

# Control of *Penicillium martensii* Development and Penicillic Acid Production by Atmospheric Gases and Temperatures

E. B. LILLEHOJ, M. S. MILBURN, AND A. CIEGLER

Northern Regional Research Laboratory, Northern Marketing and Nutrition Research Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

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The effects of various gaseous environments and temperatures on development of *Penicillium martensii* NRRL 3612 and production of penicillic acid (PA) were determined. Accumulation of PA in mold-inoculated corn was measured following incubation under air; 20% CO<sub>2</sub>, 20% O<sub>2</sub>, 60% N<sub>2</sub>; 40% CO<sub>2</sub>, 20% O<sub>2</sub>, 40% N<sub>2</sub>; and 60% CO<sub>2</sub>, 20% O<sub>2</sub>, 20% N<sub>2</sub>. Although reduced temperature initially inhibited PA production, at the end of the trial the largest quantity of PA (120 µg/g of corn) was found in air-incubated corn at the lowest test temperature (5 C). Atmospheres enriched with 60% CO<sub>2</sub> reduced PA accumulation below a detectable level at 5 and 10 C after a 4-week incubation period. Spore germination tests were carried out in a liquid growth medium incubated for 16 hr under several test conditions. Germ tube outgrowth at 30 C ranged from 36% in air to 2% in 60% CO<sub>2</sub>, whereas no germination was observed in CO<sub>2</sub>-enriched gases at 10 C. When spore respiration rates were measured in air and O<sub>2</sub> in a liquid growth medium, complete removal of CO<sub>2</sub> from the reaction atmosphere did not reduce O<sub>2</sub> uptake.

Harvesting high-moisture corn by picker-sheller is an agricultural practice widely accepted. The corn shelled in this procedure is uniquely susceptible to mold infection. Not only is the high-moisture corn a fungal substrate per se, but field-shelled corn harvested at 30% moisture sustains up to 2.5 times the kernel damage as corn harvested at 20% moisture (17). Damaged kernels enhance fungal infection because the site of seed injury provides easy access for microbial penetration.

Postharvest mold spoilage can be controlled by storage at reduced temperatures (19), which has certain commercial advantages. The latest studies show that during storage of high-moisture yellow dent corn for 6 months at 1 C, the mold *Penicillium martensii* Biourge flourishes as the predominant microbe on the stored product (11). This mold has been implicated as one of the organisms associated with the blue-green discoloration of corn germ commonly referred to as blue-eye (4, 11).

The presence of *P. martensii* on stored corn constitutes a particular hazard. Kurtzman and Ciegler (11) found that this organism develops on high-moisture corn at low tempera-

ture elaborates large quantities of the mycotoxin penicillic acid (PA). Isolation and toxic identification of PA were initially achieved by Alsberg and Black (1); later studies identified the carcinogenic properties of the substance (7).

Mold development on stored grain has been controlled through modification of its gaseous environment. Storage under CO<sub>2</sub> is an effective anaerobic method (9, 10). However, fermentation that ensues produces odors and flavors in the stored product which may be undesirable. As an alternative to control mold growth by means of anaerobic storage, Christensen and colleagues (2, 14) examined the effects of various aerobic gas mixtures on stored grains. Reduction of O<sub>2</sub> to 0.2% in N<sub>2</sub> reduced mold growth but increased anaerobic processes, whereas enriching atmospheric CO<sub>2</sub> to 18.6% in 21% O<sub>2</sub> blocked mold development aerobically (14).

Although high levels of CO<sub>2</sub> inhibit mold growth, low concentrations of the gas can stimulate it (5, 16). Fungal spore germination is particularly sensitive to CO<sub>2</sub> with inhibitory or stimulatory levels determined by test condi-

tions and strain variation (5, 16). Reducing temperature enhances CO<sub>2</sub>-mediated inhibition of molds, a technique that has been successfully exploited by CO<sub>2</sub>-temperature methods for preventing deterioration of fruits and other foodstuffs (16).

