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Toxicity of (+)- and (−)-Gossypol to the Plant Pathogen, *Rhizoctonia solani*

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The dimeric sesquiterpene gossypol occurs naturally in cottonseed and other parts of the cotton plant. Gossypol exists as enantiomers because of the restricted rotation around the central binaphthyl bond. The (−)-enantiomer is toxic to nonruminant animals while the (+)-enantiomer exhibits little, if any, toxicity to these animals. Developing cotton plants with low levels of the (−)-gossypol could expand the use of cottonseed as a feed source. Gossypol also may play a role in protecting the plant from pathogens. The relative toxicity of (+)- and (−)-gossypol to plant pathogens has not been reported. We measured the concentration of (+)- and (−)-gossypol in roots from cotton seedlings that were treated with the biocontrol agent *Trichoderma virens* that induces biosynthesis of gossypol and related terpenoids in cotton roots. (−)-Gossypol was the minor enantiomer in control and treated roots, but levels were slightly higher in roots from *T. virens*-treated seed. We also determined the toxicity of the gossypol enantiomers and the racemate to the seedling disease pathogen *Rhizoctonia solani*. The (+)- and (−)-enantiomers of gossypol and the racemate are equally effective in inhibiting growth of this pathogen. The lethal doses of the gossypols required to kill the pathogen appeared to be similar, but their toxicities are significantly less than those of related cotton and kenaf sesquiterpenes. The results indicate that altering the enantiomeric ratio in cotton roots will not adversely affect the resistance of seedlings to the seedling pathogen *R. solani*.

KEYWORDS: ***Gossypium hirsutum*; cotton; *Rhizoctonia solani*; racemic gossypol; (+) and (−)-gossypol; phytoalexins; host defense; *Trichoderma virens***

INTRODUCTION

The finding that gossypol was an effective antifertility agent for males (*1*) led to a worldwide effort to delineate the biological activity of this compound. Gossypol occurs as two stable enantiomers due to restricted rotation around the carbon−carbon binaphthyl bond (**Scheme 1**). With the growing recognition in the pharmaceutical industry that drug enantiomers can exhibit vastly different biological activities, part of the research effort on the biological activity of gossypol was directed at separating the (+)- and (−)-enantiomers of gossypol and evaluating their effectiveness as antifertility agents for males. Matlin and Zhou (*2*) isolated sufficient quantities of both enantiomers for biological testing, and Lindberg et al. (*3*) reported that only the (−)-enantiomer had antifertility activity. This finding set off a flurry of investigations into the biological activity of each enantiomer in a wide assortment of assays. Several groups have studied the effect of (+)-gossypol and (−)-gossypol on various types of cancer cells in vitro (*4*−*11*) and in whole animals (*3*, *12*−*15*). In all of these studies, the (−)-enantiomer was found to be more active than the (+)-enantiomer. In anti-HIV-1 (*16*, *17*) and antiamoebic (*18*, *19*) activity studies, the results were the same: the (−)-enantiomer was more active. In whole chicken studies (*15*), chicks fed a diet containing 5% cottonseed containing 38% (−)-gossypol were significantly smaller than those fed diets with no cottonseed, 5% glandless cottonseed (i.e., contains no gossypol), or 5% cottonseed containing 17% (−)-gossypol. The above studies overwhelmingly suggest that (−)-gossypol is the more biologically active enantiomer. However, on the enzyme level, the comparative activity of (−)- and (+)-gossypol is dependent on the enzyme under consideration. For example, only the (−)-enantiomer was found to inhibit lactate dehydrogenase isoenzyme C4 activity (*20*) and rat testicular adenylate cyclase activity (*21*); however, both enantiomers inhibited 11-β-hydroxysteroid dehydrogenase (*22*) and protein kinase A and C (*23*).

The relative toxicity of the gossypol enantiomers to plant pathogens has not been reported. To increase its use as a nonruminant animal feed source, efforts are underway to alter the enantiomeric ratio of gossypol in cottonseed to lower the (−)-gossypol level. Such efforts will probably alter the enan-

