

# Relationship of Shelled Corn Fungal Susceptibility to Carbon Dioxide Evolution and Kernel Attributes<sup>1</sup>

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## ABSTRACT

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Fifty-one samples of shelled corn were rewetted to 21% wb moisture content (MC) and evaluated for susceptibility to fungal invasion using ergosterol measurements and a test kit that measures carbon dioxide (CO<sub>2</sub>) evolution. The sample attributes measured were percent germination, electrolyte leakage after soaking in deionized water, percent fines, and percent kernel infection. The difference in ergosterol content before and after incubation at 24°C was used as the standard measure of fungal growth. Differences in CO<sub>2</sub> evolution among the samples were consistent with expectations for fungal growth based on storage history. The coefficients of determination ( $r^2$ ) for the linear regression of ergosterol differ-

ence with CO<sub>2</sub> kit readings were 0.46–0.60. All were statistically significant ( $\alpha = 0.001$ ) and  $r^2$  values were slightly greater when four high-oil corn samples were removed. These results indicate that the CO<sub>2</sub> test can be used to assess fungal susceptibility of rewetted shelled corn, which may also be indicative of its susceptibility before rewetting. The linear regressions of kernel attributes with ergosterol difference ( $48 < n < 51$ ) that were statistically significant included percent germination,  $r^2 = 0.49$  ( $\alpha = 0.001$ ); electrolyte leakage,  $r^2 = 0.27$  ( $\alpha = 0.001$ ); and percent fines determined with a 4.76-mm sieve,  $r^2 = 0.12$  ( $\alpha = 0.05$ ).

One of the major causes of quality deterioration during storage of shelled corn is invasion and growth of fungi. Shelled corn traded in the United States is usually comingled at the elevator or processing facility and there is little or no information on its prior storage history. Managers of elevators or storage facilities typically assess the likelihood of spoilage from fungi on the basis of visual appearance and wet basis (wb) moisture content (MC). Once it is placed in storage, fungal growth is detected by monitoring the temperature increases associated with heat released by the fungal respiration. Fungal respiration also produces carbon dioxide (CO<sub>2</sub>) and therefore, as demonstrated recently by Ileleji et al (2006), real time CO<sub>2</sub> monitoring of stored shelled corn in storage structures can be used to detect fungal growth.

Fungal susceptibility, as it is used in this report, is defined as the likelihood that fungi will grow on shelled corn during subsequent storage or shipment if it is subjected to conditions conducive to fungal growth. Thus, corn with high fungal susceptibility is at greater risk for fungal growth when exposed to such conditions. For example, when corn is kept in storage for extended periods of time (10 months to several years), moisture condensation caused by unfavorable headspace conditions (Montross et al 2002) and increases in grain temperature from factors such as solar radiation or high ambient temperatures can create conditions conducive to fungal growth. Also, when U.S. corn is exported overseas, it may pass through subtropical and tropical climates. This exposes the cargo to temperatures >30°C and air at high relative humidity, which can promote fungal growth, particularly in highly susceptible corn lots. When corn is shipped to countries with tropical climates, fungal growth during subsequent storage is a common problem.

The likelihood of spoilage associated with these scenarios could be reduced if there were reliable methods available for assessing corn fungal susceptibility. We believe that the rate at which fungi grow when corn is incubated at moistures and temperatures conducive to fungal growth is a good indicator of its susceptibility to fungal invasion if it encounters “stressful” ambient conditions during transport and storage. By combining fungal

susceptibility testing with real time CO<sub>2</sub> monitoring, the grain industry could better manage its stored shelled corn.

Fungal susceptibility of shelled corn can be quantified by monitoring the rate of fungal growth in a representative sample incubated at a specified temperature and moisture. One approach to this quantification is measurement of ergosterol or other compounds associated with fungal growth. Ergosterol is the sterol component in nearly all fungi and it is not produced by higher plants (Burnett 1976). Seitz et al (1979) proposed that ergosterol be used as a sensitive indicator of the presence of fungi in corn and it has subsequently been used to measure fungal biomass in grains, oilseeds, and nonfood materials (Bjurman 1994; Dhingra et al 1998; Dowell et al 1999; Panasen et al 1999; Trung et al 2001; Abramson and Smith 2003; Marin et al 2005; Mille-Lindblom 2005).

In a previous study of shelled corn by Moog et al (2004), the correlations of initial ergosterol with CO<sub>2</sub> production and other indices of fungal growth were relatively low. They concluded that initial ergosterol was inadequate for assessing susceptibility of rewetted shelled corn to fungal invasion. A better measure of fungal growth is the change in ergosterol content before and after incubation. This eliminates the influence of the ergosterol from the hyphae of dead fungi, such as field fungi that do not usually grow in stored grain. Also, Seitz et al (1977) and Schnurer (1993) observed that sporulation does not cause ergosterol to increase. Therefore, it is better to make the second ergosterol measurement in the earlier stages of fungal growth before any significant sporulation occurs.

Another technique that quantifies fungal growth is measurement of CO<sub>2</sub> production by a sample during incubation kept at a constant temperature and MC. Saul and Steele (1966), were among the first to evaluate the effects of MC, temperature, and kernel damage on CO<sub>2</sub> production and associated dry matter loss. Their data, along with results of others was the basis for an ASABE Standard for estimating allowable storage time for shelled corn based on MC and temperature (Bern et al 2002; ASABE 2006a).

