Aggressiveness of *Stagonospora nodorum* Isolates Obtained from Wheat in the Northern Great Plains

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ABSTRACT

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Isolates of *Stagonospora nodorum*, obtained from diseased wheat leaves collected in fields in Montana, North Dakota, and South Dakota, were tested on detached seedling leaves of wheat and found to be pathogenic. Differences among isolates chosen at random were detected in the first phase of the inoculations. Isolates associated with severe and mild symptom severity, as measured by lesion size, were selected in phase two. Isolates associated with severe and mild symptom severity were statistically differentiated in phase three. The ability of isolates to cause significant differences in symptom severity was interpreted as differences in aggressiveness. The differences among isolates detected with detached leaf experiments was confirmed with glasshouse inoculations of seedling wheat plants. The high aggressive isolates consistently produced higher symptom severity on wheat seedlings, as measured by percentage necrosis, than low aggressive isolates. Cultivars were consistently differentiated with isolates associated with different levels of aggressiveness. With unselected isolates, the cultivar $\times$ isolate interaction was nonsignificant, indicating a lack of specificity. With high and low aggressive isolates, the cultivar $\times$ isolate interaction was significant. Considering that the magnitude of the mean squares for interactions were rather low compared to the main effects, it is speculated either that specificity is detected with the present isolates but only at a low level, or that specificity is apparent only when isolate extremes are tested. With studying isolates in phases rather than random comparisons, fungal isolates associated the high and low aggressiveness can be identified and, once identified, isolates from different hosts or geographical areas can be compared in an efficient and meaningful manner.

Additional keywords: *Leptosphaeria nodorum*, *Phaeosphaeria nodorum*, *Septoria nodorum*

Phaeosphaeria nodorum (E. Müller) Hedjaroude (= *Leptosphaeria nodorum* E. Müller), anamorph: *Stagonospora nodorum* (Berk.) Castellani et E.G. Germano (= *Septoria nodorum* (Berk.) Berk. in Berk. & Broome) is a pathogen of wheat (*Triticum aestivum* L.) and other gramineous hosts (21). The variation of this fungus has been discussed in several reviews (4-7,12,13,20). Shipton et al. (20) concluded that good evidence for physiological specialization was lacking. Similarly, King et

al. (7) suggested that resistance is non-specific and that isolates differ in aggressiveness. Johnson (6) indicated that there were no reports of stable and repeatable interactions demonstrating specificity or reports of resistant cultivars succumbing to a new race of *S. nodorum*.

Even though difference among isolates could be detected, the lack of significant cultivar-isolate interactions suggests the lack of physiological specialization (2,17,18,24). Ruffy et al. (14) found significant cultivar $\times$ isolate interactions but did not recommend classification of isolates into races. Allingham and Jackson (1) classified reactions to 282 isolates into resistance patterns, but the patterns did not conform to conventional race differentiation. Scharen and Eyal (15) did not attempt to classify pathogenicity patterns into defined physiological races after inoculating 10 winter and spring wheat cultivars with 14 different isolates. Scharen et al. (16) reported that the cultivar $\times$ isolate interaction from testing 33 isolates of *S. nodorum* on 38 wheat and triticale cultivars was nonsignificant, but with additional analyses of the interacting matrix, they hypothesized 21 different interaction genes involved in the matrix.

The present study was undertaken to investigate the variation of *S. nodorum* in the

northern Great Plains area, to examine the possibility of significant cultivar $\times$ isolate interactions, and to test a method for selecting and comparing isolates. Inoculation experiments were divided into phases to determine the variation of *S. nodorum*, rather than a random comparison of isolates. Isolates were identified that were associated with high and low levels of symptom severity, the consistency of isolate performance was tested, and isolates were compared to maximize any potential cultivar $\times$ isolate interactions.