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Scheme 1

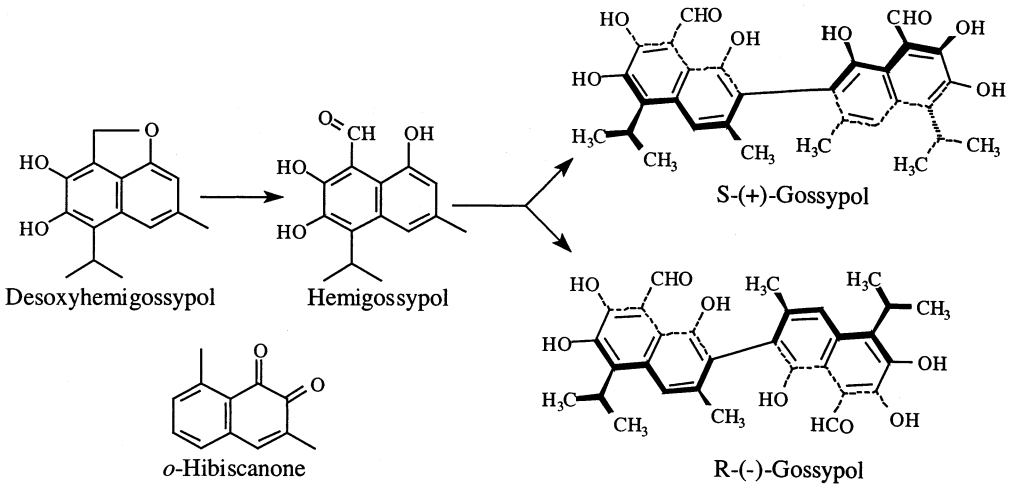

Table 1. Effect of the Biocontrol Agent *Trichoderma virens* on Individual Terpenoid Concentrations[a] and the Percent of the Total Terpenoids That Is Gossypol in Cotton Roots (Stoneville 213) with and without *Rhizoctonia solani* Inoculation

treatment	dHG	HG	gossypol	% gossypol
untreated control	1.75	1.58	33.30	92
R. solani	3.33	4.00	29.00	80
T. virens	13.97	29.30	109.26	72
R. solani + *T. virens*	22.62	39.78	94.84	60

[a] Concentration is expressed as μg/gm of tissue.

tiomeric ratio in other parts of the plant. Given the apparent tendency for (−)-gossypol to be more biologically active than (+)-gossypol, decreasing the level of the (−)-enantiomer in the roots could have a negative impact on plant resistance to diseases such as *Rhizoctonia solani*. Howell et al. (*24*) believe that root terpenoids play an important role in protecting cotton from this pathogen. They showed that the biocontrol agent *Trichoderma virens* induces the biosynthesis of cotton root terpenoids including gossypol, desoxyhemigossypol (dHG), and hemigossypol (HG) (**Scheme 1**), with the concentration of each terpenoid increasing when the seed was treated with this biocontrol agent (**Table 1**). Gossypol was the predominant compound regardless of the treatment, but the enantiomeric ratios were not reported. Should the toxicity of gossypol toward *R. solani* be dependent on its enantiomeric form, increasing the (+)-to-(−) ratio in the cotton roots could reduce resistance to this seedling pathogen.

With this in mind, we have measured the ratios of (+)- and (−)-gossypol in *T. virens* and control seedling roots. We also ascertained the toxicity of (+)-, (−)-, and racemic gossypol to *R. solani*, determining both the inhibitory and lethal concentrations. For a comparison, we also established the toxicities of other phytoalexins from cotton and kenaf (*Hibiscus cannabinus*). Herein, we report our findings and discuss the potential ramifications regarding manipulation of both the concentration of gossypol with respect to the other terpenoids and the relative ratio of the enantiomers.

MATERIALS AND METHODS

Plants. The cotton cultivar, Stoneville 213, was utilized. Seeds were treated with *T. virens* or with *R. solani* as previously described (*24*).

Determination of Gossypol in Cotton Roots. Root tissue was suspended in a solution of acetone:1% aqueous ascorbic acid (90:10) for 60 h at 2 °C. The volume of the resulting extract was reduced under vacuum to a minimum. The residue was dissolved in 2.0 mL of derivatizing reagent [acetonitrile/glacial acetic acid/D-alaninol (88:10:2)] and heated in a water bath at 70 °C for 30 min. After cooling to room temperature, the derivatized sample was analyzed via HPLC on a computer-controlled Hewlett-Packard 1090 liquid chromatograph equipped with a diode array detector and a GLSciences Inertsil ODS-3 (3 × 150 mm) column maintained at 40 °C. The mobile phase was an isocratic mix of 80% acetonitrile and 20% 10 mM KH_2PO_4 (pH adjusted to 3.0 with H_3PO_4) run at 0.6 mL/min for 8 min. The chromatographic signal was monitored at 254 nm (bandwidth 20 nm, ref 550 nm), and spectra were recorded from 220 to 400 nm. Under these conditions, the retention times for the (+)- and (−)-gossypol derivatives were 3.2 and 4.8 min, respectively. As the UV spectra and absorptivity values for the two enantiomer derivatives are identical, the ratio between the (+)- and (−)-derivative peak areas was taken as the enantiomeric ratio. Extraction and chromatographic procedures did not result in racemization.

