CHITIN AS A MEASURE OF FUNGAL GROWTH IN STORED CORN AND SOYBEAN SEED[1]

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ABSTRACT

A method of measuring the degree of fungal invasion of stored soybean and corn was developed using the analysis of chitin, measured as glucosamine, as a criterion. Soybeans at moisture contents of 10, 15.8, 19.8, and 22.3% and corn at moisture contents of 11.5, 17.6, 20.6, and 23.2% were stored for 1 month at 26°C and analyses were made weekly. The percentage of seed that yielded fungi after surface disinfection of the seed was used to confirm the information gained from chitin analyses. The chitin content of hand-picked, properly stored, wholesome corn (100–178 μg/seed) was substantially greater than that of properly stored, wholesome soybeans (20–43 μg/seed). Likewise, after storage of corn at 23.2% moisture content and storage of soybeans at 22.3% moisture content for 4 weeks, mold development in corn measured as chitin content averaged 979 μg/g seed, and that in soybean averaged 261 μg/g seed. Fungi appeared to be inhibited on soybean seed. We conclude that chitin content is an accurate and reliable measure of fungal invasion of stored seeds, and results obtained in the laboratory were substantiated by those obtained in a field case of stored corn which had molded. The method is relatively rapid (4–6 hr) when compared to the plating-out method (5–7 days) of measuring fungus invasion. The method has applicability in measuring the storability of corn and soybean seeds.

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The quality of stored cereal seeds has generally been determined by qualitative determination of the number of kinds of fungi found in the seeds after surface disinfection with sodium hypochlorite (1). This method usually takes 5 to 7 days to obtain results, and measures viable mycelium and describes the kind of fungi present; it does not reflect nonviable mycelium. The method is time-honored and usually dependable but is not rapid enough for the grain industry. With this in mind, a chemical method was devised which takes about 4 to 5 hr for analysis; it is based on the chemical determination of chitin, a constituent of fungus cell walls. It has an advantage in that it will reflect total mycelium (viable and nonviable) based on chitin content. One disadvantage is that it does not identify the fungi involved. The method described may be useful to the grain industry in rapidly determining the quality of grain.

Chitin is a constituent of the cell walls of most fungi in cereal grains and can be used as a measure of the total fungal growth, since little or no chitin-like materials occur in sound cereal grains. Golubchuk *et al.* (2) studied the chitin content of hydrolyzed crude fiber preparations of 5 varieties of wheat with varying degrees of fungus invasion. They concluded that the measurement of chitin offers promise in the evaluation of deterioration of wheat in storage, but the analytical procedure needed improvement. Arima and Uozumi (3) successfully used chitin analysis as a measure of mycelial weight in koji. The method used in this study was based on the method of chitin detection in fungus-infected host tissue devised by Ride and Drysdale (4,5). They chose the alkaline method of hydrolysis, as opposed to acid, resulting in the formation of chitosan. The

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objective of this study was to explore and adapt this method for use in measuring the degree of fungus infestation in cereal grains and in ascertaining risk of storage.

Chitin, a polymer of N-acetyl-D-glucosamine, is commonly found in the exoskeleton of insects as well as in spores and mycelium of fungi. In the analytical method devised, the polymer is not measured directly but rather is hydrolyzed to glucosamine, deaminated to its corresponding aldehyde and measured colorimetrically. Glucosamine is found in bacterial spores and seed glycoproteins and in theory would interfere with the determination of chitin. In practice, however, the residual amounts of glucosamine present due to infestation by bacteria were too small to be detected by this method or were nonexistent.

In this work, changes in the chitin content of corn and soybean seeds stored at various moisture contents are compared to the percentage of seeds that yield fungi after surface disinfection and subsequent incubation on an appropriate nutrient medium.

MATERIALS AND METHODS

Duplicate samples of corn and soybeans (1.5 to 2.0 kg) at four different moisture contents were kept under identical temperature conditions but different moisture contents for 28 days. Initially, several 2-kg lots of soybean seed (*Glycine max* (L.) Merr. 'Chippewa') and hybrid corn (*Zea mays* L.) were moistened in plastic bags with various amounts of distilled water, kept in a cold room at 4° C for 2 days, and mixed at intervals to equalize the moisture content. On the third day, they were placed in sealed plastic containers over saturated solutions of ammonium sulfate. These solutions were chosen to maintain the relative humidity of the interseed air at 80 to 85%. In addition, these containers were stored in a Hotpack growth chamber at 26° C for 28 days and continually flushed with moist air at 80 to 85% relative humidity; the concentrations of oxygen and carbon dioxide were kept at atmospheric levels.