Extensive studies have been carried out on the control of *Aspergillus flavus* and aflatoxin production through modification of gaseous environment and temperatures (6, 8; 12, 15). Landers et al. (12) observed a decrease in mold growth and sporulation with each 20% increase in CO<sub>2</sub>, beginning with 20% up to 80% and a reduction in aflatoxin production when CO<sub>2</sub> was increased from 0.03 to 100%. Sanders et al. (15) observed a decrease in *A. flavus* growth and aflatoxin production on peanuts in 20% CO<sub>2</sub>, whereas Epstein et al. (8) found that reducing temperatures in conjunction with 10% CO<sub>2</sub> inhibited mold growth and toxin synthesis on a liquid medium and on corn.

To provide information for a practical method of mold control in high-moisture corn stored at low temperature, we examined the effects of various gases and temperatures on *P. martensii* development and PA production.

#### MATERIALS AND METHODS

**Mold-inoculated corn in various gases and temperatures.** For 1 hr 250 g of yellow dent corn (14% moisture) was steeped in water. After removal of excess water, the corn was autoclaved in 2.8-liter Fernbach flasks for 20 min and then inoculated with 5.0 ml of an aqueous spore suspension containing  $1.5 \times 10^{10}$  conidia of *P. martensii* Biourge NRRL 3612 (11). The spores were uniformly distributed by gently shaking the flasks. The inoculated corn was transferred to a 1.1-liter culture vessel similar in design to the one described by Landers et al. (12) except the wire baskets were omitted. Gas mixtures were saturated with water by bubbling through temperature-controlled water flasks before introduction into a plenum chamber below the grain. The upper portion of the chamber was constructed to provide a uniform gas flow through the grain column. The culture vessel was immersed in a temperature-regulated bath. Gases were obtained from Matheson Gas Products Company, Joliet, Ill., as certified mixtures with an accuracy of 1%. Gas-flow rates were controlled with Matheson automatic regulators and monitored with precision bore Flowrators (Fischer and Porter Co., Hatboro, Pa.). Culture vessels were flushed with 1,000 ml of the specified gas/min for the first hour of the experiment, after which the rate was lowered to 100 ml/min for the rest of the test.

**PA isolation and assay.** At appropriate times 25 g of mold-inoculated corn was removed from the culture vessel and extracted with 250 ml of chloroform-methanol (90:10, v/v) in a Waring Blendor for 3 min. The recovered solvent was filtered through

anhydrous sodium sulfate and then reduced to 10 ml under vacuum. Quantitative PA determinations were made on thin-layer chromatographic plates according to the method described by Ciegler and Kurtzman (3).

**Spore germination and respiration.** Spores were obtained from *P. martensii* cultures grown on potato dextrose agar for 10 days at 28 C. Germination and respiration studies were carried out in Warburg flasks containing 0.001 M magnesium sulfate, 0.001 M ammonium nitrate, 0.01 M sodium phosphate (pH 7.0), 0.01 M glucose, and 10<sup>9</sup> spores/ml in a final volume of 3.0 ml; 0.2 ml of alkali was used in the center wells when appropriate. Flasks were incubated in a Gilson respirometer constant temperature bath (G.M.E. Inc., Middleton, Wisc.). Spore germination averages were determined microscopically (13, 20). Respiration was measured by standard manometric techniques with final values corrected for endogenous gas exchange (18).

#### RESULTS

**PA level in corn.** The accumulation of PA was followed during a 4-week period on *P. martensii*-inoculated corn under various temperatures and gaseous environments. Figure 1a presents information on the PA levels detected in corn incubated in air at 5 to 20 C. Extensive mold development and grain deterioration were observed at 15 and 20 C but not at 5 and 10 C. At 5 C no toxin was found after the initial week of the test, but a significant increase occurred during the next 3 weeks. The 120 µg of PA/g of corn detected after 4 weeks in air at 5 C was the highest found in any test condition. The striking differences between PA levels in 20% CO<sub>2</sub>, 20% O<sub>2</sub>, and 60% N<sub>2</sub> (Fig. 1b) and air were (i) the absence of a PA reduction at 15 and 20 C during the third and fourth week in 20% CO<sub>2</sub> and (ii) the inhibition of PA accumulation throughout the test at 5 C in 20% CO<sub>2</sub>. The pattern of PA accumulation in 40% CO<sub>2</sub>, 20% O<sub>2</sub>, and 40% (Fig. 1c) N<sub>2</sub> is similar to that observed in 20% CO<sub>2</sub> with the exception of a slight diminution in the quantities of toxin produced. The increase in CO<sub>2</sub> from 20 to 40% inhibited PA accumulation 21% at 20 C and 33% at 15 C. Increasing CO<sub>2</sub> from 40 to 60% (Fig. 1d) inhibited PA accumulation 21% at 20 C and 44% at 15 C, and no PA was detected at either 5 or 10 C. Overall, there is a general reduction in PA concentration following incremental increases in the CO<sub>2</sub> level. The CO<sub>2</sub>-mediated inhibition of PA is particularly significant at reduced temperatures since complete blockage of toxin production can be achieved over a 4-week period at 5 and 10 C.

