Use of a Selective Medium for Isolation of *Stagonospora nodorum* from Barley Seed

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ABSTRACT

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We developed a selective medium designated SNAB (*Stagonospora nodorum* agar for barley) to improve the isolation of the barley biotype of *S. nodorum* from barley seed and to improve the accuracy of seed assays. The medium contains (per liter of deionized water) Difco potato-dextrose agar (10 g), Bacto Peptone (2 g), oxgall (1.5 g), agar (12 g), chloroneb (5 mg), cupric hydroxide (10 mg), dichloran (7.5 mg) dissolved in 20% ethanol, and CGA-449 50 WS (CIBA-GEIGY Corp.) (1 mg). It also contains chloramphenicol (3.13 mg), erythromycin (3.13 mg), tetracycline hydrochloride (12.5 mg) dissolved in 20% ethanol, neomycin sulfate (10 mg), which suppresses bacteria, and paraquat (67 μl at 29% a.i.), which reduces seed germination. Seeds were surface-sterilized and then incubated on SNAB or oxgall agar at 20 C with a 12-h photoperiod for 12 days. Recovery of the barley and wheat biotypes of *S. nodorum* was significantly higher on SNAB than on oxgall agar. Both biotypes sporulated on SNAB but not on oxgall agar. Isolation of the barley biotype was improved after seed was stored for 6 mo, probably because of a decline in other seed fungi. Both biotypes were isolated from barley seed from Maryland and North Carolina, but only the wheat biotype was isolated from seed from Arkansas. Both biotypes sporulated on and were isolated from the lemma, palea, and pericarp. Both biotypes were recovered from glumes approximately twice as often as from the caryopsis.

Stagonospora nodorum (Berk.) Castellani & E. G. Germano (=*Septoria nodorum* (Berk.) Berk. in Berk. & Broome) (teleomorph *Phaeosphaeria nodorum* (E. Müller) Hadjaroude) (=*Leptosphaeria nodorum* E. Müller) causes a leaf and glume blotch of barley (*Hordeum vulgare* L.). The disease occasionally causes economic losses in northern Europe (7,16) and is found in the southeastern United States (5,15). Earlier reports, based on a limited number of isolates of the fungus from barley in Europe, indicated that barley isolates are similar in colony morphology to those from wheat (*Triticum aestivum* L. em. Thell) but are more virulent on barley than wheat (7,11,15,16). Isolates distinctively pathogenic to barley but avirulent to wheat have been reported in the southeastern United States (5). These isolates produce characteristic violet colonies with black pycnidia that ooze conidia in a dull white cirrhus; the periphery of each colony contains white aerial mycelium. The isolates do not fluoresce under near-ultraviolet (near-UV) light on oxgall agar or other media (5). Typical isolates from wheat vary from white to pink or orange on various culture media but are never violet. They fluoresce on oxgall agar and other media under near-UV light and are pathogenic to wheat but not to barley. These two distinct forms of *S. nodorum* have been designated the wheat and barley biotypes (5). Additional characteristics that distinguish the two biotypes have been reported recently (13).

Protocols developed for the assay of *S. nodorum* from wheat seed also have been used to assay barley seed (5,6,9). High levels of seed infection on barley, as determined by colony fluorescence and presence of pycnidia, were noted during a freezing blotter assay (9). However, isolates were not tested for pathogenicity on wheat or barley. There are no other reports of the isolation of *S. nodorum* from barley seed.

We used oxgall agar to assay barley seed for *S. nodorum*, but the growth of saprophytic and other pathogenic fungi is extensive on this medium. Because an accurate assessment of the level of seed infection and longevity of seedborne pathogens is important in disease epidemiology, we developed an agar medium to improve the recovery of the barley biotype of *S. nodorum* from barley seed. During the investigation, we found that both the barley and the wheat biotypes of *S. nodorum* were recovered from barley seed. This paper presents data on the efficacy of the medium for isolating both biotypes and the use of the medium for identifying sites of infection in barley seeds and seedlings.

