

Mycotoxin and Odor Formation in Barley Stored at 16 and 20% Moisture in Manitoba¹

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ABSTRACT

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Half-bushel parcels of barley at 16 and 20% moisture content (MC) were placed inside bulks of dry stored oats in a farm granary for 66 weeks so that we could study quality changes. Temperature, moisture, O₂ and CO₂ levels, microfloral incidence and abundance, seed germination, free fatty acids, mycotoxins (aflatoxins, sterigmatocystin, ochratoxin A, citrinin, penicillic acid, and patulin), and volatiles in the intergranular air were monitored biweekly and at longer intervals during storage. In the 20% MC barley, ochratoxin A was detected by six weeks and sterigmatocystin by 20 weeks. In 16% MC barley, sterigmatocystin was detected by week 62 but no ochratoxin was found; levels of sterigmatocystin were less than a third of those in 20% MC barley. No other mycotoxins were detected. *Penicillium*

verrucosum var. *cyclopium* was associated with production of ochratoxin A. *Aspergillus versicolor* and species of the *A. glaucus* group were found in samples containing sterigmatocystin in both 16 and 20% MC barley. The total volatiles reached maximum levels by week 6 and then decreased. The fungal volatiles 3-methyl-1-butanol, 3-octanone, 1-octanol, and 1-octan-3-ol were detected in the 16% MC barley. Except for 1-octan-3-ol, these were also detected in the 20% MC barley, which produced total volatiles at consistently higher levels. Barley stored at 20% MC was characterized by higher temperature, moisture content, fungal propagules, CO₂, free fatty acids, total volatiles, and lower levels of germination and O₂ as compared to 16% MC barley.

The development of mycotoxins in stored cereals is not well understood and is of particular importance for grain-exporting countries interested in maintaining high-quality grain. Although direct cause-effect relationships have not been established in most cases, some of the factors suggested to affect mycotoxin formation are: moisture, temperature, time, mechanical damage, O₂ and CO₂ levels, nature of substrate, fungal infestation, fungal strain differences, spore load, microbiological interactions, and invertebrate vectors (Hesseltine 1976). In an exploratory granary study, Abramson et al (1980) studied changes in temperature, moisture content, O₂ and CO₂ levels, microfloral incidence and abundance, seed germination, free fatty acids, and fungal volatiles during formation of ochratoxin A in wheat and barley at 21% moisture content (MC). In a laboratory study of 20.5% MC wheat deterioration, Abramson et al (1982) examined the effect of temperature on the formation of ochratoxin A, and on changes in moisture content and levels of O₂ and CO₂, microfloral incidence and abundance, seed germination, and free fatty acids.

Mycotoxins produced by species of *Penicillium* and *Fusarium*

have been found in Canadian barley. Ochratoxin A has been reported (Abramson et al 1980), and *Fusarium* mycotoxins detected in this crop include T-2 toxin (Puls and Greenway 1976) and deoxynivalenol (Neish and Cohen 1982).

Aspergillus spp., *Penicillium* spp., and other Fungi Imperfecti commonly infect stored cereals and oilseeds, and produce characteristic odor compounds. Kaminski et al (1974, 1975) grew various fungi on sterile coarse-ground wheat meal, and identified several volatile components of fungal origin, including 3-methyl-1-butanol, 3-octanone, 1-octanol, 1-octan-3-ol, and 2-octan-1-ol. Abramson et al (1980) identified the first three of these volatiles in moist (~21% MC) barley, wheat, and oats undergoing spoilage in a farm granary in Manitoba. Richard-Mollard et al (1976) have studied the development of 3-methyl-1-butanol, 3-octanone, 3-octanol, 1-octan-3-ol, and other volatiles in corn in airtight storage in France, and suggested their usefulness as early indicators of microbial spoilage.

We were concerned with ecological aspects of stored grain deterioration and had two objectives in this investigation: to study the formation of mycotoxins during storage of barley at 16 and 20% MC for 66 weeks and to explore the appearance of fungal volatiles during the spoilage process.

