

Ochratoxin Production by the *Aspergillus ochraceus* Group and *Aspergillus alliaceus*

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Ochratoxin A is a toxic and carcinogenic fungal secondary metabolite; its presence in foods is increasingly regulated. Various fungi are known to produce ochratoxins, but it is not known which species produce ochratoxins consistently and which species cause ochratoxin contamination of various crops. We isolated fungi in the *Aspergillus ochraceus* group (section *Circumdati*) and *Aspergillus alliaceus* from tree nut orchards, nuts, and figs in California. A total of 72 isolates were grown in potato dextrose broth and yeast extract-sucrose broth for 10 days at 30°C and tested for production of ochratoxin A in vitro by high-pressure liquid chromatography. Among isolates from California figs, tree nuts, and orchards, *A. ochraceus* and *Aspergillus melleus* were the most common species. No field isolates of *A. ochraceus* or *A. melleus* produced ochratoxin A above the level of detection (0.01 µg/ml). All *A. alliaceus* isolates produced ochratoxin A, up to 30 µg/ml. We examined 50,000 figs for fungal infections and measured ochratoxin content in figs with visible fungal colonies. Pooled figs infected with *A. alliaceus* contained ochratoxin A, figs infected with the *A. ochraceus* group had little or none, and figs infected with *Penicillium* had none. These results suggest that the little-known species *A. alliaceus* is an important ochratoxin-producing fungus in California and that it may be responsible for the ochratoxin contamination occasionally observed in figs.

Ochratoxins are fungal secondary metabolites that contaminate grains, legumes, coffee, dried fruits, beer and wine, and meats (11, 20, 22). They are potent nephrotoxins, carcinogens, teratogens, and immunotoxins in rats and possibly in humans (5; R. Walker, paper presented at the Third Joint FAO/WHO/UNEP International Conference on Mycotoxins, Tunis, Tunisia, 1999, <http://www.fao.org/WAICENT/FAOINFO/Economic/ESN/mycoto/papers%5Cmyco5b.pdf>). Ochratoxin A is commonly found in human milk and blood samples (2). Provisional tolerable daily intake levels in foods are extremely low, from 1 to 16 ng/kg of body weight (5). The presence of ochratoxins in imported foods is being increasingly regulated in many countries (6, 22, 30; R. Walker, paper presented at the Third Joint FAO/WHO/UNEP International Conference on Mycotoxins, Tunis, Tunisia, 1999, <http://www.fao.org/WAICENT/FAOINFO/Economic/ESN/mycoto/papers%5Cmyco5b.pdf>). European Union countries are imposing limits on ochratoxin A content in coffee and foods, in some cases as stringent as 5 µg/kg (5 ppb) (5, 22).

Fungi from two genera are known to produce ochratoxins: the *Aspergillus* species *A. ochraceus*, *A. melleus*, *A. auricomus*, *A. ostianus*, *A. petrakii*, *A. sclerotiorum*, and *A. sulfureus*, all in section *Circumdati* (also called the *A. ochraceus* group); *A. alliaceus* and *A. albertensis*, formerly placed in section *Circumdati* but recently shown to be more closely related to section *Flavi* (18); *A. niger* and *A. carbonarius* (in section *Nigri*); *A. glaucus* (or *Eurotium herbariorum* [section *Aspergillus*]); and *Penicillium verrucosum* (1, 4, 12, 18, 19, 24, 26). Several of these

species are synonymous or poorly defined, which complicates analysis of ochratoxin contamination of crops (18, 28).

Few of these species are known to contaminate foods with ochratoxin A. According to Pitt (20), ochratoxin A “is produced by *Penicillium verrucosum* in cereal grains in cold climates, by *A. carbonarius* in grapes, wines and vine fruits, and by *Aspergillus ochraceus* sometimes in coffee beans.” However, because few geographic areas have been studied, because many fungi are capable of producing ochratoxins, and because of taxonomic problems in section *Circumdati*, it is not always clear which species are responsible for ochratoxin contamination of crops and commodities. This is particularly true of North America, where studies on ochratoxins in crops have focused on *Penicillium verrucosum* contamination of cereals (17, 20).

