Interactions of Saprophytic Yeasts with a *nor* Mutant of *Aspergillus flavus*

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The *nor* mutant of *Aspergillus flavus* has a defective norsolorinic acid reductase, and thus the aflatoxin biosynthetic pathway is blocked, resulting in the accumulation of norsolorinic acid, a bright red-orange pigment. We developed a visual agar plate assay to monitor yeast strains for their ability to inhibit aflatoxin production by visually scoring the accumulation of this pigment of the *nor* mutant. We identified yeast strains that reduced the red-orange pigment accumulation in the *nor* mutant. These yeasts also reduced aflatoxin accumulation by a toxigenic strain of *A. flavus*. These yeasts may be useful for reducing aflatoxin contamination of food commodities.

Aflatoxins constitute a group of toxic carcinogenic metabolites produced by *Aspergillus flavus* Link: Fr., *A. parasiticus* Spear, and *A. nomius* Kurtzman (5, 8, 21). Aflatoxin B₁ (AFB₁), the most toxic of this group, is the most potent carcinogen known. Contamination of tree nuts, peanuts, corn, and cottonseed by aflatoxins is a serious food safety hazard to both humans and animals. *A. flavus* is the most common aflatoxin-producing fungus on corn, cotton, tree nuts, and peanuts (6, 7, 9, 10, 18, 23). This fungus is a wound-invading pathogen that infects plants damaged by insects, animals, early splits, and mechanical harvesting. Under conditions extremely favorable for infection, this fungus can colonize corn kernels directly (10, 18, 22). Any established infection of *A. flavus* will result in rapid accumulation of aflatoxins in the harvested commodity under conditions of warm temperatures and high humidity. The domestic market of food for human consumption presently allows a maximum level of 20 ppb for AFB₁. Even very low levels of infection by *A. flavus* can result in aflatoxin levels exceeding this standard. In a worst-case scenario, the commodity may be banned from domestic and export markets. The presence of aflatoxins in a food commodity reduces the economic return to both the growers and processors.

Saprophytic yeasts are common on the surface of plant leaves and fruits. Some yeasts effectively compete with post-harvest fungal pathogens, such as *Penicillium expansum* and *Botrytis cinerea* (11, 14, 19, 24). The potential of saprophytic yeasts to be used in a variety of application for reducing aflatoxin contamination in food has not been hitherto extensively explored.

More than 25 genes are involved in the biosynthesis of aflatoxins via the polyketide pathway. A regulatory gene, *afIR*, is required for the expression of several identified genes in the pathway (2, 3, 15, 16, 25). At least 16 enzyme-catalyzed steps are required to complete the synthesis of AFB₁ from norsolorinic acid, the first stable intermediate. We used *A. flavus* Papa 827, a *nor* mutant (17), as an indicator strain to visually monitor the interactions of yeasts with *A. flavus*. The *nor* mutant of *A. flavus* has a mutation in the gene coding for norsolorinic acid reductase, and aflatoxin biosynthesis is blocked

at this step. This mutant strain accumulates norsolorinic acid, a bright red-orange pigment, that can be easily seen (1, 12). The rationale for using the *nor* mutant as an indicator strain is that accumulation of the red-orange pigment during growth implies that the aflatoxin biosynthetic pathway is operating up to the formation of norsolorinic acid. Thus, if no visible pigment is produced, it implies that aflatoxin biosynthesis is inhibited at an earlier step in the polyketide pathway and that this inhibition also may occur in field strains of aflatoxigenic *A. flavus*.

Aspergillus flavus Papa 827 is a white-spored mutant that accumulates norsolorinic acid, a red-orange pigment. The toxigenic strain *A. flavus* 42-12 (NRRL-25347), isolated from a pistachio nut, was used to examine inhibition of aflatoxin production by yeasts. This strain produces AFB₁ and traces of AFB₂. The fungal strains were maintained on potato-dextrose agar (PDA) (Difco Laboratories, Detroit, Mich.). Fungal spores were resuspended in 0.05% Tween 80, and the number of spores in the suspension was determined microscopically with a hemacytometer.