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MATERIALS AND METHODS

Barley Samples and Chemical Standards

Certified No. 2 Canada barley (*Hordeum vulgare* L. cv. Bonanza), from crop year 1979, was obtained from Manitoba Pool Elevators, Winnipeg, Manitoba. Standards of aflatoxins B₁, B₂, G₁, G₂, sterigmatocystin, ochratoxin A, citrinin, penicillic acid, and patulin were purchased from Supelco, Inc., Bellefonte, PA, and from Applied Science Laboratories, Inc., State College, PA. Standards of 3-methyl-1-butanol (98%), 1-octan-3-ol (98%), 3-octanone (99%), and 1-octanol (99%) were obtained from the Aldrich Chemical Co., Milwaukee, WI.

Preparation, Implantation, and Sampling of Barley Parcels

Portions of the barley were adjusted to approximately 16 and 20% MC wet weight basis (actual values 16.4 and 20.4%) by adding sterile distilled water and mixing for 2 hr in an electric tumbler. After further equilibration of the moisturized seed at 2.5°C for 24 hr in sealed plastic bags, duplicate parcels, each containing 11 kg of barley, were then prepared from each moisture treatment. Each parcel was packaged in double bags 81.3 cm long and 45.7 cm wide made of polyethylene film 0.08 mm thick that had a gas sampling tube and thermocouple (Abramson et al 1980). Because storage fungi are aerobic, an aerobic environment was ensured by perforating the bottom third of each bag 130 times with a 16-gauge needle, and closing the top of the bag with a cotton plug.

A weatherproof plywood granary 3.65 m long, 3.07 m wide, and 2.41 m to roof eaves, at Glenlea, Manitoba, was filled with oats (*Avena sativa* L. cv. Random) of 13.1–14.0% MC to a depth of 150 cm. The plywood floor of the granary was supported 10 cm above ground level by wooden skids. Each parcel of barley was implanted with the top approximately 15 cm below the surface of the oats and 1 m from the center of the bin. Parcels were left undisturbed, except during sampling, during May 1980 through September 1981.

Samples of 425 g were taken biweekly from the parcels for measurement of moisture content, fat acidity value (FAV), mycotoxins, seed germination and fungi for the first 16 weeks, and every four weeks thereafter. With the advent of cold weather, and reduced biological activity, sampling was discontinued between weeks 24 and 50.

Measurement of Variables

The temperature of the granary oat bulk was monitored using a copper-constantan thermocouple and a digital indicator (Digimite, Thermo Electric, Saddle Brook, NJ) at the center of the granary and 0.5 m below the surface of the oats. Temperatures in the barley were measured using a similar thermocouple at the center of each bag. Moisture contents were determined after oven drying 10-g samples of barley for 20 hr at 130°C (ASAE 1975). FAV figures were determined according to the procedure of the AACC (1962) and percent increase above zero time value was calculated.

The germinability and fungal infestation of the barley seeds were determined in duplicate samples of 25 seeds by the filter paper method (Wallace and Sinha 1962) and by using filter paper and 7.5% (w/v) NaCl solution (Mills et al 1978). The total fungal propagule count was made according to the method of Bothast et al (1974). In brief, 11 g of seed were shaken in 99 ml of sterile 0.1% (w/v) peptone and 10 g of sterile sand; from this, further dilutions without sand were prepared. One-milliliter amounts of solution from the appropriate dilution were pipetted onto plates of yeast extract agar containing 30 ppm tetracycline. Plates were incubated at 28°C and counted after three days.

Mycotoxin Screening

Samples of 50 g were taken by hand from the upper surface of the barley parcels at zero time and after 24 and 66 weeks, and screened for aflatoxins B₁, B₂, G₁, G₂, sterigmatocystin, ochratoxin A, citrinin, penicillic acid, and patulin. Samples were ground to pass through 0.85-mm apertures, extracted, purified, and analyzed by thin-layer chromatography (Wilson et al 1976). For citrinin, the thin-layer solvent system of Hald and Krogh (1973) was used because it produced compact fluorescent spots for the citrinin

standards. The procedure of Josefsson and Moller (1977) was used for sterigmatocystin.

Mycotoxin Analysis

All samples taken from the barley samples were analyzed for ochratoxin A and sterigmatocystin by the methods of the AOAC (1975). Each sample residue represented the extract of 10 g of barley, and was analyzed by high performance liquid chromatography.