MATERIALS AND METHODS

Development of the medium. SNAW medium (*S. nodorum* agar for wheat), developed to improve recovery of *S. nodorum* from wheat seed (10), was superior to oxgall agar for recovery of *S. nodorum* from barley seed in preliminary trials. In vitro tests indicated that the barley biotype was more tolerant to dichloran and cupric hydroxide than the wheat biotype. Therefore, we modified the formulation of SNAW, and we altered the protocol for isolation to improve recovery of the barley biotype. Concentrations of dichloran and cupric hydroxide were increased. During development of SNAW, concentrations of the fungicides that highly inhibited *Fusarium* spp. also inhibited the wheat biotype of *S. nodorum*. However, the barley biotype was more tolerant to CGA-449 (CIBA-GEIGY Corp., Greensboro, NC), which inhibited *Fusarium* spp. Therefore, CGA-449 was added to the new formulation. Barley seed germinated within 3–4 days, which caused many seeds to lose contact with the agar surface, and recovery of *S. nodorum* was reduced. Therefore, we added paraquat to inhibit seed germination. The new formulation was designated SNAB (*S. nodorum* agar for barley). We used the following basal nutrients to support general growth and sporulation: (per liter of deionized water) potato-dextrose agar (Difco Laboratories, Detroit, MI) (10 g), Bacto Peptone (Difco) (2 g), oxgall (1.5 g), and agar (12 g). The fungicides used, which suppress growth of seedborne fungi other than *S. nodorum*, were chloroneb (5 mg), cupric hydroxide (10 mg), dichloran (7.5 mg) dissolved in 20% ethanol, and CGA-449 50 WS (1 mg). The antibiotics, which suppress bacterial growth, were chloramphenicol (3.13 mg), erythromycin (3.13 mg), tetracycline hydrochloride (12.5 mg) dissolved in 20% ethanol, and neomycin sulfate (10 mg). To inhibit germination of barley seed, paraquat (67 μl at 29% a.i.) was

included. Fungicides, antibiotics, and paraquat were added after the medium was autoclaved and cooled to 45 C. The amount of agar was less than that previously reported; the cost was lower but the results were not affected (4).

Barley seed assays. Six seed lots of barley harvested from plants with moderate to severe leaf and glume blotch symptoms at four locations in Georgia were chosen for the assay of *S. nodorum*. SNAB was compared to oxgall agar, which was developed as a partially selective medium for isolating *S. nodorum* from wheat seed. Oxgall agar was prepared as described (12), except that streptomycin sulfate (100 mg/L) and paraquat (67 μl L^{-1}) were added after the medium had been autoclaved. Four hundred seeds per seed lot were assayed on SNAB and oxgall agar in four 100-seed replicates. Seeds were assayed on each medium at harvest and after 6 mo of storage. Five of the seed lots were stored at 5 C, and the sixth, cultivar Wysor, was stored at room temperature during this period. Three seed lots were assayed on each medium at one time because of space limitations and the time required for recording data.

We conducted a second experiment to determine the prevalence of *S. nodorum* on barley seed throughout the southern United States. Two hundred seeds per lot from two seed lots from Arkansas, one from Maryland, and three from North Carolina were assayed on each medium.

All seeds were surface-sterilized in 0.5% sodium hypochlorite for 4 min, rinsed twice in sterile water, and air-dried before the assay. Ten seeds were placed in each 9-cm-diameter petri plate. Plates were placed in incubators at 20 C with a 12-h photoperiod provided by cool-white fluorescent lights (40 $\mu E \cdot m^{-2} \cdot s^{-1}$). Seeds were incubated for 12 days.

After 6 days, we examined fungal colonies for fluorescence under a near-UV light source (model UVSL-58, UV Products, Ltd., San Gabriel, CA) to distinguish the wheat biotype from the barley biotype of *S. nodorum*. Colonies of each biotype were identified by typical colony characteristics and the presence of sporulating pycnidia 7–12 days after seeds were plated. We assessed the fungal colonies visible around each seed and evaluated the amount of growth that was *S. nodorum*. If the colony around a seed was >50% *S. nodorum*, that seed was recorded as having a "clean" colony. To determine the percentage of infected seeds with clean colonies in each seed lot, the number of seeds with clean colonies was divided by the total number of infected seeds (10).