Our objectives in this study were (i) to determine the frequency of different members of the *A. ochraceus* group in tree nuts and figs in California, (ii) to determine which species produce ochratoxin A and to estimate the frequency of ochratoxinogenic isolates among these species, and (iii) to determine which species are responsible for ochratoxin in figs. Since California nut and fig crops have never been reported to contain the levels of ochratoxin found in wheat and other crops (10, 11, 17, 20), we hypothesized that populations of ochratoxin-producing species would be dominated by atoxigenic strains.

MATERIALS AND METHODS

Most fungi were isolated from tree nut and fig orchards in California, either from nuts, leaves, soil, or air. Others were isolated from packaged tree nuts bought in California. Reference isolates were obtained from the Northern Regional Research Laboratory (NRRL) and American Type Culture Collection (ATCC) culture collections. Most fungi from nuts were isolated on salt agar (6% NaCl, 2% agar), which is useful for isolating *Aspergillus* from tree nuts (9), and

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TABLE 1. Ochratoxin A production by *Aspergillus* isolates in two liquid media

| Species and no. of isolates | Source | Location | Ochratoxin production ^a (µg/ml) on: | |
|-----------------------------|------------------------|----------------|--|-----------|
| | | | PDB | YES |
| <i>A. ochraceus</i> | | | | |
| 1 | NRRL 398 ^T | | — | — |
| 1 | ATCC 22947 | | 0.04 ± 0.05 | 1.6 ± 2.2 |
| 1 | ATCC 91619 | | — | — |
| 20 | Tree nuts | California | — | — |
| 7 | Cottonseed | California | — | — |
| 7 | Fig | California | — | — |
| 1 | Soil | California | — | — |
| 1 | Hazelnut | Washington | — | — |
| 1 | Walnut inflorescence | France | — | — |
| <i>A. melleus</i> | | | | |
| 1 | NRRL 5103 ^T | India | — | — |
| 8 | Tree nuts | California | — | — |
| 7 | Fig | California | — | — |
| 1 | Hazelnut | Washington | — | — |
| <i>A. alliaceus</i> | | | | |
| 1 | NRRL 4181 ^T | Australia | — | — |
| 1 | NRRL 315 | Blister beetle | 2.5 | 8.9 |
| 6 | Fig | California | 4.2 ± 1.9 | 11 ± 10 |
| <i>A. elegans</i> | | | | |
| 4 | Tree nuts | California | — | — |
| <i>A. sclerotiorum</i> | | | | |
| 1 | NRRL 4901 | | — | — |
| 2 | Fig | California | — | — |

^a Each isolate was grown at least once in PDB and once in YES. The test was repeated two to four times for isolates that produced detectable levels of ochratoxin for confirmation.

most fungi from soil were isolated on dichloran rose bengal chloramphenicol agar (DRBC) (14). For identification and morphological observations, fungi were cultured on Czapek-Dox agar (Difco Laboratories, Inc., Detroit, Mich.), potato dextrose agar (Difco), and malt extract agar (Difco). *Aspergilli* were identified based on standard taxonomic systems (3, 15, 21).

To determine whether the *A. ochraceus* group or *Penicillium verrucosum* was responsible for ochratoxin contamination of crops, we collected dried figs from 10 commercial orchards in Fresno, Madera, and Merced counties. Ten thousand figs were collected in 1997, and 20,000 each in 1998 and 1999. Figs were halved and examined with a dissecting microscope for the presence of fungal colonies (10). Because the large sample size made it impractical to analyze each fig separately, figs were pooled for ochratoxin analysis. For each year, all figs visibly infected with *Penicillium* spp. were pooled for analysis. Figs infected with the *A. ochraceus* group were pooled by orchard for analysis. *Penicillium* isolates were not identified to species level.