Loss of germ viability and kernel integrity make it easier for fungi to invade corn kernels and, therefore, measurements of these traits may be indirect indicators of fungal susceptibility. Germ viability can be determined using germination tests. One measure of kernel integrity is the presence of fine material. Assuming that the fine material present in a sample of corn is produced primarily by physical damage to the kernels, the percentage of fine material in a sample can indicate the extent to which kernels have sustained damage that provides a pathway for fungi to easily penetrate into the kernels.

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Another measure of kernel integrity, electrolyte leakage, has been used by the seed industry for measuring seed deterioration. When kernels are submerged in deionized water, the leaching of electrolytes from the cells to the surrounding water is an indicator of membrane deterioration. This leakage of electrolytes increases the conductivity of the water. Marks and Strohshine (1998) hypothesized that the test could be used to indicate susceptibility to fungal invasion because fungi would have better access to kernel nutrients when cell membrane integrity has been lost. They found that CO<sub>2</sub> evolution rate from samples of shelled corn rewetted to 20.5% MC was correlated ( $r^2 = 0.62$ ) with the conductivity of deionized water after kernels were soaked for 15 min.

Recently, Woods End Laboratories (Mt. Vernon, ME) proposed that Solvita test kits could be used to determine susceptibility of shelled corn and other grains to invasion of storage fungi (Brinton 2004). The kits are currently utilized to monitor CO<sub>2</sub> production by microorganisms in soil or compost and are therefore suited for similar determinations on grain samples. The kits are relatively inexpensive and easy to use. Preliminary tests at Purdue University (Strohshine 2002) and Iowa State University (Chitrakar et al 2006) indicated that the kits could reveal differences in CO<sub>2</sub> production by samples of rewetted corn after two to three days of incubation at room temperature in air-tight glass containers. The number of samples that can be tested simultaneously is limited only by the number of jars that can be tested.

This study was conducted to determine whether CO<sub>2</sub> production by samples of shelled corn rewetted to 21% MC was a good indicator of the susceptibility of these samples to invasion by storage fungi. In addition, the relationship between fungal growth and percent infected kernels in the samples, along with the influences of kernel integrity on fungal susceptibility were investigated. The Solvita test kits were used to evaluate CO<sub>2</sub> production with the intention of determining whether they could be used by the grain industry for fungal susceptibility testing and, eventually, as a tool for better managing shelled corn during storage and transport. In conducting the evaluation, the ergosterol production by the sample during incubation was used as the standard measure of fungal growth. The specific objectives of this study were to 1) determine whether the CO<sub>2</sub> test kit gives a sensitive measurement of susceptibility to fungal invasion of shelled corn that has been rewetted to 21% MC and incubated at 24°C using correlations with ergosterol production; 2) examine the extent to which kernel integrity influences the differences in fungal susceptibility as indicated by correlations of factors with ergosterol production by the rewetted shelled corn (percent kernel infection, percent germination, percent fine material, and electrolyte leakage accompanying soaking in deionized water).

## MATERIALS AND METHODS

A total of 68 samples of shelled corn, previously stored under a variety of conditions, were tested. However, only 51 could be used for the analyses reported in this study. Of those 51 samples, 43 were obtained from bins at Purdue's Post-Harvest Education and Research Center (PHERC), and the remaining eight were collected from commercial facilities. The MC of the samples at the time of collection was <14% with the exception of sample 50, which was at 15.7% MC when it was collected. When the samples from bins or commercial facilities arrived at the laboratory, they were stored at either 2 or 6°C until needed for evaluation. Sample 50 was tested within two weeks of the date on which it was gathered.

### CO<sub>2</sub> Measurement

The CO<sub>2</sub> produced by the samples was determined using a test kit manufactured by Woods End Laboratories. Shelled corn (320 g) was rewetted with deionized water to  $21 \pm 0.5\%$  MC and equilibrated in air-tight plastic bottles with screw-on caps for 24 hr at

$24 \pm 1^\circ\text{C}$ . Subsamples (100 g) of rewetted corn were placed in separate glass jars (472 mL) with screw-on metal lids. (Note: The standard Solvita test kit uses a plastic container.) Each jar lid was fitted with a thin rubber disk that functioned as a seal when the lid was tightened. The jars were stored in the 24°C room for another 24 hr. The remainder of the rewetted sample was used for moisture determinations.

The Solvita test kits detect the CO<sub>2</sub> content of the air in the jar by means of an indicator gel affixed to a white plastic paddle insert. A picture of the test kit is included in Chitrakar et al (2006). The plastic paddle is thin (3 mm) and rectangular (44 mm × 29 mm) with a tapered plastic spike 38 mm long protruding from one edge, like the handle of a paddle. A band of gel indicator is affixed to one face of the paddle that changes color in response to increases in ambient CO<sub>2</sub> concentration. If the respiration of fungi growing on the corn kernels becomes the primary source of CO<sub>2</sub> from the sample, the color of the gel changes in response to fungal growth.

