Toxigenic Aspergillus and Penicillium Isolates from Weevil-Damaged Chestnuts

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Aspergillus and Penicillium were among the most common genera of fungi isolated on malt-salt agar from weevil-damaged Chinese chestnut kernels (16.8 and 40.7% occurrence, respectively). Chloroform extracts of 21 of 50 Aspergillus isolates and 18 of 50 representative Penicillium isolates, grown for 4 weeks at 21.1 C on artificial medium, were toxic to day-old cockerels. Twelve of the toxic Aspergillus isolates were identified as A. wentii, eight as A. flavus, and one as A. flavus var. columnaris. Nine of the toxic Penicillium isolates were identified as P. terrestrre, three as P. steckii, two each as P. citrinum and P. funiculsum, and one each as P. herquei (Series) and P. roqueforti (Series). Acute diarrhea was associated with the toxicity of A. wentii and muscular tremors with the toxicity of P. terrestrre, one isolate of P. steckii, and one of P. funiculsum.

Because of its resistance to Endothia blight (1), the Chinese chestnut (Castanea mollissima Blume) was introduced into the United States for hybridizing with the nearly extinct American chestnut (C. dentata [Marsh.] Borkle). Annual chestnut production in the state of Georgia is about 60,000 to 70,000 lb (ca. 27,215.52 to 31,751.44 kg) (personal communications, R. L. Livingston, University of Georgia) and is marketed principally in the Appalachian area of the eastern United States—roughly, the center of the original range of the American chestnut. Production estimates for other states are unavailable.

Chesnuts are a perishable commodity, easily spoiled by fungi and insects. Mature nuts are allowed to drop from trees and may lie for several days or weeks until gathered. Decay development may begin while they are on the ground (5). Commercially, chestnuts may be kept in refrigerated storage for several months before marketing. Losses due to fungi frequently occur, particularly at the consumer level (15). In experimental storage studies Hammar (6) found that spoilage ranged from 5 to 10% after 1 month and 15 to 60% after 7 months at 2 C. Wright (16) reported that 62% of kernels examined soon after harvest contained visible fungus infections. The most common fungi isolated from decayed tissues were Phoma castaneae Oud. and Pestalotia spp. Of minor importance were species of Phompis, Penicillium, Alternaria, Fusarium, Rhizopus, and others. Researchers in Italy and France have found that the most common genera of decay fungi isolated from European chestnut (Castanea sativa Mill.) kernels in storage were Penicillium, Fusarium, Phoma, Aspergillus (A. niger), and Rhizopus (2, 8, 12). There have been no reports, to our knowledge, on the mycoflora of weevil-damaged chestnuts.

The chestnut weevil (Curculio sayi Gyllenhal), commonly called the small chestnut weevil, is a major threat to chestnut production because it attacks the nut kernels (9). Adults infest trees from April to late June and deposit eggs in nearly mature nuts during August and September. The larvae feed on kernel tissues, then emerge by cutting through the shell. Infested nuts may contain several weevil larvae, or, if larvae have already emerged, may contain weevil burrows filled with excrement. Weevil-damaged nuts are likely to harbor a wide variety of mycoflora and be subject to spoilage. Nuts from which weevils have emerged are generally culled from the packing operations by flotation. Nuts containing weevils, however, are not separated by the flotation process. Weevils then emerge while chestnuts are in storage or transit, and damaged nuts enter the market channels. Although such nuts are generally discarded by the consumer, some might be incorporated into processed chestnut products or food combinations. The potential for consumption of spoiled chestnuts is increased by the absence of visible mold on many kernels with incipient fungal infections.

Fungi of the genera Penicillium and Aspergillus have been associated with toxin production on many agricultural commodities (14, 17). Other fungi, especially those of the genera Alternaria and Fusarium, have also been re-
ported as toxin producers (3, 4). This study was to
determine the incidence of some mycotoxin-
producing fungi on weevil-damaged chestnuts
and was limited to Penicillium, the most fre-
cently isolated genus, and to Aspergillus, the
genus most often associated with mycotoxin
contamination of foodstuffs.

MATERIALS AND METHODS
Isolation of fungi. Freshly gathered chestnuts
were obtained in October 1972 and 1973 from or-
chards in central Georgia. Chestnuts with weevil-
emergence holes were selected and stored for 1
week at 3 C. Kernel pieces containing sections of weevil
burrows were surface sterilized for 3 min in 0.5%
sodium hypochlorite solution containing 3% ethyl
alcohol, rinsed in sterile water, and plated on malt-
salt agar. Fungus colonies developing from kernel
pieces after 3 weeks at 21 C were classified by gen-
erg. Those not readily identifiable were placed in a
miscellaneous category. All Aspergillus and Penicil-
ilium colonies were transferred to potato-dextrose
agar slants by mass transfer, allowed to grow at
21 C for 2 weeks, and stored at 3 C.

