Histochemistry and Isolation of Gossypol and Related Terpenoids in Roots of Cotton Seedlings

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Accepted for publication 30 April 1974.

ABSTRACT

Gossypol (8,8′-dicarboxaldehyde,1,1′,6,6′,7,7′-hexahydroxy, 5,5′-diisoprophyl,3,3′-dimethyl,2,2′-binaphthalene) and four related terpenoid aldehydes, 6-methoxygossypol, 6,6′-dimethoxygossypol, hemigossypol (1,6,7-trihydroxy, 5-isopropyl, 3-methyl,8-naphthaldehyde), and 6-methoxyhemigossypol were identified in roots of 1-wk-old Acala 4-42 cotton seedlings. Histochemical procedures revealed the localization of these terpenoids in the epidermis and in scattered cortical parenchyma cells of the healthy tap root. The compounds were not detected in the first 3 cm back of the root tip. Gossypol and related terpenoid aldehydes appear to occur in cytoplasmic granules in the root epidermis and cortex. No lysigenous, terpenoid-containing glands were detected in seedling root tissues. The possible interactions of the five terpenoids with pathogens of cotton are discussed.

Phytopathology 64:1297-1302.

Additional Key Words: Verticillium, Gossypium, cytochemistry, polyphenol, phytoalexin, antibiotic.

Gossypol occurs in greater concn in cotton roots than in seeds (20). Smaller amounts occur in other plant parts. In seeds and aerial parts of healthy cotton plants, gossypol and related terpenoid pigments are localized in lysigenous glands (10); however, little or nothing is known about the localization of these pigments in roots.

Recent studies (6, 24, 25) have stressed the possible function of gossypol-related compounds in resistance of cotton to Verticillium wilt. Inoculation of stelar tissue of cotton stems with *Verticillium dahliae* Reinke & Berth. induced levels of gossypol-related compounds that were toxic to the pathogen (7). These compounds were not detected in healthy stelar tissue. Inoculation with *Verticillium* induced the biosynthesis of gossypol-related terpenoids in both susceptible and resistant stelar tissue, but the terpenoids accumulated more rapidly in resistant than in susceptible cotton cultivars (24).

In the field, infection and initial host response to *Verticillium* occurs in cotton root tissue. Beckman (3, 4) has shown that the critical reactions determining resistance to a similar disease, Fusarium wilt of banana, occur in the root stele within a few days after inoculation. Thus, data on the localization of the fungitoxic gossypol-related terpenoids in relation to the root tissues initially penetrated and subsequently colonized by the pathogen, is needed to more completely assess the function of these compounds in resistance to Verticillium and Fusarium wilts.

This paper reports the histochemical localization, isolation, and identification of gossypol and related terpenoid aldehydes in the roots of healthy cotton seedlings.

MATERIALS AND METHODS.—The cotton cultivar Acala 4-42 (*Gossypium hirsutum* L.), which is tolerant to Verticillium wilt, was used throughout these studies. Acid-delinted seeds were heated in distilled water at 80 C for 1 min to induce uniform germination. Seeds were then placed in paper germination towels, and the losely rolled towels were placed upright in 1-liter beakers containing 100 ml of Hoagland's solution (15). Seed rolls were incubated for 1 wk in a controlled environment chamber at 28 C and with a 14:10-h, light (21,500 lux.):dark regime. After the first 3 days, seedlings were repositioned in the towels to expose the cotyledons to light. Root systems of the 1-wk-old plants were harvested for extraction and histochemical studies.

Terpenoids were extracted by grinding 1 kg of fresh roots in 4 liters of cold 95% ethanol in a blender for 2 min. The mixture was filtered, and the residue was ground again in 2 liters of 95% ethanol for 2 min and allowed to stand for 30 min before filtering. The combined filtrates were thoroughly mixed with two volumes of 50% saturated aqueous NaCl solution and one volume of ethyl acetate in a separatory funnel. The lower aqueous phase was discarded, and the ethyl acetate phase was washed twice with an equal volume of a 50% saturated aqueous NaCl solution and finally with saturated NaCl solution. The ethyl acetate extract was then dried with 10% (w/v) anhydrous sodium sulfate for 20 min and reduced in volume in a rotary evaporator at 30 C. When the volume was reduced to about 10 ml (only an orange-brown viscous liquid remained), the material was dissolved in 200 ml of an ethyl acetate-hexane (1:3, v/v) mixture and filtered through a 3 × 5 cm column of SilicAR CC-7 (Mallinckrodt Chemical Works). The column was washed with an additional 200 ml of the same solvent, and the total eluate was evaporated at 30 C. The residue was either dissolved in 10 ml of ethyl acetate and stored at −20 C, or the solution was used immediately for thin-layer chromatography.

