An Improved Selective Medium for the Assay of *Septoria nodorum* from Wheat Seed

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ABSTRACT

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An agar medium (designated SNAW), developed to improve the recovery of *Septoria nodorum* from wheat seed, was compared with oxgall agar, the most selective agar medium currently used. Metabolites produced by *S. nodorum* on oxgall agar fluoresce under near ultraviolet light, but the fungus does not sporulate. Growth of other seedborne fungi is only partially suppressed. On SNAW, *S. nodorum* fluoresces and sporulates within 7 days. Growth of most other seedborne fungi was reduced by greater than 95%, but *Fusarium* spp. were reduced only 74%. SNAW contained 10 g Difco potato-dextrose agar, 15 g agar, 1.5 g oxgall, and 1 g peptone per liter of deionized distilled water. After autoclaving, chloroneb (5 mg/L), cupric hydroxide (5 mg/L), dicloran (5 mg/L), chloramphenicol (3.13 mg/L), erythromycin (3.13 mg/L), tetracycline hydrochloride (12.5 mg/L), and neomycin sulfate (10 mg/L) were added to inhibit bacteria and seedborne fungi other than *S. nodorum*. SNAW and oxgall agar were compared by assaying wheat seed lots differing in degree of colonization by *S. nodorum* and the population of other fungi. The number of fluorescing colonies 4 days after plating on SNAW increased 38%, and the total number of seeds from which *S. nodorum* was recovered increased by 48% on SNAW, compared with oxgall agar. The percentage of "clean colonies" (number of seeds with more than 50% of the area around the seeds colonized by *S. nodorum*/total number of seeds with *S. nodorum*) was also 40% higher on SNAW than on oxgall agar. SNAW offers significant improvement over oxgall agar in recovery of *S. nodorum* from wheat seed, but maximum recovery is not achieved because of competition from other seedborne fungi, especially *Fusarium* spp.

Additional keywords: fungicide, *Leptosphaeria nodorum, Stagonospora nodorum, Triticum aestivum.*

Seedborne *Septoria nodorum* (Berk.) Berk. in Berk. & Broome (= *Stagonospora nodorum* (Berk.) Castellani & E. G. Germano) (teleomorph *Leptosphaeria nodorum* E. Müller = *Phaeosphaeria nodorum* (E. Müller) Hedjaroude) is an important source of inoculum in the epidemiology of Septoria nodorum blotch of wheat (*Triticum aestivum* L. em. Thell) (1,9). Seed infection levels can exceed 60%, especially in humid regions, such as the southeastern United States (2,3). Numerous methods have been used to assay wheat seed for *S. nodorum*, including several wet-blotter and agar-media methods (5–8,10,11). These methods employ one or more criteria for identification of *S. nodorum*, such as the presence of pycnidia, symptoms on the coleoptile of germinating seeds, and autofluorescence of soluble metabolites produced by the fungus. Observation of typical pycnidia is the most reliable means of identification, but it is also the most laborious. Therefore, methods that promote the distinctive fluorescence under near ultraviolet light are used most widely. The oxgall agar assay is equal to or more accurate and less time-consuming than other methods (3,4,7); however, this method has several shortcomings. Autofluorescence of individual colonies of *S. nodorum* is variable in intensity and duration, and some other fungi may exhibit fluorescence similar to *S. nodorum*. *S. nodorum* does not sporulate on oxgall agar, and there is considerable growth of other seedborne fungi, such as *Alternaria, Fusarium, Epicoccum*, and *Helminthosporium*. Because *S. nodorum* grows more slowly than most other seedborne fungi, it is often overgrown, and the true level of seed infection is underestimated (3).

To develop a new selective medium, several goals were established. We sought to develop a medium that was more selective for *S. nodorum* by reducing significantly the growth of other seedborne fungi, which promoted development of distinctive sporulating colonies to improve accurate identification, and which retained the autofluorescence of *S. nodorum*. A medium and an assay procedure were developed to achieve these goals, which were also economical in use of materials, time, and labor.

