Penicillic Acid Production by Blue-Eye Fungi on Various Agricultural Commodities

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Of 10 Penicillium species reported to cause blue-eye disease of corn, four (P. martensii, P. palitans, P. cyclopium, P. puberulum) were found capable of producing the mycotoxin penicillic acid on various agricultural commodities. Commodities with high protein contents did not support toxin synthesis. The extent of toxin production varied with the strain of mold, the commodity, and the temperature; low temperatures (1 to 10 C) favored toxin accumulation.

In modern agricultural practice, high-moisture corn is frequently harvested by picker-sheller. It has been established that the higher the moisture, the greater the damage to kernels during shelling. The combination of high moisture, damaged kernels, and warm temperatures that often prevail in autumn makes such corn particularly vulnerable to molding. Although much of the corn is dried at or shortly after harvest to 12 to 14% moisture, holding periods may occur during which mold growth can be initiated. Sometimes the mold appears as a blue-green discoloration of the germ, a condition commonly referred to as blue-eye (7).

Reportedly this disease of corn is caused by a wide variety of fungi, primarily species of Penicillium (7, 9, 10) but also including some aspergilli (8). Semeniuk and associates (10) state that the blue-eye fungi were the only ones isolated from shelled corn, stored in bins during the winter, that were capable of growth at 0.5 and 9 C. Blue-eye disease is commonly observed in corn stored during the winter in the Midwest where temperatures often hover around freezing. Kurtzman and Ciegler (Bacteriol. Proc., p. 8, 1970) found that, in blue-eye-diseased corn caused by P. martensii, large quantities of penicillic acid were produced when high-moisture corn was stored at low temperatures (1 to 10 C). Penicillic acid is known to be toxic to mammals and has proved carcinogenic to rats (4). Since blue-eye disease may occur in combine-harvested corn, consideration needs to be given to the possible production of penicillic acid as representing a potential mycotoxin hazard to humans and livestock consuming this grain.

The purpose of this investigation was to determine the capability of the various fungi implicated in blue-eye disease to produce penicillic acid on corn and other agricultural commodities particularly at a low temperature.

MATERIALS AND METHODS

Cultures. All cultures used in this investigation were obtained from the Agricultural Research Service Culture Collection at the Northern Regional Research Laboratory. They were maintained on either potato-dextrose-agar or malt-yeast extract-agar slants stored at 10 C.

Production of toxin. Thirty-five grams of grain or other agricultural commodity was placed in 300-ml Erlenmeyer flasks. To each flask was added 28 ml of distilled water, except for rice and cottonseed to which 14 ml of distilled water was added; the flasks were then autoclaved for 15 min at 121 C. Each flask was inoculated with 1 ml of spore suspension made by suspending spores of the various molds from 10- to 14-day-old slant cultures in 50 ml of sterile distilled water. Flasks were incubated without agitation at 1 and 20 C. All samples were prepared in duplicate, and each flask was assayed in duplicate.

Extraction and assay for penicillic acid. The toxin penicillic acid was assayed fluorodensitometrically by the method of Ciegler and Kurtzman (J. Chromatogr., in press). Briefly, the method involves thin-layer chromatography of the unknown with known amounts of standard on silica gel (solvent, chloroform-ethyl acetate-formic acid, 60:40:1, v/v) followed by exposure of the plate to ammonia fumes. The penicillic acid-ammonia derivative is excited at 350 nm and fluoresces at 440 nm. The degree of fluorescence was determined with a Photovolt densitometer (model 530) equipped with an automatic scanning thin-layer plate stage and a recorder equipped with an integrator. A standard curve is prepared for each analysis, a linear plot being followed between 1 and 9 µg of penicillic acid. The concentration of unknown is determined from the standard curve taking into account the dilutions involved. The penicillic acid used for standards was produced by fermentation as previously described (C. P. Kurtzman and A. Ciegler, Bacteriol. Proc., p. 8, 1970).
For all assays, the contents of an entire flask were extracted with 250 ml of chloroform-methanol (90: 10, v/v) in a Waring Blender for 3 min. The homogenate was filtered through anhydrous sodium sulfate, and the first 50 ml of solvent recovered was analyzed for penicillic acid.