For ochratoxin A, residues were dissolved in 750 µl of acetonitrile using sonication, and were clarified by centrifugation at 300 × g for 10 min. Aliquots of 75 µl were analyzed using an automatic Hewlett-Packard 1084B system equipped with a 250 × 4.6 mm column of 5 µm Altex Ultrasphere ODS and a Schoeffel FS970 fluorescence detector. For detection, the irradiation monochromator was set at 333 nm, and a 470-nm emission filter was used. Ochratoxin A and its methyl ester were determined using 65% methanol and 35% aqueous acetic acid (4.26% v/v) at 1.7 ml/min. The methyl ester was prepared by treatment with BF₃-methanol (Scott et al 1972). Samples were taken up in 150 µl of acetonitrile, and 50-µl aliquots were injected.

For sterigmatocystin, samples were dissolved in 750 µl of methanol and centrifuged as above. Aliquots of 40 µl were analyzed using a 250 × 4.0-mm column of 10 µm Merck Lichrospher Si-100 RP-18 at 50°C, and a Hewlett-Packard 79875A scanning ultraviolet detector. For detection, sample and reference wavelengths were set at 250 and 540 nm, respectively. Sterigmatocystin was resolved using 60% methanol and 40% water at 1.5 ml/min. For identity confirmation, the fluorescent acetylation product of sterigmatocystin was prepared by treatment with pyridine and acetic anhydride (AOAC 1975). Samples were dissolved in 150 µl of methanol, and aliquots of 10 µl were analyzed using the above column at 50°C, fluorescence irradiation at 308 nm, and a 418-nm emission filter. A methanol-water gradient at 1.5 ml/min employed 50% methanol for 9 min, and increase of 10% methanol per min for 5 min, and 100% methanol for 5 min. An additional confirmation was performed by scanning the peak of interest in the samples from 190 to 540 nm, and comparing the spectra to that of the authentic sterigmatocystin in the same solvent systems.

Gas Chromatography

Levels of O₂ and CO₂ in the barley parcels were measured as previously described (Abramson et al 1980) using columns of Linde type 5A molecular sieves and Porapak Q, respectively.

Volatile compounds of fungal origin were collected in adsorption traps as previously described (Abramson et al 1980). Samples were analyzed using an improved version of the apparatus. Briefly, a thermal-desorption introduction system built as described by Murray (1977) was installed in a Varian 2100 gas chromatograph, and samples were desorbed and analyzed as follows. Each trap was purged with He at 25°C for 1 min at a gas flow rate of 1.5 ml/min, and then was subjected to volatiles desorption under He at 150°C for 9 min. The volatiles were recondensed at the temperature of liquid N₂ for 10 min in a 2-cm precolumn packed with 0.17–0.25-mm-diameter silanized glass beads. Upon application of a 160°C brass probe to the precolumn, the volatiles were swept onto a 50-m-long 0.25-mm i.d. stainless steel capillary column wall-coated with Carbowax 20 M (Analabs, North Haven, CT) under He pressure of 220 kPa. The following oven temperature program was used: 80°C for 5 min, an increase of 2°C/min for 25 min, and 130°C for 5 min. Response of the flame ionization detector was recorded using a Hewlett-Packard 3380S computing integrator.

Qualitative standards were run by placing 0.1 ml each of 3-methyl-1-butanol, 1-octan-3-ol, 3-octanone, and 1-octanol in a 50-ml flask, and sweeping the vapors for 5 sec into a trap with 99.999% N₂ at 60 ml/min.

RESULTS AND DISCUSSION

Temperature

Temperatures at the granary center and mean temperatures of the parcels of barley during storage are shown in Fig. 1A. The bin

center temperature decreased from 18–22°C in May–August to 3°C in October. The bin center temperature increased from 4°C the following April to 18–22°C during July–September. The barley remained within 2°C of the bin center until week 62 when the 20% MC barley alone was heating. By this time, it was 2°C warmer than the 16% MC barley and 6°C warmer by week 66.

Moisture Content

In 16% MC barley, the moisture content remained virtually unchanged over 66 weeks (Fig. 1B). In the 20% MC barley the moisture content increased after week 14, fluctuated around 20.5–21% until week 58, and then sharply increased, reaching 23% by week 66.