Before the test paddles were placed in the jars (47 hr after addition of water), the CO<sub>2</sub> content of the air in the jars was restored to ambient conditions by removing the lid for 1 hr. For the next 8 hr, the color of the gel indicator was determined hourly without removing the paddles from the jar. In a room well illuminated by fluorescent lighting, the color of the paddle was compared with the colors on a card provided by the manufacturer. The colors on this card are numbered (1, 2, 2.5, 3, 3.5, 4, and 5) and can be used to assign a color number to each reading. At 71 hr after rewetting, the lids were again removed for 1 hr and the first paddle was removed. A new CO<sub>2</sub> paddle was placed in the jar at 72 hr and the lids were secured on the jar. Paddle color readings were recorded hourly for the next 8 hr.

After each test was completed, triplicate 15-g samples were taken from each jar for MC determination using the 72-hr whole kernel oven method (ASABE 2006b). Only those samples with final MC values within 0.5% of the target moisture ( $21 \pm 0.5\%$ ) were used for subsequent analyses. The remainder of each sample was placed in a wire basket and dried to 14% wb moisture in a convection oven at 40°C. The slope of the plot of color number versus time was determined for each of four intervals (48–51, 48–52, 72–75, and 72–76 hr) by fitting data points to a straight line using linear regression. These indices were designated as slope 48–51, 48–52, 72–75, and 72–76, respectively. These time intervals were chosen because it was during these intervals that the color number was changing most rapidly. In addition, the sums of the color number readings for 48–51, 48–52, 48–53, 72–75, 72–76, and 72–77 hr were determined. These sums are directly proportional to the cumulative CO<sub>2</sub> evolved during the specified time intervals. By the end of the test, the maximum color number (5.0) was attained by  $\approx 10\%$  of the samples.

After completion of the CO<sub>2</sub> tests, subsamples of corn from each jar were dried to  $\leq 14\%$  MC in a forced convection oven set at 40°C. Drying progress was monitored by calculating the loss in weight needed to reduce MC from 21 to 14% and then periodically removing the samples from the oven and weighing them. Upon completion of drying, 40–70 g of shelled corn was placed in doubled plastic bags (one within another) for shipment to the USDA-ARS Grain Marketing and Production Research Center (GMPRC) in Manhattan, KS. At the GMPRC, the samples were ground and two representative 10-g subsamples were analyzed for ergosterol content using the procedure described below. The average of the two determinations was reported as the final ergosterol content of the sample from which the subsample was removed. In addition, a 50-g representative subsample was taken from all of the samples before they were wetted for the CO<sub>2</sub> test. Each subsample was placed in a doubled plastic bag and sent to the GMPRC where they were prepared for analysis using the procedure described above. All samples sent to GMPRC had MC values <14%. Therefore, there should have been no additional fungal

growth between the time of bagging and the analysis. The initial and final ergosterol contents of these samples were used to calculate the difference in ergosterol before and after testing.

### Ergosterol Analysis

The ergosterol analysis procedure was a modification of a method described by Seitz et al (1979). Whole grain samples were ground before analysis using a Falling Number AB grinder (model KT-30, Stockholm, Sweden) on setting 2. Briefly, an extract was obtained by mixing 50 mL of methanol with 10 g of ground sample and shaking vigorously for 45 min. Then 2.5 g of KOH was added to 25 mL of extract and the mixture was heated at 65–70°C for 30 min. After cooling, 10 mL of water was added, and three successive extractions were made using 10 mL of petroleum ether (PE). The extract was dried and the residue dissolved in 1 mL of methanol, then this sample was analyzed using HPLC with UV detection at 282 nm. The Luna-C18 reverse-phase column (Phenomenex, Torrance, CA) was 150 mm long and had an inside diameter of 4.6 mm. Particle size was 5 µm. The mobile phase was 93.3:6.7 methanol-to-water, and the flow rate was 1.2 mL/min. The column temperature was 60°C.

### Percent Germination

The percent germination of the samples was determined using a variation of the paper towel method used by the seed industry. Two hundred seeds were placed in a large shallow plastic tray that was lined with paper towels. Samples were wetted with deionized water and then covered with paper towels that were subsequently wetted. The trays were covered with clear plastic wrap to reduce evaporation, kept at room temperature (24°C), and periodically moistened. After seven days, the percent of kernels in each sample that germinated was determined.

### Kernel Infection

Kernels were surface-sterilized by exposure to 6% NaOCl (bleach) for 1 min and washed twice with deionized water for 1 min. Ten kernels were placed in each of 20 petri dishes that contained 6% malt salt agar medium (Tuite et al 1985) and the dishes were incubated at 29°C. After seven days of incubation, the number of kernels invaded by fungi was determined for each Petri dish and the percentages of infected kernels on each of the 20 Petri dishes were averaged.

### Percent Fine Material

The extent of kernel damage was evaluated by sieving and by electrolyte leakage. For the sieve tests, 100-g samples were shaken

for 30 sec over a 6.35-mm (1/64”) diameter round-hole sieve, and the kernels remaining on the sieve (classified as whole kernels) were weighed. The kernels that passed through the first sieve were again sieved over a 4.76-mm (12/64”) diameter round-hole sieve. After immature kernels and foreign material were removed, the weights of the material remaining on the sieve, the material that passed through the sieve, and the material removed from the sieve were determined. Three trials were conducted on each sample and the three percentages of fines removed were averaged for each of the two sieves.

