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STIMULATION OF SESQUITERPENE ALDEHYDE FORMATION IN *GOSSYPIUM ARBOREUM* CELL SUSPENSION CULTURES BY CONIDIA OF *VERTICILLIUM DAHLIAE*

PETER HEINSTEIN

Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907

ABSTRACT.—*Gossypium arboreum* cell suspension cultures, which were selected for rapid growth and absence of secondary product formation, were used to test the possibility that secondary product formation can be induced by the addition of a phytopathogenic fungus to these cultures. When 10^4 conidia of the fungus *Verticillium dahliae* (strain 277, nonvirulent to cotton) were added to an 8-ml cell suspension culture of *G. arboreum*, the formation of gossypol and hemigossypol—the major secondary natural products of *Gossypium* species—was increased approximately eightyfold in 72 h of incubation. Higher concentrations of conidia or longer incubation times caused a decrease in the formation of these secondary natural products, most likely due to the destruction of the cotton cells by the fungus, as indicated by a decrease in the cell mass accumulation. This was supported by the fact that the *V. dahliae* strain T9, a strain virulent to cotton at a concentration of 10^3 conidia caused cessation of growth and a decrease in cell mass accumulation after 48 h. However, viable conidia are not required for elicitation of secondary natural product formation. Medium in which *V. dahliae* had been growing or heat-denatured conidia did stimulate gossypol and hemigossypol formation comparable to viable conidia. The results show that stress conditions, as for example phytopathogenic fungi, can be used to elicit secondary natural product formation in plant cell suspension cultures.

Previously, it was shown that cell suspension cultures of *Gossypium* species were able to synthesize the sesquiterpene aldehydes hemigossypol and gossypol (1). However, an inverse relationship of growth to hemigossypol and gossypol formation was observed (1), and indeed, when *Gossypium hirsutum* L. Paymaster 3030, Tamcot Sp 37, and Acala SJ 5 were continuously subcultured for four years, the ability to produce hemigossypol and gossypol was drastically reduced. Various medium manipulations, including variation in hormone concentrations, did not restore the ability of these cultures to synthesize hemigossypol and gossypol.[1] Similar observations have been reported in other plant cell suspension cultures, which, immediately after initiation of callus and suspension culture, were able to produce secondary natural products, but upon subsequent subculturing, lost the ability to do so (2).

In view of the fact that many secondary natural products are phytoalexins (3), including gossypol (4), and are produced by plants upon physical, chemical, and biological injury, it appeared of interest to test the possibility that phytopathogenic fungi can stimulate the formation of secondary natural products in cell suspension cultures. Based on the previous observation that, in cotton seedlings, formation of gossypol and related sesquiterpene aldehydes is increased upon infection with conidia of *Verticillium dahliae* Kleb., a wilt-producing fungus affecting a variety of plants (5), including cotton (4), *Gossypium arboreum* L. cell suspension cultures were initiated and treated with conidia of *V. dahliae* and spent fungal medium to test the possibility that sesquiterpene aldehyde formation can be stimulated. Parts of the results herein, namely, preliminary experiments showing induction of sesquiterpene aldehyde formation in *G. arboreum* cell suspension cultures by conidia of *V. dahliae*, have been previously reported (6).

RESULTS

ASSAY.—Selection for rapid growth of *G. arboreum* cell suspension cultures re-

[1]P. Heinstein, unpublished observations.

sulted in the elimination of any significant sesquiterpene aldehyde formation in these cultures. Similar adaptive changes have been obtained with *G. hirsutum* Tamcot Sp 37 and *G. hirsutum* Paymaster 3030 cell suspension cultures (1). This inverse relationship of good growth to poor secondary natural product formation has been observed in a number of other plant cell cultures (7). In some cases, the production of secondary natural products can be stimulated by subjecting the plant cell cultures to biological stress (7). This was found to be true for the sesquiterpene aldehyde formation in *G. arboreum* cells and supports the hypothesis that the sesquiterpene aldehyde pigments in the cotton plant are phytoalexins (8).

The assay, developed to quantitate the interaction of the plant cell with a pathogenic fungus, allowed measurement of sesquiterpene aldehyde formation in a MeOH extract, as well as the growth of host-plant cells in one incubation, by using the MeOH extract residue of the cells for dry-weight determination.