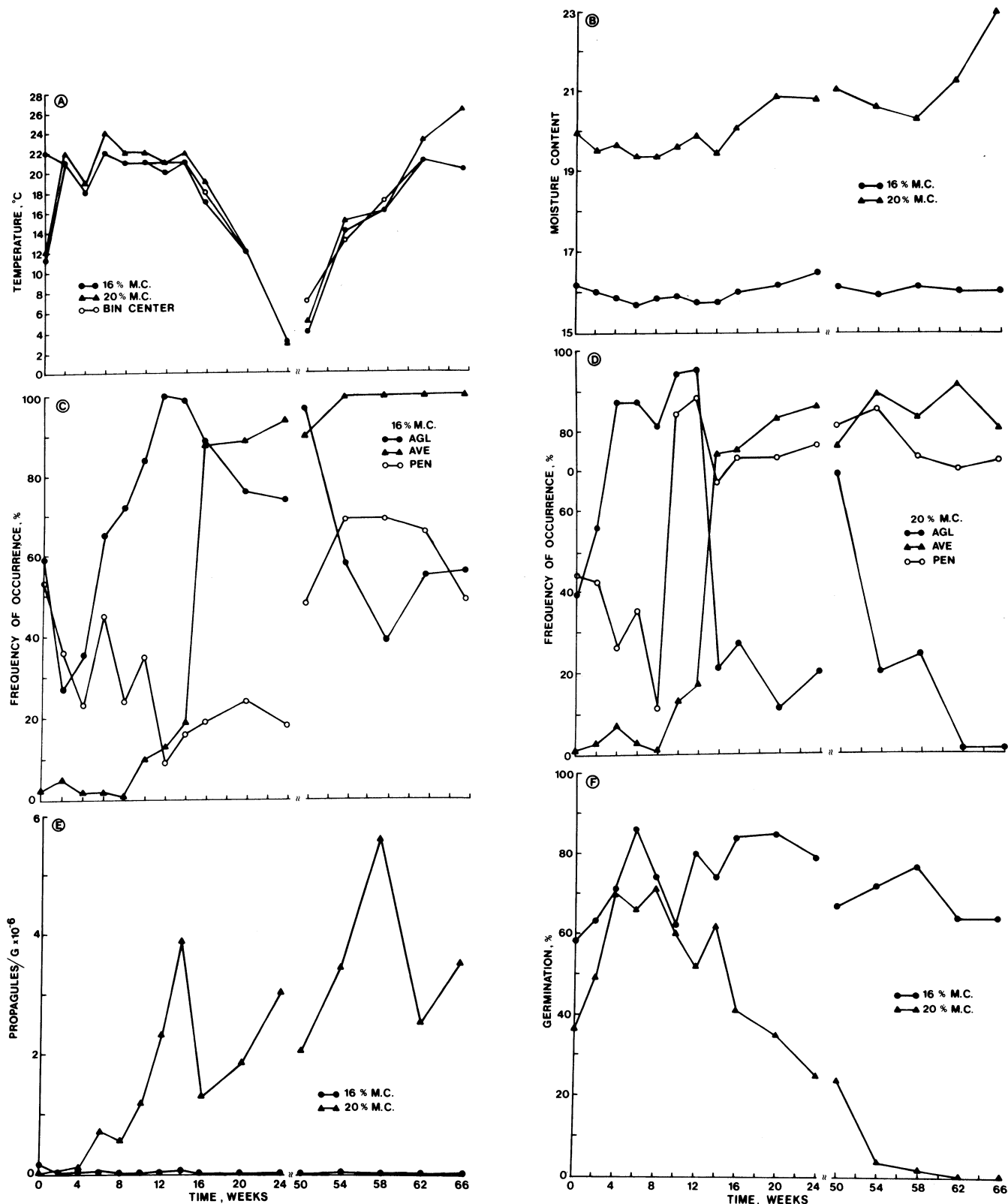


Fig. 1. Changes in biotic and abiotic variables in stored barley within a Manitoba granary during May 1980–September 1981. A, temperature; B, moisture content; C, D, fungal infestation; E, microfloral propagule count; F, germination. PEN = *Penicillium* spp., AGL = *Aspergillus glaucus* gr. spp., AVE = *Aspergillus versicolor*.