### Electrolyte Leakage

Electrolyte leakage was determined on either two or three subsamples from each sample. Either four or six 500-mL beakers were filled with 400 mL of deionized water and then placed in a water bath maintained at 25°C. After the water in the beakers had equilibrated to 25°C, two or three 100-g subsamples of kernels were removed from each sample and successively poured into beakers at 1-min intervals. For the next 30 min, the conductance of the water was measured every 5 min using a conductance meter (model 35, YSI Instruments, Yellow Springs, OH). Before each conductance measurement, the beaker was stirred five times by moving a glass rod slowly around the edge (Marks and Strohline 1998).

### Statistical Analysis

The data were analyzed using the SAS software v.9.1 (SAS Institute, Cary, NC). The means, standard deviations, minimum and maximum values of each attribute were calculated using the MEANS procedure. The REG procedure was used for the linear regression of factors with ergosterol difference and the significance of each correlation was determined using the CORR procedure.

## RESULTS AND DISCUSSION

Samples used for this research were gathered from a variety of locations and had diverse storage histories. This diversity permitted a more thorough evaluation of the CO<sub>2</sub> kit's performance. Not all of the samples tested were used for the regression analyses. Seven samples were excluded because the MC after rewetting was outside what was considered to be an acceptable range (21 ± 0.5%). Another 10 samples were excluded because differences in ergosterol were negative. This apparent impossibility can be explained by the fact that ergosterol content can vary among replicate tests on the same sample. Unusually high initial ergosterol readings can occur when the subsample used for determining the

TABLE I  
Mean Values and Ranges of Measurements Conducted on 51 Samples Used for Correlations Between Ergosterol and CO<sub>2</sub> Kit Parameters or Kernel Attributes

Factor	Minimum	Maximum	Mean	SD	CV
Ergosterol difference <sup>a</sup> (ppm)	0.02	6.16	1.36	1.24	0.913
% Kernel infection	0.0	100.0	35.38	24.54	0.694
% Germination	0.0	98.0	63.46	29.62	0.467
% Fines 6.35-mm sieve	0.61	5.32	1.76	0.87	0.494
% Fines 4.76-mm sieve	0.10	2.55	0.87	0.60	0.695
Electrolyte leakage(10 min)	32.70	90.67	58.28	12.40	0.213
Electrolyte leakage(15 min)	36.83	108.37	65.42	14.82	0.228
CO <sub>2</sub> paddle readings					
Slope 48–51 hr	0.48	1.35	0.82	0.23	0.279
Slope 48–52 hr	0.43	1.30	0.83	0.25	0.296
51st hr reading	1.25	4.25	2.43	0.77	0.317
52nd hr reading	1.50	4.83	3.21	0.98	0.306
Slope 72–75 hr	0.40	1.65	0.88	0.31	0.351
Slope 72–76 hr	0.40	1.40	0.87	0.29	0.330
74th hr reading	1.00	2.50	1.65	0.50	0.301
75th hr reading	1.00	5.00	2.59	0.98	0.378

<sup>a</sup> Difference between ergosterol content after rewetting and incubation in CO<sub>2</sub> test kit and ergosterol content before rewetting for testing.

initial ergosterol of the sample, which is taken just before rewetting of the sample, contains one or more kernels that are heavily invaded by mold.

The means, ranges, and standard deviations of the attributes of the 51 samples that could be used are shown in Table I. The mean initial ergosterol content (not shown in Table I) was 1.5 ppm. However, the upper limit of initial ergosterol was 6.2 ppm, which indicates there was abundant fungal growth in some of the samples. In general, germs or entire kernels in samples with high ergosterol were discolored and they often had visible fungal growth on exposed parts of the kernels. Several samples had been stored for only a short period of time, yet were relatively high in ergosterol. This can be attributed to invasion by field fungi before harvest or to poor storage conditions immediately after harvest. The mean percentage of the kernels infected by osmophilic storage fungi was 32%, indicating that fungi had already invaded at least some of the kernels in most samples. The average percentage of kernels that germinated in the individual samples (63%) was relatively low. Germination usually decreases as time in storage and kernel temperature during drying increase. A low average percent germination is not surprising because many of the samples were taken from corn that had been stored several years or dried in a high-temperature dryer.

The first objective of this study was to determine whether the CO<sub>2</sub> evolution rate of a sample is a good indicator of its susceptibility to fungal growth. Results of the CO<sub>2</sub> kit paddle readings from tests on eight of the samples with diverse storage histories are shown in Fig. 1. Information on the storage history of these samples is presented in Table II. The differences in CO<sub>2</sub> evolution

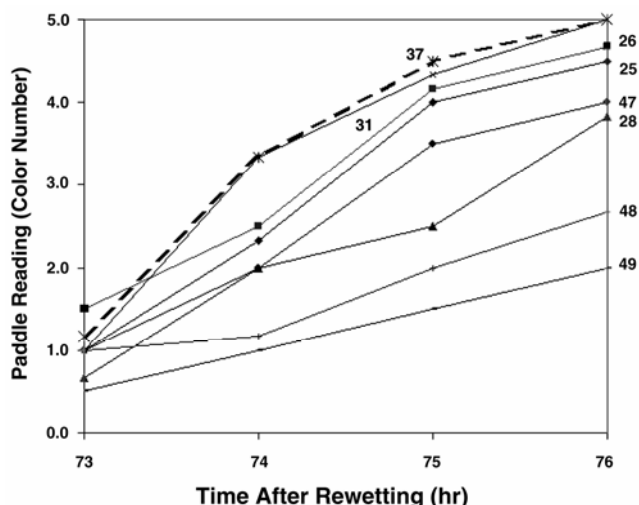


Fig. 1. CO<sub>2</sub> paddle readings (color number) vs. time for tests on samples of shelled corn. Samples were selected from a variety of storage conditions (see Table II).

