Aflatoxin and Cyclopiazonic Acid Production by a Sclerotium-Producing Aspergillus tamarii Strain

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The production of aflatoxins B₁ and B₂ by Aspergillus tamarii (subgenus Circumdati section Flavi) is reported for the first time. The fungus was isolated from soil collected from a tea (Camellia sinensis) field in Miyazaki Prefecture, Japan. Three single-spore cultures, NRRL 25517, NRRL 25518, and NRRL 25519, were derived from subcultures of the original isolate 19 (MZ2). Each of these single-spore cultures of A. tamarii produced aflatoxins B₁ and B₂ and cyclopiazonic acid, as well as black, pear-shaped sclerotia. The demonstration of aflatoxin production by A. tamarii is examined in connection with A. tamarii phylogenetic relationships, chemical ecology, and potential use in food fermentations.

In a recent study of Aspergillus flavus group (subgenus Circumdati section Flavi) isolates from soil samples collected from Japanese tea fields, isolate 19 (MZ2) from Miyazaki Prefecture produced substantial quantities of aflatoxin B₁ and cyclopiazonic acid in liquid media (13). While the colonies of aflatoxin-producing species A. flavus Link:Fr., Aspergillus parasiticus Speare, and Aspergillus nomius Kurtzman et al. were bright greenish yellow on Czapek agar (14, 20), mature colonies of isolate 19 were dark olive to olive brown, the colors of cultures of Aspergillus tamarii Kita (20). This evidence confirms that isolate 19 is an unrecognized form of A. tamarii which produces aflatoxins B₁ and B₂, cyclopiazonic acid (CPA), and sclerotia.

MATERIALS AND METHODS

The culture of A. tamarii isolate 19 (MZ2) was collected in 1993 by Itou and Goto (12) from soil and experimental tea field in Miyazaki Prefecture, Japan. Like most tea fields in Japan, the soil had become acidified (pH = 4.0) through a long history of fertilizer applications. To confirm the original observation of aflatoxin production by isolate 19, three subcultures, MZ2D, MZ2E, and MZ2F, were prepared by mass conidial transfer from the original malt extract agar culture slant. To eliminate the possibility that the cultures were contaminated with an aflatoxin-producing species (i.e., A. flavus, A. parasiticus, or A. nomius), single-spore isolations were performed with MZ2D, MZ2E, and MZ2F. Cultures developing from single-spore isolates of MZ2D SS#1 (NRRL 25517), MZ2E SS#2 (NRRL 25518), and MZ2F SS#3 (NRRL 25519) were grown for 21 days (25°C) on Czapek agar slants in a dark incubator. Each culture had the same olive-brown colony appearance as isolate 19, including the presence of numerous black, pear-shaped sclerotia. The cultures were deposited with the Agricultural Research Culture Collection (formerly called the Northern Regional Research Laboratory [NRRL], Peoria, Ill.).

Observations of macroscopic colony characters of Aspergillus tamarii NRRL 25517, NRRL 25518, and NRRL 25519 were made from three-point inoculations on Czapek agar (20) in petri dishes following incubation for 7, 14, and 21 days at 25°C. Czapek agar slants were also inoculated with each strain of A. tamarii and incubated in total darkness for 10 days at 25°C. Colony colors were determined with the color charts of Ridgway (22).