(+)-, (−)- , and (±)-Gossypol. A fractional crystallization process was used to resolve the gossypol enantiomers (*25*, *26*). Racemic gossypol was obtained from Sigma Chemical Co. (94.5% pure). The gossypol samples were analyzed by HPLC as described above to confirm enantiomeric content. The racemic gossypol was 50.5% (−)-gossypol and 49.5% (+)-gossypol, while the samples prepared by the method of Dowd et al. (*25*) were >99.5% enantiomerically pure.

ED_{50} Bioassay Procedure for *R. solani*. The ED_{50} bioassay employed is an adaptation of the turbimetric procedure previously reported for evaluating the inhibition of *Verticillium dahliae* conidia by cotton phytoalexins (*27*). Plugs of *R. solani* (strain J-1) were grown on potato dextrose broth containing 50 mg/L of rifampicin at 26−28 °C for 3−4 days. The broth was decanted, and 5.00 g of fungal matter was weighed out and then macerated in 100 mL of deionized water for 1−1.5 min. The resulting suspension was filtered through a 180-μm stainless steel sieve stacked on top of a 70-μm sieve. After the fungus caught on the sieves was rinsed with 400 mL of water, the fungal matter on the 70-μm sieve was carefully washed off into a flask using 200 mL of a buffered defined medium composed of 150 mM sodium−potassium phosphate buffer (pH 6.3) with 30 mM $NH_4H_2PO_4$, 1.0 mM $MgSO_4 \cdot H_2O$, and 125 mM D-glucose. This fungal suspension was stirred at moderate speed for 10 h at room temperature to allow the fungus to recover from the maceration process. Toward the end of the recovery period, test solutions were prepared in dimethyl sulfoxide (DMSO) and medium by dissolving a weighed amount of pure phytoalexin at double the amount required to give the highest concentration to be assayed. The compounds were first dissolved in DMSO at 2 times the level required in the final assay and then diluted with buffered medium. The less concentrated solutions were prepared by diluting the most concentrated one with medium/DMSO. At the end of the 10-h fungal recovery period, the fungal suspension was

Table 2. Percent of Gossypol Enantiomers in Stoneville 213 Cottonseed and Seedling Roots from Untreated and *T. virens*-Treated Seed

tissue/treatment	% (+)-gossypol	% (−)-gossypol
cottonseed	66.8 ± 1.2	33.2 ± 1.2
roots, untreated seed	75.6 ± 2.3[a]	24.4 ± 2.3[a]
roots, *T. virens*-treated seed	67.1 ± 0.6[a]	32.9 ± 0.6[a]

[a] These values for roots within columns are statistically different as evaluated by Student's *t* test analysis.

filtered through a 212-μm stainless steel sieve and the filtrate stirred at moderate speed. Test solutions were prepared by mixing equal volumes of the phytoalexin solution and fungal suspension. For each compound tested, a control was prepared by mixing equal volumes of fungal suspension and the medium/DMSO solution used in that assay. The test solutions and the associated control were pipetted into rows of 10 wells in a 96-well flat-bottom microtiter plate. A final row of 10 wells was filled with just medium to be used for a blank. The plates were incubated in dark, moist conditions at 26−28 °C. After 72 h of incubation and every 12 h thereafter, the optical densities of the well solutions were read using a Molecular Devices Emax plate reader set at 750 nm until the optical density of the control was between 0.065 and 0.100. At this point, a final reading was taken, and the earlier OD data were discarded. The fractional effect at each phytoalexin concentration was calculated from the test solution OD and the control OD. From the set of fractional effect values and phytoalexin concentrations, the ED_{50} value (the dose required to inhibit fungal growth by 50%) was computed (*28*). Within a single bioassay experiment, three replicate plates were tested. Additionally, at least four bioassay experiments were carried out for each compound.