MATERIALS AND METHODS

Basal medium and antibiotics. Oxgall promotes fluorescence around colonies of *S. nodorum* but inhibits sporulation. Nutrients in the seeds were not sufficient to support a distinctively bright fluorescence while maintaining sporulation by *S. nodorum* on an agar substrate (data not shown). To promote sporulation but retain fluorescence, it was necessary to reduce the concentration of oxgall in the medium. It was also necessary to maintain nutrients at a low level to prevent excessive growth of seedborne fungi other than *S. nodorum*. After preliminary testing, 10 g potato-dextrose agar (Difco Laboratories, Detroit, MI), 15 g agar, 1.5 g oxgall, and 1 g peptone per liter of deionized water comprised the basal medium that met these criteria.

To suppress bacterial growth, chloramphenicol (3.13 mg/L), erythromycin (3.13 mg/L), tetracycline hydrochloride (12.5 mg/L) dissolved in 20% ethyl alcohol, and neomycin sulfate (10 mg/L) were added after autoclaving when the agar had cooled to 45 C.

Evaluation of fungicides. To suppress other seedborne fungi while minimizing reduction in growth of *S. nodorum*, 53 fungicides were assayed singly and in combination in a minimal agar medium (MA). MA contained 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.2 g $NaNO_3$, 10 g sucrose, and 20 g agar per liter of water. Eleven wheat seed isolates belonging to the genera *Epicoccum, Alternaria, Fusarium, Helminthosporium*, and *Phoma* and three isolates of *S. nodorum* were assayed for fungicide sensitivity. Radial growth was measured after 7 days on MA amended or unamended with fungicides at incubation conditions identical to that used for seed assays (see next section). The best combinations of fungicides were also tested on a seed lot having a high incidence of *S. nodorum*.

Wheat seed assay for *S. nodorum*. The new medium, designated SNAW (*S. nodorum* agar for wheat) was compared with oxgall agar (10), modified by the addition of streptomycin sulfate (100 mg/L) after autoclaving for recovery of *S. nodorum* from seed. The five seed lots of the four wheat cultivars used were: Blueboy, harvested in 1988; Florida 301 (Fl 301), harvested in 1989; Georgia

100, harvested in 1988 and 1989 (GA 100-1 and GA 100-2, respectively); and Holley, harvested in 1989. These seed lots, harvested at different locations in Georgia with diverse environmental conditions, provided varied infection levels of *S. nodorum* and populations of other seedborne fungi. Four hundred seeds per lot in four 100-seed replicates (10 seeds per dish, 10 dishes per replicate) were assayed on each medium at 20 C in a growth chamber under cool-white fluorescent lights, with a 12-h photoperiod (40 μE m^{-2} sec^{-1}). The experimental design was a randomized complete block. Four shelves in the growth chamber served as replicate blocks. All seed lots were assayed twice on each medium.

Seeds were examined and marked for fluorescing colonies of *S. nodorum* under an 18.4-watt near ultraviolet light (model UVSL-58, UV Products, San Gabriel, CA) 4 and 6 days after plating. Fluorescence was recorded on a 0–6 visual scale, where 0 = no fluorescence and 6 = very intense fluorescence. Total colonies of *S. nodorum* were recorded 7 days after plating. The presence of pycnidial initials and sporulating pycnidia of *S. nodorum* visible without magnification was considered positive for sporulation. Colonies were designated "clean" when more than 50% of the fungal colony around a seed was *S. nodorum*. In the results, the percentage of clean colonies is reported as 100 (number of seeds with clean colonies/total number of seeds with *S. nodorum*). Colonies of *S. nodorum* were enumerated by marking the sites of fluorescent colonies 4 and 6 days after plating and by observing growth habit and noting the presence of pycnidia 7 days after plating. Presence of pycnidia with conidia was confirmed with the aid of a dissecting microscope at 30–50×. The percent increase of SNAW over oxgall agar for each of the above parameters was calculated as 100 [(SNAW/oxgall agar) − 1].

TABLE 1. Degree of fluorescence at 4 days and percent sporulating colonies of *Septoria nodorum* at 7 days after plating cultivar Holley wheat seeds on four media (100 seeds per medium) and incubating at 20 C with a 12-h photoperiod

Medium	Fluorescence[a,b]	Sporulation[a] (%)
Oxgall agar	5.4 a	0.0 b
Basal medium	4.8 b	48.5 a
SNAW[c]	4.3 b	45.0 a
Minimal agar	0.6 c	45.5 a

[a] Means within columns followed by the same letter are not significantly different ($P < 0.01$) according to Duncan's new multiple range test.
[b] 0–6 scale, where 0 = no fluorescence and 6 = intense fluorescence.
[c] Containing fungicides and antibiotics as described in the text.

