Biocontrol of *Aspergillus flavus* by *Pichia anomala*

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Aflatoxins are extremely potent natural carcinogens and a major food safety concern because of potential contamination of food commodities. Threshold levels set by the U.S. Food and Drug Administration for aflatoxins in foods for domestic consumption are less than 20 parts/billion (ppb). However, the European Union (EU) and Japan have set threshold levels at less than 4 ppb. These low tolerance levels increase potential for rejection of agricultural commodities from exporting countries. The major aflatoxin-producing fungus, *Aspergillus flavus* is ubiquitous in agricultural soils and has a broad ecological niche. The use of yeast as antagonists against Mycotoxin fungi is a promising approach to manage aflatoxin contamination via biological control. Research to enhance the effectiveness and mass production of biocontrol yeast will facilitate commercial product development for practical application. *Pichia anomala* WRL-076 has been demonstrated to reduce the spore production of *A. flavus* in both lab conditions and field experiments. Thus, *P. anomala* is likely to provide an economical means of managing aflatoxin contamination in food chain.

**Keywords** aflatoxin, *Aspergillus flavus*, yeast, bicontrol mechanisms, food safety

1. Introduction

The use of chemical fungicides has resulted in development of pest resistance and resurgence. In addition, use of fungicides in certain agricultural systems is impractical due to the expense, risk of environmental pollution, and negative effects to human health [1]. Biological control of insect pests, plant pathogens, and weeds is the only major alternative to the use of pesticides in agriculture and forestry. Accordingly, microorganisms naturally present in agricultural ecosystems are being studied as environmentally compatible alternatives to traditional chemical methods for controlling plant diseases and fungi associated with mycotoxin production. Biological control can reduce the harmful effect of phytopathogenic or mycotoxigenic fungi while having a minimal impact on the environment [2, 3].

The Food and Agriculture Organization (FAO) of the United Nations estimates that 25% of the world’s food crops are affected by mycotoxins. Contamination by mycotoxins such as aflatoxin in tree nuts, peanuts, corn and cottonseed is a serious food safety hazard to both humans and animals. Saprophytic yeasts have been studied as biological control agents for the control of various fungal pathogens, including *Botrytis cinerea*, *Penicillium* spp., and *Monilinia fructicola* in apples, grapes, pears, peaches, sweet cherries, and citrus fruit. Furthermore, selected yeast strains can also be applied in combination with suitable fungicides to maximize the efficacy of biocontrol while reducing the amount of fungicides on food products [2, 4, 5]. The potential of saprophytic yeasts to be used in applications for reducing aflatoxin contamination in food is the focus of this review.

2. Mycotoxins in food

Mycotoxins are naturally occurring toxins produced by filamentous fungi that affect many agricultural crops. Over 300 mycotoxins have been identified, of which about 20 have been shown to occur naturally in food at sufficient levels to pose food safety concerns [6, 7]. The majority of these toxins are produced by fungi of the genera, *Aspergillus*, *Penicillium* and *Fusarium*. The most commonly occurring mycotoxins are aflatoxins (B1, B2, G1, G2, M1), ochratoxin A, patulin, citrinin, sterigmatocystin, fumonisins (B1, B2 and B3), zearalenone, T-2 and HT-2 toxins, nivalenol and deoxynivalenol. Of these, the greatest threat to human health is aflatoxin B1, which contains a double bond in the terminal furan ring. This bond is commonly oxidized by hepatic enzymes into an epoxide that can intercalate into DNA. Aflatoxin B1 has hepatocarcinogenic potential, especially in individuals infected with hepatitis B [8, 9]. Other mycotoxins also present a health threat. For instance, ochratoxin A (OTA) has been shown to cause cancer of the kidneys in animals. Exposure to high levels of fumonisins (B1, B2 and B3) has also been reported to cause liver and kidney damage in experimental animals.

Consequently, mycotoxin content in food is monitored and regulated in many countries around the world. Currently, more than 100 countries have regulations regarding levels of mycotoxins in the food and feed industry [10]. In the U.S., the Food and Drug Administration (FDA) has set the maximum total aflatoxin limit for tree nuts that are intended for human consumption at 20 ng/g (20 ppb) [11]. The EU, a major importer of California tree nuts and dry fruits, has in the past applied tolerance levels as low as 2 ppb for aflatoxin B1 and 4 ppb total aflatoxins (European Commission 2005; European Commission 2006). These limits have recently been somewhat relaxed, with current allowable limits of 8 ppb for aflatoxin B1 and 10 ppb total aflatoxin (Commission Regulation (EU) No 165/2010). The European Commission’s Scientific Committee for Food has set a limit at 5 ppb OTA in dried fruit intended for direct consumption. EU
regulatory limits for fumonisins in maize range from 0.2 to 2 ppm, and U.S. FDA recommended limits are 2 to 4 ppm [12].