LD_{100} Bioassay Procedure for *R. solani*. The LD_{100} bioassay is an adaptation of that previously reported by Mace et al. (*29*), which evaluates the lethal effects of compounds toward fungal mycelia. Plugs of *R. solani* (strain J-1) were grown on potato dextrose broth containing 50 mg/L of rifampicin at 26−28 °C for 3−4 days. The broth was decanted, and 1.20 g of fungal matter was weighed out and then macerated for 1−1.5 min in 150 mL of buffered defined medium (composition described above in the ED_{50} Bioassay paragraph, above). Solutions with different concentrations of phytoalexin in medium/DMSO were prepared, again, as described above. Equal volumes of fungal suspension and phytoalexin solution were pipetted into the wells of 24-well flat-bottom microtiter plates. A set of 12 wells was filled for each concentration. Equal volumes of suspension and the medium/DMSO used to prepare the solutions were added to a final set of 12 wells of suspension to be used as the control. The dishes were incubated in dark, moist conditions at 26−28 °C for 48 h. Afterward, the contents in each well were spread on their own plate of potato dextrose agar containing 100 mg/L of chloramphenicol and 50 mg/L of tetracycline (PDA). After 4−5 days of incubation at room temperature in ambient light conditions, the PDA plates were examined visually for fungal growth. At each phytoalexin concentration, the percentage of plates without any live fungus was calculated relative to the number of control plates with live fungus. The lowest concentration at which no fungal growth occurred was reported as the LD_{100} for the phytoalexin. Each phytoalexin was bioassayed at least three times.

RESULTS AND DISCUSSION

The ratio of (−)- to (+)-gossypol in cottonseed and in the roots of seedlings germinated from seed that had or had not been treated with *T. virens* (isolate G4) was determined. (+)-Gossypol was found to be the predominant enantiomer in the seed and in both the untreated and treated roots (**Table 2**). Interestingly, the (−)-enantiomer was induced to a greater extent in roots from seed treated with *T. virens* as compared to untreated seed.

A turbimetric (ED_{50}) bioassay was used to measure the inhibitory effects of the various terpenoids on the growth of *R. solani*. The ED_{50} values obtained from the bioassays are listed in **Table 3**. Unexpectedly, the inhibitory effects of (±)-gossypol, (+)-gossypol, and (−)-gossypol were found to be the same. Comparing the ED_{50} values, gossypol is significantly less inhibitory than dHG, dMHG, and MHG. However, on a molar basis, HG and gossypol have the same activity. This suggests that HG and gossypol act at the same site and that dimerization of HG to gossypol in the cotton plant reduces resistance because there are fewer active molecules available.

Table 3. Concentrations of Cotton or Kenaf Phytoalexins Required To Reduce Growth of *Rhizoctonia solani* by 50% (ED_{50})

compound	ED_{50} (μg/mL)[a]	ED_{50} (μM)	DMSO[b] (%)
desoxhemigossypol-6-methyl ether	0.65 ± 0.09	2.52	1
desoxyhemigossypol	0.86 ± 0.09	3.52	1
hemigossypol-6-methyl ether	3.60 ± 0.58	13.14	1
hemigossypol	5.56 ± 0.53	21.38	2
(+)-gossypol	12.38 ± 0.75	23.90	3
(−)-gossypol	12.59 ± 1.13	24.31	3
(±)-gossypol	13.00 ± 0.53	25.10	3
o-hibiscanone	0.70 ± 0.06	3.76	1

[a] Values are averages of at least four replicate experiments. [b] Concentration of dimethyl sulfoxide utilized in the bioassay.

Table 4. Concentration of Cotton or Kenaf Terpenoids Required To Kill All Propagules of *Rhizoctonia solani* (LD_{100})

compound	LD_{100} (μg/mL)[a]	LD_{100} (μM)	DMSO[b] (%)
desoxhemigossypol-6-methyl ether	10.0	40	1
desoxyhemigossypol	10.0	41	1
hemigossypol-6-methyl ether	15.0	55	1
hemigossypol	20–25	77–96	2
(+)-gossypol	>55	>106	3
(−)-gossypol	>55	>106	3
(±)-gossypol	>55	>106	3
o-hibiscanone	5.0	27	1

[a] Values are from three replicate experiments. [b] Concentration of dimethyl sulfoxide utilized in the bioassay.