rate among these samples were consistent with expectations based on factors that affect the likelihood of fungal growth, such as time in storage and MC during storage (Perez et al 1982). Samples that had a good appearance and had been stored less than one year (no. 48 and 49) had relatively low ergosterol levels (<1.60 ppm), low percent kernel infections (<25 %), and produced the least amount of CO<sub>2</sub>. By contrast, the samples that produced the most CO<sub>2</sub> (no. 25, 26, 31, and 37) had previously been stored for over a year in conditions conducive to fungal growth (Table II).

Regression analysis was used to compare ergosterol differences of the 51 samples to individual color numbers at 50 to 52 hr and 74 to 76 hr, along with the values for the slopes and sums. The relationships with the highest values of *r*<sup>2</sup> are shown in Table III. The difference in ergosterol is an indicator of the mass of fungi that has been produced during the CO<sub>2</sub> test and it seems reasonable that CO<sub>2</sub> production should be proportional to the increase in fungal mass, at least during the initial stages of fungal growth before sporulation occurs. All of the correlations with differences in ergosterol (including those not shown) were statistically significant and the coefficients of determination (*r*<sup>2</sup>) for the best correlations (Table III) were 0.46–0.60. Plots of the sum of CO<sub>2</sub> kit readings for the readings at 72 through 75 hr and the reading at 50 hr after rewetting versus ergosterol difference (*r*<sup>2</sup> = 0.58 and 0.56, respectively) are presented in Fig. 2.

When this report was reviewed for publication, one reviewer asked whether hermetic sealing of the jars could have depleted oxygen and affected fungal growth. This possibility was investigated using a series of calculations in which the color number 3 hr after insertion of the paddle into the jars (51 and 75 hr) was converted to percent CO<sub>2</sub>. Color numbers of 4.0 and 5.0 correspond to 1.5 and 3.0% CO<sub>2</sub>, respectively, in the jars. Assuming respiration of a simple sugar, the mg of CO<sub>2</sub> released, calculated from the % CO<sub>2</sub> in the jar, will equal the mg of O<sub>2</sub> consumed. If the air in the jars initially contains 21% O<sub>2</sub>, the %O<sub>2</sub> remaining in the jars after 23 hr (the maximum time the jars were sealed) can

TABLE III  
Coefficients of Determination (*r*<sup>2</sup>) for Linear Regressions of CO<sub>2</sub> Kit Color Indices with Differences in Ergosterol of Samples

Factor	<i>r</i> <sup>2</sup> for All Samples (n = 51) <sup>a,c</sup>	<i>r</i> <sup>2</sup> for Selected Samples (n = 47) <sup>b,c</sup>
Sum 48–52 hr	0.549	0.555
Slope 48–52 hr	0.464	0.479
50th hr reading	0.559	0.527
51st hr reading	0.506	0.522
Sum 72–75 hr	0.575	0.599
Slope 72–75 hr	0.489	0.595
74th hr reading	0.536	0.550
75th hr reading	0.537	0.580

<sup>a</sup> Moisture content criteria (21 ± 0.5%).

<sup>b</sup> Excludes results from four samples with high-oil corn.

<sup>c</sup> Correlation is statistically significant at α = 0.001.

TABLE II  
Information on Storage History of Samples Used for CO<sub>2</sub> Kit Tests Shown in Fig. 1

Sample	Source <sup>a</sup>	MC (% wb)	History
25	PHERC	13.0	Nonaerated bin; sample taken from top of the bin; Bt corn; condensation on the surface of the kernels; stored for ≈3 yr.
26	PHERC	12.7	Nonaerated bin, sample taken from bottom of the bin; Bt corn; stored for ≈3 yr.
28	PHERC	13.0	Bin aerated with ambient air using a fan; sample taken from bottom of the bin; Bt corn; stored for ≈3 yr.
31	PHERC	11.8	Nonaerated bin; sample taken from top of the bin; condensation on top layer of grain; stored for ≈3 yr.
37	PHERC	11.1	Bin aerated with air cooled by a chiller; sample taken from top of the bin; records indicated water had leaked into this bin during the storage period; stored for ≈3 yr.
47	Commercial trader	14.7	Obtained from corn being exported; sample was received in July of 2003; estimated time of storage 9 months.
48	Commercial trader	15.3	Obtained from corn being exported; sample was received in July 2003; estimated time of storage 9 months.
49	Commercial trader	14.5	Obtained from corn being exported; sample was received in July 2003; estimated time of storage 9 months.

