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EFFECTS OF OPTICALLY ACTIVE GOSSYPOL ON CONIDIA GERMINATION AND GROWTH OF *Aspergillus flavus*

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Abstract

Racemic, (–)-, and (+)-gossypol were evaluated over a concentration range of 0 to 400 µg per mL for effects on several *Aspergillus flavus* growth parameters. In the concentration range tested, racemic gossypol did not stimulate conidial germination on water agar alone. In a germination stimulatory environment (potato dextrose medium), racemic gossypol concentrations of ≥ 200 µg per mL decreased the number of germinated conidia, germ tube length and conidiophore development, and increased frequency of hyphal branching. (–)-Gossypol elicited the same fungal responses as racemic gossypol, but at lower concentrations (≥ 50 µg/mL). (+)-Gossypol was less active than racemic gossypol. Activity differences exhibited by the different enantiomers of gossypol were quantitative in nature and activity of racemic gossypol equaled the sum of activities of the individual optical isomers. The observed inhibitory effects on *A. flavus* development were temporal, and were partially reversed with longer incubation. Thus, *A. flavus* appeared to adapt to the presence of gossypol to overcome its inhibitory growth effects.

Introduction

Gossypol is a terpenoid compound produced by cotton (*Gossypium hirsutum*) that is principally localized in lysigenous glands found throughout aerial portions and in non-glandular root tissue of the plant. Cottonseed kernels contain, on average, about 1.3% gossypol by weight (National Cotton Variety Tests, 2001). Gossypol contributes to plant defenses through anti-insect activity and may be involved in other plant defense functions. *Aspergillus flavus* is a fungal pathogen of cottonseed that is capable of production of the potent mycotoxin aflatoxin. Infection of cottonseed by *A. flavus* can lead to aflatoxin contamination, greatly reducing the commercial value of the seed. Since the fungus may encounter high concentrations of gossypol during cottonseed invasion, an investigation was initiated into effects of this terpenoid material on *A. flavus* growth parameters.

Methods and Materials

Aspergillus flavus AF13 (ATCC 96044) was isolated from soil in southwestern Arizona (Yuma area) and maintained on a 5% V-8 vegetable juice (Campbell Soup Co., Camden, NJ) agar medium at 31°C (Cotty, 1989).

Gossypol was tested in a conidia germination assay in a concentration range of 0 to 400 µg per mL. Fungal conidia were incubated on agarose "pads" with varying concentrations of gossypol. A 1% (w/w) solution of agarose (Type I, low EEO, Sigma Chemical Co., St. Louis, MO) was prepared in 50 mM HEPES, pH 7.4 with 0.2% (w/w) $MgSO_4$ by briefly boiling (1 min) and then equilibrating to 60°C in a water bath for 15 min. Appropriate volumes of gossypol stock solutions (2.0 mg/mL) in acetone were added to the 60°C agarose solution to achieve the desired concentration. About 200 µL of gossypol/agarose solution was applied within a ring of petroleum jelly 1.5 cm in diameter on a microscope slide and allowed to gel at room temperature in a sterile environment. A suspension of *A. flavus* conidia at a concentration of 1.3×10^6 spores per mL was prepared in 0.5% potato dextrose broth (pdb; Difco); 50 µL of this conidial suspension was applied to each agarose/gossypol "pad". The fungal-inoculated "pads" were incubated at 31°C in a high humidity chamber for 24 or 48 h. Control incubations contained either no gossypol with pdb or 200 µg per mL gossypol (racemic) with no pdb. Fungal germination was visualized with lactophenol/cotton blue stain (Becton-Dickeinson Microbiological Systems, Cockeysville, MD) and analyzed microscopically at 400X. Measurements were made with a calibrated ocular micrometer. The following fungal parameters were monitored: 1.) presence of a mycelial mass, 2.) number of conidiophores, 3.) number of non-germinated conidia, 4.) germ tube length, 5.) distance from conidial wall to first hyphal branch point. Experiments with racemic and each enantiomer of gossypol were performed a minimum of three times. The data presented here is representative of those experiments.

Racemic gossypol was prepared as previously reported (Dowd and Pelitire, 2001). The preparation of (+)- and (−)-gossypol was by a fractional crystallization process. Small single crystals of enantiomorphic gossypol-acetone (1:3) were prepared by crystallization from solutions of gossypol acetic acid in acetone at 4°C (Dowd et al., 1999; Dowd et al., 2001). These seed crystals were used to produce large single crystals, each weighing about 50 mg, by controlling crystallization conditions to inhibit the nucleation and growth of the unseeded form. To determine the enantiomeric form of each product crystal, a small sample was derivatized with *R*-2-amino-1-propanol (D-alaninol) and analyzed by HPLC on a C-18 reverse-phase column (Kim et al., 1996). Crystals of the same form and acceptable purity were combined, and the occluded acetone was removed by storing the samples under vacuum at room temperature (unpublished results, M. Dowd). HPLC analysis of the final products indicated that the preparations were >99.5% optically pure.

Results and Discussion

Aspergillus flavus conidia, when placed in a nutritional stimulatory environment (pdb), germinated in 6 to 8 h at 31°C. After incubation for 24 h, a complete mycelial matrix had formed, and conidiophores with maturing conidia were present (Table 1). Essentially all viable conidia had germinated and hyphal branching was infrequent and, when present, occurred at a considerable distance from the conidial wall (>400 µm). Fungal conidia incubated on water agar with or without gossypol (200 µg/mL) did not germinate after an 8-h incubation period. Thus, *A. flavus* conidia required nutritional stimulatory conditions to germinate in 6 to 8 h, and presence of gossypol was not stimulatory to conidial germination.