3. Aspergillus flavus

Aspergillus flavus has a broad ecological niche, thus has a worldwide distribution. It can reproduce abundantly resulting from the production of numerous airborne conidia. The spores can easily disperse by air. Environment has a great impact on mould growth, with humidity being the most important variable [13]. Genetic diversity of A. flavus populations has been demonstrated by vegetative compatibility grouping (VCG) and DNA fingerprinting [14-16]. The spores of A. flavus can infect wounded plant tissues [17, 18]. Infection of corn, peanuts, cotton seeds, almonds, or pistachios by A. flavus creates the potential for production of aflatoxins. Although in many cases the sources of infections are unknown. The mold spores can be found in the soil or in the air. Aflatoxin contamination is aggravated by factors such as insect damage, drought, and high temperatures.

A. flavus is second only to A. fumigatus as the cause of human invasive aspergillosis by exposing to fungal spores. The fungus is the second leading cause of invasive aspergillosis and is the most common cause of superficial infection. Disease associated with A. flavus include chronic granulomatous sinusitis, keratitis, cutaneous aspergillosis, wound infections and osteomyelitis [13].

The toxic effect of aflatoxins on animal and human health is referred to as aflatoxicosis. Although acute aflatoxicosis in humans is rare, several lethal outbreaks have been reported. In 2004, an aflatoxicosis outbreak in rural Kenya resulted in 317 cases and 125 deaths. Contaminated maize was responsible for the outbreak and officials found aflatoxin B1 levels as high as 4.4 parts per million (ppm), 220 times the Kenyan regulatory threshold [19, 20].

4. Yeast species useful for biological control

Yeast species are promising biocontrol agents because they do not produce allergenic spores and they are usually non-pathogenic. Saprophytic yeasts have been studied as potential biological control agents for the control of various fungal pathogens, including Botrytis cinerea, Penicillium spp. Biological control yeasts have been mainly used to manage postharvest losses due to these fungal infections in apples, grapes, pears, peach, sweet cherries, and citrus. A number of commercial yeast products have been developed in recent years. The yeast Metschnikowia fructicola is the active ingredient in the commercial biocontrol product Shermer, which is marketed for the control of postharvest diseases of fruits and vegetables. Candida oleophila and Candida sake are active ingredients of commercial products of BioNext and Candifruit respectively. Furthermore the selected yeast strains can be applied in combination with suitable fungicides to maximize the efficacy of biocontrol while reducing the amount of fungicides on food products [2, 5]. Yeast species can develop quickly in leaf, fruit and flower surfaces, excluding the other microorganism growth by means of competition for space and nutrient. The use of yeasts in postharvest biocontrol formulations apparently presents advantages over other organisms. Yeasts are easy to cultivate, fast growing and are present in a variety of environmental niches [2, 21].

5. Screen yeast strains antagonistic to Aspergillus flavus

A bioassay has been developed to screen for effective yeasts inhibiting both the growth of the A. flavus and aflatoxin production [22]. The bioassay can simultaneously score for growth inhibition and aflatoxin production of the fungus (Figure 1). Several species of yeast have been identified as potential effective biocontrol agents. Saprophytic yeasts were isolated from fruits of almond, pistachio, and walnut trees. The six selected isolates were identified as 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). 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.1, B and C respectively). Yeasts WRL-084 and WRL-015 were less inhibitory, and red-orange color was observed in the fungal mycelium (Fig.1, D and E). Yeasts WRL-024 and WRL-053 were not very inhibitory, and large amounts of norsolorinic acid accumulated in the mycelium (Fig.1, F and G).
6. Inhibition of aflatoxin production by yeast strains

Spores of *A. flavus* (strain 42-12, toxigenic wild type) were inoculated between two streaks of yeast on PDA plates and incubated for 7 days. Fungal discs were cored from agar plates and extracted for aflatoxin analysis by HPLC ChemStation (Agilent, Palo Alto, CA, USA). When grown on PDA, strain 42-12 produced 4,800 ng of aflatoxin per four discs. Aflatoxin production was drastically reduced by 99% (WRL-076) and 96% (WRL-038). Significantly reduction of aflatoxin biosynthesis were observed in yeast stains WRL-015, WRL-053, and WRL-084 [22].