<sup>a</sup> Purdue's Post-Harvest Education and Research Center (PHERC) samples taken from bins used in tests by Ilejeji et al (2004).

be calculated. For color numbers 4.0 and 4.75 at 3 hr of incubation, there would be 9.3 and 1.5% O<sub>2</sub> in the jars at 23 hr, respectively. If color number 5.0 is attained, then all of the O<sub>2</sub> could have been consumed at 23 hr. According to Paster (1990) and Banks and Annis (1990), many storage fungi can grow at elevated CO<sub>2</sub> concentrations (up to 50%) and oxygen concentrations as low as 2%, while some can grow at oxygen concentrations as low as 0.5%. Of the samples used for this study, only one attained a color number >4.5 at 51 hr and only two attained a color number >4.5 at 75 hr. The one sample that reached a color number of 5.0 at 51 hr (during the day 2 test) attained a color number of 4.5 at 75 hr (during the day 3 test). Therefore, there was no indication that depletion of O<sub>2</sub> affected results of our experiments. Nevertheless, the effects of oxygen depletion and procedures for preventing it should be investigated in future studies.

Although some studies have attributed the primary source of CO<sub>2</sub> production by stored seeds to microbial activity rather than the respiration of the seed (Milner and Giddes 1945; Christensen and Kauffman 1969; Muir and White 1998), the CO<sub>2</sub> contributed by seed respiration can still influence results. The effect of seed respiration was investigated by examining the plot of CO<sub>2</sub> kit color number for the reading at 74 hr versus the percent germination for 51 samples (Fig. 3). Note that the slope is negative, indicating that for most samples, the color number decreased as percent germination increased. If seed respiration had a dominant effect on CO<sub>2</sub> evolution, then the slope of the regression line should have been positive.

The color numbers can be converted to an equivalent percent CO<sub>2</sub> using data available from the test kit manufacturer. The color numbers predicted by the regression line for 0 and 100% germination, respectively, are 2.28 and 1.23. These correspond to 0.48 and 0.23% CO<sub>2</sub> in the incubation jars. This means that three days after rewetting, after the first 2 hr of incubation (74 hr), the samples containing mostly nonviable seeds released, on the average, twice as much CO<sub>2</sub> as those in which most of the seeds were viable.

Three of the samples with germinations >70% had relatively high color numbers (>4, the data points in the upper right corner of the plot). Examination of sample attributes revealed that two were high-oil hybrids. High-oil corn hybrids typically have germs that are a larger proportion of the total kernel weight (Weber 1987 [citing Flora and Wiley 1972; Arnold et al 1974; Roundy 1976]) and therefore it seems likely that viable high-oil seeds would produce more CO<sub>2</sub>. There were two more high-oil corn samples among the 51 samples. One had 22% germination and its data

point was very close to the regression line. The fourth sample had 1.5% germination and its data point was below the line. Before these four samples were removed, the value of regression was  $r^2 = 0.37$ , and after they were removed, it increased to  $r^2 = 0.45$ . Table III includes the  $r^2$  values for regressions of the CO<sub>2</sub> kit readings versus ergosterol difference after the four high-oil samples were removed. With the exception of the 50th hr reading, the  $r^2$  values increased after exclusion of the four high-oil samples. The values of  $r^2$  for the factors derived from the third day readings were greater than most of those calculated for the second day readings, and the increases in  $r^2$  as a result of removal of the four high-oil hybrids were also greater. A possible explanation is that the final ergosterol was determined after 72 hr of incubation and there may have been changes between 50 and 72 hr that affected the correlation.

As noted previously, some of the samples were excluded from the regression analyses because final MC values exceeded 21.5%. That group included six high-oil corn samples with MC values of 21.51–22.04%. All of the points for the five high-oil samples with >33% germination were well above the expanded data set's regression line for color number versus percent germination. However, the data point for the remaining sample, which had 4.0% germination, was very near the regression line. The greater CO<sub>2</sub> release by the viable high-oil seeds could be attributed to seed respiration, a higher inherent susceptibility to fungal invasion (Ileleji et al 2003) or to a slightly higher (0.5%) MC (Chitrakar et al 2006).

A review of the data presented by Ileleji et al (2003) indicates that fungal growth and CO<sub>2</sub> production by high-oil corn hybrids at a given time should be, on the average, ≈10–15% greater than they are for normal corn. The effect of MC can be estimated using an equation presented by Chitrakar et al (2006). Two of the six high-oil samples had MC values of 22%, while the remainder of the high-oil samples had MC values of 21.51–22.72%. For a 1% increase in moisture, the percent CO<sub>2</sub> in the test kit jar should increase by a factor of 1.3. In these tests, after only 3 hr of incubation, the % CO<sub>2</sub> in the jars for high-oil samples with higher germination percentages (≥33%) were 2.7–5.0 times the average CO<sub>2</sub> content for a sample with the same percent germination, as predicted using the regression equation fit to the expanded data set. Thus the CO<sub>2</sub> production by the high-oil samples was greater than what would be expected if only greater susceptibility and higher MC are considered.

CO<sub>2</sub> production was also observed for a pair of samples, one with nearly 100% germination (HG1) and the other with almost

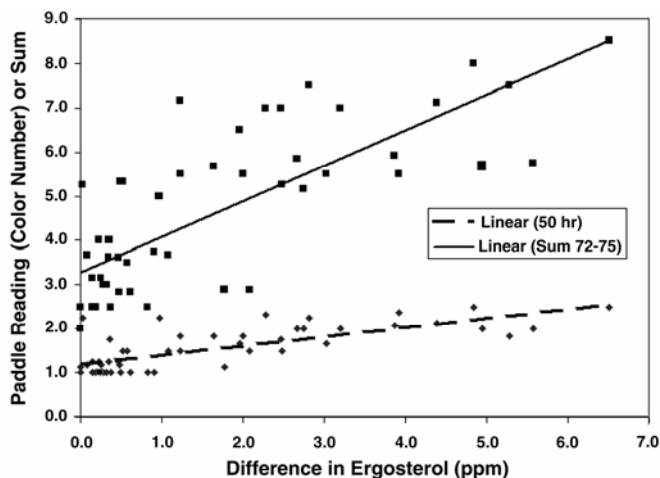


Fig. 2. CO<sub>2</sub> kit paddle color readings vs. the difference between final and initial levels of ergosterol. Readings are color numbers at the 50th hr (◆) and sum of the readings for 72–75 hr after rewetting (■).

