Note

A New Criterion by Which to Distinguish *Aspergillus sojae*, a Koji-mold, from Related Taxa Producing Echinulate Conidia

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In Japan, *Aspergillus sojae* Sakaguchi et Yamada ex Murakami has been used commercially in the production of shoyu (fermented soy sauce). This fungus is morphologically similar to *Aspergillus oryzae* (Ahlburg) Cohn. A striking characteristic of *A. sojae* is the presence of echinulate conidia, and strains of this species do not have the ability to produce aflatoxin.

In 1944, Sakaguchi¹⁾ isolated from shoyu-koji some strains producing echinulate conidia which were not placed in the *Aspergillus flavus-oryzae* series circumscribed by Thom and Church,^{2,3)} and in 1944, based upon these isolates, *Aspergillus sojae* was first proposed without the Latin diagnosis by Sakaguchi et Yamada.¹⁾ In 1965, Raper and Fennell⁴⁾ placed *A. sojae* as a synonym of *A. parasiticus* Speare in the *A. flavus* group. But the majority of *A. parasiticus* strains have been recognized as having an aflatoxin-producing ability. In 1971, Murakami⁵⁾ classified the species producing echinulate conidia in the *A. flavus* group (sensu Raper and Fennell⁴⁾) within *A. sojae*, *A. parasiticus*, and *A. toxicarius* Murakami on the basis of some morphological (e.g., metulae: present or absent) and physiological (e.g., pink coloring of the conidia on Czapek agar slant culture, containing 0.05% anisaldehyde = *p*-methoxybenzaldehyde) characteristics.

However, it has been necessary to establish another method for differentiating more clearly between *A. sojae* and related taxa (e.g., *A. parasiticus* and *A. toxicarius*) because a large number of strains belonging to *A. parasiticus* and *A. toxicarius* are known to produce aflatoxin.

Method to identify aflatoxin-producing molds and aflatoxin-non-producing molds have been provided by Hara et al.,⁶⁾ Bothast et al.,⁷⁾ and Wicklaw et al.⁸⁾

A new criterion by which to distinguish *A. sojae* from related taxa, sometimes classified as *A. parasiticus* and *A. toxicarius*, is presented here.

Twenty-nine strains were obtained from the National Research Institute of Brewing (RIB), Tax Administration Agency, Tokyo, Japan, and fifteen strains from the American Type Culture Collection (ATCC), Maryland, U.S.A.

A modified Czapek medium containing the following constituents per liter of distilled water was used: Sucrose, 30 g; NaNO₃, 3 g; K₂HPO₄·7H₂O, 1 g; KCl, 0.5 g; MgSO₄·7H₂O, 0.5 g; FeSO₄·7H₂O, 0.1 g; ZnSO₄·7H₂O, 0.2 g. The pH of the medium was adjusted to 7.0. The conidia of each strain from a malt-extract agar slant were inoculated into 50 ml of the medium in a 150-ml Erlenmeyer flask. The cultivation was carried out at 30°C for 10 days. The production of a yellow pigment was judged by observation of the cultured broth with the naked eye.

Twenty-one strains, each of which is classified as *A. parasiticus* or *A. toxicarius*, produced a considerable amount of yellow pigment which show a maximum absorption at 410 nm as shown in Fig. 1. Exceptions to this are the three negative strains, ATCC 1018, 18501, and 26850. These three strains have been listed as *A. parasiticus* in the ATCC Catalogue.⁹⁾ However, in our experiment, it was confirmed that each of these three strains was not an aflatoxin producer, and that the conidial wall of each strain was not echinulate. For this reason, it is considered that ATCC 1018, 18501, and 26850 should not be regarded as members of *A. parasiticus* producing echinulate conidia. On the other hand, all strains of *A. sojae* tested did not produce the yellow pigment. But five strains (RIB 1024, 1041, 1042, 1046, 1047) of *A. sojae* tested, produced a brown pigment. The spectrum of this brown pigment was quite different from that of the yellow pigment as shown in Fig. 1. The above results are summarized in Table I. The correlation between the quantity of the yellow pigment with that of aflatoxin was uncertain.

