Evaluation of Ergosterol as an Indicator of Infestation of Barley Seed by *Drechslera graminea*

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ABSTRACT

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Twelve seed lots of barley were examined for the presence of *Drechslera graminea*, the cause of barley stripe. The level of infestation by *D. graminea* was estimated by each of the following methods: plating seed on barley leaf pieces set on water agar and identifying infested seed by the growth and sporulation of *D. graminea*; growing plants from seed under greenhouse conditions conducive to disease development and rating plants for the occurrence of barley stripe; and quantifying the concentration of ergosterol extracted from surface-sterilized seed after incubation for 46 hr. Infestation levels as low as 1% could be detected by all three methods. The grow-out method provided the most accurate estimate of the level of infestation by *D. graminea* but at least 6 wk were required to obtain results. Infestation levels indicated by the seed-plating method correlated well with the grow-out data, and results were available after 7 days. The concentration of ergosterol extractable from a seed sample was porportional to the infestation level indicated by the grow-out data, and the ergosterol assay gave results within 48 hr.

Additional key words: *Hordeum vulgare*.

Drechslera graminea (Rab.) Shoem., a seedborne pathogen of barley, causes barley leaf stripe. Following germination of the seed, the fungus systemically infects the preemergent seedling (7). Because infested seed is the only source of inoculum, seed treatments, such as mercurial fungicides, were an effective means of disease control. Since the use of mercurials was discontinued, the incidence of barley stripe has increased substantially (5). As a result, commercial seed lots may have high levels of infestation with *D. graminea*. The identification of seed lots that contain infested seed can be a useful tool in disease management.

D. graminea in barley seed can be detected by growing plants from seed under conditions conducive to disease development (the grow-out method) (10), or by plating seed on a suitable medium and identifying infested seed by the growth and sporulation of *D. graminea* (3,10). Both of these procedures are slow to yield results; 7 days for the seed plating method and at least 6 wk for the grow-out method. We have examined the possibility of using a more rapid procedure based on the presence of ergosterol in infested seed (1). Ergosterol is a sterol produced by a number of fungi (11); it is not a native constituent of cereal grain (8). Ergosterol has been used to quantify the extent of saprophytic fungal invasion of cereal grain (8).

This study was undertaken to examine the utility of using ergosterol as an indicator of infestation of barley seed by *D. graminea*. Twelve seed lots were examined for this purpose; the infestation level of each seed lot was estimated by the seed-plating method, the grow-out method, and by the concentration of extractable ergosterol. A preliminary report of these results has been published (2).

MATERIALS AND METHODS

Three samples of each seed lot (100 seeds per sample) were assayed for the presence of *D. graminea* by the seed plating method. Seeds were pretreated to reduce surface contaminants by immersion in a 1% solution of sodium hypochlorite for 10 min, drained, and placed on wetted filter paper in glass petri dishes.

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After 24 hr at room temperature, seeds were frozen at −20 C for 24 hr (3). Seeds were then returned to room temperature and transferred to barley leaf piece agar (BLPA) (10). After 3 days at room temperature, the seeds were exposed to 24 hr of cool-white fluorescent light, followed by 24 hr of darkness at 15 C (10). Infested seeds were identified by the growth and sporulation of *D. graminea*.

In the grow-out method, three samples of 100 seeds from each seed lot were sown in flats of moist soil. These flats were kept in the dark at 6 C for 24 days and then moved to a greenhouse. Plants were rated for barley stripe 3–4 wk later.

The ergosterol content of each experimental seed lot was determined by analyzing two 20-gm samples (~400 seeds per sample) of surface sterilized seed. Surface sterilization to remove saprophytic fungi was accomplished by the following series of treatments. First, seed was heated at 90 C for 1 hr (4). After heating, the seeds were placed directly into 100 ml of a solution containing 95% ethanol (74 ml), 5.25% sodium hypochlorite (20 ml), and 50% benzalkonium chloride (6 ml). Seeds were stirred in this solution for 30 sec. They were drained and then immersed in a 1% solution of sodium hypochlorite with 0.1% Tween-20 and stirred for 2 min. After draining, the seeds were transferred to a 30% solution of H_2O_2 for 20 min with stirring. Seeds were then placed in a stainless steel mesh strainer and rinsed with 500 ml of sterile deionized water. The seeds were placed on two sheets of Whatman #1 filter paper wetted with 4.5 ml of sterile deionized water, in glass petri dishes, ~50 seeds per dish. Seeds were allowed to imbibe water at room temperature for 3–4 hr then were frozen at −20 C for 4 hr. After the seeds were returned to room temperature they were incubated for an additional 38–40 hr prior to extraction. The incubation period facilitated detection of *D. graminea* in seed lots with low infestation levels.