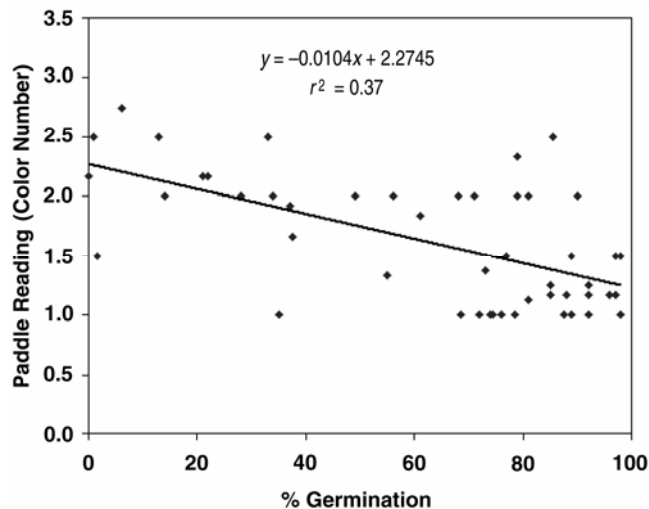


Fig. 3. Plot of CO<sub>2</sub> kit paddle color readings vs. % germination for 51 samples. Readings were color numbers at 74 hr after rewetting.

0% germination (LG1) starting at 24 hr after addition of water (Moog 2006). At 24–32 hr, HG1 produced as much CO<sub>2</sub> as LG1, where CO<sub>2</sub> production should have been primarily from fungal growth. The next day, at 48–56 hr, CO<sub>2</sub> production by HG1 dropped to 37% of the previous day's value while CO<sub>2</sub> production by LG1 was nearly the same (93% of the previous day's value).

It is our opinion that these results indicate germ respiration can make a noticeable contribution to CO<sub>2</sub> production and that it can affect the CO<sub>2</sub> test kit results. However we also believe that it does not obscure the ability of the test kit to identify samples with high fungal susceptibility. Thus a correction of the CO<sub>2</sub> kit color results for germ viability could improve the kit's ability to measure fungal susceptibility. However, a thorough investigation of the effect of germ respiration and the development of a correction procedure are beyond the scope of this study.

Regression analysis was also used to compare correlations between ergosterol content and each of the various kernel attributes measured. The results are summarized in Table IV. As explained in the table footnotes, several samples with an ergosterol difference >7 ppm were excluded from three of the analyses. These samples were outliers in that they were noticeably below the regression lines.

Percent germination had the highest correlation ( $r^2 = 0.49$ ) among the regressions. Tuite and Foster (1979) stated that a good gauge of the likelihood that a given lot of grain can be safely stored is its ability to maintain a high germination percentage during continued storage. Fungal invasion is one of the major factors that causes germination to decrease. Results of several studies indicate that fungi preferentially invade the germs of the corn kernels. Qasem and Christiansen (1960) and Tuite et al (1985) found that damage to kernels in the vicinity of the germ promoted fungal invasion. Tsuruta et al (1981) examined invaded kernels using electron microscopy and observed that fungal hyphae generally invaded the kernel through the tip cap or the pericarp covering the germ and that there were many hyphae in or near the germ. On the other hand, other factors, most notably high temperature drying, also decrease the percent germination. This may explain why seven samples with initial ergosterol readings <2 ppm also had <20% germination. These seven samples were obtained either from bins that had been aerated with cool air from a grain chiller or from corn lots that appeared to be of high quality and were being exported.

Physical damage to the kernel such as damage to the seed coat, cracks in the endosperm, and pieces broken from the kernel can increase its susceptibility to invasion by fungi and could also increase the rate of water penetration into the kernel and thereby facilitate efflux of electrolytes. The regression of electrolyte leakage versus ergosterol difference had a positive slope, indicating that fungal growth was greater in samples that had greater electrolyte efflux. Although the coefficient of determination for this regression was relatively low ( $r^2 < 0.29$ ), it was the second highest

among the relationships tested. The correlation at 15 min was better than the correlation at 10 min. The additional soaking time may have allowed more water penetration into the kernel facilitating the leakage of electrolytes from damaged kernels. Although Marks and Stroshine (1998) presented evidence that MC could affect electrolyte leakage, their results were not conclusive. Furthermore, the MC of the samples used in these tests was relatively consistent (13–15%). These variations in moisture should have had only a minor effect on the results (Marks 1993).

