Ergosterol as a Measure of Fungal Growth

L. M. Seitz, D. B. Sauer, R. Burroughs, H. E. Mohr, and J. D. Hubbard

U.S. Grain Marketing Research Laboratory, Science and Education Administration, Agricultural Research, U.S. Department of Agriculture, Manhattan, KS 66502 (first, second, fourth, and fifth authors) and Department of Grain Science and Industry, Kansas State Univerisity, Manhattan 66506 (third author).

Contribution 78-321-J, Kansas Agricultural Experiment Station, Manhattan 66502.

Accepted for publication 16 May 1979.

ABSTRACT

SEITZ, L. M., D. B. SAUER, R. BURROUGHS, H. E. MOHR, and J. D. HUBBARD. 1979. Ergosterol as a measure of fungal growth. Phytopathology 69: 1202-1203.

An ergosterol assay was more sensitive, rapid, and convenient than a chitin assay in measuring *Alternaria* and *Aspergillus* growth on milled rice. This finding plus the fact that ergosterol is relatively specific to fungi as the predominant sterol suggest that the ergosterol assay can quantitatively measure total fungal invasion in other substrates.

Additional key words: Alternaria alternata, Aspergillus amstelodami, A. candidus, A. flavus, A. glaucus, A. ochraceus, A. repens, A. ruber, Fusarium moniliforme, F. roseum, Penicillium brevi-compactum.

When a fungal biomass cannot be separated from a solid substrate, growth of the fungus can be monitored by measuring a chemical component. Chitin, a cell wall constituent of most fungi, is the component most commonly measured (2,6). Ergosterol has not been widely used to monitor fungal growth, even though it is the predominant sterol component of most fungi and is either absent or a minor constituent in most higher plants (5,8). A rapid ergosterol assay by high-pressure liquid chromatography (HPLC) we developed to measure fungal invasion in grain (7) is more effective than a chitin assay for monitoring growth in solid substrate cultures. Our investigations, although limited to fungal growth in cereal grains, suggest that the ergosterol assay might be used to measure fungal invasion in other natural materials.

MATERIALS AND METHODS

The fungi were cultured as follows: Air-dried, enriched, milled rice (100g) were dispensed into 300-ml Erlenmeyer flasks. After moisture content was adjusted to 35% for *Alternaria alternata* and 30% for *Aspergillus flavus* and *A. amstelodami*, each flask was autoclaved 30 min, cooled, inoculated, and weighed. Flasks were incubated at 25 C. At each sampling, two flasks were weighed and the contents air-dried on trays in a fume hood and analyzed separately.

The ergosterol assay was similar to that described previously (7). Briefly, 30–50 g of substrate was extracted with methanol, the methanol extract was saponified for 30 min, and the saponified mixture was extracted with petroleum ether (bp 60–70 C). The small silica gel column used previously (7) was eliminated to save time. Instead, the residue from evaporated petroleum ether extract was dissolved in methylene chloride-isopropanol (99:1, v/v) and filtered through a glass fiber filter (double thickness) in a Swinney filter holder. Volume was adjusted to 0.5–8 ml depending on stage of growth, and 10 μl was injected into the HPLC. Ergosterol was detected by absorption of 282 nm light. The detector was set at 0.1 absorbance unit full scale, which was only one-tenth of its maximum sensitivity. *A. alternata* cultures were analyzed with the normal-phase HPLC system (7), ie, μ-Porasil column (Waters Associates Inc., Milford, MA 01757) with methylene chloride-isopropanol (99:1, v/v) eluant. For analyses of the *A. flavus* and *A. glaucus* cultures, as well as for other recent analyses of wheat and grain sorghum, we used a reversed-phase system consisting of a μ-Bondapak-C_{18} column (Waters Associates Inc.) and 5% water in methanol. At a 1.67 ml per minute flow rate, ergosterol was eluted at 9 min.

Chitin was assayed by using an amino acid analyzer to measure glucosamine liberated from chitin. Substrate (10 g) was digested in 40 ml of HCl for 3 hr at 110 C, and the digested mixture was vacuum-filtered through a glass fiber disk. The filter was washed with distilled water, and the sample was diluted with distilled water to 250 ml in a volumetric flask. A 5-ml sample was evaporated to dryness on a rotary evaporator. The residue was diluted to an appropriate volume (2–10 ml, depending on stage of growth) with 0.2 *N* sodium citrate buffer of pH 2.2 and filtered through 0.22-μm membrane filter before analysis. The volume injected into the analyzer was 250 μl.

