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Fluorescent Viability Stains to Probe the Metabolic Status of Aflatoxigenic Fungus in Dual Culture of *Aspergillus flavus* and *Pichia anomala*

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Abstract The metabolic activity of the aflatoxigenic fungus, *Aspergillus flavus* co-cultured with the biocontrol yeast, *Pichia anomala* was examined using several viability stains. Both the FUN-1 stain and the combined use of $DiBAC_4(5)$ with CDFA-AM stains were applied in this study. The results suggest that the ATP-generating system in *A. flavus* was inactivated as the ratio of yeasts to fungi increased in the dual culture. A decrease in hyphal membrane potential and esterase activity was substantiated by the combined stains of $DiBAC_4(5)$ and CDFA-AM. Reduced metabolic function in conjunction with cell wall damage of *A. flavus* hindered the growth and biomass production of this fungus. Viability stains such as FUN-1 and $DiBAC_4(5)$ with CDFA-AM may assist in elucidating the biocontrol mechanism by allowing for the visualization of the antagonistic effect of yeast species on target fungi in situ, as well as for screening potent biocontrol yeast agents against fungal pathogens.

Keywords Aflatoxin · Yeast · Fungus · Fluorescence · Mechanism of biocontrol

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Introduction

Fluorescent dyes that measure different cellular physiological states are useful for a comprehensive understanding of the metabolic status of the cells. For example, the DNA intercalating fluorescent red dye, propidium iodide (PI) and the green fluorescent dye oxonol (OX) have been used for measuring membrane integrity and membrane potential, respectively, in dead or damaged yeast cells. Fluorogenic esterase substrates such as 5-chloromethylfluorescein diacetate (CMFDA) have been commonly used for measuring membrane esterase activity which is only active in intact cells [1, 2].

The FUN-1 [2-chloro-4-(2,3 dihydro-3-methyl (benzo-1,3-thiozol-2-yl)-methylidene)-1-phenylquinolinium iodide] stain is a membrane-permeant, halogenated cyanine compound that binds to nucleic acids. Biochemical processing of the dye by metabolically active yeast cells yields cylindrical intra-vacuolar structures (CIVS) that are markedly red shifted in fluorescent emission and spectrally distinct from the nucleic acid bound form of the dye. The formation of CIVS structure requires ATP [3]. The FUN-1 stain has been applied to assess the damage to target pathogens caused by antifungal agents in several species of *Aspergillus* species [4–6]. These studies show that FUN-1 is useful as a viability stain to monitor the metabolic status of fungi.

Oxonol stains are lipophilic anionic compounds capable, unlike most ionic molecules, of entering the

plasma membrane barrier. The fluorescence of $DiBAC_4(3)$ [Bis-(1,3-dibutylbarbituric acid) trimethine oxonol] is strongly dependent upon the surrounding environment [7]. The plasma membrane in viable cells will normally exhibit a potential gradient such that the exterior is more positively charged and the oxonol will accumulate toward the outer surface [2]. Under this condition, the fluorescence is suppressed. However, when the cell membrane potential collapses, whether in response to a cytotoxic event or otherwise, the stain enters the cell and exhibits green fluorescence. In the case of $DiBAC_4(5)$ [Bis-(1,3-dibutylbarbituric acid) pentamethine oxonol], a structural analog of the commonly used $DiBAC_4(3)$ a red fluorescence is exhibited. The CFDA-AM [5-carboxyfluorescein diacetate, acetoxymethyl ester] probe is taken up by viable cells and upon hydrolysis by intracellular esterases, forms carboxyfluorescein. The carboxyfluorescein is retained in the cell and exhibits bright, green fluorescence in the cytoplasm. The process of hydrolysis is disrupted when the cell is damaged. Therefore, it serves as a marker for cell membrane integrity. The CFDA-AM probe may be used to assess fungal cell injury induced by active antifungal agents.

In this study, we examined the metabolic activity of the aflatoxigenic fungus, *Aspergillus flavus* co-cultured with a biocontrol yeast, *Pichia anomala*. FUN-1, $DiBAC_4(5)$ and CFDA-AM were used as viability stains to investigate the effect of yeast on *A. flavus*. Our two-color vital staining scheme involved the red fluorescent membrane potential-sensitive oxonol stain with the esterase-dependent green fluorogenic probe, and the vitality of *A. flavus* fungal hyphae could be clearly differentiated even at the hyphal segment level.