Thin-layer chromatography (TLC) was performed on 0.5-mm layers of polyamide powder (E. Merck Chem. Co.) or Silicar TLC-7GF (Mallinckrodt Chemical Works). Plates were spread, allowed to dry overnight, and used without further activation. All chromatographic procedures were conducted in dark or with minimum light to avoid light-catalyzed oxidation of the terpenoid aldehydes. The terpenoids in ethyl acetate were spotted on plates and the solvent was removed by blowing nitrogen over the spotted area for a few minutes. Plates were then either sprayed immediately with chromogenic reagents, or areas containing the terpenoids were quickly removed with a razor blade. Terpenoids were eluted from silica gel with ethyl ether, and from polyamide with a methanol:formic acid (98:2) mixture. Chromogenic reagents used for terpenoid aldehydes on TLC plates were: a saturated solution of $SbCl_3$ in 60% $HClO_4$, a saturated solution of 2,4-dinitrophenylhydrazine in 2N HCl, and 1% phloroglucinol in a mixture of ethanol:concd HCL (1:1, v/v). Details for the purification and crystallization of individual terpenoid aldehydes are published elsewhere (9, 23).

Each terpenoid reported was purified to crystalline purity and its structure confirmed by mass spectrophotometry in a Varian MAT CH-7 instrument, by nuclear magnetic resonance spectrophotometry in a JEOL MH-100 instrument, and by infrared spectrophotometry with a Beckman 18A instrument. Melting points were obtained on a Kofler hot stage.

Unless otherwise specified, tap roots were used for the preparation of fresh, free-hand sections for histochemical studies. Pith sticks were used to support root segments during sectioning. Fresh root sections were immediately placed in the histochemical reagents.

The saturated solution of $SbCl_3$ on 60% $HClO_4$ used on TLC plates was also used to localize histochemically gossypol and related terpenoids in fresh root sections. Fresh root sections (approx. 100-μm thick) or whole mounts were placed in a few drops of the $SbCl_3$-$HClO_4$ reagent on a glass slide, incubated 10 min (approx. 23 C), covered with a cover slip, and photographed. The $SbCl_3$-$HClO_4$ reagent was originally developed for the detection of steroids in crude plant materials (14); however, it was found to possess a high degree of specificity for gossypol and related terpenoids in cotton roots. A bright-red complex, presumably an Sb-terpenoid chelate, is formed by the $SbCl_3$ reagent at sites of localization of gossypol and related terpenoids in root sections.

The saturated solution of 2,4-dinitrophenylhydrazine (DNP) in 2N HCl (6) used on TLC plates was also used to localize terpenoid aldehydes in fresh root sections. Root sections were incubated in this reagent for 10 min and rinsed in distilled water before observations were made. Aromatic aldehydes such as gossypol yield an orange-red colored 2,4-dinitrophenylhydrazone after treatment with the DNP reagent (21).