For ochratoxin analyses in vitro, fungi were grown in 50 ml of liquid medium in 125-ml Erlenmeyer flasks. Two media were used: potato dextrose broth (PDB; Difco) and yeast extract-sucrose broth (YES; 2% yeast extract, 15% sucrose) (26). Cultures were incubated without agitation for 10 days at 30°C in the dark. Then 2 ml of culture fluid was removed from each flask, filtered through a 0.2-µm syringe filter, and extracted with 2 ml of chloroform. The organic phase was collected, evaporated, and resuspended in 500 µl of methanol. Then 20 µl was injected into a high-performance liquid chromatograph (HPLC model 1050; Hewlett Packard, Palo Alto, Calif.) with model 1046A fluorescence detector (Hewlett Packard). The HPLC was run on a VYDAC 218TP54 C₁₈ column (4.6 by 250 mm; VYDAC/The Separations Group, Inc., Hesperia, Calif.), with methanol-H₂O-H₃PO₄ (87%), 70:30:0.1, as the mobile phase and a flow rate of 1 ml/min. Excitation was at 333 nm, with detection at 418 nm. Peak areas were calculated from a standard curve based on concentrations from 0.005 to 15 µg/ml of an ochratoxin A standard (Sigma Chemical Co., St. Louis, Mo.). Each isolate was grown at least once in PDB and once in YES. Isolates that produced detectable levels of ochratoxins were tested again for confirmation. The limit of detection for ochratoxin A was 10 ng/ml. Ochratoxin analysis of figs was as previously described (10).

To compare ochratoxin production on grain with that in liquid culture, a subset of 10 isolates, including both ochratoxin producers and nonproducers, was cho-

sen. Twenty grams of hard red winter wheat was soaked in 25 ml of water overnight in 125-ml Erlenmeyer flasks, autoclaved for 1 h, and inoculated. Flasks were incubated at 30°C for 10 days; at 3 days, flasks were shaken to redistribute inoculum. To extract ochratoxin A, the contents of each flask were ground in a blender for 1 min with 72 ml of acetonitrile-water (60:40) and filtered. The acetonitrile was removed in a rotary evaporator, and water was added to 30 ml. The extract was partitioned with 30 ml of chloroform, the chloroform was evaporated, and the extract was resuspended in 1 ml of methanol. Wheat extractions were done in triplicate. Detection and detection limits were as described above.

RESULTS

The most commonly isolated species was *A. ochraceus*, comprising 57% of the 63 isolates from California (Table 1). *Aspergillus melleus* comprised 24% of isolates from California; *A. alliaceus*, 10%; *A. elegans*, 6%; and *A. sclerotiorum*, 3%.

Ochratoxin A. None of 58 field isolates of *A. ochraceus* or *A. melleus* produced ochratoxin A in either liquid medium above the limit of detection (0.01 µg/ml) (Table 1). Seven of these isolates were also grown on wheat grain and did not produce ochratoxin A at detectable levels on this substrate either. However, *A. ochraceus* isolate ATCC 22947 produced 0.04 µg/ml in PDB, 1.6 µg/ml in YES, and 2.4 µg/g on wheat.

The six isolates of *A. alliaceus* from figs produced ochratoxin A at 2.5 to 6.5 µg/ml in PDA (\bar{x} = 4.2) and 3.1 to 30 µg/ml in YES (\bar{x} = 11.0) (Table 1); one (isolate 791) was tested on wheat and produced 13 µg/g. The type isolate of *A. alliaceus* (NRRL 4181, from Australian soil) did not produce ochratoxin A, and NRRL 315 produced 2.5 µg/ml in PDB and 8.9 µg/ml

TABLE 2. Ochratoxin A content of figs with colonies of *Aspergillus* and *Penicillium*^a

| Yr | Ochratoxin A, $\mu\text{g/g}$ (% of figs infected) | | |
|------|--|---------------------------|--------------------|
| | <i>A. alliaceus</i> ^b | <i>A. ochraceus</i> group | <i>Penicillium</i> |
| 1997 | 1.85 (0.1) | 0.003 (0.1) | 0 (0.6) |
| 1998 | — (0.0) | 0 (0.1) | 0 (0.5) |
| 1999 | 0.81 (0.1) | 0.005 (0.1) | 0 (1.1) |

^a Ten orchards were sampled each year; a total of 100 figs per orchard were collected in 1997, and 200 per orchard in 1998 and 1999. The limit of detection of ochratoxin A was 1 ng/g. For *A. alliaceus* and *A. ochraceus*, all visibly infected figs from each orchard were pooled; for *Penicillium*, figs from all orchards were pooled for each year.