In those samples in which penicillic acid was not found, the 50 ml of solvent extract was evaporated to dryness, the residual solids were placed in solution or suspension in 3 ml of propylene glycol, and 0.1 and 0.2 ml of the glycol solution was injected into 20-g mice as a test for the potential presence of other mycotoxins.

RESULTS AND DISCUSSION

All Penicillium species chosen for this survey have been implicated in blue-eye disease of corn with the exception of P. granulatum NRRL 1575 (1, 8-10). This organism was included because, in a previous unpublished investigation, we determined that it also produced penicillic acid in Czapek-Dox broth. Two of the cultures, P. trzebinskii NRRL 732 and P. ochraceum NRRL 870, were isolated from corn that had been blue-eye-damaged in the field. Two cultures of P. martensis, NRRL 3747 and 3612, were isolated from high-moisture corn that became blue-eyed after large-scale experimental storage under refrigeration.

Data in Table 1 reveal that only 5 of the 16 strains tested produced penicillic acid. The production of this toxin was confirmed by thin-layer chromatography, by derivative formation with phenylhydrazine to form the hydrazone (C. P. Kurtzman and A. Ciegler, Bacteriol. Proc., p. 8, 1970), and by mouse assay.

In general, as we reported earlier (C. P. Kurtzman and A. Ciegler, Bacteriol. Proc., p. 8, 1970), low temperature (1 C) favors the accumulation of penicillic acid although the rate of toxin formation is more rapid at a higher temperature (20 C). The exception to this was P. cyclopium NRRL 1888, which produced a greater quantity of toxin at 20 C than at 1 C. Strain specificity is also involved in the amount and type of toxin produced. P. martensis NRRL 3612 consistently produced more penicillic acid than did P. martensis NRRL 3747, but neither strain produced the tremorgenic mycotoxin (3), a capability shown by P. martensis NRRL 2034, which does not produce penicillic acid. Similar observations apply to strains of P. palitans: P. palitans NRRL 3672 produces penicillic acid but no tremorgen; the converse is true for NRRL 3468, whereas NRRL 966 produces neither toxin and appears to be nontoxicogenic as determined by mouse assay. Thus, it would not be judicious to predict whether one type of mycotoxin or another might be produced on a given grain based on a mold profile.

Penicillic acid production on some commodities. Drying most grains and other agricultural commodities to a low-moisture level is a generally accepted practice for safe storage of grains. However, several new procedures including

| Penicillium culture | NRRL no. | Penicillic acid (mg/g of corn) |
|--------------------|----------|-------------------------------|
|                    |          | At 20 C                       | At 1 C                       |
|                    |          | 1 wk 2 wk 3 wk 4 wk          | 3 wk 5 wk 7 wk 9 wk 13 wk   |
| P. martensis       | 3747     | 0.14 0.60 0.64 3.3            | 0.08 0.25 0.71 4.1 10.25    |
| P. martensis       | 3612     | 0.60 0.86 1.60 2.88 2.13      | -a 0.33 1.70 2.96 4.63      |
| P. rugulosum       | 1045     | 0.94 2.05 2.58 2.88 2.13      | -b 0.33 1.70 2.96 4.63      |
| P. palitans        | 3468     |                               | -b 0.33 1.70 2.96 4.63      |
| P. palitans        | 966      |                               |                               |
| P. chrysogenum     | 3672     |                               |                               |
| P. notatum         | 807      |                               |                               |
| P. viridicatum     | 821      |                               |                               |
| P. cyclopium       | 963      |                               |                               |
| P. cyclopium       | 942      |                               |                               |
| P. cyclopium       | 1888     |                               |                               |
| P. trzebinskii     | 732      |                               |                               |
| P. ochraceum       | 870      |                               |                               |
| P. ochraceum       | 871      |                               |                               |
| P. puberulum       | 3564     |                               |                               |
| P. granulatum      | 1575     | 1.35 1.4 1.8 2.08 3.05 5.81   |                               |