**Spore germination and respiration.** Since increased CO<sub>2</sub> levels and reduced tempera-

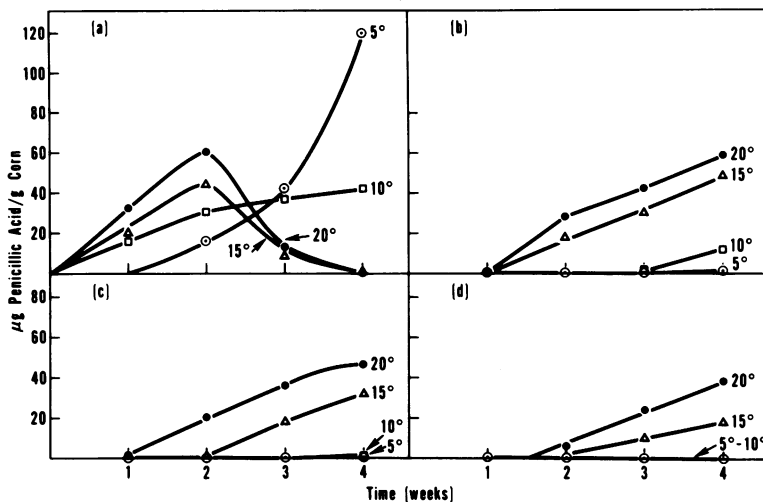


FIG. 1. Effect of temperature and gaseous environment on production of penicillic acid (PA) by *Penicillium martensii* on corn. Gases employed: (a) air; (b) 20% CO<sub>2</sub>, 20% O<sub>2</sub>, 60% N<sub>2</sub>; (c) 40% CO<sub>2</sub>, 20% O<sub>2</sub>, 40% N<sub>2</sub>; (d) 60% CO<sub>2</sub>, 20% O<sub>2</sub>, 20% N<sub>2</sub>. Culture vessels were flushed with the appropriate gas at 100 cc/min during the trial. PA was extracted from moldy corn with chloroform-methanol (90:10, v/v) and subsequently analyzed with quantitative thin-layer chromatographic methods.

tures dramatically reduced PA production on corn, tests were carried out to determine the effect of these environmental conditions on germination and respiration of *P. martensii* conidia. The experiments were designed to elucidate the nature of the inhibitory process of increased CO<sub>2</sub> and reduced temperature on spore germination.

Figure 2 contains information on the mean germination percentage of spores observed after 16 hr in a growth medium incubated under various gaseous and temperature conditions. Gas mixtures were the same as those used in previous tests on PA accumulation in corn: (i) air, (ii) 20% CO<sub>2</sub>, (iii) 40% CO<sub>2</sub>, and (iv) 60% CO<sub>2</sub>. No germination was detected at the temperature extremes in CO<sub>2</sub>-enriched gases, and only limited germination was observed at 10 and 40 C in air. However, at the optimal temperature of 30 C, germ tube outgrowth was found in all the gas mixtures ranging from 36% in air to 2% in 60% CO<sub>2</sub>. No germination was seen in pure CO<sub>2</sub> during a similar incubation period.

Respiratory patterns of *P. martensii* spores in a growth medium were determined at 5 to 40 C in (i) air and (ii) O<sub>2</sub> (Fig. 3).