RESULTS

Inhibition of seedborne fungi other than *S. nodorum*. After preliminary testing of fungicides, three compounds, chloroneb, cupric hydroxide, and dicloran (dissolved in 20% ethyl alcohol), were most effective in inhibiting growth of other seedborne fungi and least inhibitory to *S. nodorum* when tested in vitro individually. In general, inhibition of radial growth was least in *Fusarium*, followed by *Alternaria, Epicoccum, Helminthosporium*, and *Phoma*. A combination of the three fungicides inhibited most other seedborne fungi more than 95%; growth of *Fusarium* spp. was inhibited an average of 74%. This combination also resulted in the highest inhibition of *S. nodorum*. These compounds were evaluated further against a wheat seed lot that had a high incidence of *S. nodorum*. The combination of chloroneb, cupric hydroxide, and dicloran (5 mg each) gave maximal recovery of sporulating colonies of *S. nodorum* and provided the best control of other seedborne fungi within 7 days after plating seeds. The combination of three fungicides did not inhibit *S. nodorum* in seed as greatly as in the in vitro tests.

Fluorescence and sporulation. Intensity of fluorescence by *S. nodorum* was significantly ($P < 0.01$) greater on oxgall agar than on the basal medium, SNAW, or MA (Table 1). There was no significant difference in the degree of fluorescence observed between the basal medium and SNAW. Although fluorescence was significantly brighter on oxgall agar compared with SNAW, the intensity on SNAW was bright enough to identify colonies of *S. nodorum* accurately.

Sporulation on SNAW was not significantly different ($P < 0.01$) from the basal medium or MA (Table 1). There was no sporulation on oxgall agar. The percentage of sporulating colonies of *S. nodorum* increased from 46% at 7 days to 59% at 10 days on all media except oxgall agar. There was 55% sporulation of colonies of *S. nodorum* in 7 days when continuous light was used (data not shown).

Assay of seed lots for *S. nodorum*. Significantly ($P < 0.01$) more fungal colonies around seeds fluoresced on SNAW than on oxgall agar for all seed lots tested except GA 100-1 (Table 2). The percent increase in fluorescing colonies on SNAW over oxgall agar ranged from 21 to 195% (mean, 38%) and was significant ($P < 0.01$) for each seed lot except GA 100-1. The recovery of total colonies of *S. nodorum* was significantly higher ($P < 0.01$) for all seed lots on SNAW compared with oxgall agar. The percent increase ranged from 32% for cultivar Holley to 69% for Fl 301. Recovery of *S. nodorum* averaged over all seed lots was significantly higher ($P < 0.01$) on SNAW (44%) than on oxgall agar (29%) (Table 2). The percentage of clean colonies of *S. nodorum* was also significantly ($P < 0.01$) higher for all seed lots except GA 100-1 on SNAW than on oxgall agar

TABLE 2. Percent wheat seeds from five cultivars that produced fluorescent colonies in 4 days, total colonies of *Septoria nodorum*, and clean colonies of *S. nodorum* recovered 7 days after plating on SNAW and oxgall agar

	Percentage of seeds[a]					
Medium	Blueboy	Fl 301	GA 100-1	GA 100-2	Holley	Mean
Fluorescent colonies						
SNAW	14**	33**	14	65**	67**	39**
Oxgall agar	5	21	11	54	50	28
LSD	3.9	7.6	NS	4.0	8.7	3.3
Increase (%)[b]	195	52	31	21	35	38
Total colonies						
SNAW	32**	29**	27**	68**	62**	44**
Oxgall agar	22	17	17	43	47	29
LSD	5.8	5.0	7.3	8.1	8.0	2.9
Increase (%)[b]	44	69	63	59	32	48
Clean colonies						
SNAW	97**	46**	69	64**	75**	70**
Oxgall agar	79	22	58	40	52	50
LSD	12.3	10.6	NS	12.6	6.7	6.0
Increase (%)[b]	23	104	18	61	44	40

[a] Means of two assays, with 400 seeds per lot per assay on each medium; values are rounded to the nearest whole number; ** = significant at $P < 0.01$ and NS = non-significant at $P < 0.05$.
[b] Increase (%) is calculated from percentage of seeds values before rounding.