MATERIALS AND METHODS

Isolates of *S. nodorum* were obtained from naturally infected wheat leaves collected on farms in the northern Great Plains, a semi-arid region. Sections from these leaves, about 3 cm long, were surface-sterilized for 3 min in a 1% sodium hypochlorite solution containing a surfactant, rinsed in sterile distilled water, plated on water agar in plastic petri dishes, and incubated under a 12-h photoperiod (cool-white fluorescent tubes) at 21°C. Mass transfers of cirrhi were made to V-8 juice agar (V8A; 18% V8 juice, 2% agar, and 2 g of calcium carbonate per liter; 22) with an antibiotic (gentamicin sulfate, 50 ppm) to eliminate bacterial contaminants. After sporulation, transfers from individual pycnidia were made and incubated under constant light. For long term storage, conidia from isolates were suspended by pouring a sterile 15% glycerol solution on a sporulating culture and gently rubbing the culture surface with an L-shaped glass rod. The conidial suspension was pipetted into freezer vials and maintained in a freezer at -90°C (9). To revive isolates for inoculation experiments, transfers were made directly from the vials and streaked down the center of V8A plates. The remainder of the frozen suspension was maintained in the freezer for future use.

To produce inoculum, isolates were grown on V8A in a controlled-temperature room (21°C) 30 cm below continuous fluorescent light (40W cool-white fluorescent tubes) for 7 to 14 days. Spores were harvested by flooding the surface of the plates with sterile distilled water, gently rubbing with a rubber spatula, and rinsing with water. The washings were poured into a blender, blended for 30 s, and filtered through four layers of cheesecloth.

Seven wheat cultivars, having a range of reaction to *S. nodorum*, were used in the inoculations: Butte 86 (ND597), Eureka

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(C.I. 17738), Fortuna (C.I. 13596), Kitt (C.I. 17297), Red Chief (C.I. 12109), and Olaf (C.I. 15930). Wheat seeds were planted in a peat moss-vermiculite mixture (1:1, vol/vol) to which a balanced macro- and micro-nutrient fertilizer was added. In the first phase, the plants were grown in a glasshouse; later, they were grown in a controlled environment chamber with high intensity lights and a 14-h photoperiod. Temperatures were 23°C during the light and 17°C during the dark.

Detached leaf inoculations. A laboratory technique of inoculating detached seedling leaves (3), was used which was effective in differentiating isolates of *S. nodorum* (8). Isolates were compared on different leaves of the same cultivar in the same microenvironment (petri dish) and the petri dish, as an experimental unit, was replicated four times in an incubator.

Six or seven detached seedling leaves from each of the seven cultivars, 5 cm in length, were placed adaxial side up on agar containing 150 ppm of benzimidazole in 100 mm square plastic petri dishes (15 mm depth), and inoculum from one isolate of *S. nodorum* was applied to the center of each leaf with an automatic pipette (5 µl). Inoculum concentrations of 1 to 2 × 10⁶ spores per milliliter plus a surfactant (Tween 20, polyoxyethylene sorbitan monolaurate, 2 drops/50 ml) were used. Comparable inoculum concentrations were determined within each experiment by counting spore concentrations of each isolate with a hemacytometer. Occasionally, when a spore concentration was not adequate for an individual isolate, the isolate was dropped from the experiment. Inoculated leaves were incubated at 21°C with a 12-h photoperiod. Leaves were visually rated 7 to 10 days after inoculation for

symptom severity, which was measured by lesion length and percentage necrosis.

The ability of isolates to cause significant differences in symptom severity with the detached leaf experiments, as measured by lesion length or percentage necrosis, was interpreted as differences in aggressiveness. Aggressiveness is defined as the amount of disease caused by a pathogen upon a host that contains only race nonspecific resistance to pathogenesis (19). The apparent-high aggressive isolates associated with lesions that are statistically longer and larger than lesions associated with other isolates in a particular experiment are referred to as high aggressive isolates, and the apparent-low aggressive isolates associated with the shorter and smaller lesions are referred to as low or less aggressive isolates, even though lesion length is a continuous quantitative variable.

Glasshouse inoculations. Isolates associated with large or small lesions in detached leaf inoculations were compared in glasshouse inoculations of seedling plants. Seeds of the same wheat cultivars listed above were planted in plastic containers (4 cm diameter by 21 cm depth) containing the same potting media and fertilizer as above. The containers, with three to four plants each, were used to replicate the experiments five times. Plants were grown in a glasshouse with a 12-h light and temperature cycle. Sodium vapor lamps (400W) were used to supplement the natural daylight and to maintain a 12-h light period if needed. Temperatures ranged from 24°C during the light to 13°C during the dark.