Each of the three single-spore isolates was examined for the ability to produce aflatoxins on glucose yeast extract (GY) liquid medium and to produce CPA on modified Czapek liquid medium, as described by Itou and Goto (12). Minerals were added to each of these media in the following amounts per liter: Na₂B₄O₇·10H₂O, 0.7 mg; (NH₄)₂MoO₄·2H₂O, 4H₂O, 0.5 mg; CuSO₄·5H₂O, 0.3 mg; and ZnSO₄·7H₂O, 17.6 mg. Aflatoxin levels were analyzed by both thin-layer chromatography (TLC) (30) and high-performance liquid chromatography (HPLC) (16). A semiquantitative TLC method was used for initial screening for aflatoxin production. The isolates were inoculated into 10 ml of GY liquid medium in a test tube (18 by 180 mm) (tube plugged with cotton) and incubated in the dark for 7 days at 27°C (29). To extract aflatoxins, 400 to 500 mg of KCl and 5 ml of methanol were first added to 5 g of culture medium and then extracted twice with 3 ml of chloroform. The chloroform extract was dried under a stream of nitrogen gas. The residue was dissolved in benzene-acetonitrile (98:2 vol/vol) and spotted on a TLC plate (catalog no. 5721: Merck). The plate was developed with toluidine—ethyl acetate—acetic acid formic acid (63:1 vol/vol) (TFA) in a glass tank. Aflatoxins were detected under long-wavelength (365-nm wavelength) UV light.

An HPLC method (16) was used for the quantitative analysis of aflatoxins. The fungus was grown on 30 g of polished, short grain japonica-type rice moistened with a 15-ml solution of minerals (listed above) in 300-ml Ehretmann flasks and incubated for 7 days at 27°C. Aflatoxins were extracted with chloroform and separated from other components by using a silica gel-aluminum oxide column (7). The aflatoxin-containing fraction was evaporated to dryness, and the residue was dissolved in benzene-acetonitrile (98:2 vol/vol) and spotted on a TLC plate (catalog no. 5721: Merck). The plate was developed with normal-phase HPLC with fluorescence detector (16). The HPLC system used to perform these analyses consisted of an LC-6A pump (Shimadzu Co.), RF550 fluorescence detector (excitation wavelength, 365 nm; emission wavelength, 425 nm) (Shimadzu Co.), C-R5A reporting integrator (Shimadzu Co.), Rhodine 7125 sample injector with 20-μl sample loop (Rhodine Inc.), and Develosil 60-5 column and guard column (Nomura Chemical Co.). For the confirmation of aflatoxins, two-dimensional TLC and mass spectrometry were used. The liquid medium contained 33.4 g of modified Czapek Dox medium (Unipath Co.), 30 g of glucose, 1.5 mg of methionine, 33 mg of yeast extract, 10 mg of vitamin B₁, 10 mg of vitamin B₆, 10 mg of vitamin B₂, 0.1 mg of vitamin B₃, 0.01 mg of vitamin B₇, 0.03 mg of vitamin B₉, 0.01 mg of vitamin B₁₂, 1 mg of biotin, 0.1 mg of folic acid, 1 mg of inositol, 0.5 mg of niacin, 0.5 mg of pyridoxine, 0.5 mg of thiamine, and 1 mg of vitamin K. The isolate was inoculated into 10 ml of GY liquid medium in a test tube (18 by 180 mm) (tube plugged with cotton) in the dark for 10 days at 25°C. The liquid medium consisted of 33.4 g of modified Czapek Dox medium (Unipath Co.), 30 g of glucose (see above), and 1 liter of distilled water. The sample extracts for CPA analysis were prepared by a mini-test tube method (8). Sample extracts were then analyzed by HPLC with monitoring by UV absorption at 284 nm. The HPLC system used for CPA analysis consisted of an LC-6A pump, SPD-6A UV detector (284-nm wavelength) (Shimadzu Co.), SSCP3000 column oven at 50°C (Senshu Kagaku Co.), C-R5A reporting integrator, Rhodine 7125 sample injector, and a Develosil 60-5 column (4.6 by 150 mm) with a guard column (4 by 10 mm) (Nomura Chemical Co.). The mobile phase was a mixture of ethylacetate—2-propanol—35% aqueous ammonia (55:20:5, vol/vol) (8). Results were treated with those for an authentic standard of CPA purchased from Sigma Chemical Co.