(Table 2).

Fluorescence of colonies of *S. nodorum* often declines within 5–7 days after plating seeds on oxgall agar. Therefore, we compared the number of fluorescing colonies on the sixth versus the fourth day after plating seeds on the two media. The difference in number of fluorescing colonies between the sixth and the fourth day was significantly lower on SNAW than on oxgall agar for Fl 301 ($P < 0.01$) but not for Blueboy. A comparison was also made between the total colonies of *S. nodorum* identified after 7 days and the number of fluorescent colonies on the fourth day after plating seeds on the two media. No significant difference was found between SNAW and oxgall agar for either cultivar. However, on both media, the total number of colonies of *S. nodorum* was significantly higher than the number identified by fluorescence on the fourth day after plating.

DISCUSSION

Radial growth of *S. nodorum* was inhibited less than that of several other seedborne fungi in minimal medium amended with fungicides singly or in combination. Inhibition of in vitro radial growth of *Fusarium* was greater in the combinations chloroneb and dicloran and cupric hydroxide and dicloran than in the three-fungicide combination. However, we used the combination of chloroneb, cupric hydroxide, and dicloran because of the greater ability to recover *S. nodorum* from seeds in SNAW medium and enhanced inhibition of other seed fungi.

Oxgall agar was developed to detect *S. nodorum* on the basis of fluorescence (10). However, because *S. nodorum* does not sporulate on oxgall agar and often its growth is suppressed by ubiquitous seedborne fungi such as *Alternaria* and *Epicoccum*, fluorescence is often concealed (3,4,7). Although intensity of fluoresence of *S. nodorum* was less on SNAW than on oxgall agar, the intensity was sufficiently high to identify *S. nodorum* (Table 1). There was also a small (but significant) improvement in the duration of fluorescence on SNAW compared with oxgall medium.

Pycnidial initials developed around the middle of about 50% of the colonies of *S. nodorum* on SNAW within 7 days. The number of sporulating colonies increased during 3–4 additional days of incubation. Continuous light during incubation increased the number of sporulating colonies of *S. nodorum*; however, colony size was reduced.

Nonsporulating colonies of *S. nodorum* were identified by fluorescence in 4 or 6 days and by distinctive cultural characteristics. Colonies of *S. nodorum* on SNAW were generally yellow-brown when observed from the underside of the petri dishes. Hyphae at the edge of the colony grew conspicuously radiate on the surface of SNAW and characteristically were different from most other seedborne fungi associated with wheat seed. However, fluorescence and sporulation of colonies of *S. nodorum* differed from seed to seed on SNAW, indicating that there were variations among isolates (2,5). Variation may also be due to colony size as affected by competition from other seedborne fungi.

The percentage of fluorescent colonies, total colonies of *S. nodorum* recovered, and clean colonies all increased to varied degrees on SNAW compared with oxgall agar for all seed lots tested (Table 2). Increased detection of *S. nodorum* is related to reduction of competing seed fungi, especially *Alternaria* and *Epicoccum*.

Cunfer (3) reported a significant decline in seedborne fungi other than *S. nodorum* within 1 yr after harvest, which resulted in increased recovery of *S. nodorum* on oxgall agar. In the current study, the percentage of clean colonies on oxgall agar was higher in the two seed lots stored for more than 1 yr (Blueboy and GA 100-1) than in seed lots harvested in 1989 (Table 2). However, on SNAW, the number of clean colonies for GA 100-1 (1988) was similar to that for GA 100-2 and Holley, both harvested in 1989. These results indicate that SNAW inhibits other seedborne fungi to a significant extent on newly harvested seed, thus improving the accuracy of seed assays conducted shortly after harvest.

SNAW offers significant advantage over oxgall agar, both in percent recovery and potentially in accuracy of identification of *S. nodorum*, because the fungus sporulates quickly on it. Inhibition of other seedborne fungi is not complete, however, and contamination by *Fusarium* spp. may obscure the true level of seed infection by *S. nodorum*. Nevertheless, recovery was improved significantly even in seed lots highly contaminated by secondary seedborne fungi.

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