Seed samples (50–80 g) were removed from each container at the start of the experiment and at weekly intervals. Each sample was analyzed for moisture content, chitin content, percentage of seed yielding fungi after surface disinfection, and identification of the fungi isolated.

Percentage of Seed that Yields Fungi after Surface Disinfection (PSYF)

One-hundred seeds were surface disinfected in 0.5% NaOCl, rinsed in sterile deionized water, and plated out on tomato juice salt agar (6) at 23° C for 7 days. The number of seeds infected was recorded in per cent and identified with standard taxonomic keys (7,8).

Seed Moisture Content

The moisture contents of whole soybeans and corn seeds were determined using the method described by Roberts (9). The weight of whole seeds (about 50 g) was taken before and after drying at 105° C for 16 hr.

Chitin Assay

The method used is a modification of the one described by Ride and Drysdale (4,5). The seed samples were dried at 105° C (72 hr) and stored at room

temperature in tightly capped glass bottles until used. In this preliminary laboratory study, the seeds were dried for this prolonged period (dried to constant weight) in order to minimize any variation due to difference in seed moisture content. In the analytical method as described, seeds at 12–16% moisture can be ground directly; others are dried at 105°C for 0.5 hr. The extraction and analysis are carried out as follows:

A. Sample preparation for hydrolysis of chitin to chitosan.
1. Grind 50-g seed samples in a Stein Mill for 1.5 min.
2. Place 0.2 g of the finely divided seed into a 30-ml plastic autoclavable centrifuge tube.
3. Add 4 ml of KOH (120 g KOH/100 ml H_2O) to soybeans and 5 ml to corn.
4. Autoclave the test tube and contents for 15 min and then cool to 0° C.

B. 5. Ice-cold 70% ethanol (8 ml) is added to each tube and subsequently layered with Celite (1 ml); this facilitates precipitation of chitosan. Celite concentration: 1 g/20 ml 70% ethanol.
6. The tubes are then centrifuged (12,000 rpm, 10 min, 0°C) and the supernatant solution decanted.
7. The pellet is resuspended by adding 8 ml of ice-cold 40% ethanol to each tube and stirring thoroughly.
8. Step 6 is repeated.
9. The pellet is resuspended in distilled water (8 ml) and the pH adjusted to $2.0^{\pm 0.2}$.
10. Step 6 is repeated

C. Colorimetric assay for chitosan based on glucosamine.
11. One and one-half milliliters of $KHSO_4$ (5% w/v) and 1.5 ml $NaNO_2$ (5% w/v are added to each tube and the total volume brought to 4.5 ml with distilled water. The precipitate is stirred into solution.
12. A 1.5-ml aliquot is withdrawn from each tube and placed into separate test tubes containing 0.5 ml $NH_4SO_3NH_2$ (12% w/v). The tubes are thoroughly shaken.
13. One-half milliliter of 3-methyl-2-benzothiozolone hydrazone (MBTH; 0.5% w/v) was added and completely mixed.
14. A set of glucosamine-HCl standards giving a final concentration range of 10 to 150 μg per tube (total volume equals 3 ml) is prepared. Water is substituted for glucosamine-HCl in the blank.
15. The tubes containing the experimental samples and the standards are incubated in a boiling water bath for 4 min, removed, and cooled in an ice bath.
16. One-half milliliter $FeCl_3$ (0.5% w/v) is added to each tube and allowed to stand for 30 min at room temperature before reading; alternatively, the mixture may be centrifuged or filtered.
17. The absorbance is read at 650 nm in a Beckman spectrophotometer or a Coleman colorimeter. The chitin content is estimated from the standard curve of glucosamine-HCl read at 650 nm.