Ten days after planting, plants were inoculated by spraying a conidial suspension (2 to 3 × 10⁶ conidia per millimeter) plus a

surfactant onto the leaves with an atomizer (hand-held pressurized Sure Shot sprayer, Milwaukee Sprayer Mfg. Co., Inc., Milwaukee, WI). Comparable numbers of spores and separate sprayers were used for each isolate. To avoid cross contamination of isolates, inoculations were conducted in a fume hood that was washed with running water after each inoculation. After inoculation, all plants were maintained in a high-humidity chamber for 48 h (11) and then moved to a glasshouse bench. Ten days after inoculation, plants were clipped above the first leaf and the first leaves were assessed for percentage necrosis of the leaf blade tissue.

Experimental design. A split-plot design with five to seven isolates inoculated onto leaves of one cultivar per petri dish was used for the detached leaf inoculations. For each experiment, analyses of variance were conducted on the arcsine-transformed percentage necrosis data and the lesion length data (SAS version 6.08, SAS Institute, Cary, NC). When data from an experiment had missing values, as when an inoculation droplet did not stay at the point of inoculation but spread along the edge of the leaf, data were analyzed with a general linear model (SAS). If a distilled water control was in the experiment for comparison, the control data were not included in the statistical analysis, but the control mean for an individual experiment was included with the data presented in the tables. The statistical analyses of these experiments were used to obtain a general pattern or statistical trend for isolate and cultivar main effects, and cultivar × isolate interactions. Because the results obtained from the percentage necrosis data were similar to the results obtained with the lesion length data for the detached leaf experiments, only the lesion length data are presented. For each glasshouse inoculation experiment, an analysis of variance was conducted on the arcsine-transformed percentage necrosis data. Statistical comparisons within all experiments were made with the Student-Newman-Keuls test (SAS).

Statistical differences in symptom severity, measured by lesion length and percentage necrosis, caused by different isolates over the same period of time in the same experiment, were used to determine differences among isolates. Inoculation experiments were divided into four phases.

Phase one, initial inoculations. Five to seven isolates chosen at random were compared in individual experiments to determine if isolates could be statistically differentiated. Twenty-six isolates from regional wheat fields were compared in experiments W1 through W4. Isolates from Montana were compared in experiments W1 and W2, isolates from North Dakota and South Dakota in W3, and isolates from North Dakota in W4 (Table 1). Twenty-eight isolates from local fields at Mandan,

Table 1. Analyses of variance for lesion length on detached wheat leaves following inoculation by wheat isolates of *Stagonospora nodorum* from North Dakota, South Dakota, and Montana

Experiment	Isolate designation ^y	Phase	Source of variation ^x			
			Replicate	Cultivar	Isolate	Cultivar × isolate
W1	U	1	*	**	**	NS
W2	U	1	NS	NS	**	NS
W3	U	1	NS	**	NS	NS
W4	U	1	NS	**	**	NS
W5	L	2	NS	**	**	NS
W6	A	2	NS	**	**	NS
W7 ^z	A-L	3	**	**	**	**
W8 ^z	A-L	3	NS	**	**	NS
W9 ^z	A-L	3	NS	**	**	NS
W10	A-L	3	NS	**	**	**
W11	A-L	3	NS	NS	**	**
W12	A-L	3	NS	**	**	**
W13	A-L	3	NS	**	**	**
GH1	A-L	3	NS	**	**	**
GH2	A-L	3	NS	**	**	**

^x NS = not significant at *P* = 0.05, * = significant at *P* = 0.05, and ** = significant at *P* = 0.01.

^y U = unselected isolate; A = isolate that produced large lesions in an earlier study and considered to be highly aggressive; L = isolate that produced small lesions in an earlier study and considered to be less aggressive.

^z Comparison of one isolate that produced large lesions with another isolate that produced small lesions. Each isolate was used three times in each study.