Although the regression of ergosterol difference with percent fines determined with the 4.76-mm sieve was statistically significant, the  $r^2$  was relatively low (0.12). The regression for the 6.35-mm sieve was not statistically significant. This may be related to the size of the particles in the fine material removed by the sieve. The larger pieces that were removed by the 6.35-mm sieve were mainly composed of endosperm while the smaller pieces removed by the 4.76-mm sieve included pieces of germs. Smaller and softer germ could easily break into smaller pieces during handling. These small pieces of germ may be a good source of sucrose, protein, oil, and other nutrients and may therefore have promoted fungal growth.

The correlation of kernel infection with ergosterol difference was not statistically significant (Table IV). Although kernel plating indicates whether fungi have been able to penetrate into the interior of the kernels, Tuite and Foster (1979) noted that it does not indicate the extent of the invasion. Cantone et al (1983) reported a relatively low correlation between kernel infection and ergosterol ( $r^2 = 0.28$ ). However, Moog et al (2004) reported that for 54 samples compared in their study, kernel infection was relatively well correlated ( $r^2 = 0.58$ ) with initial ergosterol. A more in-depth examination of the plated kernels revealed that many of the kernels in samples taken from corn stored <8 months were infected by *Fusarium* spp. Although this species usually does not grow in storage in the presence of storage fungi, refrigeration of the samples may have allowed the *Fusarium* to survive. It is possible that *Fusarium* growth was responsible for the lack of correlation between kernel infection and ergosterol difference.

## CONCLUSIONS

The results presented in this study indicate that the CO<sub>2</sub> test kit is able to distinguish differences in susceptibility to invasion by storage fungi among samples of shelled corn rewetted to 21% MC. In general, the variations in CO<sub>2</sub> production among the samples tested were consistent with expectations regarding fungal growth based on known storage history. The difference in ergosterol content of samples before and after testing was used as the standard measurement quantifying fungal growth in the rewetted samples. The correlations between ergosterol difference and CO<sub>2</sub> kit readings and indices derived from these readings were statistically significant ( $\alpha = 0.001$ ) and the values of  $r^2$  for the better correlations were 0.46–0.60. The best ( $r^2 = 0.60$ ) was for the CO<sub>2</sub> kit color 75 hr after rewetting versus the difference in ergosterol.

There were also statistically significant correlations between four attributes and ergosterol difference (in order of decreasing  $r^2$  values): percent germination ( $r^2 = 0.47$ ,  $\alpha = 0.001$ ); electrolyte leakage after 15 min of soaking ( $r^2 = 0.27$ ,  $\alpha = 0.001$ ); electrolyte leakage after 10 min of soaking ( $r^2 = 0.24$ ,  $\alpha = 0.001$ ); and % fines determined with a 4.76-mm round-hole sieve ( $r^2 = 0.12$ ,  $\alpha = 0.05$ ).

Correlations of percent kernel infection by plating and percent fines determined using a 6.35-mm sieve were not statistically significant. It may be possible to combine results of several of these kernel attribute tests to obtain a relatively quick (<15 min) but approximate assessment of shelled corn fungal susceptibility. Samples that appear to be susceptible to fungal invasion could then be evaluated more thoroughly using a more precise technique such as the CO<sub>2</sub> test kit.

TABLE IV

Coefficients of Determination ( $r^2$ ) for Linear Regressions of Kernel Attributes with Differences in Ergosterol of Samples

Factor	$n^a$	$r^2$ (probability) <sup>b</sup>
% Kernel infection	48	0.051 (0.163) N
% Germination	49 <sup>c</sup>	0.487 (0.001) N <sup>d</sup>
% Passing through 6.35-mm sieve	51	0.082 (0.252) P
% Passing through 4.76-mm sieve	51	0.120 (0.015) P <sup>e</sup>
Electrolyte leakage(10 min) <sup>f</sup>	47 <sup>c</sup>	0.240 (0.001) P <sup>d</sup>
Electrolyte leakage(15 min) <sup>f</sup>	47 <sup>c</sup>	0.273 (0.001) P <sup>d</sup>

<sup>a</sup> Number of samples in the regression.

<sup>b</sup> P indicates a positive correlation; N indicates a negative correlation.

<sup>c</sup> Only samples with ergosterol differences <7 ppm were considered.

<sup>d</sup> Correlations were statistically significant at  $\alpha = 0.005$ .

<sup>e</sup> Correlations were statistically significant at  $\alpha = 0.05$ .

<sup>f</sup> Units in millisiemens/m.

One of the uncertainties associated with the CO<sub>2</sub> evolution tests is that they do not distinguish between the CO<sub>2</sub> produced by the respiration of the seed and by respiration of fungi. However, there can also be a degree of uncertainty in ergosterol measurements. The presence or absence of several heavily invaded kernels can cause variations among replicate subsamples taken from the same sample. Given the limitations of these two tests, level of agreement between them was good. We believe that the CO<sub>2</sub> test kit can be a useful tool for measuring the fungal susceptibility of shelled corn. Additional research should be conducted to refine the methodology (e.g., investigate effects of O<sub>2</sub> depletion in the test kit jars and CO<sub>2</sub> contributed by seed respiration) and to determine how the test results should be used in managing stored grain. The test is relatively simple and low cost and could be used for testing a large number of samples. One disadvantage is that it would require up to three days to obtain results. However, it may be possible to increase the accuracy of the CO<sub>2</sub> kit determination and reduce to two days the time required to complete the test by developing a method of correcting results for seed respiration.

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