The finding that gossypol was inhibitory to *R. solani* was somewhat surprising, as lethal-dose bioassays performed previously in our laboratory had shown that (±)-gossypol was relatively nontoxic to mycelia of the cotton plant pathogen, *Verticillium dahliae*. As the inhibitory efficacy and lethal toxicity of a compound can differ significantly, we utilized an adaptation of the *V. dahliae* lethal-dose bioassay to evaluate the lethal toxicities of gossypol toward *R. solani*. Unfortunately, limited solubility of gossypol in the medium/DMSO used in the bioassay prevented the establishment of LD_{100} values for the gossypols. The bioassay, however, did reveal that the LD_{100} values are in excess of 55 μg/mL, and thus, (−)-, (+)-, and (±)-gossypol do not contribute significantly toward killing the pathogen. In **Table 4**, the LD_{100} for gossypol is compared with that obtained for other cotton phytoalexins toward *R. solani*. Clearly, the LD_{100} values show that the sesquiterpenoids are significantly more toxic than the racemate or individual enantiomers of gossypol. This result is in general agreement with our earlier studies with *V. dahliae*.

Our bioassays show that, while gossypol is not strongly lethal toward *R. solani*, (−)-, (+)-, and (±)-gossypol inhibit the growth of this pathogen equally. This latter result was surprising, given the preponderance of data showing that (−)-gossypol is the biologically active enantiomer in mammalian systems. At the same time, the result indicates that altering the gossypol enantiomeric ratio in cotton roots should not affect resistance

to *R. solani*. Since altering the ratio of (−)- to (+)-gossypol in cottonseed could change this ratio in other tissues of the cotton plant, our findings show that this should not reduce disease resistance. This expands the options available for making cottonseed nontoxic to nonruminant animals to include enantiomeric selection along with total gossypol elimination. Success in such endeavors opens the way for cottonseed to be used as a feed source for these animals.

These bioassays also show that the inhibitory efficacy and lethal toxicity of gossypol are less than those of the other sesquiterpene phytoalexins, except for HG. The LD_{100} results show a similar trend. Unexpectedly, the results suggest that the difference in toxicity of gossypol and HG is actually due to a reduction in the number of molecules of phytoalexin available to act against the fungus instead of an alteration in the molecular toxic potential. The dimerization of HG to gossypol is detrimental to the overall potency of the phytoalexin activity. Fortunately, gossypol does accumulate within the plant tissue instead of undergoing significant decomposition, thus somewhat diminishing the negative effect of this conversion on the resistance of cotton to *R. solani*. Reducing or eliminating the conversion of HG to gossypol, however, should increase the potency of the phytoalexin mix and thus increase the resistance of cotton to *R. solani*.

o-Hibiscanone, a phytoalexin produced by kenaf (*Hibiscus cannabinus*) (*30*), is thought to be derived from δ-cadinene via an isoprenoid pathway similar to that found in cotton for the biosynthesis of the sesquiterpenoids. *o*-Hibiscanone also has been found to be approximately 8 times more toxic to *V. dahliae* than desoxyhemigossypol, the most potent phytoalexin produced by cotton (*30*, *31*). Therefore, this compound is considered a likely candidate in efforts to enhance resistance of cotton to wilt fungi. The question was whether this effort would inadvertently and negatively affect the resistance of cotton to seedling disease pathogens. The results from both the inhibition and lethal toxicity bioassays (**Tables 3** and **4**, respectively) indicate that *o*-hibiscanone is equally or more toxic to *R. solani* than the cotton phytoalexins. This suggests that adding *o*-hibiscanone to the phytoalexin mix in cotton roots should not negatively affect resistance to the seedling disease pathogen and would probably enhance resistance.

In earlier work, we showed that the (+)-enantiomer of gossypol does not significantly affect the growth of chicken broilers (*15*). Findings reported here indicate that incorporating this trait into cotton would not reduce resistance to the seedling disease pathogen *R. solani*. Therefore, introducing a trait that increases the (+)-gossypol/(−)-gossypol ratio in cotton appears to be a worthy goal. The seed from moco cottons from Brazil are known to produce high levels of (+)-gossypol (*32*), and it has been found that the ratio of (+)- to (−)-gossypol in some varieties of moco cottons are as high as 97:3 (*33*). Bell et al. (*33*) have demonstrated that the percentage of (+)-gossypol in cottonseed can be increased in cotton cultivars through a back cross program. To be certain that this effort does not end up adversely affecting the resistance of cotton to insect pests, we are currently investigating the toxicity of (+)- and (−)-gossypol to a phytophagous insect.

This study has also found that dimerization of hemigossypol to gossypol reduces the overall potency of the phytoalexin mix to *R. solani*. One expects, therefore, that down-regulation of this reaction would significantly increase resistance to this pathogen. The kenaf phytoalexin *o*-hibiscanone remains a potentially useful resource to increase resistance of cotton to both seedling and wilt pathogens.

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