The means of eight replicates were used for each chitin value reported in this

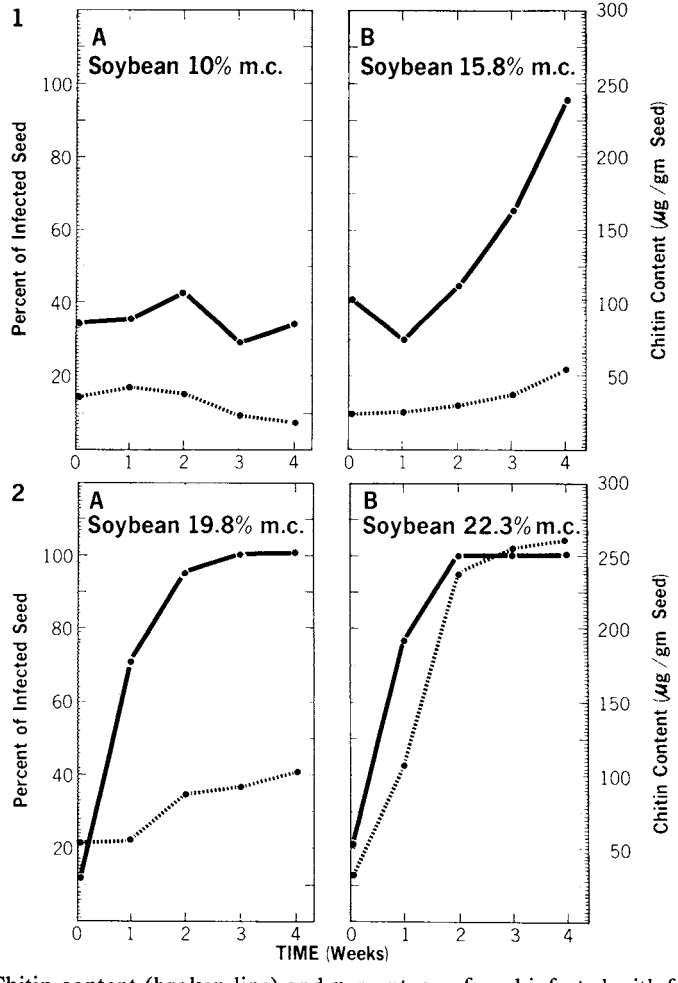

Fig. 1. Chitin content (broken line) and percentage of seed infected with fungi (solid line) of soybean seeds stored at various moisture contents for 1 month. The results are averages of duplicate samples. 1A: 10% moisture content; actual chitin values proceeding from 0 time to 4 weeks: $36.5^{\pm 6.4}$, $42.6^{\pm 7.1}$, $38.9^{\pm 7.3}$, $24.0^{\pm 6.3}$, and $19.5^{\pm 3.9}$. Fungi isolated: *Aspergillus glaucus, Alternaria* sp. and *Phoma* sp. Percentage of seed that yielded fungi (PSYF) after surface disinfection at end of experiment was 33. 1B: 15.8% moisture content; actual chitin values are $24.7^{\pm 7.0}$, $26.0^{\pm 6.4}$, $29.4^{\pm 8.8}$, $36.6^{\pm 3.6}$, and $54.8^{\pm 6.2}$. Fungi isolated *Aspergillus glaucus* (92-98%), *Alternaria* sp., *Phoma* sp. and *Aspergillus candidus*. PSYF = 92-98%. Fig. 2. Chitin content (broken line) and percentage of seed infected with fungi (solid line) of soybean seeds stored at various moisture contents for 1 month. The results are averages of duplicate samples. 2A: 19.8% moisture content: actual chitin values are $54.0^{\pm 8.6}$, $56.8^{\pm 10.2}$, $87.6^{\pm 18.9}$, $92.1^{\pm 10.7}$, and $102^{\pm 16.8}$. Fungi isolated: *Aspergillus glaucus* (100%), *A. candidus, Alternaria* sp., *Penicillium* sp. and *Phoma* sp. PSYF = 100%. 2B: 22.3% moisture content; actual chitin values are $32.8^{\pm 4.6}$, $107.8^{\pm 14.5}$, $238.6^{\pm 36.0}$, $254.8^{\pm 15.5}$, and $261.5^{\pm 27}$. Fungi isolated: *Aspergillus glaucus* (98%), *A. candidus, Penicillium* sp., *Alternaria* sp. and *Phoma* sp. PSYF = 100%.