The experimental design for both experiments was a randomized complete block. Each shelf of an incubator held a replicate of each seed lot on each medium. Each experiment was conducted twice. We analyzed the results for each treatment on the two media by using PROC TTEST (SAS Institute, Cary, NC) following the arcsine-square root transformation for stabilizing variances. Data for each parameter are reported as untransformed percentages.

Location of *S. nodorum* in barley seed and symptoms on seedlings. We chose three seed lots, one each from Arkansas, Georgia, and North Carolina, to determine the location of *S. nodorum* in the seed. To facilitate the removal of the lemma and palea, 12 seeds from each lot were soaked overnight in water at 5 C. The glume tissue and caryopses were surface-sterilized in 0.5% sodium hypochlorite for 2 min and rinsed in sterile water. Glumes and caryopses from each seed were plated on SNAB. A second set of 12 glumes and caryopses were plated on SNAW. Samples were incubated, and data were recorded as in previous experiments. Barley seeds from the above seed lots were germinated at 18 C on wet blotters and observed for symptoms and signs of *S. nodorum* on coleoptiles and leaves.

RESULTS

Isolation of the wheat biotype of *S. nodorum* from seed from Georgia. The wheat biotype of *S. nodorum* was isolated from seed in all six lots of barley. Colonies of the wheat biotype on barley seed fluoresced blue-green 4–6 days after seeds were plated on SNAB; the color was similar but slightly less intense than that on oxgall agar (12). Colony characteristics and production of pycnidia with pink cirrhi were the same as on SNAW (10). Differences between the two assay dates within each of the parameters measured for the six seed lots were not significantly different. Therefore, data from the two dates were combined. SNAB significantly improved recovery ($P < 0.01$) of the wheat biotype of *S. nodorum* over that obtained on oxgall agar from all cultivars except Venus (Table 1). The incidence of seed infection was <3% on Venus.

The percentage of clean colonies of the wheat biotype also was significantly higher on SNAB than on oxgall agar for GA 535/Miller, Milton, and Wysor (Table 1). Recovery of the wheat biotype from Wysor was very low on SNAB and significantly better than oxgall agar only because the fungus was not isolated on that medium. Lack of significant improvement in the development of clean colonies on SNAB from Venus may also be attributable to the very low level of infection by the wheat biotype on this cultivar. SNAB increased the incidence of clean colonies ($P < 0.01$) from 36 to 55% compared with oxgall agar when averaged over all seed lots (Table 1).

Isolation of the barley biotype of *S. nodorum* from seeds from Georgia. The barley biotype produced dark violet colonies with sparse white aerial mycelium at the margin on SNAB. Dark pycnidia with dull white cirrhi developed in concentric rings within 6–10 days, probably in response to the alternating light and dark cycles. Colonies were nearly black when viewed from the underside and were nonfluorescent under near-UV light.

The recovery of the barley biotype on SNAB was higher at the second assay date for GA 535/Miller, Sussex, and Wysor, but similar to the incidence on the first date for the other three

TABLE 1. Comparison of SNAB and oxgall agar for isolation of the wheat biotype of *Stagonospora nodorum* from six lots of barley seed

	Colony occurrence (% of seeds assayed)[a]					
	Fluorescent colonies		Total colonies		Clean colonies (% of total)[b]	
Barley cultivar	SNAB	Oxgall	SNAB	Oxgall	SNAB	Oxgall
GA535/Miller	8.9**[c]	3.0	11.1**	3.5	55.1*	23.5
Kline/Gangbori	11.1**	4.1	12.5**	3.3	83.6	66.3
Milton	7.9**	2.4	9.4**	2.8	55.4**	17.5
Sussex	6.8**	2.6	6.3**	2.9	62.1	60.0
Venus	2.4	0.8	2.3	1.1	19.5	5.6
Wysor	0.8**	0	1.3**	0	37.5**	0
Mean[d]	6.3**	2.1	7.1**	2.3	55.2**	36.6