Microflora

Penicillium spp., *Aspergillus glaucus* gr. spp., *A. versicolor* (Vuill.) Tiraboschi, *Alternaria alternata* (Fr.) Keissler, *Wallemia sebi* (Fr.) v. Arx, and bacteria were isolated. The frequency of occurrence of the first three postharvest fungi measured as a percentage of the seeds infested is shown for both moistures in Fig. 1C and D. *A. glaucus* gr. spp. included *Eurotium chevalieri* Mang., *E. repens* de Bary, *E. amstelodami* Mang., and *E. rubrum* Konig et al.

Penicillium, mainly *P. verrucosum* var. *cyclopium* (Westling) Samson, Stolk and Hadlok, reached maximum values in the 16% MC barley by 54 weeks and in the 20% MC barley by 12 weeks. Western Canadian strains of this fungus have been shown to be producers of ochratoxin A (Mills and Abramson 1982). *A. glaucus* spp. reached a maximum by 12 weeks in barley at both moistures, and declined to 56% at 16% MC and to 1% at 20% MC by week 66. *A. versicolor* increased sharply between weeks 14 and 16 in the 16% MC barley and between weeks 12 and 14 in the 16% MC barley. The 16% MC barley showed a 100% infestation with *A. versicolor* between weeks 54 and 66, whereas the 20% MC barley showed 80–90% infestation during this time. Except at week 50, this species predominated at both barley moistures.

A. alternata declined from levels of 80–90% to 0% in barley at both moistures by 50 weeks, but recurred at weeks 54 and 62 in the 16% MC barley. In the 16% MC barley, *W. sebi* increased to 37% by week 54 and 41% by week 66; in the 20% MC barley it appeared at a 4% infestation level by week 4 and disappeared afterwards. Bacteria appeared for the first time on barley at both moistures at week 10, and increased to 36% at 16% MC and to 59% at 20% by week 66.

The total fungal propagule count (Fig. 1E) in the 16% MC barley remained virtually unchanged at the initial level of 1.9×10^5 propagules per gram. In the 20% MC barley, this count reached a maximum of 5.7×10^6 propagules per gram by week 58.

Seed Germination

Germination of the 16% MC barley fluctuated between 62 and 85% but remained at 64% at the end of 62 weeks (Fig. 1F). At 20% MC, seed germination progressively decreased to zero by week 62.

Levels of O₂ and CO₂

In the 16% MC barley, gas levels in the intergranular air of the parcels showed little change from the initial values (Fig. 2A). The low O₂ value at week 66 may be caused by sampling error. The 20% MC barley showed consistently lower O₂ levels and higher CO₂ levels. The greatest decrease in O₂ and increase in CO₂ occurred between weeks 58 and 66 of storage in 20% MC barley.

FAV Increase

The FAV increased progressively throughout the experiment, with a more pronounced increase in the 20% MC barley (Fig. 2B). The pattern of FAV increase, ie, a sharp rise, followed by a leveling off, followed by another sharp rise, is similar to that observed earlier by Sinha and Wallace (1977) for 8–9% MC rapeseed stored in a steel bin in Manitoba. Final actual FAV figures for the 16 and 20% MC barley were 65 and 253 mg of KOH per 100 g of dry seed, respectively.

Mycotoxins

Screening results of the 24- and 66-week samples indicated detectable levels of ochratoxin A and sterigmatocystin by week 24 in the 20% MC barley, and of the latter toxin by week 66 in the 16% MC barley. No other mycotoxins were found.

Ochratoxin A was not found in the 16% MC barley but was detected in the 20% MC barley by week 6 (Fig. 2C). After an initial fluctuation in test results, a steady rise occurred in levels of this mycotoxin from weeks 14 to 62, reaching 242 ppb by week 66. Because ochratoxin A is highly toxic, causing liver and kidney damage (Scott 1977), the mode of its production in barley in a "typical" farm granary is important.