To measure total sesquiterpene aldehyde formation, the phloroglucinol assay (9) was chosen. A comparison of the total sesquiterpene aldehydes in the MeOH extract of *G. arboreum* cells, incubated for 120 h with 10^3 *V. dahliae* 277 conidia, with the sum of the tlc separated (10) sesquiterpene aldehydes showed that $92\pm5\%$ of the total phloroglucinol positive material could be accounted for by the sum of gossypol and hemigossypol and their derivatives. It appeared, therefore, that negligible amounts of nonsesquiterpene aldehyde material, which was phloroglucinol positive, was extracted from the *G. arboreum* cells. Typically, tlc separations showed that 42% of the synthesized sesquiterpene aldehydes appeared to be gossypol, 35% hemigossypol, and the remainder, methylated derivatives of gossypol and hemigossypol (11). However, this distribution depended considerably on the size of *V. dahliae* inoculum used and the length of incubation.

Dilution of cell suspension cultures of parsley into fresh medium or H_2O resulted in the induction of the enzymes in the phenylpropanoid biosynthetic pathway and, therefore, increased formation of phenylpropanoids (12). A similar effect on sesquiterpene aldehyde formation, upon dilution into fresh medium, was found to occur in *G. arboreum* cell suspensions. However, the dilution effect was less pronounced than in parsley cell suspensions, and only a twofold increase in sesquiterpene aldehyde concentration was observed in *G. arboreum* cultures after 10 h from the initial dilution. Furthermore, sesquiterpene aldehyde concentration was reduced to pre-dilution concentration after 24 h incubation from initial dilution. To eliminate any effects of dilution upon growth, or sesquiterpene aldehyde formation in *G. arboreum* cell suspension cultures, fresh inocula were incubated for 36 h before fungal conidia or fungal medium were added to initiate an experiment.

The contribution to the dry weight by the *V. dahliae* conidia was insignificant. In preliminary experiments, inoculation of 10^5 conidia of *V. dahliae* 277 or T9 into 8 ml of Linsmaier and Skoog (LS) medium, without *G. arboreum* cells, resulted in an increase in dry weight of 0.035 g/liter after 120 h incubation; therefore, *V. dahliae* T9 or 277 cannot grow in LS medium. The MeOH extract of the above *V. dahliae* 277 or T9 cultures did not give a phloroglucinol positive color reaction and showed no measurable absorption at 550 nm after reaction with phloroglucinol. Similarly, dilution of 1 ml of medium in which *V. dahliae* 277 had been growing for 8 days, but was removed through Millipore filtration into 8 ml of LS medium, did not yield any measurable absorption at 550 nm.

EFFECTS ON SESQUITERPENE ALDEHYDE FORMATION.—Addition of increasing concentrations of *V. dahliae* conidia, from 10^2 to 10^4 conidia per 8 ml *G. arboreum* cell suspension caused an increased formation of sesquiterpene aldehydes (Figures 1 and 2). Optimum sesquiterpene aldehyde formation was obtained with a *V. dahliae* 277 conidia

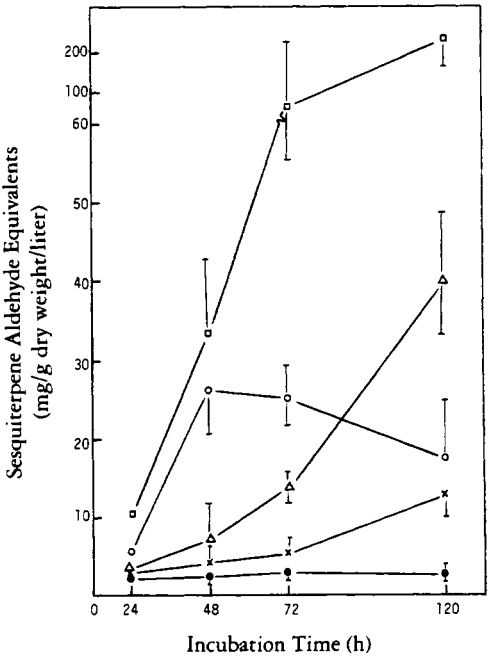

FIGURE 1. Effect of *Verticillium dahliae* 277 conidia concentration on the formation of sesquiterpene aldehydes in *Gossypium arboreum* cell suspension cultures. *G. arboreum* cells (8 ml) were inoculated with 0 (●—●), 4.2×10^2 (×—×), 4.2×10^3 (△—△), 4.2×10^4 (□—□), and 4.2×10^5 conidia (○—○).

concentration of 10^3 to 10^4 conidia in 8 ml of a plant cell suspension. *V. dahliae* 277 conidia concentration of 10^5 per 8 ml incubation showed a marked decrease (Figure 1) of sesquiterpene aldehyde formation after being in contact with *G. arboreum* cells for 48 h.