Bioassay for toxicity. Cultures for bioassay were
grown on shredded wheat (7) or on fresh or on auto-
claved medium. Autoclaved chestnut medium, pre-
pared in a 500-ml flask, consisted of 50 g of quar-
tered, fresh chestnuts and 10 ml of water, which were
autoclaved at 15 lb/in² and 121 C. Fresh chestnut
medium was prepared by adding 50 g of surface-
sterilized quartered kernels to an autoclaved flask
containing 10 ml of water. Media were inoculated by
mass transfer of spores from the potato-dextrose
agar slants, and cultures were grown for 4 weeks at
21 C.

Cultures were extracted for bioassay by the
method based on that of Kirksey and Cole (7). Cul-
tures were blended with 200 ml of chloroform in a
Waring blender for 45 s, and the homogenates were
filtered through a 1-cm pad of sodium sulfate on a
90-mm Buchner funnel. Chloroform filtrates were
transferred to 150-ml beakers containing 5.5 ml of
corn oil and placed on a steam plate for 3 h to com-
pletely evaporate the chloroform. Five 1-day-old
DeKalb 151 cockerels were dosed by crop intubation
with 1 ml of corn oil which contained extract.
Checks were dosed with corn oil to which only pure
chloroform had been added and then evaporated.
Cockerel mortality, expressed as survival ratios,
and any clinical symptoms were recorded over a 5-
day observation period. If mortality was over 50% or
if survivors exhibited unusual clinical symptoms
one or two additional bioassays were conducted. If
confirmatory tests were also positive, the extracts
were considered toxic. Cultures were rated for de-
gree of toxicity: less than 50% mortality but with
debilitated survivors equals low toxicity; more than
50% but less than 90% mortality equals moderate
toxicity; and over 90% mortality equals high toxic-
ity.

Identification of toxic isolates. Cultures which
produced toxic extracts were transferred to diagnost-
ic media (10) for taxonomic identification. All taxo-
nomic identities at the species level were considered
definitive if major cultural and microscopy charac-
teristics of an isolate agreed with published descrip-
tions (10, 11). When one or more characteristics of an
isolate were at variance with descriptions, identifica-
tion was at series level only. All Aspergillus isolates
and only those Penicillium isolates shown to be toxic
were identified at species level.

RESULTS
Penicillium spp. were the fungi most fre-
cently isolated (40.7% occurrence) from wee-
vil-damaged chestnuts (Table 1). Next, in order of
frequency of occurrence, were Rhizopus, Al-
ternaria, and Aspergillus, each comprising
about 17% of the total mycoflora isolated. Fusar-
ium constituted 6.4% of the colonies isolated, and
fungi of unidentified and miscellaneous genera constituted 1.3%.

Twenty-one of the 50 Aspergillus cultures
isolated from chestnuts were toxic to day-old
cockerels (Table 5). Most of the isolates were A.

| Table 1. Percentage of occurrence of fungi isolated from weevil-damaged Chinese chestnuts on malt-salt agar |
|---------------------------------------------------------------|
| Genera           | No. isolated | Occurrence (%) |
|------------------|--------------|----------------|
| Penicillium      | 121          | 40.7           |
| Rhizopus         | 52           | 17.5           |
| Alternaria       | 51           | 17.2           |
| Aspergillus      | 50           | 16.8           |
| Fusarium         | 19           | 6.4            |
| Miscellaneous    | 4            | 1.3            |

| Table 2. Toxicity to day-old cockerels of chloroform extracts of Aspergillus and Penicillium cultures isolated from weevil-damaged Chinese chestnuts and grown on shredded wheat medium |
|---------------------------------------------------------------------------------------------------------------|
| Isolates     | No. bioassayed | No. toxic | No. of toxic cultures causing clinical symptoms |
|--------------|----------------|-----------|-----------------------------------------------|
|              |                |           | Diarrhea | Tremors |
| Aspergillus spp. | 50             | 21        | 11      | 0       |
| A. wentii     | 26             | 12        | 11      | 0       |
| A. flavus     | 11             | 8         | 0       | 0       |
| A. oryzae     | 7              | 0         | 0       | 0       |
| A. flavus var. colonaris | 4             | 1         | 0       | 0       |
| A. niger      | 2              | 0         | 0       | 0       |
| Penicillium spp. | 50             | 18        | 0       | 12      |

* Isolate considered toxic if mortality was over 50% or if debilitative clinical symptoms developed in survivors in three repetitive tests.
A. wentii, 12 of which were toxic. The toxicity of A. wentii caused acute diarrhea, loss of appetite, and general debilitation or mortality. The toxicity of these cultures was generally low to moderate. A. wentii isolates CA 8, CA 10, CA 13, CA 25, CA 26, and CA 28 were of low toxicity, each causing a cumulative average mortality of less than 50% (Table 3). The remainder of the A. wentii isolates (CA 9, CA 15, CA 19, CA 43, CA 45, and CA 51) were moderately toxic, causing over 50% but less than 90% mortality.