Sterigmatocystin was not detected until week 62 in the 16% MC barley and week 20 in the 20% MC barley (Fig. 2D). Levels of this

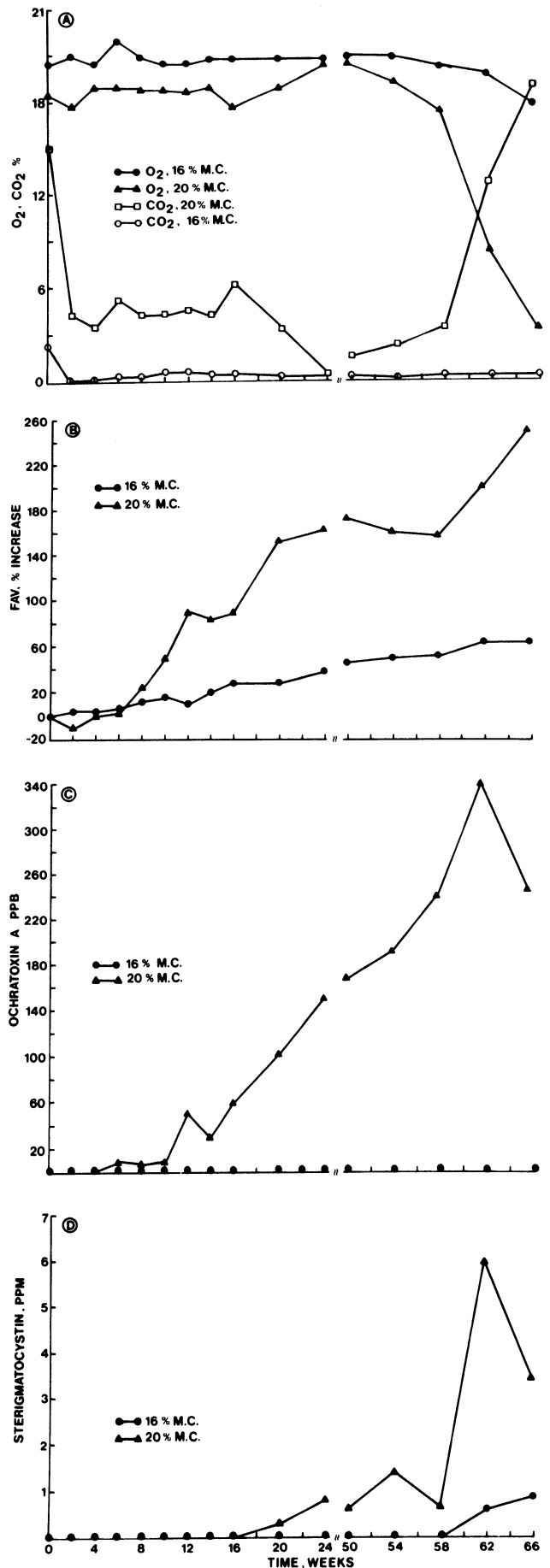


Fig. 2. Changes in biotic and abiotic variables in stored barley within a Manitoba granary during May 1980–September 1981. A, oxygen and carbon dioxide; B, FAV increase; C, ochratoxin A; D, sterigmatocystin.