The stimulation of sesquiterpene aldehyde formation in cell suspensions appeared to be independent of *V. dahliae* virulence (Figure 2). The *V. dahliae* T9 strain, which in vivo causes complete defoliation and death of cotton plants, stimulated sesquiterpene aldehyde formation by the same magnitude as the nonvirulent *V. dahliae* strain 277. A completely nonpathogenic yeast, *Saccharomyces cerevisiae*, could induce sesquiterpene aldehyde formation by *G. arboreum* cell suspension, although conidial concentrations of 10^5 per 8 ml of plant cell suspension were required for sesquiterpene aldehyde formation comparable to 10^4 conidia of *V. dahliae* 277 (Figure 3).

Medium in which *V. dahliae* 277 had been growing for 6 days, and from which *V. dahliae* 277 conidia were removed through Millipore filtration, could initiate sesquiterpene aldehyde formation in *G. arboreum* cell suspension cultures (Figure 3). However, the capacity to initiate sesquiterpene aldehyde formation of spent medium was much less than that of intact conidia, inasmuch as 1 ml of spent medium would correspond to approximately 10^9 conidia. The activity to initiate sesquiterpene aldehyde formation contained in the spent *V. dahliae* media appeared to be heat labile (Figure 3), but autoclaving *V. dahliae* 277 conidia for 3 min resulted in no loss of sesquiterpene-aldehyde-synthesis-initiating activity (Figure 3). Fresh Czapek-Dox medium did not initiate ses-

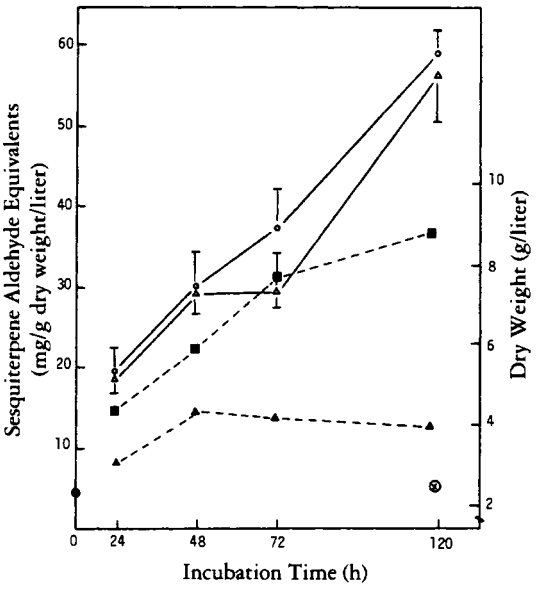

FIGURE 2. Effect of different strains of *Verticillium dahliae* on growth and on sesquiterpene aldehyde formation in *Gossypium arboreum* cell suspension cultures. To 8 ml of *G. arboreum* cell suspensions were added 4.2×10^3 conidia of strain 277 and sesquiterpene, aldehydes (○—○) or cell mass (■---■) determined. Similarly, 4.2×10^3 conidia of strain T9 was added to 8 ml of *G. arboreum* cells and, after the time indicated, sesquiterpene aldehyde (△—△) and cell mass (▲---▲) was determined. Sesquiterpene aldehyde concentration in control incubation ⊗. Initial *G. arboreum* inoculum ●.

quiterpene aldehyde formation in *G. arboreum* cell suspension cultures (Figure 3). However, compared with the control value after 120 h of incubation (Figure 1), the sesquiterpene aldehyde concentration was slightly increased by fresh Czapek-Dox medium (Figure 3).

In a few experiments, the MeOH extract of the stimulated *G. arboreum* cells was concentrated to 0.5-1 ml, and an aliquot of the sesquiterpene aldehyde was separated by tlc (11). Two major components were identified as hemigossypol (HG) and gossypol (G) by the color of their phloroglucinol derivatives and comparison of Rf values with standards (11). The results of a typical experiment are shown in Figure 4. A number of other minor components have not yet been identified but appear to be methylated derivatives of gossypol and hemigossypol by Rf value and comparison with literature values (10,11). *V. dahliae* 277 conidia, at a concentration of 10^2 conidia per 8 ml of *G. arboreum* cell suspensions, increased formation of these sesquiterpene aldehydes after an apparent lag period of 24 h (Figure 4). Hemigossypol formation was increased the most, followed by gossypol. At 10^3 conidia per 8 ml plant cell suspension, the lag period was decreased to 8 to 12 h, and the initial rate of formation for both sesquiterpene aldehydes was considerably increased up to 48 h of incubation. However, the formation of sesquiterpene aldehyde was lower after a 120-h incubation with 10^3 conidia, as compared to 10^2 conidia.