The remaining toxic Aspergillus cultures belonged to the A. flavus group. Eight of the 11 A. flavus isolates and one of the four A. flavus var. columnaris isolates were toxic (Table 2). Toxicity of A. flavus was considered moderate to high. Extracts of isolates CA 21, CA 46, CA 54, CA 55, and CA 38 (A. flavus var. columnaris) caused over 90% mortality, and isolates CA 14, CA 22, CA 48, and CA 52 were moderately toxic (Table 3).

None of the A. oryzae or A. niger cultures isolates from chestnuts were toxic (Table 2).

Eighteen of the 50 bioassayed Penicillium

| Species                      | Accession no. | No. of survivors/no. dosed for three bioassays | Mortality (%) | Toxicity |
|------------------------------|--------------|-----------------------------------------------|---------------|---------|
| A. wentii                   | CA 8         | 0/5                                           | 3/5^a         | 5/5^a   | 47        | Low      |
| A. wentii                   | CA 9         | 0/5                                           | 4/5^a         | 0/5     | 73        | Moderate |
| A. wentii                   | CA 10        | 0/5                                           | 4/5^a         | 5/5^a   | 40        | Low      |
| A. wentii                   | CA 13        | 3/5^a                                         | 3/5^a         | 4/5^a   | 33        | Low      |
| A. wentii                   | CA 15        | 1/5                                           | 1/5^a         | 5/5^a   | 53        | Moderate |
| A. wentii                   | CA 19        | 1/5                                           | 4/5^a         | 0/5     | 66        | Moderate |
| A. wentii                   | CA 25        | 0/5                                           | 4/5^a         | 4/5^a   | 47        | Low      |
| A. wentii                   | CA 26        | 0/5                                           | 4/5^a         | 4/5^a   | 47        | Low      |
| A. wentii                   | CA 28        | 2/5                                           | 2/5           | 2/5     | 40        | Low      |
| A. wentii                   | CA 43        | 0/5                                           | 4/5^a         | 0/5     | 73        | Moderate |
| A. wentii                   | CA 45        | 4/5^a                                         | 2/5           | 0/5     | 60        | Moderate |
| A. wentii                   | CA 51        | 0/5                                           | 0/5           | 3/5     | 80        | Moderate |
| A. flavus                   | CA 14        | 0/5                                           | 1/5           | 4/5^a   | 66        | Moderate |
| A. flavus                   | CA 21        | 0/5                                           | 0/5           | 4/5^a   | 73        | Moderate |
| A. flavus                   | CA 22        | 1/5                                           | 0/5           | 1/5     | 66        | Moderate |
| A. flavus                   | CA 46        | 1/5                                           | 0/5           | 0/5     | 93        | High     |
| A. flavus                   | CA 48        | 1/5                                           | 0/5           | 1/5     | 87        | Moderate |
| A. flavus                   | CA 52        | 0/5                                           | 0/5           | 2/5     | 87        | Moderate |
| A. flavus                   | CA 54        | 0/5                                           | 0/5           | 100     | 100       | High     |
| A. flavus                   | CA 55        | 0/5                                           | 0/5           | 100     | 100       | High     |
| A. flavus var. columnaris   | CA 38        | 0/5                                           | 0/5           | 100     | 100       | High     |
| A. terrestrum               | CP 3         | 0/5                                           | 3/4^a         | 0/5     | 79        | Moderate |
| A. terrestrum               | CP 4         | 0/5                                           | 0/5           | 3/4     | 79        | Moderate |
| A. terrestrum               | CP 28        | 0/5                                           | 1/5^a         | 0/5     | 93        | High     |
| A. terrestrum               | CP 30        | 5/5^a                                         | 0/5           | 0/5     | 60        | Moderate |
| A. terrestrum               | CP 34        | 0/5                                           | 0/5           | 0/5     | 100       | High     |
| A. terrestrum               | CP 37        | 0/5                                           | 0/5           | 0/5     | 100       | High     |
| A. terrestrum               | CP 38        | 2/4^a                                         | 2/5           | 2/5     | 57        | Moderate |
| A. terrestrum               | CP 39        | 4/5^a                                         | 0/5           | 0/5     | 40        | Low      |
| A. terrestrum               | CP 43        | 2/5^a                                         | 0/5           | 0/5     | 87        | Moderate |
| P. steckii                  | CP 18        | 0/5                                           | 5/5^a         | 0/4     | 64        | Moderate |
| P. steckii                  | CP 19        | 2/5                                           | 0/5           | 0/5     | 87        | Moderate |
| P. steckii                  | CP 26        | 0/4                                           | 0/5           | 0/5     | 100       | High     |
| P. citrinum                 | CP 2         | 0/5                                           | 0/5           | 0/5     | 100       | High     |
| P. citrinum                 | CP 41        | 0/5                                           | 0/5           | 0/4     | 100       | High     |
| P. funiculosum              | CP 21        | 0/5                                           | 0/5           | 0/5     | 100       | High     |
| P. funiculosum              | CP 25        | 1/5                                          | 2/5           | 1/4     | 71        | Moderate |
| P. herquei (Series)         | CP 35        | 2/5                                          | 2/5           | 2/5     | 47        | Low      |
| P. roqueforti (Series)      | CP 14        | 1/4                                          | 1/5           | 0/4     | 85        | Moderate |