Materials and Methods

Microorganisms and Culturing Media

Aspergillus flavus, strain CA18 and *Pichia anomala*, strain WRL-076 were used in this study. The fungal and yeast cultures were maintained on potato dextrose agar (PDA). To inoculate PDB (potato dextrose broth), fungal spores and yeast cells were resuspended in Tween 80 solution (0.9 % NaCl and 0.01% Tween 80) and counted using a Beckman Coulter Multisizer II (Beckman Coulter, Fullerton, CA, USA) or a hemocytometer.

Chemicals

FUN-1 [2-chloro-4-(2,3 dihydro-3-methyl (benzo-1, 3-thiozol-2-yl)-methylidene)-1-phenylquinolinium iodide] was purchased from Invitrogen (Carlsbad, CA, USA); Tween-80, glucose and HEPES [N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid] were purchased from Sigma Chemical Co. St. Louis, MO). CFDA-AM [5-carboxyfluorescein diacetate, acetoxymethyl ester] and $DiBAC_4(5)$ [Bis-(1,3-dibutylbarbituric acid) pentamethine oxonol] were purchased from Ana Spec Inc. (San Jose, CA, USA).

Experimental Design

Flasks containing 50 ml potato dextrose broth (PDB) were inoculated with yeast and each flask was inoculated with 1 ml of fungal spore suspension at 10^6/ml, and the final concentration of the yeast cells in the medium was adjusted according to the yeast to fungus ratios (Y:F) of 1:1, 5:1, 10:1, 30:1, and 50:1. The flasks were incubated at 28°C for 48 h with gentle shaking. Hyphae of *A. flavus* were harvested and separated from the yeast cells by filtering through the Cellector tissue sieve with 38.1-μm filter (VWR Scientific, Brisbane, CA, USA). As a positive control for viability, fungal spores were inoculated into 50 ml PDB without the yeast cells. Dead hyphae were produced by boiling hyphae for 30 min. Both live and dead fungal hyphae were used as controls in the staining studies.

Fluorescent Staining

The fungal hyphae harvested by sieve screening were gently rinsed with sterile deionized water before being transferred to a 1.5-ml microfuge tube containing 1 ml sterile deionized water. The hyphal suspension was then centrifuged in an Eppendorf microfuge for 5 min. The supernatant was removed, and FUN-1 in HEPES buffer was added to the hyphae and incubated at 28°C in the dark for 30 min.

The stained hyphae were rinsed twice with HEPES buffer, spotted on a slide and viewed under a Leica DMRB epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a fluorescein filter. The fluorescein filter enabled the visualization of both green fluorescent hyphae and red fluorescent CIVS. Images were captured with a Sony DKC-5000

digital color camera (Sony Electronics Inc., Tokyo, Japan).

Stock solutions of staining chemicals were prepared in dimethyl sulfoxide (DMSO): 2.5 mg/ml for CFDA-AM and 1 mg/ml for $DiBAC_4(5)$. All samples were stained in PBS (phosphate-buffered saline) unless otherwise indicated. Staining was performed by addition of 1ul of each stain per 1 ml of sample followed by incubation for 30 min at room temperature in the dark. Samples were thereafter kept on ice until observation under the microscopic. The samples stained with CFDA-AM and $DiBAC_4(5)$ were viewed with a fluorescein filter under a Zeiss Axioskop epifluorescence microscope (Carl Zeiss Inc. Thornwood, New York). Images were captured with a Sony AxioCam MR (Sony Electronics Inc, Tokyo, Japan).

Effect of *P. anomala* on *A. flavus* Fungal Biomass

Aspergillus flavus was co-cultured in PDB with *P. anomala* at various ratio and the hyphae collected as described above. The hyphae were then rinsed with distilled water, transferred to a pre-weighed filter paper and dried in an oven at 65°C overnight. The weight of each dried hyphal sample was measured in order to assess the inhibitory effect of *P. anomala* on the *A. flavus* biomass.