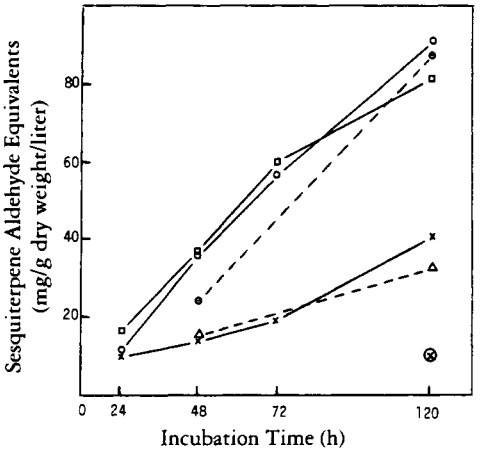

FIGURE 3. Effect of *Verticillium dahliae* 277 medium (1 ml) (○—○), heat-denatured (15 min, 120°) *V. dahliae* 277 medium (1 ml) (×—×), 4×10^4 heat-denatured (3 min, 120°) *V. dahliae* 277 conidia (□—□), and *Saccharomyces cerevisiae* conidia (4.8×10^4 conidia per 8 ml, △---△; 4.8×10^5 conidia per 8 ml, ⊙ ---⊙) on sesquiterpene aldehyde formation in *Gossypium arboreum* cell suspension cultures (8 ml). Control incubation with Czapek Dox medium ⊗.

A more-sustained stimulation of the formation of both sesquiterpene aldehydes in *G. arboreum* cell suspension cultures was obtained when these cultures were stimulated with 1 ml of spent *V. dahliae* 277 medium. The rate of gossypol and hemigossypol formation did not decrease after 72 h under these conditions (results not shown).

EFFECTS ON GROWTH.—Addition of *V. dahliae* 277 conidia to *G. arboreum* cell suspension cultures caused a decrease of dry weight accumulation and, therefore, decreased cell growth (Figure 5). This decrease in growth was significant when 4.2×10^4 conidia were added to 8 ml of *G. arboreum* cell suspensions. Growth of the plant cells ceased after 72 h of incubation when the conidia concentration was increased to 4.2×10^5 conidia per 8 ml plant cell suspensions (Figure 5). In general, the inhibition of growth of *G. arboreum* cell suspensions was related to the virulence of the *V. dahliae* strain (Figure 2). Strain T9, a highly virulent strain at a concentration of 4.2×10^3 conidia per 8 ml, caused complete cessation of growth and apparently cell lysis, as judged from the decrease in cell mass, of *G. arboreum* cell suspension after a 48-h incubation.

No reduction in growth of *G. arboreum* cell suspension cultures was observed when *V. dahliae* conidia (strain 277, 10^4 conidia per 8 ml plant cell suspension) was autoclaved for 20 min at 19 psi and 125°. *S. cerevisiae*, at 10^5 conidia per *G. arboreum* cell suspension, and medium in which *V. dahliae* had been growing did not have any inhibitory effect on plant cell growth.

DISCUSSION

Previously (13), it has been shown that a relationship exists between sesquiterpene aldehyde accumulation in intact *Gossypium* plantlets and infection with the wilt-producing fungus, *V. dahliae*. These observations implied that gossypol, hemigossypol,