RESULTS AND DISCUSSION

Growth of *A. alternata, A. flavus,* and *A. amstelodami* (*A. glaucus* group) on moist, milled rice at 25 C was monitored by ergosterol and chitin assays (Fig. 1). Ergosterol assay detected growth of each fungus 24 hr after inoculation. Chitin assay, however, did not detect growth of *A. alternata* and *A. flavus* until 40–48 hr and did not adequately measure growth of *A. amstelodami.* After 48 hr, ergosterol content paralleled chitin content in the *A. alternata* and *A. flavus* cultures.

Three additional isolates of *A. flavus* and two other species in the *A. glaucus* group (*A. repens* and *A. ruber*) produced ergosterol and chitin curves similar to those for *A. flavus* and *A. amstelodami*, respectively. Other important grain fungi that were strong ergosterol producers when grown 3 days or longer on milled rice at 30% moisture and 25 C were *Aspergillus candidus, A. ochraceus, Penicillium brevi-compactum, Fusarium moniliforme,* and *F. roseum.*

Species belonging to the *A. glaucus* group produced much less ergosterol in milled rice than did the other fungi. The ergosterol content (Fig. 1) indicated that *A. amstelodami* grew fairly rapidly during the first 3–4 days, but total growth was much less than that of *A. alternata* and *A. flavus.* Tests with liquid cultures of the three fungi indicated that the ergosterol contents of the fungi were similar (Table 1), suggesting that the lower ergosterol production by *A.*

amstelodami reflected less invasion (growth) by that species. Growth of species of the *A. glaucus* group differed markedly from that of the other fungi. *A. glaucus* did not cause moisture condensation in the flasks, extensive clumping of kernels, or severe loss of kernel integrity, as did the other fungi. Substrate utilization by *A. alternata* and *A. flavus* could be measured by weight loss on day 3 and reached about 33% by day 28. With *A. glaucus,* however, weight loss was not measurable until day 14 and reached only 2% by day 28. These results are consistent with reports that wheat samples containing 30% moisture were heated much less by growth of *A. glaucus* than by growth of *A. flavus* (1) and that *A. glaucus* reduced germinability of wheat (4) and pea seeds (3) much more slowly than did *A. flavus*.

TABLE 1. Weight and ergosterol content of fungal mass grown in liquid media

Fungus[a]	Incubation (days)	Fungal mass (mg/flask)	Ergosterol (mg/g air-dried wt)
Alternaria alternata[b]	5.0	108	3.8
	10.0	173	4.4
Aspergillus flavus[c]	2.1	65	2.3
	7.0	110	3.3
A. amstelodami[d]	3.6	26	5.9
	8.6	28	4.9

[a] Isolates are identical to those depicted in Fig. 1.
[b] 5% malt extract, 100 ml in 300-ml flask.
[c] 5% malt extract, 30 ml in 300-ml flask.
[d] 3% malt extract and 10% sucrose, 30 ml in 300-ml flask.