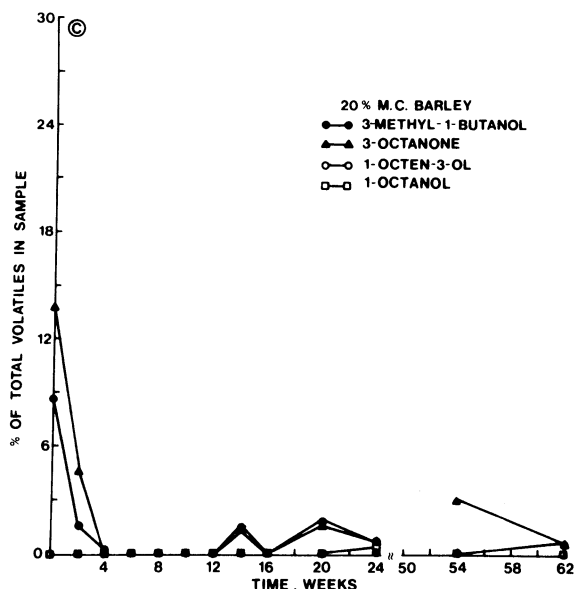
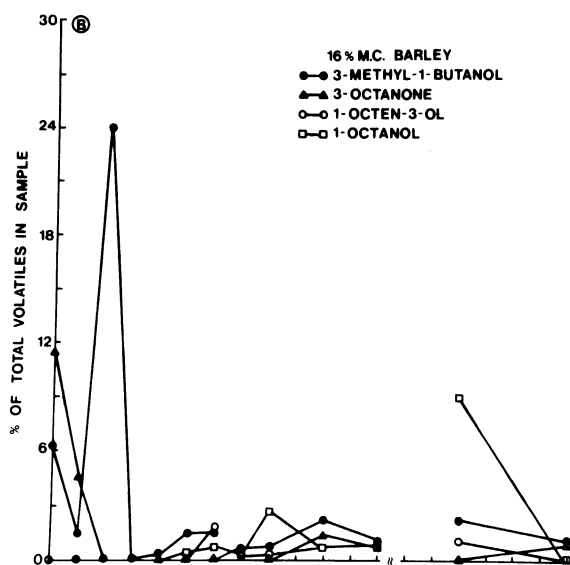
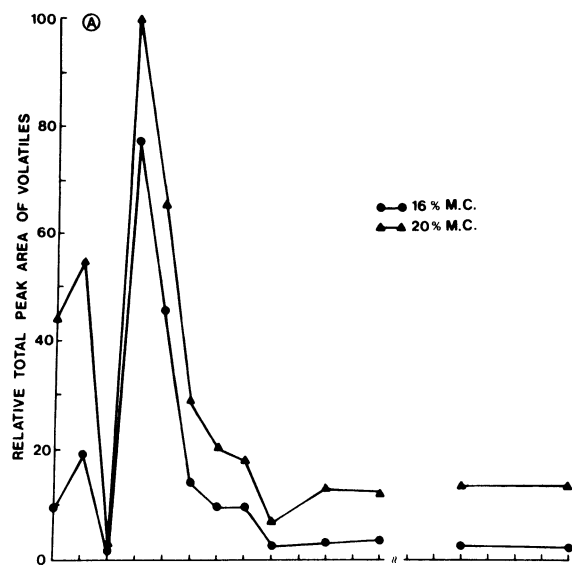


Fig. 3. Changes in intergranular air volatiles in stored barley within a Manitoba granary during May 1980–August 1981. A, relative total peak area; B,C, known fungal odor components as percentages of the total volatiles.

toxin rose gradually, and reached 3.45 ppm in the 20% MC barley as compared to 0.85 ppm in the 16% MC barley by week 66. The incidence of this toxin in barley is significant because of its carcinogenicity. Sterigmatocystin is a metabolite of several grain storage fungi such as *A. versicolor* and *A. nidulans* (Eidam) Wint. and of preharvest cereal fungi such as *Bipolaris*, and has been found in wheat and various feeds (Scott 1978).

Fungal Volatiles

Changes in the total volatiles, which would be responsible for the odor of the barley in 400 ml of intergranular air taken from the implanted parcels over 62 weeks are indicated in Fig. 3A. Samples of intergranular air were taken less frequently after 24 weeks and were not taken beyond 62 weeks because of the diminishing amount of barley. The amounts of total volatiles are a summation of all peak areas measured, and were calculated relative to the amount of total volatiles in the 20% MC barley at week 6, which was taken as 100.

In both the 16 and 20% MC barley, the total volatiles peaked at weeks 2 and 6 and declined sharply afterwards. This is consistent with the results of previous analyses of volatiles of 20.5% MC barley in a Manitoba granary during June–October (Abramson et al 1980) where the total volatiles peaked around week 7. The 20% MC barley consistently produced more volatiles than the 16% MC (Fig. 3A); this difference became more pronounced after week 16. Thereafter, the 16% MC barley continued to produce negligible amounts of volatiles. Changes in the relative percentages of intergranular air accounted for by the known fungal volatiles 3-methyl-1-butanol, 3-octanone, 1-octen-3-ol, and 1-octanol are illustrated for the 16 and 20% MC barley in Fig. 3B and C, respectively. Both 3-methyl-1-butanol and 3-octanone were initially present in barley at both moisture contents, declined to zero levels by week 6, and reappeared by week 14. In the 16% MC barley, both 1-octen-3-ol and 1-octanol were also present, appearing at weeks 14 and 10, respectively. In the 20% MC barley, only 1-octanol was additionally present, appearing by week 24. This is consistent with previous findings (Abramson et al 1980) that only 3-methyl-1-butanol, 3-octanone, and 1-octanol are present in the volatiles of 20.5% MC barley during granary storage. At both barley moistures, only 3-methyl-1-butanol and 3-octanone remained after week 62.

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