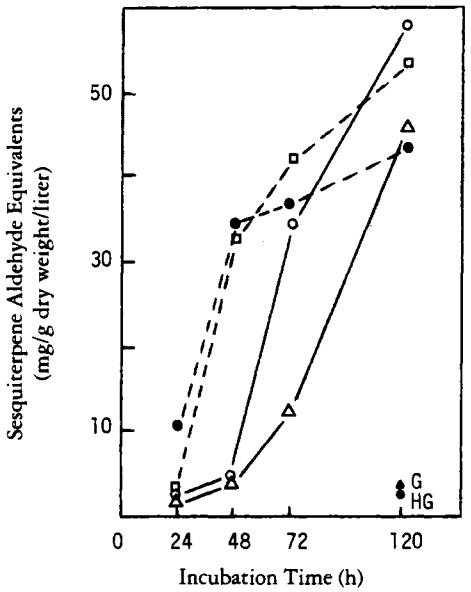

FIGURE 4. Effect of *Verticillium dahliae* 277 conidia on hemigossypol and gossypol formation, *V. dahliae* 277, 8×10^2 and 8×10^3 conidia, were added to 8 ml of *Gossypium arboreum* cell suspension cultures. Sesquiterpene aldehydes were extracted and separated by tlc. Hemigossypol produced upon inoculation with 8×10^2 conidia (●---●) and 8×10^3 conidia (□---□); gossypol produced upon inoculation with 8×10^2 conidia (△—△) and 8×10^3 (○—○). Control incubations without conidia produced little hemigossypol (●HG) or gossypol (▲G).

and their derivatives are phytoalexins (4). Therefore, subjection of *G. arboreum* cell suspension cultures to stress, as for example *V. dahliae*, should cause an increased accumulation of sesquiterpene aldehydes in culture. This, indeed, was found to be the case and agrees with observations in the literature for other pathogen-host interactions in vivo (4).

Addition of up to 4×10^4 conidia of *V. dahliae* to an 8-ml *G. arboreum* cell suspension culture caused a comparable increase in sesquiterpene aldehyde formation of up to a 72-h incubation time. However, a further increase of condidia or a longer incubation time slowed the rate of formation or actually decreased the concentration of sesquiterpene aldehydes in these cultures (Figure 1). The decreased rate of sesquiterpene aldehyde formation can be attributed to a decrease in cell mass accumulation (Figure 5) at a conidia concentration of 10^4 per 8 ml of cell suspension. The decrease in sesquiterpene aldehyde concentration implied degradation. However, this decrease may simply reflect *G. arboreum* cell lysis, as supported by the arrest of cell growth (Figure 5) at 10^5 *V. dahliae* 277 conidia per cultures. At higher concentrations of conidia of *V. dahliae* 277 (results not shown), or when the virulent T9 strain of *V. dahliae* was used at a concentration of 4×10^3 conidia, an actual decrease of cell mass was observed (Figure 2), substantiating the observation that at higher conidia concentrations or longer incubation

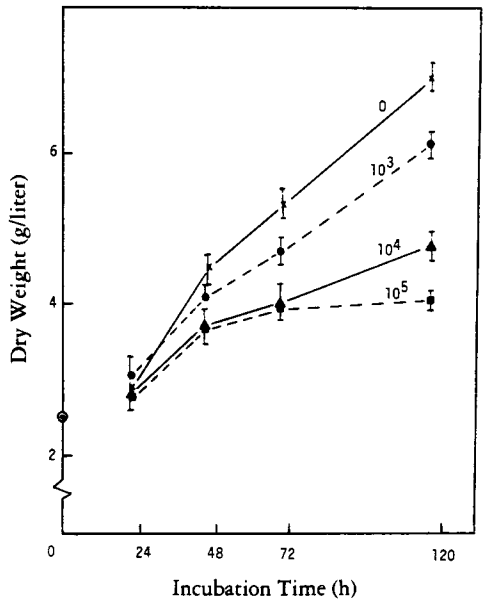

FIGURE 5. Effect of *Verticillium dahliae* 277 conidia concentration on growth of *Gossypium arboreum* cell suspensions. *V. dahliae* 277 conidia at a concentration of 4.2×10^3 (●—●), 4.2×10^4 (△—△), and 4.2×10^5 (□—□) conidia per 8 ml of *G. arboreum* cell suspension cultures. Control incubations (×—×) contained water instead of conidia. (⊙) Weight of the initial inoculum.

times, sesquiterpene aldehyde formation and concentration in *G. arboreum* cell suspension cultures is actually decreased. This reduction in growth, and therefore in the rate of sesquiterpene aldehyde formation, was not apparent when heat denatured *V. dahliae* 277 conidia or a nonpathogenic fungus, as *S. cereviciae* at 10^5 conidia per 8 ml suspension culture, were used.