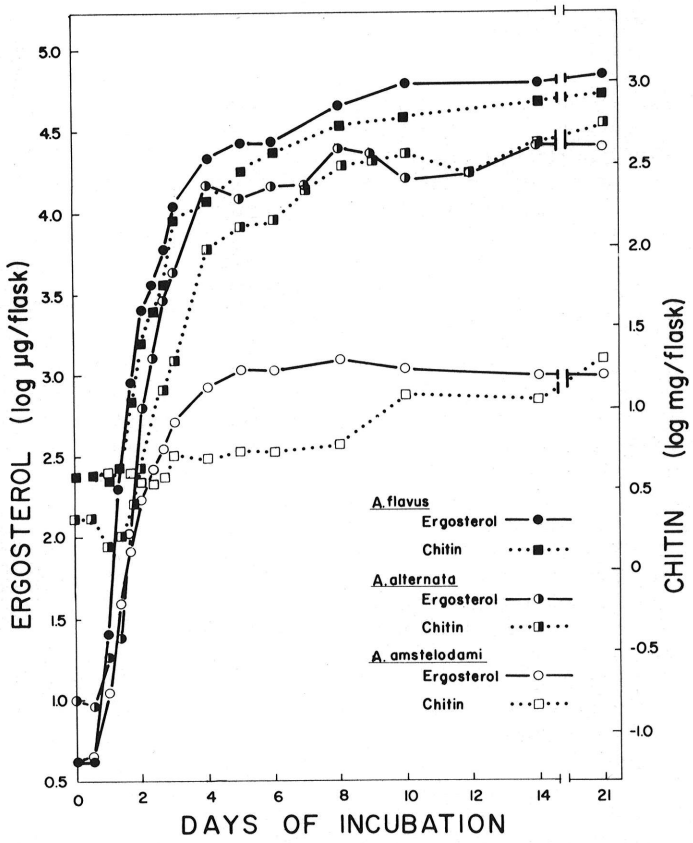

Fig. 1. Ergosterol and chitin contents in cultures of *Aspergillus flavus* (30% moisture), *Alternaria alternata* (35% moisture), and *Aspergillus amstelodami* (30% moisture) on milled rice at 25 C. Each point represents the average of results from duplicate flasks. Coefficient of variation of duplicates averaged (ergosterol and chitin, respectively): 12.1 and 7.4% for *A. flavus,* 12.3 and 9.2% for *A. alternata*, and 10.4 and 6.2% for *A. amstelodami.*

Comparison of the initial amount of a fungal component in a substrate with the increase in amount caused by fungal growth determines the suitability of that component as an indicator of early or limited fungal growth. On that basis, the assay for ergosterol was more sensitive than that for chitin (hydrolyzed to glucosamine) in the milled rice cultures. We extracted up to 50 g of material to assure a representative sample, but with appropriate adjustments, either assay could be done on samples of about 100 mg. Analytic sensitivities of the assays were adequate to detect ergosterol and glucosamine levels of 0.04–0.09 µg per gram and 0.02–0.04 mg per gram, respectively, in the milled rice substrate before onset of fungal growth. The ergosterol assay also measured slight preharvest invasion in wheat, corn, and grain sorghum (7).

Extensive preharvest fungal invasion would limit the ability of either ergosterol or chitin assay to detect early fungal growth in grains during storage. In substrates with previous fungal invasion, growth of a weak invader such as *A. glaucus* could be reliably detected only by an assay for a component specific to that fungus. Possibly, other fungal sterols normally absent in plants could be assayed to measure fungal growth, but such assays might require more complex instrumentation, such as gas chromatography combined with mass spectroscopy.

The ergosterol assay's capability of detecting early growth would be valuable in studying secondary metabolite synthesis in relation to fungal growth on solid substrates. Two of the four *A. flavus* isolates, including the one depicted in Fig. 1, were aflatoxigenic. Both aflatoxin B_1 and chitin were detected 12–16 hr after ergosterol was first detected. Alternariol produced by *A. alternata* was measurable about 48 and 24 hr after earliest detection of ergosterol and chitin, respectively.

We found that ergosterol has several advantages over chitin in measuring fungal growth on grains. The ergosterol assay was more sensitive to early growth and was easier and faster (about 1 hr compared with 4–6 hr) than the chitin assay. Because the popularity of HPLC has increased during the past several years, many laboratories have adequate equipment for the ergosterol assay. Chitin analysis by ion-exchange chromatography (9) requires an amino acid analyzer or similar specialized equipment. Colorimetric determinations for chitin require only commonly available equipment but have many steps and may be subject to more interferences than chromatographic methods.

LITERATURE CITED

1. CHRISTENSEN, C. M., and D. R. GORDON. 1948. The mold flora of stored wheat and corn and its relation to heating of moist grain. Cereal Chem. 25:40-51.
2. DONALD, W. W., and C. J. MIROCHA. 1977. Chitin as a measure of fungal growth in stored corn and soybean seed. Cereal Chem. 54:466-474.
3. FIELDS, R. W., and T. H. KING. 1962. Influence of storage fungi on deterioration of stored pea seeds. Phytopathology 52:336-339.
4. MILNER, M., C. M. CHRISTENSEN, and W. F. GEDDES. 1947. Grain storage studies. V. Chemical and microbiological studies on "sick" wheat. Cereal Chem. 24:23-38.
5. NES, W. R. 1977. The biochemistry of plant sterols. Pages 233-324 in: R. Paoletti and D. Kritchevsky, eds. Vol. 15. Advances in Lipid Research. Academic Press, New York. 360 pp.
6. RIDE, J. P., and R. B. DRYSDALE. 1972. A rapid method for the chemical estimation of filamentous fungi in plant tissue. Physiol. Plant Pathol. 2:7-15.
7. SEITZ, L. M., H. E. MOHR, R. BURROUGHS, and D. B. SAUER. 1977. Ergosterol as an idicator of fungal invasion in grains. Cereal Chem. 54:1207-1217.
8. WEETE, J. D. 1974. Fungal Lipid Biochemistry: Distribution and Metabolism. Plenum Press, New York. 393 pp.
9. WU, L., and M. A. STAHMANN. 1975. Chromatographic estimation of fungal mass in plant materials. Phytopathology 65:1032-1034.