a No assay run.
b No obvious fungal growth.
reproduction are being designed or are in use to store high-moisture corn and other cereals (5). High-moisture corn is more palatable to cattle than dry corn; it is also preferable for some industrial applications. Since blue-eye disease of corn appears to be particularly selected at low temperatures, we examined the four species found to produce penicillic acid on corn at 1 C (Table 1) for their ability to synthesize this toxin on a limited number of additional farm commodities at 20 and 1 C. Good growth and sporulation of the four species were observed on all commodities tested. In general, commodities with a high protein concentration (peanuts, soybeans, cottonseed) did not support penicillic acid synthesis by the test fungi at either temperature, whereas those substances rich in starch did when fermented at 20 C (Table 2). However, even with the starchy substrates, there is considerable variation with respect to the fungus, the grain, and the amount of toxin produced. Sorghum provided the best substrate for penicillic acid production by all four organisms at 20 and 1 C, whereas barley, oats, and wheat were poor substrates. Curiously, rice supported production by only one strain, *P. cyclopium* NRRL 1888.

At 20 C, penicillic acid in affected commodities tends to gradually disappear, whereas at 1 C it slowly accumulates reaching levels higher than at 20 C. The reason for the loss of toxin at the higher temperature has not yet been determined. Better production of mycotoxins at low temperatures is not unusual and has been recorded for a number of fungi (2, 6, 11).

When penicillic acid was not detected in any given flask during the survey, except for *P. palitans* NRRL 3468 which has been shown to produce tremorgenic toxins (3), the residue from the solvent extract was injected intraperitoneally into mice to test for the potential presence of other mycotoxins; none was found. Because of the ready growth of mold, even where toxin was not synthesized, refrigeration at 1 C of high-moisture grains does not appear to be a suitable substitute at the moment for drying grains before storage.

**Interfering substances.** Substances that might seriously interfere with the penicillic acid assay from the various commodities used or that might be mistaken for the bright-blue fluorescent-ammoniated derivative of penicillic acid were not encountered, with the possible exception of soybeans. A pale-green fluorescing substance, extracted from soybeans, behaved somewhat like penicillic acid on thin-layer chromatographic

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**Table 2. Effect of time and temperature on penicillic acid production by Penicillium spp. on various agricultural commodities**

| Commodity  | 20 C | 1 C  |
|------------|------|------|
|            | 1 wk | 3 wk | 6 wk | 6 wk | 12 wk |
| Rice (white) | -   | 2.11 | 1.67 | 0.21 | 0.43  | 1.07  | 2.8  |
|           | A    | B    | C    | D    | A    | B    | C    | D    |
| Barley     | 0.08 | 0.09 | 0.03 | 0.35 | 0.25 | 0.38 | 1.1  | 1.7  |
| Sorghum    | 0.59 | 0.32 | 0.39 | 0.67 | 0.95 | 0.79 | 1.56 | 0.6  |
| Oats       | 0.13 | 0.06 | 0.04 | 0.03 | 0.09 | 0.05 | 0.03 | 0.03 |
| Wheat      | 0.13 | 0.06 | 0.04 | 0.03 | 0.09 | 0.05 | 0.03 | 0.03 |

* Penicillic acid was not detected in peanuts, cottonseed, and soybeans.
* A, *P. martensii* NRRL 3747; B, *P. cyclopium* NRRL 1888; C, *P. puberulum* NRRL 3564; D, *P. palitans* NRRL 3672.
* Dash indicates no penicillic acid was detected. No penicillic acid was detected after 3 weeks in the commodities incubated at 1 C.
* Values expressed as milligrams of penicillic acid per gram of substrate.
FIG. 2. Thin-layer chromatogram of penicillic acid and an unknown compound from soybeans. Developing solvent, chloroform-ethyl acetate-formic acid (60:40:1,v/v). Detecting agent, ammonia fumes. (1) Penicillic acid; (2) unknown substance from soybeans; (3) mixture of penicillic acid and unknown substance from soybeans; (4) penicillic acid.

plates. It did not fluoresce before treatment of the plates with ammonia, fluoresced green instead of blue, had a different excitation and emission spectrum (Fig. 1) than penicillic acid (penicillic acid: excitation, 350 nm; emission, 440 nm; unknown: excitation, 352 nm; emission, 480 nm), and had a slightly lower Rf in the thin-layer chromatographic system used, 0.41 versus 0.45 (Fig. 2). Hence, the assay as developed should be suitable to detect penicillic acid in agricultural commodities.

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