We used the following bioassay protocol: (i) the yeast isolate was grown in a flask containing potato dextrose broth (Difco) for 24 h on a shaker at 28°C, and the fresh culture was used for inoculation; (ii) spore suspensions of the *nor* mutant were prepared (10⁴ spores/ml); (iii) two streaks of 20-μl yeast cultures were applied 15 mm from the center line of a petri dish containing PDA; (iv) 4 h later, 20 μl of the *A. flavus* spore suspension was inoculated along the center line of the PDA agar; (v) the petri dish was sealed with Parafilm M (American National Can, Neenah, Wis.) and incubated at 28°C for 10 days.

We determined the effects of yeast isolates on *A. flavus* by visually estimating the amount of red-orange color formed by the *nor* mutant (Fig. 1). A score of + + + + was assigned to the bright orange pigment level of control (Fig. 1A), and a score of – was assigned to an almost imperceptible level of pigment (Fig. 1B and C). The growth of the *nor* mutant was restricted to and reduced within the boundary of the two streaks of the yeast zone in the bioassay (Fig. 1). As shown in Fig. 1A, the fungal colony expanded toward the edges of the PDA in the petri dish in the absence of the yeasts.

The amount of norsolorinic acid produced was quantified spectrophotometrically to verify the reliability of the visual bioassay. The bright red-orange pigment in the mycelium was

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FIG. 1. A visual system for monitoring interactions of yeasts and *A. flavus*. *nor* mutant *A. flavus* Papa 827 is used as an indicator strain. Details of the experiment are given in the text. (A) *nor* mutant in the absence of yeasts; (B) WRL-076; (C) WRL-038; (D) WRL-084; (E) WRL-015; (F) WRL-024; (G) WRL-053.

extracted by alkaline methanol (methanol–1 N NaOH [90:10, vol/vol]). Five agar discs (7-mm diameter) of fungal cultures were cored and transferred to a centrifuge tube containing 10 ml of solvent. Norsolorinic acid concentration in the supernatant was determined spectrophotometrically at a wavelength of 560 nm in a Beckman DU 640 spectrophotometer. Purified norsolorinic acid was dissolved in alkaline methanol and used as a standard. The visual scores correlate well with the concentration of norsolorinic acid (Table 1).

Saprophytic yeasts were isolated from fruits of almond, pistachio, and walnut trees. The six selected isolates, *Candida guilliermondii* WRL-015, *Cryptococcus laurentii* WRL-024, *Candida krusei* WRL-038, *Rhodotorula mucilaginosa* WRL-053, *Pichia anomala* WRL-076, and *Candida oleophila* WRL-084, exhibited a wide range of activity inhibitory toward norsolorinic acid biosynthesis and accumulation (Fig. 1 and Table 1). Yeasts WRL-076 and WRL-038 were the most inhibitory to aflatoxin biosynthesis; the mycelium of the *nor* mutant in the center of the PDA inhibitor plate was completely white (Fig. 1B and C, respectively). Yeasts WRL-084 and WRL-015 were less inhibitory, and red-orange color was observed in the fungal mycelium (Fig. 1D and E, respectively). Yeasts WRL-024 and WRL-053 were not very inhibitory, and large amounts of norsolorinic acid accumulated in the mycelium (Fig. 1F and G, respectively).

The norsolorinic acid synthesized and accumulated by the

nor mutant is probably localized on the membrane inside the fungal hypha (12, 16). It is unlikely that the yeast streaks on the agar can degrade this compound, since it does not diffuse out from the *nor* mutant. We also determined the pH of the agar near the yeast streaks. To do this, five agar discs near the streak of yeast were cored, transferred to a microcentrifuge tube, and mixed with 0.5 ml of distilled H₂O. After centrifugation at 14,000 rpm in an Eppendorf centrifuge for 10 min at 4°C, the pH of the supernatant was measured in a pH meter (Table 1). The pH readings near the most effective yeasts, WRL-038 and WRL-076, were 5.2 and 4.4, respectively. Acidic pH favors aflatoxin synthesis in toxigenic strains of *Aspergillus* spp. and norsolorinic accumulation in the *nor* mutant (4, 13). The *nor* mutant of *A. flavus* Papa 827 grown between the streaks of these two yeasts was expected to produce and accumulate larger quantities of norsolorinic acid so that more red-orange pigment would be seen in the agar. However, the opposite was observed. The fungal cultures had very little visible red-orange color (Fig. 1B and C). Therefore, the pH of the agar surrounding the *nor* mutant was not a determining factor for inhibiting the production of norsolorinic acid. Instead, we think that diffusible metabolites produced by the yeasts mediate the inhibitory effect.