[a] Each value is the mean of two assays of 400 seeds on each medium. Colony fluorescence was measured after 6 days of incubation.
[b] Calculated from the number of infected seeds with colonies that are ≥50% *S. nodorum* divided by the total number of seeds with *S. nodorum*.
[c] Within each pair of treatments, * = significant at $P \geq 0.05$ or ** = significant at $P \geq 0.01$, according to the unpaired Student's t test after arcsine-square root transformation of the data.
[d] Mean of six seed lots.

seed lots (Table 2). Recovery on oxgall agar was higher on the second date for all seed lots except Venus. Because these date-related differences were significant, data for each assay date are reported separately. Incidence of the barley biotype of *S. nodorum* from each of the six seed lots was significantly higher on SNAB than on oxgall agar on all assay dates except the second assay from Milton (Table 2). When results were averaged over all seed lots, the barley biotype was recovered at a higher percentage ($P < 0.01$ and $P < 0.05$) on SNAB than on oxgall agar on each of the two assay dates. Overall, the barley biotype was identified from 23.4% of seeds on SNAB compared with 12.8% on oxgall agar ($P < 0.01$).

When data were analyzed by individual seed lot on each assay date, the percentage of clean colonies on both media was similar for four of the six seed lots. The percentage of clean colonies was significantly higher on SNAB than oxgall agar only for Milton on the first assay date and for Wysor on the second date (Table 2). However, when data were combined over all seed lots for the second assay date and over both assay dates (Table 2), there was a greater number ($P < 0.05$) of clean colonies of the barley biotype on SNAB than on oxgall agar.

TABLE 2. Comparison of SNAB and oxgall agar for isolation of the barley biotype of *Stagonospora nodorum* from six lots of barley seed

		Total colonies (% of seeds assayed)[a]		Clean colonies (% of total)[b]	
Barley cultivar	Assay	SNAB	Oxgall	SNAB	Oxgall
GA535/Miller	1	10.8**[c]	4.3	43.8	53.8
	2	27.4*	13.8	45.7	37.6
Kline/Gangbori	1	24.3*	7.3	67.1	68.8
	2	27.3**	13.5	72.1	42.3
Milton	1	17.8**	4.5	36.8**	0
	2	19.0	14.3	67.9	40.6
Sussex	1	16.8*	6.3	43.1	63.8
	2	30.5*	17.5	45.2	31.5
Venus	1	10.0*	3.0	44.4	64.6
	2	11.3**	4.3	31.3	12.5
Wysor	1	20.0**	9.5	49.6	14.9
	2	65.5*	55.8	85.8**	71.4
Mean, first assay		16.6**	5.8	47.5	44.3
Mean, second assay		30.1*	19.8	58.0**	39.3
Mean, both assays		23.4**	12.8	52.7*	41.8

[a] Four hundred seeds were assayed on each date for each seed lot and medium.
[b] Calculated from the number of infected seeds with colonies that are ≥50% *S. nodorum* divided by the total number of seeds with *S. nodorum*.
[c] Within each barley selection and assay date, differences between media are * = significant at $P \geq 0.05$ or ** = significant at $P \geq 0.01$, according to the unpaired Student's t test after arcsine-square root transformation of the data.

Isolation of *S. nodorum* from seeds from Arkansas, Maryland, and North Carolina. The wheat biotype was isolated from seeds of six lots (Table 3). Recovery on SNAB was significantly higher ($P < 0.01$) than on oxgall agar from four seed lots. The barley biotype was isolated from all three seed lots from North Carolina (Table 3). The barley biotype was not recovered from seed lots from Arkansas and was recovered from only two seed lots from Maryland on each medium. Recovery on SNAB was higher ($P < 0.01$) for two of the three seed lots from North Carolina and for the mean for these three lots. The percentage of clean colonies of the wheat biotype was higher ($P < 0.05$) on SNAB, but the percentage of clean colonies of the barley biotype was higher on oxgall agar. There were more clean colonies of the barley biotype than the wheat biotype on both media.