Maximum respiration rates during a 5-hr test were used in determining Q values. Gas exchange activity was greatest at 30 C with marked reduction at 5, 10, and 40 C. The response of spore respiration to temperature approximates the pattern observed in germina-

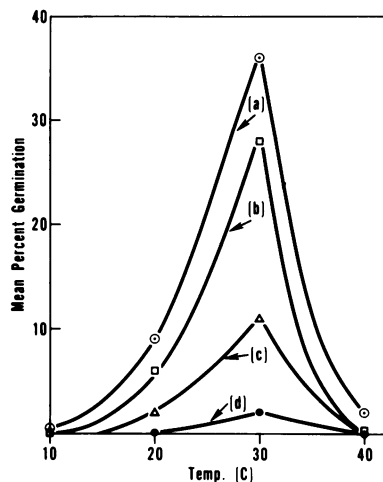


FIG. 2. Mean germination of *P. martensii* spores after 16 hr at 30 C. Gases employed: (a) air; (b) 20% CO<sub>2</sub>, 20% O<sub>2</sub>, 60% N<sub>2</sub>; (c) 40% CO<sub>2</sub>, 20% O<sub>2</sub>, 40% N<sub>2</sub>; (d) 60% CO<sub>2</sub>, 20% O<sub>2</sub>, 20% N<sub>2</sub>. Spores were incubated in a glucose-salts growth medium in Warburg flasks. Flasks were gently shaken in a constant-temperature bath and flushed with 5 ml of the test gas/min. Spore germination was determined microscopically by counting germ tube outgrowth of 150 spores from each of three separate flasks from each treatment.

tion tests (Fig. 2). Since the respiratory rates in air and O<sub>2</sub> were similar, it appeared that CO<sub>2</sub> was not a fastidious requirement for spore metabolism during germination.

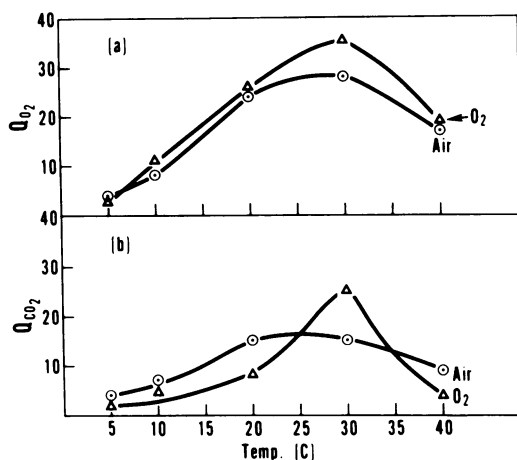


FIG. 3. Mean germination percentage of *P. martensii* spores in air at 10 C. Spores were incubated in a glucose-salts growth medium in Warburg flasks. Flasks were gently shaken in a constant-temperature bath and flushed with 5 ml of air/min. Spore germination was determined microscopically.

### DISCUSSION

The PA formed in mold-infected corn incubated in air demonstrates the hazard inherent in low-temperature corn storage. The largest quantity of toxin was found at the lowest test temperature (5 C) after 4 weeks. Unquestionably, reduced temperatures permit PA accumulation on moldy corn stored in air. Modification of the gaseous environment as a mechanism for controlling toxin production showed that an increase of CO<sub>2</sub> blocked PA synthesis at 5 C and reduced the toxin level at 10 C.

Examination of *P. martensii* spore germination in various conditions showed that maximum germ tube outgrowth occurred at 30 C with no significant development at 10 C in any of the test gases. Although optimal germination occurred at 30 C, the process in 60% CO<sub>2</sub> was reduced 95% relative to that observed in air. Respiration rates of *P. martensii* spores demonstrated that gas exchange was similar in O<sub>2</sub> and air. Since spore germination was not blocked by the absence of CO<sub>2</sub>, the possibility was reduced that removal of CO<sub>2</sub> from the storage gas would effectively control the mold. However, the experiments on *P. martensii* growth and PA accumulation provided presumptive evidence that increasing the CO<sub>2</sub> level from 20 to 60% while maintaining 20% O<sub>2</sub> would extend the period of safe corn storage at reduced temperatures. This environmental

modification clearly retards mold development and simultaneously curbs anaerobic fermentation.

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