RESULTS AND DISCUSSION

The three single-spore cultures of A. tamarii isolate 19 (NRRL 25517, NRRL 25518, and NRRL 25519) were identical.
in appearance when grown on Czapek agar in petri dishes. Colonies sporulated heavily, and the colors of the colonies were old gold to lake (Plate XVI in reference 22), shifting to isabellae (Plate XXX), orange citrine (Plate IV), dark citrine (Plate IV), or yellowish olive (Plate XXX) at maturity; with age (21 days), colonies became medial bronze (Plate IV). However, the colonies did not continue to darken and become near Prout’s brown (Plate XV) as previously described for \textit{A. tamarii} (20). The colony reverse was pale orange-brown as a result of pigments diffusing into the medium. Raper and Fennell (20) described the \textit{A. tamarii} colony reverse as “uncolored or occasionally pinkish.” When grown on Czapek agar slants (14 days), these same cultures were isabellae (Plate XXX) to orange citrine (Plate IV) near the tip of the agar slant but dark citrine (Plate IV) to olive green (Plate IV) toward the base. The colony reverse and agar were pale orange-brown. Numerous pyriform black sclerotia were produced in Czapek agar slants. The sclerotia measured 600 to 1,000 \(\mu\)m by 375 to 750 \(\mu\)m. No sclerotia were found in petri dish cultures (Czapek agar). Examination of wet mounts revealed biseriate and/or uniseriate conidial heads; conidiophores variable in length (700 to 1,500 \(\mu\)m, up to 2,500 \(\mu\)m), with smooth to sparsely echinulate walls; globose to subglobose vesicles (diameter of 45 \(\mu\)m, up to 55 \(\mu\)m); and conspicuously roughened globose conidia (diameter of 5.0 to 6.5 \(\mu\)m, up to 9.0 \(\mu\)m including tubercles and warts). The outer conidial walls became detached from the firm inner wall when pressure was applied to the coverslip. The conidial characteristics matched the description for \textit{A. tamarii} (20).

On the basis of morphological evidence alone, NRRL 25517, NRRL 25528, and NRRL 25519 can be classified as \textit{A. tamarii} (by the criteria in reference 20). The colonies are yellowish-olive green and become medal bronze (Plate IV) with age, suggestive of the so-called bronze series (24). The bronze series included strains near \textit{A. tamarii} whose colonies are yellow-green when young and yellow to brown when mature. Raper and Fennell (20) were unable to distinguish strains of \textit{A. tamarii} from strains in the bronze series and revised their description of \textit{A. tamarii} to include such strains. Horn and Greene (10) recognized two types of \textit{A. tamarii} isolates from agricultural soils from Georgia. Isolates of \textit{A. tamarii}, designated type A, were morphologically similar to NRRL 429 (WB 429), one of several \textit{A. tamarii} strains used by Raper and Fennell (20) for their description of this species. Horn et al. (11) proposed that \textit{A. tamarii} type B strains are distinctive enough to be separated from \textit{A. tamarii} and subsequently described the new species \textit{Aspergillus caelatus} (9). Colonies of \textit{A. caelatus} isolates were olive, while the colony color of \textit{A. tamarii} isolates was olive brown (10). Furthermore, \textit{A. caelatus} isolates produced a pale yellow-brown diffusible pigment in the medium, most evident on Czapek agar slants, whereas \textit{A. tamarii} isolates did not. Vesicle diameter, stipe width, and stipe length of \textit{A. caelatus} were significantly smaller than those of the conidiophore structures of \textit{A. tamarii}. Sixty-nine percent (22 of 32) of the \textit{A. caelatus} isolates formed irregularly shaped sclerotia, whereas sclerotia were not present in any of the \textit{A. tamarii} isolates. Egel et al. (5) also reported finding no sclerotia in several cultures of \textit{A. tamarii} they examined. Like \textit{A. caelatus} isolates, \textit{A. tamarii} NRRL 25517, NRRL 25518, and NRRL 25519 strains produce a pale brown pigment in Czapek agar and produce sclerotia. However, the sclerotia were uniformly small in size and distinctly pyriform in shape.