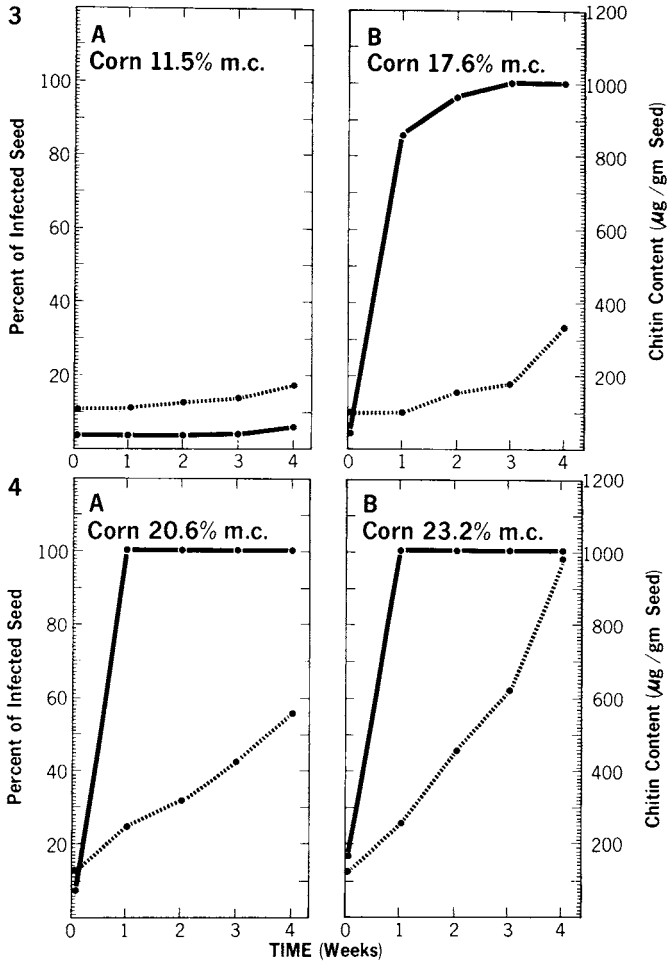

Fig. 3. Chitin content (broken line) and percentage of seed infected with fungi (solid line) of corn seed stored at various moisture contents for one month. The results are averages of duplicate samples. 3A: 11.5% moisture content; actual chitin values proceeding from 0 time to 4 weeks are: $102.9^{\pm 16.2}$, $119.9^{\pm 32.3}$, $128.5^{\pm 21.0}$, $133.6^{\pm 14.9}$, and $177.9^{\pm 35.6}$. Fungi isolated: *Aspergillus glaucus, Penicillium* sp. and *Chaetomium* sp. PSYF at end of 4 weeks equals 6%. 3B: 17.6% moisture content; actual chitin values are: $102.9^{\pm 16.2}$, $103.9^{\pm 25.1}$, $161.5^{\pm 27.5}$, $186.2^{\pm 36.9}$, and $335.9^{\pm 24.3}$. PSYF = 100%. Fig. 4. Chitin content (broken line) and percentage of seed infected with fungi (solid line) of corn seed stored at various moisture contents for one month. The results are averages of duplicate samples. 4A: 20.6% moisture content; actual chitin values are $122.3^{\pm 30.3}$, $250.9^{\pm 24.2}$, $318.9^{\pm 54.5}$, $426.0^{\pm 45.0}$, and $556.8^{\pm 78.2}$. Fungi isolated: *Aspergillus glaucus* (100%), *A. flavus, A. niger, Penicillium* sp. and *Chaetomium* sp. PSYF = 100%. 4B: 23.2% moisture content; actual chitin content values are $128.1^{\pm 26.6}$, $259.7^{\pm 16.4}$, $459.8^{\pm 70.7}$, $619.6^{\pm 122.9}$, and $978.9^{\pm 102.8}$. Fungi isolated: *Aspergillus glaucus* (38-98%), *A. flavus* (15-96%), *Penicillium* sp., *Chaetomium* sp., *A. niger*. PSYF = 100%.

communication. These values were normalized by dividing them by the value of the dry weight of the grain used in the initial extraction.