^b Some fig samples with *A. alliaceus* also had colonies of the *A. ochraceus* group; we counted these mixed samples only as *A. alliaceus*.

in YES. *A. elegans* and *A. sclerotiorum* isolates from California did not produce ochratoxin A above the limit of detection.

Differences among culture media. Ochratoxin A production was almost always higher in YES than in PDB. Morphologies were very different in the two media: in PDB most isolates produced a flat mycelium at the surface of the liquid and produced sclerotia, whereas in YES most isolates produced a very thick, deeply furrowed mycelium and fewer sclerotia.

Since higher ochratoxin production has been reported on wheat and corn than in liquid media (4, 13) and since wheat is a natural substrate for ochratoxin production (17, 20), we tested 10 isolates for ochratoxin production on autoclaved wheat. Isolates that did not produce ochratoxins at the level of detection in liquid media did not produce detectable levels of ochratoxins on wheat. Isolates that produced ochratoxins in liquid media also produced them on wheat, at comparable levels. The correlation between ochratoxin production on wheat and production in liquid media (average of PDA and YES values) was highly significant ($r^2 = 0.99$).

Fungi and ochratoxin A in figs. Among the 50,000 figs collected between 1997 and 1999, figs containing sporulating colonies of potentially ochratoxigenic fungi were rare (Table 2). *Penicillium* was more common on figs than the *A. ochraceus* group and *A. alliaceus* for all 3 years. *Aspergillus alliaceus* was found in three orchards in 1997 and two orchards in 1999 but was not found in 1998 (Table 2). Pooled sample of figs with *Penicillium* colonies did not contain ochratoxin A. Samples of figs with colonies of *A. alliaceus* contained from 0.37 to 7.86 μg of ochratoxin A per g, respectively. Of the samples of figs with the *A. ochraceus* group, eight contained no detectable ochratoxin A and the remaining samples contained <0.01 $\mu\text{g/g}$. No ochratoxin A was detected in the seven samples infected with *A. ochraceus* from 1998, the year in which no *A. alliaceus* was found.

Six of the isolates tested in vitro came from figs whose ochratoxin A content was determined previously (10). Levels of ochratoxin in the figs correlated significantly with levels produced by the isolates in pure culture, suggesting that these fungi were responsible for the ochratoxin contamination observed (linear regression, $r^2 = 1$).

DISCUSSION

Three unexpected results shed new light on production of ochratoxin A by fungi. First, none of the isolates of *A. ochra-*

ceus or *A. melleus* produced ochratoxin A above the 0.01- $\mu\text{g/ml}$ limit of detection, except for ATCC 22947. Second, all our isolates of *A. alliaceus* produced ochratoxin A in culture, some at high levels. Third, the ochratoxin content of figs was correlated with the presence of *A. alliaceus* but not with the *A. ochraceus* group or *Penicillium*.

***A. ochraceus* group.** Both *A. ochraceus* and *A. melleus* have been found in figs, pistachios, and pistachio tree litter in California, at frequencies comparable to those of *A. flavus* (8, 9, 10). Ochratoxins were first isolated from *A. ochraceus*, and *A. ochraceus* has been used as a model system to study ochratoxin production (16, 23, 25, 27, 29). It was therefore surprising that none of the *A. ochraceus* isolates that we collected produced ochratoxin A above the 0.01- $\mu\text{g/ml}$ limit of detection (Table 1).