Single-spore isolates of \textit{A. tamarii} produced aflatoxin \(B_1\) and \(B_2\) in GY liquid medium (NRRL 25517, 30 ppb of aflatoxin \(B_1\) and \(B_2\), NRRL 25518, 30 ppb of aflatoxin \(B_1\) and \(B_2\), NRRL 25519, 30 ppb of aflatoxin \(B_1\) and 0.2 ppb of aflatoxin \(B_2\), and rice medium (NRRL 25517, 48.6 ppb of aflatoxin \(B_1\) and 0.9 ppb of aflatoxin \(B_2\), NRRL 25518, 49.5 ppb of aflatoxin \(B_1\) and 2.8 ppb of aflatoxin \(B_2\); NRRL 25519, 49.6 ppb of aflatoxin \(B_1\) and 3.2 ppb of aflatoxin \(B_2\)). No aflatoxin was detected in the rice that was used as a fermentation substrate (detection limit of <1.0 ppb). Aflatoxins \(G_1\) and \(G_2\) were not detected by any of these methods of analysis (minimum level of detection of 0.5 ppb). These results can be contrasted with those of a known aflatoxin-producing strain, \textit{A. parasiticus} NRRL 2990, that was cultured on GY liquid medium and incubated under the same conditions and produced 10 ppb of aflatoxin \(B_1\), 0.3 ppb of aflatoxin \(B_2\), 30 ppb of aflatoxin \(G_1\), and 1 ppb of aflatoxin \(G_2\). These single-spore isolates also produced CPA in modified Czapek Dox liquid medium (NRRL 25517, 1.71 ppb of CPA; NRRL 25518, 2.49 ppb; NRRL 25519, 1.84 ppb). These results provide the first demonstration of aflatoxin production by a strain of \textit{A. tamarii}. Manabe et al. (17) examined nine strains of \textit{A. tamarii} for production of aflatoxin and kojic acid. None of the strains produced aflatoxin, but all of the strains produced kojic acid. The three single-spore cultures of \textit{A. tamarii} isolates 19 also produced substantial quantities of kojic acid (NRRL 25517, 1.92%; NRRL 25518, 2.38%; NRRL 25519, 2.01%) by the methods described by Manabe et al. (17). \textit{A. tamarii} strains from the American Type Culture Collection were examined for their ability to produce aflatoxins (25, 26). Analysis of culture extracts from seven \textit{A. tamarii} strains by TLC and HPLC revealed no aflatoxin. Aflatoxin production was not detected for four strains of \textit{A. tamarii}, including \textit{A. tamarii} NRRL 20818 (CBS 104.13), the ex-lectotype of the species (5). Klich and Pitt (13) reported that CPA was produced by 10 of the 13 \textit{A. tamarii} isolates they examined but did not detect aflatoxins. \textit{A. tamarii} has been reported to produce the following mycotoxins: CPA (2), fumigaclavine A (6), and kojic acid (19). The mycotoxin profile of \textit{A. tamarii} can be contrasted with those of other members of the section Flavi (6). \textit{A. flavus} produces aflatoxins \(B_1\) and \(B_2\), aspergillaric acid, CPA. 3-nitropropionic acid, and kojic acid. \textit{A. parasiticus} produces aflatoxins \(B_1\), \(B_2\), \(G_1\), \(G_2\), aspergillaric acid, and kojic acid but not CPA or 3-nitropropionic acid. \textit{A. nomius} produces aflatoxins \(B_1\), \(B_2\), \(G_1\), \(G_2\), aspergillaric acid, kojic acid, and tenuazonic acid but not CPA or 3-nitropropionic acid. Therefore, \textit{A. tamarii} NRRL 25517, NRRL 25518, and NRRL 25519 are further distinguished from \textit{A. parasiticus} and \textit{A. nomius} because the latter two species do not produce CPA. While our aflatoxin-producing \textit{A. tamarii} strains do not produce aflatoxins \(G_1\) and \(G_2\), Kurtzman et al. (14) offer evidence suggesting that \textit{A. tamarii} has only recently evolved from \textit{A. flavus} and \textit{A. nomius}. They performed pairwise comparisons of DNA complementarity (reported as a percentage) between \textit{A. tamarii} and other species known to produce aflatoxins (\textit{A. nomius}, 42%; \textit{A. flavus} var. \textit{flavus}, 52%; \textit{A. flavus} var. \textit{parasiticus}, 41%). Intraspecific DNA relatedness for all taxa exceeded 85%. Chang et al. (1) offers evidence from PCR product profiles of \textit{A. tamarii} genomic DNA suggesting the presence of the \textit{A. parasiticus} \textit{affl} gene that may be involved in the regulation of aflatoxin biosynthesis. \textit{A. tamarii} NRRL 25517, NRRL 25518, and NRRL 25519 strains all produce CPA, which further distinguishes them from isolates of \textit{A. caelatus}. Horn and Greene (10) reported that isolates of \textit{A. tamarii} consistently produced CPA and kojic acid, whereas \textit{A. caelatus} isolates produced only kojic acid.