7. Inhibition of *A. flavus* spore production

The experiments were conducted in pistachio orchards. Pistachio nut-fruits on the tree were individually wounded with a dental needle and sprayed with aqueous suspension of yeasts at 3x10⁷ cells/ml in August. The wounded nut-fruits without yeast-spray were used as controls. Five weeks after the yeast spray, wounded nut-fruits was hand picked from the tree and immediately placed to a special agar medium and incubated at 28°C for eight days. Microorganisms were eluted from the experimental samples in Tween 80 solution by shaking and sonicating in flasks. Viable counts of yeast and *A. flavus* were determined by spreading the eluted samples on dichloran rose bengal chloramphenicol (DRBC) agar plate using an Autoplate 4000 Spiral Plate (Spiral Biotech, MA, USA). To examine the variation among the nuts, each single nut collected was analysed for colonization of *A. flavus* and viable spores production. The percent of colonization by *A. flavus* on nut-fruits was 27.1% for the control and 5.1% for the yeast treated nut-fruits. The colony forming unit (CFU) of *A. flavus* spores from each single nut was enumerated. Average spore production in *A. flavus* infected nuts...
was $5.6 \times 10^6$ and $1.3 \times 10^6$ respectively, for the control and yeast sprayed pistachio nut-fruits. The experiments demonstrated that the yeast, *P. anomala* can reduce spore production of *A. flavus* in wounded the pistachio nut-fruits. A reduction of spore number was observed in the range of 77%. The total number of *A. flavus* spores produced on all wounded pistachios in the control is $1.28 \times 10^8$ and for yeast sprayed pistachios is $5.2 \times 10^6$ respectively. Therefore the *A. flavus* spore number present in the orchard environment was reduced by 96%. [24].

![Average *A. flavus* Spores on Wounded Pistachio](image)

**Fig. 3** Effect of *P. anomala* on spore production of *A. flavus*

### 8. Growth of *Pichia anomala* WRL 076 at low water activity

The ability of *P. anomala* to grow at low water activity is an attribute to its ability to survive under water stress. In laboratory experiments, PEG (polyethylene glycol) 8000 was used to adjust medium $a_w$ to 0.96, which mimicked a water stress condition of $-5.62$ MPa. Spores of *A. flavus* were inoculated 24 hrs before adding yeast cells. *P. anomala* WRL-076 could grow at this low water activity ($a_w$) and formed a film to inhibit the growth of *A. flavus* inoculated to the medium. This demonstrates that *P. anomala* can overcome water stress to inhibit the spreading of *A. flavus* [25].

![Restriction of fungal growth at low water activity by *Pichia anomala*](image)

**Fig. 4** Restriction of fungal growth at low water activity by *Pichia anomala*

### 9. Molecular mechanisms of biocontrol

Yeast and fungi were inoculated into potato dextrose broth (PDB) at yeast to fungus (Y : F) ratios of 1:1. The effects of the yeast, *P. anomala* against *A. flavus* were investigated. RT-PCR (reverse transcription polymerase chain reaction) was applied to analyze gene expressions in yeast cells. Genes for cell wall degrading enzymes, exo-β-glucnase I and II of *P. anomala* were monitored. Both *PaEXG1* and *PaEXG2* of *P. anomala* increased by co-culturing with *A. flavus*. The data indicate cell wall degradating enzymes are involved in mechanism of biocontrol.
Fig. 5 Induction of exo-β-glucanase gene transcription

Fungal hyphae were stained with the fluorescent compounds, FUN-1, DiBAC₄(5) and CDFA-AM, then viewed under an epifluorescence microscope. The results suggest that the yeast might inhibit the ATP generating system of A. flavus. The hyphal membrane was probably damaged by the lytic enzymes and lost its integrity. [26, 27].

10. Shelf-life of biocontrol yeast

An important requirement for the commercialization of biocontrol agents is the ability to economically produce large quantities of the microorganisms and the development of formulations for good shelf life of the microorganisms. Live P. anomala yeast cells are required for its broad application in food and agriculture industries. A stable liquid formulation is highly desirable for P. anomala WRL-076 because of easy dispersion in water and delivery to crops. The biocontrol yeast grows well to 10⁹ cells/ml in nutrient broth with yeast extract and glucose. One of the liquid formulations developed for P. anomala WRL-076 showed 83 percent cells were viable after cold storage for 12 months [28].

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References