While *Aspergillus ochraceus* strains clearly vary in ochratoxin production (1, 13, 23, 25, 26), comparing results among studies is difficult. For example, some early studies had high limits of detection for ochratoxins (e.g., 100 ng/ml [13]), so some ochratoxin-producing strains may have been missed. In other cases, isolates were tested by enzyme-linked immunosorbent assay, and only the presence or absence of ochratoxin A was reported (26). Also, early studies used solid media such as corn or wheat (4, 13, 29), whereas recent studies usually used liquid media (1, 19, 24, 26). The highest levels of ochratoxin A have been reported on grain (13), but there is considerable variation in results among different studies testing the same isolates on grain (4, 13). The relatively low ochratoxin A production that we observed in strains ATCC 22947 and NRRL 4181 may reflect a decline of ochratoxin production during decades of maintenance in culture.

Studies of the *A. ochraceus* group are complicated by difficulties in distinguishing *A. ochraceus* from related species. Phylogenetic studies based on internal transcribed spacer sequences (28) and large-subunit rDNA sequences (18) both showed *A. ochraceus* to be poorly delimited. In another study (27), two *A. ochraceus* clades were identified. All of the members of one clade produced no ochratoxin A, while the other clade included both ochratoxin producers and nonproducers. We hypothesize that most *A. ochraceus* strains in California belong to the first clade or to a similar group.

We likewise expected some isolates of *A. melleus* to produce detectable amounts of ochratoxin A. As with *A. ochraceus*, problems with identification of fungi and delimitation of species may explain part of this inconsistency. However, it appears that populations of *A. melleus* associated with tree nuts and figs in California are less ochratoxigenic than the *A. melleus* isolates tested previously.

***A. alliaceus*.** Unlike *A. ochraceus* and *A. melleus*, all *A. alliaceus* isolates that we tested (except the type isolate) produced ochratoxin A, at concentrations up to 30 $\mu\text{g/ml}$ (Table 1). In three previous studies, one of five isolates, two of two isolates, and six of seven isolates of *A. alliaceus* produced ochratoxin A (4, 13, 28). *A. alliaceus* is considered to be widely distributed but not common (15, 21). It has never been identified as a contributor to ochratoxin contamination of crops. *A. alliaceus* is rare in figs and tree nuts: it was isolated from $<0.008\%$ of figs and $<0.1\%$ of pistachios in California (8, 10).

Implications for agriculture. Since the common species *A. ochraceus* and *A. melleus* appear to produce little or no ochratoxin A in California, and the only species known to produce

high levels of ochratoxin A in California is the relatively rare *A. alliaceus*, we would predict very low levels of ochratoxin contamination of crops. However, ochratoxin production by the *A. niger* group in North America has not been studied, and these fungi may also produce ochratoxins (1, 20).

Differences in toxigenicity and distribution of *A. alliaceus* and *Aspergillus* section *Circumdati* may be analogous to those of the aflatoxin-producing aspergilli. Almost all *A. parasiticus* isolates produce aflatoxins (like *A. alliaceus*), whereas many *A. flavus* isolates are atoxigenic or produce very low levels (like *A. ochraceus* and *A. melleus*). However, *A. parasiticus* is much less common than *A. flavus* in most crops (6). Atoxigenic isolates of *A. flavus* are being used successfully for biocontrol of aflatoxin contamination of crops; the introduced atoxigenic isolates displace toxigenic ones, making natural populations less toxigenic (6, 7). Fortunately, in the case of ochratoxins in California crops, nonproducing (or very weakly producing) strains already appear to dominate populations of *Aspergillus* section *Circumdati*. Since much is known about aflatoxin contamination of crops, and since *A. alliaceus* has recently been shown to be related to section *Flavi* (18, 28), knowledge about aflatoxin contamination may be useful for predicting and preventing ochratoxin contamination as well.

In summary, none of our isolates of *A. ochraceus* or *A. melleus* produced ochratoxin A at the limit of detection, but all isolates of *A. alliaceus* produced it, sometimes at high levels. The presence of *A. alliaceus* was correlated with the presence of ochratoxin A in figs, whereas the presence of the *A. ochraceus* group and *Penicillium* was not. Based on our results, future studies of ochratoxin production and contamination by the aspergilli should focus on *A. alliaceus* rather than *A. ochraceus*.

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