Aflatoxin-producing species of \textit{A. flavus}, \textit{A. parasiticus}, and now \textit{A. tamarii} have all been recorded from diseased or dead insects (28). For example, \textit{A. tamarii} NRRL 428
was isolated from an unidentified insect. A. tamarii CBS 631.67 was isolated from the pupae of Prodenia litura, and A. tamarii has been recorded from diseased silkworms and pupae of the silk moth Attacus ricini (20). Aflatoxin is a potent natural insecticide (28, 30). Furthermore, all known aflatoxin-producing species, including A. tamarii, also produce kojic acid. Dowd (4) demonstrated that kojic acid synergizes the toxicity of aflatoxin B1 to lepidopteran insect pests of crops because it inhibits oxidative enzymes likely to be involved in aflatoxin B1 detoxification.

A. tamarii is also recognized as a colonist of seeds with known susceptibility to aflatoxin contamination including cotton seed (Gossypium hirsutum L.) (1), peanuts (Arachis hypogaea L.) (2, 10), pistachio nuts (Pistacia vera L.) (3), sunflower seed (Helianthus annuus L.) (13), corn grain (Zea mays L.), and rice grain (Oryza sativa L.) (Agricultural Research Service Culture Collection Records). A. tamarii is sometimes listed with Aspergillus oryzae and Aspergillus sojae as molds used for koji in traditional oriental food fermentations. For example, A. tamarii NRRL 429 was isolated from a soybean sauce called tamari. According to Thom and Church (25), “Kita believed that where tamari sauce was made empirically it owed its individuality to an Aspergillus which he named A. tamarii.” Examples of A. tamarii strains used in the production of koji include the following: JCM 2259 used in the production of Amazake-koji (18); CBS 821.72 from koji; and RIB 3001 from tamari-miso koji (21). No one has attempted to distinguish domesticated strains of A. tamarii from wild isolates, as was done for A. oryzae and A. sojae (27).

In summary, aflatoxin-producing strains of A. tamarii NRRL 25517, NRRL 25518, and NRRL 25519, when grown on Czapek agar, best fit the description of A. tamarii (20), only differing from the species as follows. (i) Colonies do not darken in age and become truly brown or Prout’s brown (22). (ii) The agar becomes a pale orange-brown color from a diffusible pigment(s). (iii) Numerous small, pyriform sclerotia are formed in slant cultures during incubation in the dark. Raper and Fennell (20) note that pyriform sclerotia are produced by some strains of A. tamarii. Although the fungus resembles A. tamarii morphologically, it may in fact be a different taxon, as indicated by its mycotoxin profile and possibly by further molecular work. In this respect, A. tamarii from koji may not present an aflatoxin risk. We concur with the views of Egel et al. (5) and Horn and Greene (10) that there is a need to better describe the variability and potential divisions within the taxon A. tamarii. The discovery of aflatoxin production by strains classified as A. tamarii should serve as a stimulus for such research.

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REFERENCES