Racemic gossypol influenced the behavior of *A. flavus* conidia. At lower concentrations (0-100 µg/mL), gossypol had no observable effect (Table 1). However, at a concentration of 150 to 200 µg per mL, distinct changes in *A. flavus* growth were noted. Development of both a mycelial matrix and conidiophores was inhibited (Table 1). In addition, the number of non-germinating conidia increased at higher concentrations (250-400 µg/mL, Table 1). Also, germ tube length decreased and the frequency of hyphal branching increased at higher gossypol concentrations (Table 1). Thus, at concentrations of 200 to 400 µg per mL, racemic gossypol was an inhibitor of conidial germination and fungal growth in general.

Incubation of *A. flavus* conidia in a (−)-gossypol concentration range of 0 to 400 µg per mL resulted in similar biological activities as observed with the racemer, but at lower concentrations. The concentration eliciting a lack of mycelial mass and conidiophore formation was half that observed with racemic gossypol (Table 2). In addition, increased numbers of non-germinating conidia, germ tube growth inhibition and increased hyphal branching occurred at lower concentrations of (−)-gossypol (Table 2). Complete inhibition of conidia germination occurred at 400 µg per mL. Thus, the (−) enantiomer of gossypol exerts the same effects on fungal growth parameters, but appears to be more potent.

Incubation of *A. flavus* conidia with (+)-gossypol resulted in fungal growth responses similar to those observed with racemic gossypol, but occurring at higher concentrations. The concentration eliciting a disappearance of a mycelial matrix and conidiophore formation was 250 to 300 µg per mL (Table 3). Similar concentrations of the (+)-enantiomer were required to observe increased numbers of non-germinating conidia, reduced germ tube length and increased hyphal branching (Table 3). In most cases, hyphal branch point distance was not determined for a sample with a mycelial matrix because this value was greater than the observation field and very difficult to ascertain for a given conidium. Thus, (+)-gossypol seemed to be less active than racemic gossypol. Indeed, the biological activity exhibited by racemic gossypol with *A. flavus* appeared to be the sum of activities of the individual optical isomers.

The inhibitory effects of gossypol on *A. flavus* growth parameters appeared to be temporal. When *A. flavus* conidia were incubated with (−)-gossypol (250-350 µg/mL) for 24 h, near total inhibition of conidial germination was observed. However, when identical incubations were carried out for 48 h, a significant number of conidia germinated (about half) and produced germ tubes of considerable length (≥ 200 µm; data not shown). Thus, the fungus appeared to adapt to the presence of gossypol with increased time.

The observed differences in the biological activities of the (+) and (−) enantiomers of gossypol appear to be quantitative. This finding is consistent with other bioassays in which gossypol has been tested (Kim et al., 1996; Shelley et al., 1999). Optical isomers of gossypol have been tested against *Rhizoctonia solani*, another fungal pathogen of cotton. In that case, gossypol was about 10 times more potent in inhibitory activity against *R. solani* than for *A. flavus*, but there were no significant quantitative differences detected between the racemate and enantiomers of the terpenoid compound (Puckhaber, et al., 2002). *Aspergillus flavus* appears to be relatively insensitive to the growth inhibitory effects of gossypol and also seems to have the ability to adapt to high gossypol concentrations. The mechanism of this adaptation would appear to be a promising area of investigation.

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Table 1. Effect of racemic gossypol on *A. flavus* conidia germination (24 h).

[Gossypol] (μg/mL)	Mycelial Mass	Conidiophores	Non-germ Conidia	Germ Tube Length (μm)	Branch Pt. Distance(μm)
0	yes	3	1	>400	N.D.[a]
50	yes	10	0	>400	N.D.[a]
100	yes	15	0	>400	N.D.[a]
150	reduced	5	1	>400	N.D.[a]
200	no	0	2	70	17
250	no	0	14	43	16
300	no	0	3	43	17
350	no	0	27	30	15
400	no	0	35	19	11

[a] N.D.= Not determined.

Table 2. Effect of (−)-gossypol on *A. flavus* conidia germination (24 h.).

[−Gossypol] (μg/mL)	Mycelial Mass	Conidiophores	Non-germ Conidia	Germ Tube Length (μm)	Branch Pt. Distance (μm)
0	Yes	1	13	>400	N.D.[a]
50	Yes	6	6	>400	N.D.[a]
100	No	0	11	88	N.D.[a]
150	No	0	33	22	N.D.[a]
200	No	0	141	25	N.D.[a]
250	No	0	23	28	N.D.[a]
300	No	0	8	45	16
350	No	0	22	33	21
400	No	0	75	0[b]	0[b]

[a] N.D. = Not determined.

[b] Conidia germination inhibited 100%

Table 3. Effects of (+)-gossypol on *A. flavus* conidia germination (24 h).

[+Gossypol] (µg/mL)	Mycelial Mass	Conidiophores	Non-germ Conidia	Germ Tube Length (µm)	Branch Pt. Distance(µm)
0	Yes	0	0	>400	N.D.[a]
50	Yes	4	0	>400	N.D.[a]
100	Yes	3	0	>400	N.D.[a]
150	Yes	3	0	>400	N.D.[a]
200	Yes	3	0	>400	N.D.[a]
250	Yes	0	3	110	N.D.[a]
300	No	0	30	43	23
350	No	0	26	25	10
400	No	0	42	48	7

[a] N.D. = Not determined.