Ergosterol was extracted and quantified by a procedure similar to that described by Seitz et al (8). It consisted of homogenizing seed in methanol, filtering through glass fiber (Whatman GFA), saponifying the filtrate, and partitioning the nonsaponifiable components into petroleum ether. The petroleum ether fraction was concentrated, by evaporation under nitrogen, to a volume of 5–10 ml and loaded onto a 1.5-ml silica cartridge (SEPAK, Waters Assoc., Milford, MS). Prior to application of the sample, the cartridge was rinsed with 5 ml of methanol followed by 5 ml of petroleum ether. Finally, the sterol fraction was eluted from the silica with methanol. The sample, in methanol, was filtered through a 0.45-μm nylon filter. The final volume was adjusted to 1 ml, and a 50-μl sample was injected into an HPLC. Ergosterol was eluted

from a 5 μm C-18 column (Alltech Assoc., Deerfield, IL) with methanol:ethanol (90:10, v:v). Eluent absorptance at 282 nm was monitored, and the peak corresponding to ergosterol was identified by its retention time. Quantification was based on a standard curve relating peak area to the concentration of authentic ergosterol (Sigma Chem. Co., St. Louis, MO 63178). Definitive characterization of ergosterol was accomplished by first fractionating the seed extract with TLC and eluting the band which cochromatographed with authentic ergosterol (8). The material thus obtained was dissolved in DMSO and methylated prior to analysis by gas chromatography-mass spectrometry (6).

RESULTS AND DISCUSSION

The levels of infestation by *D. graminea* indicated by each of the three methods for the 12 seed lots examined are shown in Table 1. Because it is based on the distinctive symptoms of this disease, the grow-out method is very specific. Provided that plants are exposed to low temperature prior to emergence, grow-out data give an accurate measure of the infestation level in the seed lots tested (10). The major disadvantage of the grow-out method is that it requires a minimum of 6 wk to obtain results.

As found in other studies (3,10), infestation levels indicated by the seed-plating method (Table 1) were significantly correlated with grow-out data ($r^2 = 0.80$). The only exception to this was seed lot 7. Twenty-three percent of the seed from lot 7 gave rise to sporulating fungal colonies counted as *D. graminea* while only 0.4% of the seed gave rise to a diseased plant in the grow-out. This discrepancy could be reconciled if the fungi growing from the seed on BLPA were actually *Drechslera teres* (Sacc.) Shoem. and not *D. graminea*. *D. teres*, the cause of net blotch, is closely related to *D. graminea*, and the two are difficult to distinguish morphologically (3,9). To test this possibility, single-spore isolations were made of *Drechslera* spp. growing out of four seeds in lot 7. Conidial suspensions, prepared from pure cultures of these isolates, were used to inoculate barley cultivars susceptible to net blotch. Typical net blotch symptoms were observed on all inoculated plants. Thus, it is probable that most of the infested seed in lot 7 carried *D. teres* rather than *D. graminea*. All isolates obtained from infested seed in lot 9 were shown to be *D. teres* as well. Detection of *D. teres* as well as *D. graminea* may or may not be desirable, depending on the importance of seedborne *D. teres* inoculum relative to other sources. The significant advantage of seed plating relative to the grow-out method is that results are obtained in considerably less time.

TABLE 1. Detection of *Drechslera graminea* in 12 seed lots of barley by three methods

Seed lot	Cultivar	Seeds (%): infested[a]	Seeds (%): diseased[b]	Ergosterol[c]
1	Summit	23	33	0.27
2	Summit	26	17	0.21
3	Kombyne	70	77	0.90
4	Kombyne	40	40	0.53
5	Kombyne	72	60	1.24
6	Kombar	0	0	0.0
7	Prato	23	0.4	0.43
8	Kombyne	1	0	0.04
9	Atlas	3	0	0.05
10	Sutter	0	0	0.0
11	Pirouette	0	0.4	0.0
12	Kombyne	9	36	0.66
Correlation coefficients (r^2)		0.79[d]	0.80[e]	0.80[f]

[a] Based on observation of sporulating *D. graminea* in incubated seed; data represent the average of three samples of 100 seeds.

[b] Based on a grow-out planting in the greenhouse; maintained at 6 C for the first 3 wk after seeding. Data represent the average of three samples of 100 seeds.

[c] The concentration (μg/g seed) of ergosterol extracted from 20-gm samples of surface sterilized seed after incubation for a total of 46 hr; data represent the average of two samples.

[d] Infested versus ergosterol.

[e] Infested verus diseased.

[f] Diseased verus ergosterol.