* All survivors underweight and affected by severe diarrhea.
* At least 50% of survivors underweight and affected by severe diarrhea.
* Clinical symptoms included sustained tremors and convulsion.

Table 3. Survival ratios for cockerels dosed with chloroform extracts of toxic Aspergillus and Penicillium cultures isolated from weevil-damaged chestnuts and grown on shredded wheat medium.
isolates were toxic (Table 2). Twelve of these 18 were associated with sustained muscular tremors and, in some cases, convulsions before death. Of the toxic Penicillium isolates, nine identified as *P. terrestre* (Series) were tremorgenic and of varying toxicity (Table 3). Three of the toxic Penicillium isolates were *P. steckii* and were moderately to highly toxic. Two were *P. citrinum* and two were *P. funiculosum*, but all one were highly toxic. The *P. funiculosum* isolates were tremorgenic. One isolate of *P. herquei* (low toxicity) and one of *P. roqueforti* (moderately toxic) were also identified.

Selected isolates of each major group of toxic fungi were cultured on autoclaved and on surface-disinfected fresh chestnuts. Of the three *A. wentii* cultures tested, CA 8 extracts from either autoclaved or fresh chestnuts were not toxic, CA 10 extracts from autoclaved but not from fresh chestnuts were toxic, and CA 13 extracts from both media were toxic (Table 4). Six of seven *A. flavus* isolates tested (including *A. flavus* var. *columinaris*) produced toxin on autoclaved and on fresh chestnuts, and one (CA 46) was toxic on autoclaved chestnuts only.

With the exception of *P. herquei* (CP 35), all extracts of *Penicillium* isolates grown on autoclaved chestnuts were toxic to day-old cockerels. On fresh chestnut medium, one isolate each (of two tested) of *P. terrestre* (CP 39), *P. steckii* (CP 18), and *P. citrinum* (CP 41) was toxic. The one isolate tested of *P. funiculosum* (CP 25) was of low toxicity, and *P. herquei* (CP 35) and *P. roqueforti* (CP 14) were not toxic when grown on fresh chestnuts.

**DISCUSSION**

A high percentage of *Penicillium* and *Aspergillus* isolates from weevil-damaged Chinese chestnuts were capable of producing mycotoxins. Forty-two percent of all aspergilli were toxic, and seven of 10 representative isolates tested produced toxin on inoculated, fresh chestnuts. Similarly, 36% of the penicillia bioassayed produced toxin on artificial media, and four of nine tested produced the toxins on fresh chestnuts. The organisms studied were fungi established in dehydrated or discolored tissues adjoining insect-damaged areas. No mycotoxins have been found on market chestnuts; however, a potential exists for toxin production in the event fungal development occurs on kernel tissues. The presence of surface contaminants also presents a potential problem if chestnut quality deteriorates in the market or in storage. The potential presence of mycotoxins in weevil-damaged chestnut kernels suggest the need for effective weevil-eradication programs in the orchards and for fastidious, quality control measures after harvest.

Most *A. wentii* isolates lost a degree of toxicity during the course of this study. Initial subcultures of original isolates were highly toxic. Subcultures taken from original isolates in storage for 6 to 8 months were less toxic although diarrheagenic symptoms were strong. Prolonged storage of these toxigenic fungi on artificial medium or repeated subculturing may have resulted in mutations or in metabolic changes affecting toxicity.

Further research is needed to test the capability of fungi other than the aspergilli and penicillia present on weevil-damaged chestnuts. Genera such as *Alternaria*, *Fusarium*, and others have been associated with mycotoxicity.

Research is now in progress to identify the toxins produced by the fungi reported in this study. The *A. wentii* toxin has been isolated and identified as emodin (13). Preliminary analyses suggest that aflatoxins and citrinin are the toxins responsible for the activity of *A. flavus* in the extracts tested.
and *P. citrinum* isolates, respectively (unpublished data).

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