After each assay, susceptible cultivars of wheat and barley were inoculated with representative isolates of both biotypes; isolates were chosen on the basis of colony morphology and fluorescence. Typical pathogenicity patterns were observed for each biotype of *S. nodorum* (i.e., wheat biotypes caused lesions on wheat but no symptoms on barley, and barley biotypes produced lesions on barley but not on wheat).

Location of *S. nodorum* in the seed. Both biotypes were isolated from glume tissue and the caryopsis (Table 4). *S. nodorum* produced abundant pycnidia in all parts of the lemma and palea. In some cases, pycnidia were more dense near the emerging shoot than elsewhere. On the caryopsis, pycnidia always were seen in the pericarp tissue and at the base of the emerging coleoptile. No pycnidia were seen in the endosperm or embryo. Pycnidia of the barley biotype were more abundant than those of the wheat biotype on the caryopsis. Both biotypes produced pycnidia around glumes and caryopses in the agar of both media. Both biotypes were recovered about twice as often on SNAB than on SNAW from all seed lots.

Symptoms on seedlings. Brown superficial streaks or irregularly oval lesions occurred on coleoptiles. Brown lesions were most common on the base. Some coleoptiles were shortened, but no knobs or distortions were observed as noted on some wheat cultivars (2). Pycnidia of both biotypes developed profusely on the lemma and palea 8–12 days after seeds were placed in moist chambers. On some seeds, both biotypes could be identified by the color of the pycnidia and the pink cirrhus of the wheat biotype or by the dull white cirrhus of the barley biotype. Pycnidia were not seen on coleoptiles or primary leaves 20 days after plating. Pycnidia of the barley biotype developed on coleptile tissue plated on SNAB.

DISCUSSION

Improved selectivity of media for isolation of *S. nodorum* from wheat and barley seeds increases our understanding of the role

TABLE 3. Isolation of the wheat and barley biotypes of *Stagonospora nodorum* on SNAB and oxgall agar from barley seed collected at three locations in the southern United States

		Wheat biotype				Barley biotype			
		Seeds with colonies (%)[a]		Clean colonies (% of total)[b]		Seeds with colonies (%)		Clean colonies (% of total)	
Barley cultivar	Source[c]	SNAB	Oxgall	SNAB	Oxgall	SNAB	Oxgall	SNAB	Oxgall
SC830366	AR	48.8[d]	40.8	39.9**	11.8	0	0	...	...
VA89-42-9	AR	55.3**	28.5	35.4**	10.8	0	0	...	...
Sussex	MD	4.8	3.0	10.5	16.7	0.5	0.5	0	0
Anson	NC	10.8**	4.3	69.8	58.8	33.0**	21.3	68.7	84.7*
MD 72025	NC	15.5**	8.5	67.2	51.0	48.3**	31.8	63.9	76.1**
Milton	NC	28.5**	17.5	73.2	69.5	51.0	46.5	77.8	82.9
Mean[e]		27.3**	17.1	47.5**	26.6	44.1**	33.2	70.2	81.2**

[a] Each value is the mean of two assays of 200 seeds on each medium.
[b] Calculated from the number of infected seeds with colonies that are ≥50% *S. nodorum* divided by the total number of seeds with *S. nodorum*.
[c] AR = Arkansas; MD = Maryland; NC = North Carolina.
[d] Within each pair of media per barley cultivar or line, * = significant at $P \leq 0.05$ or ** = significant at $P \leq 0.01$, according to the unpaired Student's t test after arcsine-square root transformation of the data.
[e] Mean of six seed lots for the wheat biotype and for the three seed lots from North Carolina for the barley biotype.