The apparent discrepancy in the total accumulation of sesquiterpene aldehydes (compare Figure 1 and Figure 2) is somewhat puzzling. A number of possible explanations can be advanced. Since these results were obtained from different experiments, a potential genetic variation (15) in the *G. arboreum* cultures upon subsequent transfers cannot be excluded, even though care was taken to select cultures with identical outward appearances, such as color and suspension consistency. Initial experiments showed that the age of the *G. arboreum* cell suspension cultures, from which the inoculum was obtained, was of importance. However, this explanation can be excluded since only 9-day-old *G. arboreum* cultures were used for all experiments. Another possible explanation for these discrepancies could be the suspension consistency of the *V. dahliae* cultures, and experiments are in progress to test this hypothesis.

The results clearly show that stress conditions, brought about by the pathogenic fungus *V. dahliae*, can be used to stimulate sesquiterpene aldehyde formation in *G. arboreum* cell suspension cultures and can possibly be applied to other plant cell suspension cultures where little or no secondary natural products are produced. However, for optimum results, selection of a specific pathogen and adjustment of the conidia concentrations, as well as length of incubation time, are required.

MATERIALS AND METHODS

CELL CULTURES.—Cell suspension cultures of *G. arboreum* cv. Nanking were initiated from aseptically germinated seeds obtained from the National Cotton Pathology Laboratory, USDA, and maintained on LS medium (16), containing 0.18 mg/liter of α-naphthalene-acetic acid and 0.22 mg/liter of 2,4-dichlorophenoxyacetic acid, described previously (1) for *G. hirsutum* cultures. Initially, the *G. arboreum* cultures produced 37 mg/liter of sesquiterpene aldehydes as measured by the phloroglucinol method (9). The production of sesquiterpene aldehydes in these cultures was reduced to a stable level of 0.002 mg/liter by selecting for rapid growth and low pigment production. This required subculturing every 10 days through dilution of one part of a 10-day-old cell suspension into 5-6 parts of fresh LS medium.

FUNGAL CULTURES.—Strains of *V. dahliae* (T9 and 277) were obtained from Dr. A.A. Bell, National Cotton Pathology Research Laboratory, USDA. *V. dahliae* T9 is considered to be a strain virulent to cotton and has been isolated from this host (17). Strain 277, isolated from diseased sugar beet tissue, is a strain nonvirulent to cotton (17).

The two strains were maintained on potato-carrot-dextrose agar (17) at 22° and subcultured every 6 weeks. For experiments, conidia were grown for 10 days in potato-carrot-dextrose (17) or in Czapek-Dox (18) liquid medium at 22° on a rotary shaker at 240 rpm. Conidia were harvested by centrifugation, washed three times with 0.1 M K_2HPO_4-KH_2PO_4, pH 6.5, and conidia concentration was determined in a Speirs-Levy chamber (A.H. Thomas Co., Philadelphia). *S. cerevisiae*, ATTC 9763, was grown at 22° in Bacto-yeast morphology broth. After harvesting and washing by centrifugation, the number of conidia was determined as above.

INCUBATION.—A typical experiment was initiated by inoculating one ml of loosely packed *G. arboreum* cells, obtained by gravity filtration from a 9-day-old culture (late-log phase) into 8 ml of fresh LS medium in a 50-ml Erlenmeyer flask. The cell suspensions were incubated in the dark at 30° and 110 rpm rotary agitation for 36 h before adding fungal conidia, heat-inactivated conidia, or medium in which *V. dahliae* had been growing (spent medium). *G. arboreum* cells were harvested from individual suspension cultures after 24, 48, 72, and 120 h by vacuum filtration.

For extraction of sesquiterpene aldehydes, freshly filtered plant cells were continuously stirred overnight with 10 ml of MeOH saturated with $NaHSO_3$. The MeOH extract was removed by filtration. The residue was dried at 60° for 16 h and used for dry weight determination. The filtrate was concentrated to 3-6 ml under a stream of N_2, depending on the amount of sesquiterpene aldehydes present, and an aliquot used for measuring sesquiterpene aldehyde concentration by adding phloroglucinol and determining absorbance at 550 nm (9). Standard curves were prepared with a mixture of equal amounts of gossypol and hemigossypol.

In some experiments, gossypol and hemigossypol, produced by the *G. arboreum* cell suspension cultures, were separated by tlc (8) and quantitated, as described previously (9), with the modification that the EtOH extract of the spots corresponding to gossypol and hemigossypol was reacted with phloroglucinol (0.1 ml of a 5% solution) and 0.2 ml of concentrated HCl in a total volume of 1 ml of 95% EtOH, and concentrations determined at 550 nm.

All experiments were statistically analyzed using the Student *t*-test.

ACKNOWLEDGMENTS

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