Results and Discussion

Determination of the Metabolic Status of Fugal Hyphae by FUN-I

The optimal concentration for staining of live *A. flavus* was 10 μM FUN-1 in HEPES buffer (PH, 7.0). Addition of glucose to the buffered staining solution was essential for the biochemical processing of the dye and therefore the production of the fluorescently red-shifted CIVS. Consequently, live *A. flavus* hyphae stained with FUN-1 showed intense red fluorescence in the vacuole when visualized by epifluorescence microscopy. The red fluorescent intravacuolar structures within the hyphae (shown in greater detail in inset) and by the relatively low and diffuse green fluorescence of the hyphae are observed in Fig. 1a. All hyphae of *A. flavus* grown in PDB without *P. anomala* were metabolically active, as evidenced by the presence of CIVS within the hyphae (Fig. 1a).

As the inoculation ratios of yeast cells to fungal spores increased, FUN-1 accumulation in the vacuoles was shown at a decreasing rate. Hyphae of *A. flavus* grown with the yeast *P. anomala* in a ratio of (Y:F, 30:1) displayed few red fluorescent vesicles while a high proportion of the hyphae had bright green fluorescence, characteristic of low metabolic activity (Fig. 1b). It is unclear whether this heterogeneous effect of the yeast on the metabolism of individual *A. flavus* hyphae was caused by the shielding of a small proportion of the hyphae within the fungal biomass from the inhibitory effect of the yeast cells, or by the heterogeneous sensitivity of the hyphae to the inhibition by the yeast within the biomass. This heterogeneity in a fungal population has been reported previously by Lass-Flör et al. [4]. In their study, FUN-1 staining revealed that a small number of viable hyphae remained viable, and thus survived exposure to high concentrations of fungicide, whereas a large proportion of the fungal population appeared dead.

Most hyphae of *A. flavus* grown in the presence of *P. anomala* at a ratio of Y:F (50:1) lacked red fluorescent vesicles and had bright green fluorescence (Fig. 1c); their appearance was similar to heat-killed hyphae (Fig. 1d), suggesting that the yeast had a strong negative effect on the viability of the fungus. Heat-killed *A. flavus* stained with FUN-1 showed only bright green fluorescent hyphae devoid of red vacuoles (Fig. 1d).

Antagonistic Effect of *P. anomala* on *A. flavus*

A combined use of the red fluorescent membrane potential-sensitive oxonol stain, $DiBAC_4(5)$ and the esterase-dependent green fluorogenic probe, CFDA-AM could clearly demonstrate that hyphae were damaged by the yeast, *P. anomala* in a segmental pattern. Live *A. flavus* hyphae grown in the absence of *P. anomala* exhibited intense green fluorescence when visualized by epifluorescence microscopy (Fig. 2a). Hyphae of *A. flavus* grown with the yeast *P. anomala* in a ratio of 5;1 and 10:1 (Y:F) displayed both red and green fluorescent on the same hypha, suggesting that part of the hypha had lost membrane potential permitting fluorescence of $DiBAC_4(5)$ stain. The red fluorescence imparted to the damaged segment of the hyphae. Other segments of the same fungal hypha appeared to remain intact, therefore the