RESULTS AND DISCUSSION

The chitin contents of sound (relatively free of fungi) soybean and corn seed differed radically from each other (Figs. 1A and 3A); that of sound soybeans ranged between 20 and 43 μg/g seed, and that of corn was 100 to 178 μg/g seed. These chitin values were determined from samples of Foundation seed which were free of obvious fungal contamination. It seems likely that the high background response for corn may be due to glucosamine or N-acetyl-D-glucosamine, constituents of seed glycoproteins (10,11). Both of these compounds can be detected by the chitin assay and the amount present is a species characteristic. Glucosamine ranges from trace amounts in barley seeds to 0.051 g/g dry weight of seed in clover (2).

The maximal amounts of chitin in moldy soybean and corn seed also differ from each other, even when they are incubated under similar environmental conditions. After 28 days under comparable environmental conditions, chitin values of 261 μg/g seed (Fig. 2B) and 980 μg/g seed were obtained for soybeans and corn (Fig. 4B), respectively. Both samples of seed were infected chiefly with *Aspergillus glaucus*. For some unknown reason, the soybean seed appeared to inhibit fungal growth more than corn did. This inhibition is shown in Fig. 5, using moisture content as the dependent variable.

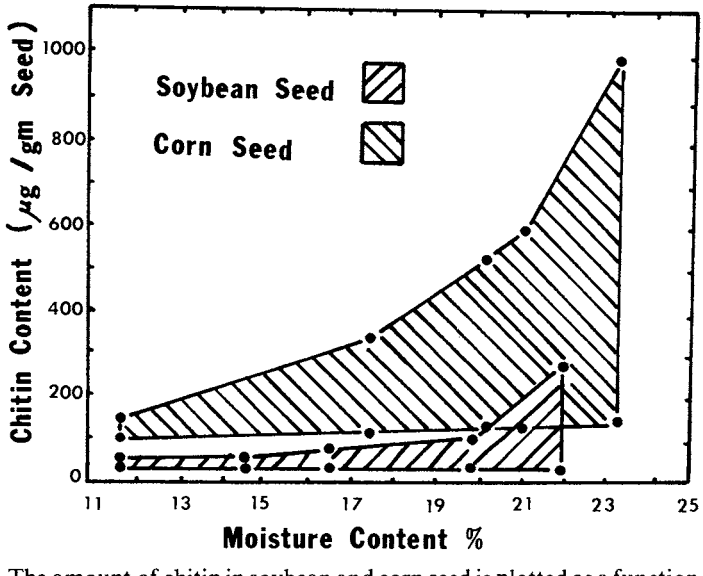

Fig. 5. The amount of chitin in soybean and corn seed is plotted as a function of the seed moisture content. The lower line for either the corn or soybean seed represents the control values on day one. Those of the upper line were taken after 4 weeks. All seed samples were stored at 26° C, atmospheric O_2 and CO_2 levels, and between 80 and 85% relative humidity.

The shape of the chitin growth curves shown in Fig. 5 suggests that fungal growth was inhibited in soybean seeds; it increased until the second week, after which it leveled off (Fig. 2B). In contrast, the chitin growth curve of moldy corn (Fig. 4B) had no such plateau; instead, growth increased linearly with time.

The plating method used measures the number of seeds in a sample which are internally infected with fungi, but does not measure the extent of fungus invasion of the seed before plating. Chitin values, on the other hand, directly estimate the total quantity of fungus mycelium and conidia in seeds as they appear in storage. It is evident that the two methods yield different information; the plating method measures the number of propagules in the seed, whereas the chitin method measures the degree of colonization based on viable and nonviable mycelium. On the other hand, chitin content does not distinguish between the species of fungi found in seeds, whereas the plating method does.

The chitin content of seed and the PSYF are related, but not absolutely; *i.e.*, one cannot use the value of one to estimate the other with reasonable accuracy. For example, as the percentage of infected soybean seeds increases, the amount of chitin present may increase only a little (Figs. 1B and 2A). This suggests that even though most of the seed sample was infected, little fungal growth had