The characteristics of the yellow pigment were examined further. The yellow pigment was extracted with 500 ml of ethylacetate from 250 ml of the cultured broth of *A. parasiticus* (RIB 1037). The extract was concentrated *in vacuo* and analyzed by thin-layer chromatography (TLC). For the TLC analysis, plates (silica gel G plates, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) were developed in benzene-methanol = 95 : 5 (v/v). Each plate was dried. Two yellow spots were observed on the TLC plate: the *R_f* value of one spot was 0.84 (pigment A), and that of the other spot was 0.14 (pigment B). The concentrated extract was subjected to preparative TLC (Kiesel Gel G, Merck & Co., Inc.). Each zone of *R_f* = 0.84 and 0.14 was scraped off and eluted with ethyl acetate. The UV-absorption spectrum of each isolated yellow pigment in ethanol was recorded with a Hitachi 323 recording spectrophotometer.

The results are shown in Fig. 2. Two kinds of yellow pigment were produced by *A. parasiticus* and *A. toxicarius*, but not produced by *A. sojae*.

FIG. 1. Visible Absorption Spectra of Pigments Produced by *Aspergillus sojae* and *Aspergillus* Species Producing Echinulate Conidia.

TABLE I. PRODUCTION OF A YELLOW PIGMENT BY STRAINS OF *Aspergillus* PRODUCING ECHINULATE CONIDIA

Strain	Pigment production	Strain	Pigment production
<i>A. sojae</i> RIB 328	—	<i>A. toxicarius</i> RIB 4001	+
<i>A. sojae</i> RIB 401	—	<i>A. toxicarius</i> RIB 4002	+
<i>A. sojae</i> RIB 405	—	<i>A. toxicarius</i> RIB 4003	++
<i>A. sojae</i> RIB 410	—	<i>A. toxicarius</i> RIB 4005	+
<i>A. sojae</i> RIB 1011	—	<i>A. toxicarius</i> RIB 4015	+
<i>A. sojae</i> RIB 1023	—	<i>A. toxicarius</i> RIB 4018	+
<i>A. sojae</i> RIB 1024	—	<i>A. parasiticus</i> RIB 1037	++++
<i>A. sojae</i> RIB 1040	—	<i>A. parasiticus</i> RIB 4032	+++
<i>A. sojae</i> RIB 1041	—	<i>A. parasiticus</i> RIB 4033	+++
<i>A. sojae</i> RIB 1042	—	<i>A. parasiticus</i> ATCC 1018*	—
<i>A. sojae</i> RIB 1044	—	<i>A. parasiticus</i> ATCC 18501*	—
<i>A. sojae</i> RIB 1045	—	<i>A. parasiticus</i> ATCC 26804	++
<i>A. sojae</i> RIB 1046	—	<i>A. parasiticus</i> ATCC 26850*	—
<i>A. sojae</i> RIB 1047	—	<i>A. parasiticus</i> ATCC 26862	+++
<i>A. sojae</i> RIB 1049	—	<i>A. parasiticus</i> ATCC 26863	++
<i>A. sojae</i> RIB 1050	—	<i>A. parasiticus</i> ATCC 26864	++
<i>A. sojae</i> RIB 1051	—	<i>A. parasiticus</i> ATCC 26865	++
<i>A. sojae</i> RIB 1052	—	<i>A. parasiticus</i> ATCC 26866	++
<i>A. sojae</i> RIB 1054	—	<i>A. parasiticus</i> ATCC 26870	+++
<i>A. sojae</i> RIB 1059	—	<i>A. parasiticus</i> ATCC 26871	++
		<i>A. parasiticus</i> ATCC 26872	+
		<i>A. parasiticus</i> ATCC 28285	+
		<i>A. parasiticus</i> ATCC 34645	++
		<i>A. parasiticus</i> ATCC 36537	+++

* In our observation, the conidial wall of each strain was not echinulate. —, not produced; +, 0.01~0.05 (optical density at 410 nm); ++, 0.06~0.10; +++, 0.11~0.15; +++, 0.16~0.25.

Medium, modified Czapek medium containing $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 7.0); culture conditions, 30°C, 10 days.

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FIG. 2. UV-Absorption Spectra of the Yellow Pigments Produced by *Aspergillus* Species Producing Echinulate Conidia.