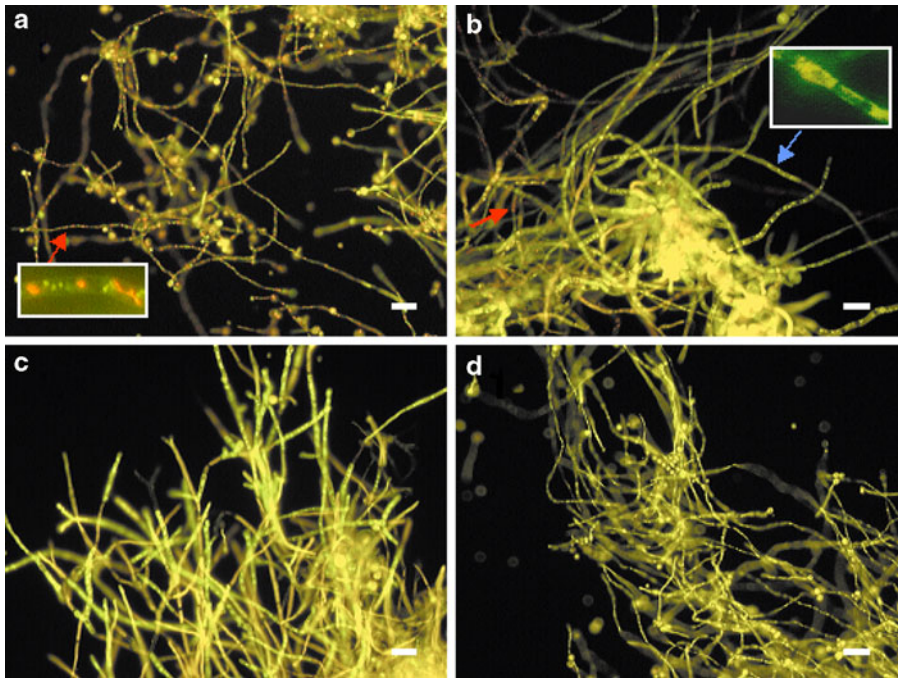

Fig. 1 Epifluorescence micrographs of live, yeast-treated and heat-killed *A. flavus* stained with FUN-1 viability stain. Viable *A. flavus* was evidenced by the red fluorescent intravacuolar structures (*red arrows*) within the hyphae (shown in greater detail in *inset*) and by the relatively low and diffuse green fluorescence of the hyphae (**a**). *A. flavus* grown with the yeast *P. anomala* in a ratio of 30:1 (Y:F) displayed few red fluorescent vesicles and a high number of bright green fluorescent hyphae (**b**). Hyphae of *A. flavus* grown with *P. anomala* at a ratio of 50:1 (Y:F) lacked red fluorescent vesicles and had bright green fluorescence (**c**), similar to the heat-killed control of *A. flavus* (**d**). The FUN-1-stained hyphae were visualized with a fluorescein filter. Magnification: 400×. *Bars* 20 μm

CFDA-AM stain was cleaved by the active esterase and produced a bright green fluorescent color (Fig. 2b). Most hyphae of *A. flavus* grown in the presence of a high number of *P. anomala* (Y:F at 10:1) were red fluorescent and had some bright green fluorescence (Fig. 2c). Fungal hyphae inactivated by heat treatment showed only red fluorescence (Fig. 2d). The data suggest that *A. flavus* grown in the presence high ratio of yeast in the dual culture loses its viability.

Reduction of Fungal Biomass

The dry weights of *A. flavus* mycelia grown in PDB at the yeast to fungal spore ratios of 0:1, 1:1, 5:1, 10:1 and 50:1 (Y:F) were 57.5 (SD, 4.1), 16.9 (SD, 2.6), 10.9 (SD, 1.1), 3.4 (SD, 0.6) and 0.3 (SD, 0.26) mg, respectively. These results demonstrate that the yeast inhibited the growth of *A. flavus* by 70.6% in the 1:1 yeast to fungus samples when compared to the control in which yeast was not present. When the ratio increased to 50:1, the dry mycelia weight decreased by 99.5%, which corresponded to a 192-fold decrease. Comparison of the biomass averages by ANOVA indicates that the antagonistic effect of *P. anomala* on *A. flavus* mycelium production was significant (Fig. 3).

The inhibitory effect of the yeast on the biomass of *A. flavus* is consistent with the results obtained with the viability stains, based on observation under the fluorescent microscope. A small proportion of the hyphae remained viable at a ratio of yeast to fungus of 30:1, while a complete lack of metabolic activity was obvious at a ratio of 50:1. This loss of metabolic activity, based on staining with FUN-1, correlates well with the near complete inhibition of *A. flavus* growth in the presence of high population densities of *P. anomala*. Additionally, dual staining with $DiBAC_4(5)$ and CFDA-AM revealed that large segments of *A. flavus* hyphae were nonviable at yeast to fungal ratio of 10 to 1.