TABLE 4. Isolation of the barley and wheat biotypes of *Stagonospora nodorum* from glumes and caryopses of barley

Cultivar	Source[a]	Wheat biotype		Barley biotype	
		Glumes	Caryopses	Glumes	Caryopses
Wysor	AR	14[b]	6	0	0
Wysor	GA	0	0	7	3
Milton	NC	12	5	16	10
Total		26	11	23	13

[a] AR = Arkansas; GA = Georgia; NC = North Carolina.
[b] Glumes and caryopses from 24 seeds of each seed lot were assayed.

of this inoculum source in the epidemiology of septoria nodorum blotch on both crops. The oxgall agar assay and some wet-blotter assay methods rely on the distinctive fluorescence that surrounds colonies of *S. nodorum* for identification (9,12). Few isolates of the barley biotype fluoresce (5,13), and they rarely sporulate on oxgall agar (5). Therefore, our observations of the barley biotype on oxgall agar relied on colony characteristics. The lack of fluorescence and sporulation on the medium increases the possibility of misidentification. The barley biotype of *S. nodorum* retains typical cultural characteristics on SNAB, and fertile pycnidia of both biotypes develop on SNAB within 7–12 days. This ensures accurate identification of each biotype from barley seed. The assay procedure takes longer than the assays of wheat seed on oxgall agar (12) or SNAW (10) but is comparable to several wet-blotter and growing-on tests used for *S. nodorum* on wheat (8,14). In all assays of seed lots with varying levels of infection by *S. nodorum* and differing microflora, SNAB was consistently better than oxgall agar for recovery of *S. nodorum*. The cost of materials for SNAB is about one-half of that for oxgall agar.

Growth of secondary seed fungi is still significant on SNAB, and improved selectivity for *S. nodorum* is needed. The incidence of seedborne fungi on barley is higher than on wheat, probably because of the presence of the attached lemma and palea on barley. The higher population of seedborne fungi on barley is also reflected by surface sterilization, which improved recovery of *S. nodorum* from barley seed but not from wheat seed (10,12).

Other seedborne fungi declined in several of the seed lots during the 6-mo interval between assays. This resulted in significant increases in recovery of the barley biotype of *S. nodorum* on the second assay date, especially for Wysor, which was stored at room temperature between assays. The other seed lots were stored at 5 C. The increase in recovery of the barley biotype while other fungi declined is identical to the pattern noted for the wheat biotype in wheat seed during storage (1). The length of survival of *S. nodorum* in barley seed is not known, but the fungus survives in wheat seed stored at 5 C for at least 11 yr (3). Both biotypes of *S. nodorum* also probably survive at least several years in barley seed.

The wheat biotype of *S. nodorum* was isolated least often from Wysor in the assays of Georgia-grown seed. Wysor seed came from a site at which wheat or other susceptible small grains had not been grown for several years. The other barley cultivars were grown at sites within 10 m of wheat. These results indicate that the incidence of the wheat biotype in barley seed is likely to be greatest when wheat and barley are grown nearby. Most isolates of the wheat biotype are weakly virulent on barley (5,13,16), and *S. nodorum* is a necrotrophic pathogen. Therefore, the wheat biotype probably invades the lemma and palea when the tissue begins to senesce during the maturation process.

The distribution of the barley biotype in seeds from several states was diverse. The recovery patterns may have been influenced more strongly by cropping history or local environmental conditions during the growing season than by geographical location. The results provide additional evidence about the distribution of the barley biotype in the United States (5). These results and those from the survey of Georgia-grown seed demonstrate that barley seed can be a reservior for the wheat biotype of *S. nodorum*.

The most extensive colonization of barley seed by *S. nodorum* appears to be in the attached lemma and palea. Pycnidial production was most profuse on this tissue, and *S. nodorum* was isolated from this tissue more often than from the caryopsis. Production of pycnidia was confined to the pericarp tissue, but additional histological study is needed to determine the extent of colonization of the barley caryopsis by *S. nodorum*. The amount of tissue colonization may be important to the length of survival in seed. Pycnidia were only observed on coleoptiles plated on SNAB. Symptoms or pycnidia of the fungus were not observed on barley seedlings in blotter tests (9). Further work is needed to determine if *S. nodorum* is transmitted from the seedling to the developing plant.

SNAB provides a significant improvement for isolation and identification of the barley and wheat biotypes of *S. nodorum* from barley seed. Because some isolates of *S. nodorum* that are pathogenic to barley are similar in culture to typical wheat isolates (11,16), SNAW may be more suitable for their recovery from seeds (10).

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