North Dakota were compared in four experiments. The local Mandan isolates were obtained from wheat cultivars grown under various field management practices.

Phase two, selection of isolates. Regional wheat isolates that produced large or small lesions in phase one were retested to verify the consistency in the size of the lesions produced. Isolates that produced small lesions were compared in experiment W5 and those that produced large lesions were compared in W6. Because isolates that caused an intermediate level of symptom severity could not always be statistically differentiated as a distinct group, they were eliminated from further study. This retesting of isolates was necessary because average size of the lesions varied among experiments.

Because local Mandan wheat isolates were not statistically differentiated in three out of four experiments in phase one, isolates producing large and small lesions were not selected for further study.

Phase three, differentiation of isolates. Isolates associated with either large or small lesions were compared to determine if isolates were consistent, and to confirm their differences based on lesion length in the same experiment. Regional wheat isolates associated with different lesion sizes were compared in nine experiments. In the three detached leaf experiments W7 through W9, one isolate associated with large lesions was compared to one isolate associated with small lesions. Eight replicates and three cultivars, Fortuna, Eureka, and Olaf, were used. Seven leaves were inoculated in each petri dish, three leaves with one isolate, three leaves with another isolate for comparison, and one leaf with a water control. In the next four detached leaf experiments, W10 through W13, several isolates associated with large lesions were compared with isolates associated with small lesions in the same experiment with seven cultivars, four replicates, and standard procedures. Isolates were also compared on seedling leaves in glasshouse experiments GH1 and GH2.

RESULTS

Phase one, initial inoculations. All isolates of *S. nodorum* caused symptoms on wheat, but they differed in the size of the lesion they produced. When regional wheat isolates were compared, statistical differences were detected among the isolates in experiments W1 through W4 (Table 1). Variation in lesion size was evident (Table 2). Although wheat isolates associated with large and small lesions were detected in phase one, few isolates produced small lesions.

In contrast, statistical differences were detected in only one of four experiments when local wheat isolates from Mandan were tested. Even when statistical differences were detected in one experiment, five of the seven isolates were statistically

similar (data not shown). Since local Mandan wheat isolates were associated with large lesions, it was difficult to detect differences among this group of isolates.

When comparing regional and Mandan isolates in phase one, differences among wheat cultivars were statistically significant in 88% (7 out of 8) of the experiments (Table 1, regional isolates). The cultivar $\times$ isolate interactions were not significant in all eight analyses of variance (Table 1).

Phase two, selection of isolates. When regional wheat isolates associated with small lesions were compared (W5) and when isolates associated with large lesions were compared (W6), statistical differences among isolates were evident in all analyses (Table 1). When comparing isolates in phase two, differences among wheat cultivars were statistically significant in all experiments and the cultivar $\times$

isolate interaction was not significant (Table 1).

Phase three, differentiation of isolates. In the W7 through W13 wheat experiments, the wheat isolates associated with large lesions produced higher symptom severity than the isolates associated with small lesions (Tables 1 and 3).

When comparing isolates in phase three, wheat cultivars were statistically differentiated in 9 out of 10 experiments (Table 1). The cultivar $\times$ isolate interaction was significant in eight out of ten analyses of variance (Table 1).

In glasshouse inoculations, isolates associated with large lesions consistently produced higher symptom severity, as measured by percentage necrosis, than the isolates associated with small lesions (Table 4). The average percentage necrosis was 58% for the high aggressive isolates,

Table 2. Comparison of lesion length on detached leaves following inoculations with unselected isolates of *Stagonospora nodorum* from wheat^y

Experiment	Isolate	Original host	Source of isolates		Lesion length (mm) ^z
			County	State	
W1	8614-3	Wheat	McCone	MT	26 a
	8630-2	Wheat	Daniels	MT	22 ab
	8631-2	Wheat	Daniels	MT	22 ab
	8617-2	Wheat	McCone	MT	19 b
	8624-2	Wheat	Roosevelt	MT	18 b
	9076	Wheat	Richland	MT	7 c
W4	8671-2	Wheat	Mountrail	ND	14 a
	8683-2	Wheat	Burke	ND	10 b
	8718-2	Wheat	Ward	ND	10 b
	8700-2	Wheat	Bottineau	ND	9 b
	8661-2	Wheat	Williams	ND	9 b
	8694	Wheat	Renville	ND	7 b
	8563	Wheat	Mercer	ND	4 c

^y Phase one inoculations, initial inoculations with unselected isolates. Six wheat cultivars used in all studies were Butte 86, Fortuna, Kitt, Red Chief, Eureka, and Olaf.