The compound isolated from seed infested with *D. graminea* was identified as ergosterol on the basis of the following properties it shared with authentic ergosterol: chromatographic behavior on silica gel thin layer plates and a reverse phase C-18 column; ultraviolet absorptance spectrum with maxima at 193 nm, 282 nm, 271 nm, and 263 nm; and mass spectrum (following methylation) consisting of a molecular ion at 410 nm and principal fragment ions at 363, 337, 253, and 211 nm.

Ergosterol was detected in incubated samples of every seed lot shown to harbor *D. graminea* by either of the other two procedures, except for seed lot 11 that had an infestation level of 0.4% according to the grow-out method. Ergosterol was found in seed lots 7 and 9, indicating the ergosterol assay does not discriminate between *D. teres* and *D. graminea*. Significantly, ergosterol was not detected in seed lots 6 and 10 which were shown to be free of infestation of *D. graminea* by both of the other methods. As shown by the data in Table 1, the concentration of ergosterol extractable from a seed sample was proportional to the infestation level indicated by the grow-out data ($r^2 = 0.78$). The ergosterol assay does not give a numerical estimate of the infestation level. Therefore, when an accurate estimation of the infestation level is needed, the grow-out and seed-plating methods are preferable to the ergosterol assay. Where the purpose of testing is to determine whether or not *D. graminea* is present above a certain threshold (3), the ergosterol assay will provide the required data. The major disadvantage of this method is its lack of specificity. Ergosterol is a common fungal metabolite, and it is a constituent of some saprophytic fungi which may colonize the surface of barley seed. For this reason, removal of superficial fungal biomass is an essential prerequisite to the application of this technique. The effectiveness of the surface sterilization procedure used in this study was verified by examining surface sterilized seed 7 days after treatment. No saprophytic fungi grew out of seed in any sample so treated. Several of the 12 seed lots examined in this way had been heavily colonized by saprophytic fungi and therefore provided a good test for the surface sterilization procedure. The high level of colonization was exemplified by the observation that saprophytic fungi grew from 100% of the seed sampled from lot 11, even after a 5-min soak in 1% sodium hypochlorite. Of course, these results do not preclude the possibility of the ergosterol assay giving a falsely positive result. Seed lots, as yet unexamined, may have seedborne mycelium of a saprophytic fungus capable of surviving this surface sterilization procedure. Our experience suggests this will occur infrequently.

When seed samples were extracted without first using the surface sterilization procedure, ergosterol was detected in all 12 of the seed lots examined even if the seeds were extracted prior to the

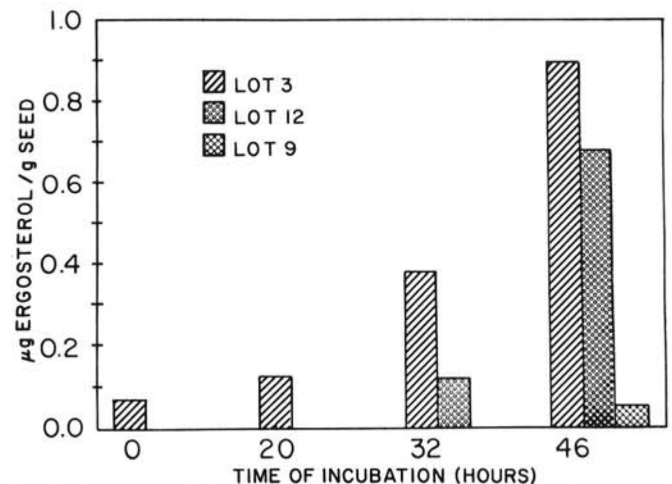

Fig. 1. The effect of incubation time on the concentration of ergosterol extractable from three barley seed lots. Seeds were incubated on moist filter paper: the first 3 hr at room temperature, the next 4 hr at −20 C, and the remainder of the incubation period at room temperature.

incubation period. Ergosterol was detectable in seed lots heavily infested with *D. graminea*, such as lots 3 and 5, immediately after surface sterilization. However, incubation was required for detection of lower infestation levels, as in lots 9 and 12 (Fig. 1). It is possible that a more sensitive method for detecting ergosterol would obviate the need for an incubation period.

The detection threshold is near 1% infestation for all three methods tested (Table 1). This detection limit could be lowered, for any of the three methods, by an increase in sample size. The primary advantage of the ergosterol assay, relative to the other two methods, is that results can be obtained in about 48 hr, which is less time than required by either the seed plating or grow-out methods.

At present, seed plating is the method most commonly used for detection of *D. graminea* in barley seed (3,4). It offers somewhat greater specificity than the ergosterol assay so it may remain the method of choice, particularly for laboratories equipped for culture work and not analytical chemistry. The ergosterol assay might prove useful where a more rapid screening procedure is required. Perhaps more important than the immediate prospects for application of the ergosterol assay, is the demonstration that a specific metabolite can be used in the detection of a seedborne fungal pathogen.

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