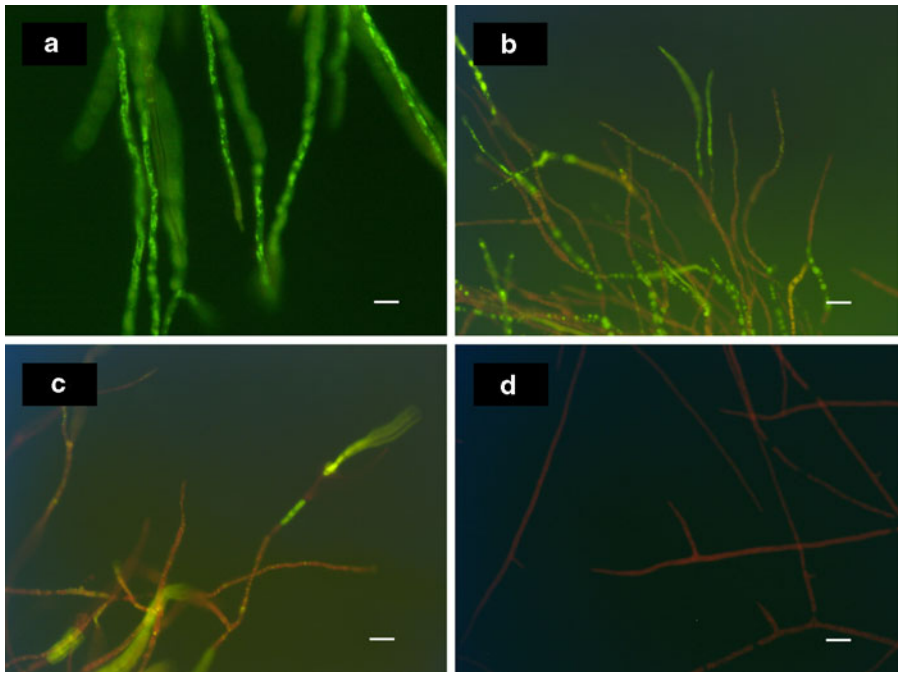

Fig. 2 Epifluorescence micrographs of live, yeast-treated and heat-killed *A. flavus* stained with DiBAC$_4$(5) and CFDA-AM viability stains. Live *A. flavus* hyphae showed intense green fluorescence (**a**). *A. flavus* grown with the yeast *P. anomala* in a ratio of 5:1 (Y:F), displayed both red and green fluorescent on the same hypha (**b**). Most hyphae of *A. flavus* grown in the presence of *P. anomala* at a ratio of 10:1 (Y: F) were red fluorescent and had very little bright green fluorescence (**c**). The appearance of heat-killed *A. flavus* hyphae stained *red* (**d**). Magnification: 400×. *Bars* 20 μm

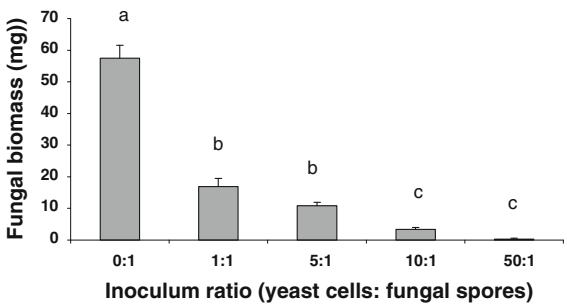

Fig. 3 Effect of *P. anomala* on *A. flavus* fungal biomass when both were co-inoculated in culture at increasing ratios of yeast cells to fungal spores. Details of the experiments were described in the method above. Each data point represents the mean dry weight of three replicate cultures and the standard deviation. Values marked by the *same letter* were not significantly different by the ANOVA analysis ($P < 0.05$)

A variety of yeast species have been used as biocontrol agents for postharvest or preharvest control of fruit diseases [8, 9]. A major mechanism of yeast biocontrol agents is their effectiveness in competing for nutrients [8]. In addition, production of the cell wall degrading enzyme, exo-β-1, 3 glucanase was implicated in the biocontrol effect of *P. anomala* strain K against *Botritis cinerea* [10]. Both FUN-1 stain and the combinatorial use of DiBAC$_4$(5) with CDFA-AM stain demonstrated that *P. anomala* not only inactivated the ATP generating system in *A. flavus* but also caused damage in hyphal cell walls and decreased membrane potentials. This study demonstrates the great potential for using viability staining in the evaluation of biocontrol yeast. As we learn more about mechanisms by which yeasts control the target fungus, more effective methods for selecting, formulating and applying biocontrol agents will emerge.

Several strains of the yeast species, *P. anomala*, have been demonstrated to control storage mold in small grains [11, 12], to reduce fruit rot in apple [10] as well as to prevent aflatoxin and ochratoxin contamination by *A. flavus* and *Penicillium roqueforti* [11–14]. The Food and Agriculture Organization (FAO) estimates that 25% of the world's food crops are affected by mycotoxins [15–17], of which the most harmful are aflatoxins (Commission of the European Community, 1998; U.S. Food and Drug Administration, 1996). Thus, *P. anomala* may be a

potential biocontrol agent to reduce mycotoxins in a variety of commodities.

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