^z Overall means within a study were compared with the Student-Newman-Keuls test; means followed by a letter in common are not significantly different at $P = 0.05$.

Table 3. Comparison of lesion length on detached leaves following inoculation with isolates of *Stagonospora nodorum* obtained from wheat^w

Experiment	Source of isolate	Isolate	Isolate designation ^x	Lesion length (mm) ^y
W7 ^z	Wheat	8683-21	A	14 a
		8683-22	A	12 b
		8683-23	A	12 b
		9076-21	L	5 c
		9076-22	L	4 c
		9076-23	L	4 c
		Control	...	1
W10	Wheat	8683-2	A	12 a
		8237-2	A	10 b
		8614-3	A	9 b
		8630-2	A	9 b
		9074	L	3 c
		8563	L	2 c
		9076	L	2 c

^w Phase three inoculations, comparison of isolates that produced large lesions with isolates that produce small lesions. Six wheat cultivars used in all studies were Butte 86, Fortuna, Kitt, Red Chief, Eureka, and Olaf.

^x A = isolates that produced large lesions in an earlier study and considered to be highly aggressive; L = isolates that produced small lesions in an earlier study and considered to be less aggressive.

^y Overall means within a study were compared with the Student-Newman-Keuls test; means followed by a letter in common are not significantly different at $P = 0.05$.

^z Comparison of one isolate that produced large lesions with another isolate that produced small lesions. Each isolate was used three times.

compared to 21% for the low aggressive isolates.

Wheat cultivars were statistically differentiated and the cultivar $\times$ isolate interaction was significant when comparing isolates (Table 1). In general, the magnitude of the mean squares for the interactions was low compared to the mean square of the main effects (Table 5), and the cultivar $\times$ isolate interactions were not strongly evident in the data.

DISCUSSION

Differences among unselected regional wheat isolates were detected in the first phase of the inoculations. Isolates associated with large and small lesions were further selected in the second phase of the inoculations. High and low aggressive isolates were consistent and were statistically differentiated in phase three of the inoculations. The differences among isolates detected with detached leaf experiments were confirmed with glasshouse inoculations of seedling wheat plants. The high aggressive isolates consistently produced higher symptom severity of wheat seedlings than low aggressive isolates. Another study of the isolates used in phase three confirmed that these isolates were standard wheat isolates of *S. nodorum* when compared with enzyme restrictions of the PCR-amplified ITS region (P. Ueng, personal communication).

Although wheat isolates associated with large and small lesions were detected, most isolates from wheat were associated with large lesions. The ability of isolates to cause significant differences in symptom severity, as measured by lesion length and percentage necrosis, was interpreted as differences in aggressiveness. Overall, differences among isolates were detected in 79% (15 out of 19) of the experiments. Excluding the local Mandan isolates, differences were detected in 93% (14 out of 15) of the experiments. The inability to detect differences among the local Mandan isolates is understandable, considering that these isolates were collected from research plots located within a radius of 2.4 kilometers; in contrast, the regional isolates were collected from a geographical semi-arid region extending over three states: Montana, North Dakota, and South Dakota. This is similar to another study with isolates of *Pyrenophora tritici-repentis* (10).

Overall, cultivars were statistically separated from one another in 89% (17 out of 19) of the experiments. This would indicate that cultivars can be consistently differentiated with a wide range of isolates associated with different levels of aggressiveness.