To measure growth inhibition of the *nor* mutant, we determined the dry weight of the fungal mass in the agar between the two streaks of yeast. Five agar discs (7-mm diameter) of fungal mycelium were cored and transferred to boiling water. The floating mycelial mat was washed once in boiling water to remove agar traces and air dried at room temperature for 16 h. The dry weight of the fungal mass depended upon the yeast strain in the petri dish (Table 1). Growth and norsolorinic acid were not correlated ($r = 0.43$ and $p = 0.18$ based on the Kendall correlation), so the yeasts must affect norsolorinic acid accumulation in some manner other than simply reducing the amount of biomass available to accumulate the compound.

We selected the toxigenic strain *A. flavus* 42-12 to test the effectiveness of the six yeasts for inhibiting aflatoxin biosynthesis in the bioassay. Four agar discs (7-mm diameter) of fungal cultures were cored in the center of a petri dish and transferred to a glass tube containing methanol. Aflatoxin was extracted and analyzed by high performance liquid chromatography (20). When grown on PDA, strain 42-12 produced 4,800 ng of aflatoxin per four discs. Aflatoxin production was drastically reduced by yeasts WRL-076 and WRL-038 and moderately reduced by WRL-015, WRL-053, and WRL-084 (Table 1). The findings are consistent with that of the visual bioassay with the

TABLE 1. Effects of saprophytic yeasts on toxigenic strain 42-12^a

Yeast	External pH	Visual score ^b	Norsolorinic acid (μg/5 discs)	Biomass (mg/5 discs)	AFB ₁ (ng/4 discs)
WRL-015	4.7 ± 0.06	+	16 ± 2.1	7.2 ± 0.7	770 ± 74
WRL-024	4.8 ± 0.07	+++	66 ± 8.3	6.8 ± 0.7	1,900 ± 170
WRL-038	5.2 ± 0.09	–	3 ± 0.4	5.5 ± 0.5	180 ± 20
WRL-053	4.9 ± 0.09	+++	60 ± 7.2	7.0 ± 0.6	360 ± 35
WRL-076	4.4 ± 0.05	–	4 ± 0.8	3.8 ± 0.4	60 ± 9
WRL-084	5.6 ± 0.16	+	21 ± 3.5	4.5 ± 0.4	410 ± 40
Control	4.8 ± 0.12	++++	112 ± 12	8.8 ± 0.7	4,800 ± 370

^a Values are means ± standard deviations ($n = 6$) of two sets of triplicate samples from two experiments

^b Visual score of red-orange pigment in fungal agar cultures shown in Fig. 1; ++++ was assigned to the bright red-orange pigment level of control, +++ was assigned to a pigment level not as high as the control, + was assigned to a low pigment level but visible, and – was assigned to an imperceptible level of pigment.

exception of WRL-053 in that the norsolorinic acid accumulated to a fairly high level in the *nor* mutant but aflatoxin production of 42-12 was much lower (Fig. 1 and Table 1).

The visual bioassay described here provides a simple and useful monitoring system for screening large numbers of yeast strains for their effectiveness in blocking early steps of the polyketide pathway in the biosynthesis of aflatoxin by *A. flavus*. The *nor* mutant also is safer for use in routine experiments than is a wild-type strain of *A. flavus*, since the mutant produces little if any aflatoxin during routine laboratory culture. This report is the first to demonstrate that yeasts can interact with *A. flavus* to reduce aflatoxin levels. Applications of these yeasts for reducing aflatoxin contamination of food commodities warrants further research.

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