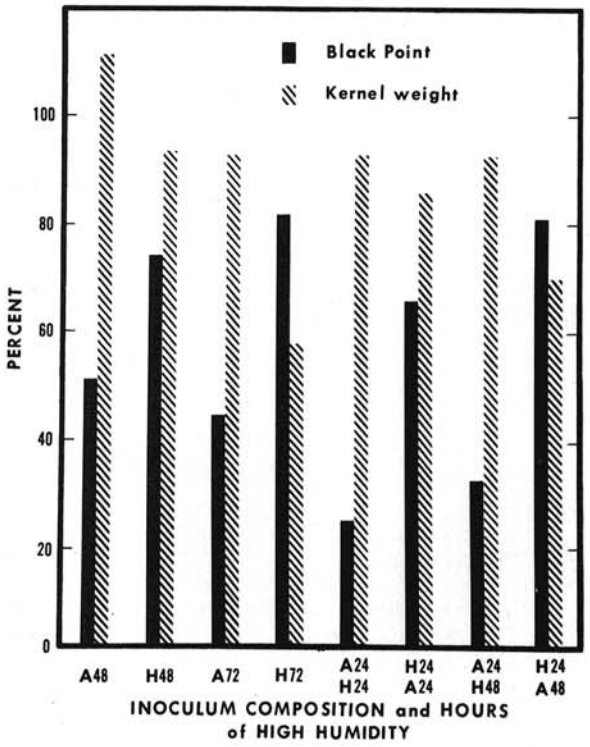

Fig. 5. The influence of inoculum composition and the duration of high relative humidity on the severity of black point and concomitant kernel weight expressed as percent of control; H = *Helminthosporium sativum*, A = *Alternaria alternata*; A over H or H over A indicates a delay between inoculations which is expressed in hours.

TABLE 2. The severity of black point of 'Golden Ball' durum caused by *Helminthosporium sativum* as influenced by spikelet and floret position on the spike

Spikelet	Severity rating[a]		
	Floret position[b]		
	A	B	C
1	1	1	2
2	12	3	12
3	32	11	32
4	47	22	47
5	77	39	69
6	72	35	73
7	81	48	83
8	85	55	88
9	84	53	90
10	84	40	86
11	80	28	85
12	77	15	77
13	70	9	69
14	56	8	63
15	37	6	35
16	10	5	11
17	0	2	5

[a] Data were collected from 60 inoculated heads; spikelets were numbered beginning with the lowest on each head. Severity was based on 0-5 rating: 0 = sterile floret; 1 = no discoloration of kernel; 2 = tip or crease of kernel discolored; 3 = 1/2 of kernel discolored; 4 = 3/4 of kernel discolored; 5 = kernel completely discolored. Severity rating was calculated according to the formula: sum of all readings × 100/60 × 5.

[b] Floret position A, B, and C refer to outer left, central, and outer right florets, respectively, in each spikelet.

TABLE 3. The inhibiting effect on growth of *Helminthosporium sativum* and *Alternaria alternata* when both organisms were transferred simultaneously to PDA or when the transfer of one organism preceded the other by 3 days

Age of culture (days)		Percent of control[a]	
Alternaria	*Helmintho-sporium*	*Alternaria*	*Helmintho-sporium*
10	10	65	62
7	10	58	74
10	7	80	56

[a] Colonies were measured along the radius from the site of inoculation to the point of most growth in the direction of the other organism. The controls consisted of colonies of each fungus grown alone.

and *A. alternata* were isolated most frequently and were found by inoculation, to cause typical black point symptoms. These two organisms are the major incitants of the black point disease in North Dakota. That conflicting reports on the relative importance of these two organisms as black point incitants have been made over the years (4, 9, 13) may be due partly to the nature and composition of the inoculum at the time the host is most susceptible.

Both organisms are capable of causing grain discoloration. Contrary to some early reports (10,12, 15) that discolored (presumably black-pointed) kernels of wheat are larger than healthy kernels, we found that when *H. sativum* predominated in a mixture of inocula, kernel size was reduced. In the field, the wet conditions under which black point is usually more severe may also result in production of larger kernels because they afford favorable growing conditions for the host. In addition, inocula may vary; with *A. alternata* tested singly as inoculum, kernel size was closer to that of the healthy kernel, whereas with *H. sativum* tested singly, kernel size was reduced. However, the fact that our tests were done in the greenhouse and involved the cultivar Golden Ball, whereas those of Machacek & Greaney (10) were done in the field and involved the 'Pentad' durum variety and sulfur-treated controls may account for the discrepancy in the results.

Natural infections in the field occur when the inocula contain both *H. sativum* and *A. alternata* (14). Isolations of these organisms may be difficult when inocula composition are varied. This was indicated by the increased difficulty of isolating either fungus, when the different inocula used in the dual inoculation tests consisted of different proportions of the two pathogens (Fig. 3, 4). Isolation methods may also favor certain organisms.

The age of kernels at inoculation is important in the severity of black point as shown by the susceptibility tests (Table 2). Central florets of each spikelet are considered to be the youngest and had the lowest severity ratings in our tests. Also, the central florets are sterile more often than lateral florets, even without inoculation. Maturation of the spikelets proceeds upwards from near the center of the spike and then toward the base. Thus, the basal spikelet was the youngest. Those spikelets near the base and the tip showed the lowest severity rating. Senescence is probably very important in the development of black point, especially when colonization occurs with a vigorous saprophytic organism. Both age of grain and relative humidity influenced colonization by *H. sativum*, a relatively virulent parasite. At the same time, colonization by *A. alternata*, a much less vigorous parasite than *H. sativum*, may have been jeopardized or at least reduced by a previous inoculation of immature grain with *H. sativum*. The difficulty of obtaining severe infection is increased then when inoculum composition favors *A. alternata*, or when *H. sativum* inoculation is preceded by *A. alternata*. The frequency of isolation of *A. alternata* when *H. sativum* was used as the initial inoculum in delayed inoculation experiments was consistently and dramatically reduced to less than 5%. The frequency of isolating *H. sativum* was reduced from 90 to 20% when it was used as the second inoculum. Rapid colonization by the initial fungus, somehow all but excludes colonization by the second fungus.

H. sativum caused at least three times more black point than *A. alternata* in single inoculations (Fig. 4). The severity of black point was not appreciably affected by inoculations with the second organism, regardless of which organism was used in the second inoculation. The disease that developed was caused by infection of the grain by the first organism.

In general, disease symptoms and severity ratings are described for infections caused by a single organism. In nature, a suscept may be attacked by many organisms. Each organism may affect the host's metabolism differently both qualitatively and quantitatively. Each attack may cause a type of biological predisposition which alters the results of subsequent infections. Symptoms and severity of a disease may differ each year due to variations in biological predisposition. In cases where apparent symptoms and severities differ, inoculation with complex inocula may be warranted in order to clarify the description of the symptoms.

Both organisms have the ability to influence the growth rate of the other organism both in culture and in grain. In simultaneous culture, Boyer (1), showed that *H. sativum* was partially or completely inhibited by *A. alternata*. We found that the growth of either organism was reduced about 35% by the second organism. Of major importance is the fact that the deterrent of growth of the second organism in vitro seems to be due to colonization ability or competition for nutrients and not to the production of diffusible inhibitors, since inhibition of one organism by the other was not demonstrated. On the other hand, though colonization ability probably is important during disease development, the in vivo production of an inhibitor is also possible.

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