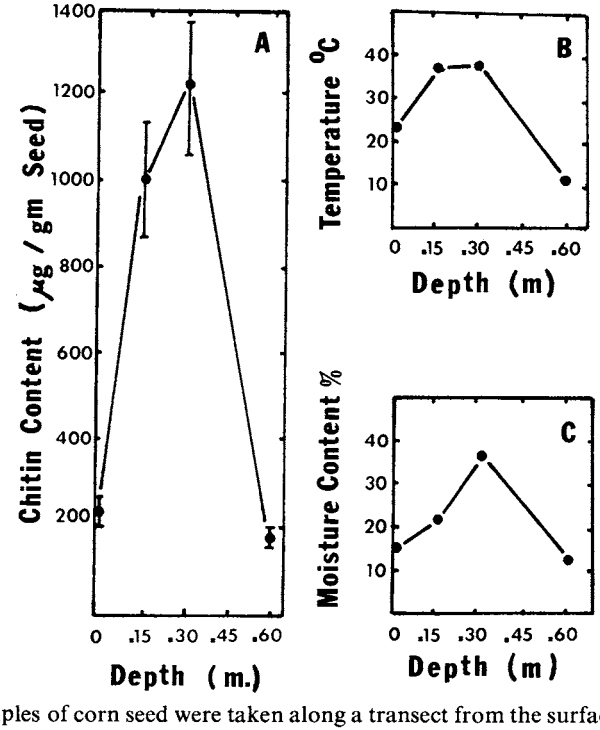

Fig. 6. Samples of corn seed were taken along a transect from the surface of a corn bin down 0.6 m through a microbially induced hotspot. The chitin content (A), temperature (B), and moisture content (C) of the seed are plotted as a function of the depth from the surfaces of the seed found in the elevator.

occurred in individual seeds. Likewise, a low value for percentage of infected seed may be misleading in the sense that although most seeds may be free from fungi, a few may be heavily invaded as measured by the amount of chitin.

When 100% of the seeds is infected with fungi, the PSYF is of little value as a relative measure of fungal growth in moldy seed. This is illustrated in Figs. 3B, 4A, and 4B where corn has a PSYF value of 100% but the chitin content continues to increase linearly.

The lower the moisture content, the slower the fungal invasion and the lower the chitin content of the seed (Figs. 1–4). This assertion is illustrated in Fig. 5 where the chitin content at the beginning and end of a month of fungal growth is graphed against moisture content. Although it is common knowledge that moisture content can limit fungal growth in seeds of cereals (1,7), as determined by the plating-out method, this is the first report of this phenomenon in corn and soybeans as determined by measure of chitin content.

A microbially induced "hotspot" in stored corn in a local commercial elevator was discovered during the course of this study. The hotspot was characterized as to size, temperature, moisture content, and chitin content of the stored seed, as illustrated in Fig. 6. The temperature at the center of the hotspot and 0.15 to 0.30 m in depth was 38° C, and the moisture content ranged from 22% at 0.15 m to 37% at 0.30 m. The chitin contents at the surface and at 0.15, 0.30, 0.45, and 0.60 m were about 200, 1000, 1200, and 190 μg/g of seed, respectively. No visible growth of fungi appeared at the surface or at 0.6 m, and the corn appeared sound when examined visually and had a moisture content of about 15%. Fungi isolated from corn at all depths were predominantly species of *Penicillium*. The center of the hotspot contained about the same chitin content (1000 μg/g) as corn stored in the laboratory at a moisture content of 23% at 26° C for 4 weeks (Fig. 4B). As the initial temperature of the stored grain was about 12° C, it appeared that the hotspot took more than 4 weeks to develop. This report constitutes the first such study of a microbially induced hotspot in a grain bin characterized according to its chitin content, and shows that the chitin values obtained under laboratory conditions can be similar to that found in a naturally occurring hotspot in a commercial elevator.

Does the chitin assay have any potential in commercial grain transactions for diagnosing grain deterioration? Conventional analysis of seed for deterioration involves determination of the percentage of infected seeds. Plating seeds may take 5–7 days for diagnosis. In contrast, the chitin assay can be done in a day (4–6 hr, depending upon the number of samples). Further, the method has potential for greater sensitivity in analyzing for glucosamine via gas chromatography, thereby decreasing the sample size needed for analysis and decreasing the time of analysis.

In summary, this study has shown that: 1) chitin analysis is a relatively rapid method that can be used to measure fungus invasion of corn and soybean seed; 2) the method can more accurately measure the degree of fungus invasion of the above seeds than the plating method; 3) the chitin method was successfully used to characterize fungus invasion of a naturally occurring hotspot in stored corn; 4) the chitin content (probably arising from glycoprotein) of sound corn is greater than that of soybean seeds; and 5) compared to corn, soybean seed appears to inhibit growth of fungi under the environmental conditions described.

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