When comparing isolates in phase one and two, the cultivar $\times$ isolate interaction was not significant in 100% (10 out of 10)

of the experiments. According to Vanderplank (23), the lack of a significant interaction would indicate that isolates differ in aggressiveness and vary independently of the cultivars tested. This is consistent with numerous reports in the literature, when variation among isolates has been reported; strong evidence that physiological specialization of *S. nodorum* is lacking (1,2,6,14,15,17,24). When comparing isolates associated with severe and mild levels of symptom severity in phase three, the cultivar $\times$ isolate interaction was significant in 78% (7 out of 9) of the experiments. This significant interaction would be expected if some level of specificity is present among the isolates (23). This is in contrast to the nonsignificance of the cultivar $\times$ isolate interaction in the two earlier phases, even though a number of the same isolates were used. Apparently, in the selection of isolate extremes, only those associated with large and small lesions influenced the significance of the cultivar $\times$ isolate interaction. By using isolate extremes, one would expect the cultivar $\times$ isolate interactions to be at their highest levels, yet the magnitude of the mean squares for the interactions were low compared to the mean square of the main effects. One can speculate either that specificity is present with the isolates tested but only at a low level, or that specificity is apparent only when isolate extremes are

Table 4. Necrosis (percentage of first leaf area) on wheat seedlings following inoculations with isolates of *Stagonospora nodorum* from wheat^y

Isolate	Isolate designation ^z	Wheat cultivars					
		Fortuna	Eureka	Butte 86	Kitt	Olaf	Red Chief
8408	A	92	90	80	66	76	16
8630-2	A	83	80	68	51	51	15
6265	A	90	76	72	45	40	20
8683-2	A	82	60	45	38	36	12
9074	L	51	42	21	28	20	8
9076	L	23	16	10	14	14	3

^y Glasshouse inoculations of seedling plants. Each datum is the average of 10 observations, five replicates in each of two experiments, GH1 and GH2.

^z A = isolate that produced large lesions in an earlier study and considered to be highly aggressive; L = isolate that produced small lesions in an earlier study and considered to be less aggressive.

Table 5. Analyses of variance for lesion length on detached wheat leaves (W10) and for percentage necrosis of leaves on seedling plants (GH1) following inoculations by isolates of *Stagonospora nodorum*^y

Experiment	Source of isolate	Source of variation	Degrees of freedom	Mean squares
W10	Wheat	Replicate	3	16 NS
		Cultivar	5	73 **
		Error a	13	8
		Isolate	6	323 **
		Cultivar $\times$ isolate	30	18 **
		Error b	78	5
		Total	135	
GH1 ^z	Wheat	Replicate	3	0.009 NS
		Cultivar	5	1.532 **
		Error a	15	0.013
		Isolate	5	1.190 **
		Cultivar $\times$ isolate	25	0.053 **
		Error b	90	0.024
		Total	143	

^y Split plot design. NS = not significant at $P = 0.05$; * = significant at $P = 0.05$; ** = significant at $P = 0.01$. The total degrees of freedom were adjusted for missing values.

^z Analysis on arcsine-transformed percentage of necrosis data.

selected. Perhaps, because of the rather vigorous saprophytic phase of the *S. nodorum* life cycle, there is no pressure on the population for the selection of highly specific types, so the population mean does not move in that direction as it does with obligate parasites.

This variation in the significance of the interactions depending on how the isolates were compared may account for some of the reported variation among isolates. Without pre-testing of isolates to determine their level of aggressiveness, the significance of the interaction could depend on the random comparison of certain isolates. There is an advantage to studying isolates in phases, as presented above, to fully understand the range of aggressiveness present within a particular group of isolates (e.g. isolates from different regions, countries, hosts) before trying to compare isolates among groups. With such a method, the isolate extremes of a fungus can be identified and studied and, once isolate extremes are identified, isolates from different hosts or geographical areas can be compared in an efficient and meaningful manner. With defined isolates, the possibility of identifying potentially significant interactions would be maximized. Otherwise, comparisons of randomly selected isolates from different groups could give mixed results, depending on the isolates selected for comparison. Without the pre-testing of isolates, one would not know if a high or low aggressive isolate is being compared to another high or low aggressive isolate. The comparison of isolates from different groups, which are identified as to their level of aggressiveness, would allow for more efficient and manageable experiments when trying to understand the overall variability of this fungus, particularly when selecting isolates for the evaluation of germplasm.

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