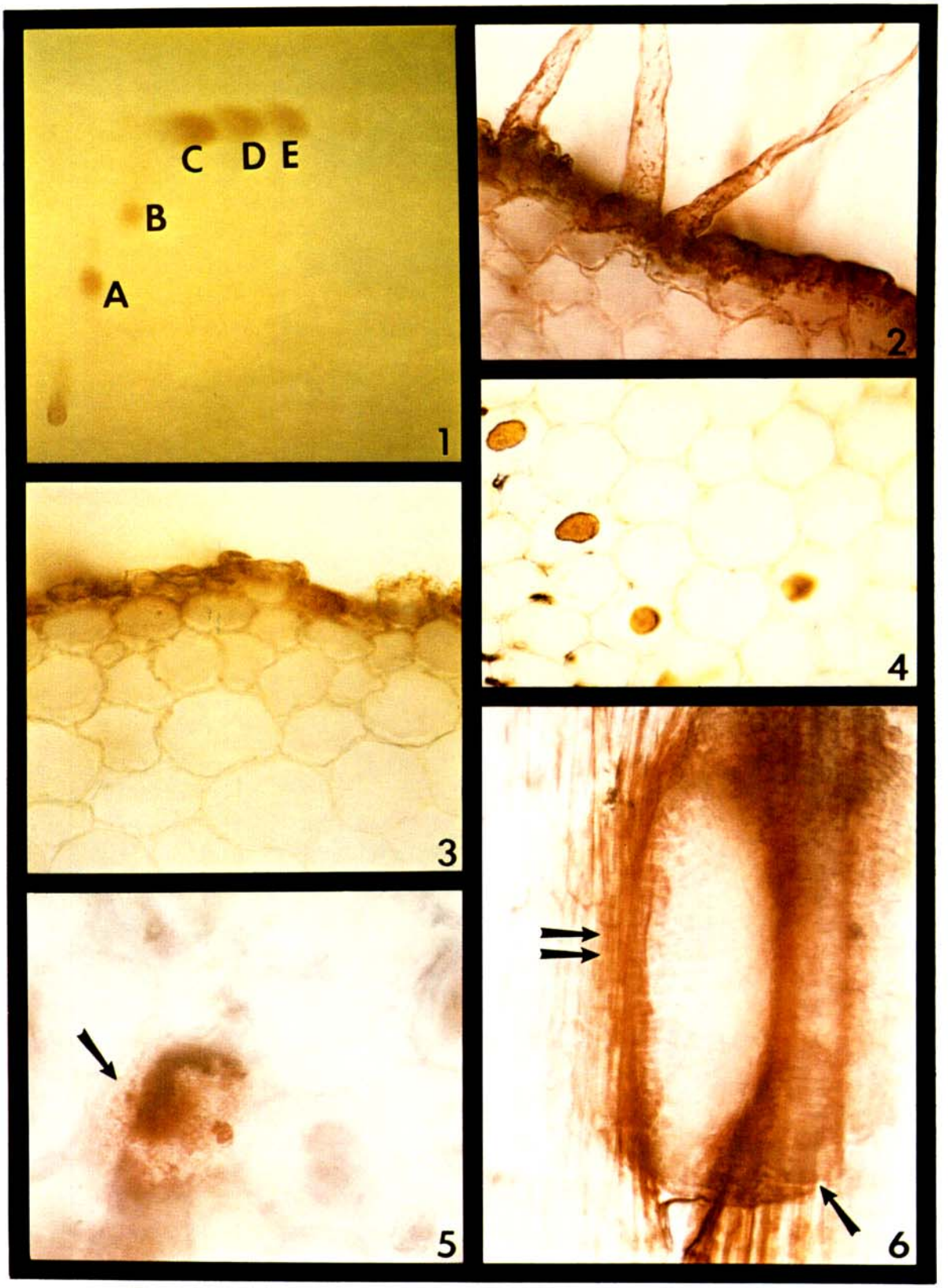

Fig. 1-6. 1) Two-dimensional TLC plate with five 2,4-dinitrophenylhydrazone (DNP) derivatives of terpenoid aldehydes extracted from 1-wk-old Acala 4-42 seedling roots. A = hemigossypol; B = 6-methoxyhemigossypol; C = gossypol; D = 6-methoxygossypol; E = 6,6'-dimethoxygossypol. **2-6)** Cross-sections **(2-5)** and whole mount **(6)** of tap roots of 1-wk-old Acala 4-42 seedlings treated with 2,4-dinitrophenylhydrazine and $SbCl_3$ reagents. Terpenoid aldehydes appear as red Sb complexes and orange 2,4-dinitrophenylhydrazone derivatives. **2)** Sb complexes of terpenoids in epidermis (including root hairs). **3)** DNP derivatives of terpenoids in epidermis. **4)** DNP complexes of terpenoids in isolated cortical parenchyma cells. Protoplasts of terpenoid-containing cells are severely plasmolyzed. **5)** Ruptured cortical parenchyma cell illustrating the fine particulate nature (arrow) of the Sb complexes of terpenoids. **6)** Root surface, illustrating emerging lateral root (single arrow) and zone of intensified terpenoid accumulation (double arrow) in the ruptured epidermis.

A 1% solution of sodium borohydride ($NaBH_4$) in 1% aqueous NaH_2PO_4 was used to reduce the aldehyde groups of gossypol-like compounds in root sections and thereby verify the in vivo occurrence of the terpenoids in the aldehyde form (16). Root sections were incubated in the solution for 10 min and washed several times in distilled water before being placed in the $SbCl_3$ or DNP histochemical reagents. TLC plates also were sprayed with $NaBH_4$ solution and allowed to stand 10 min before being sprayed with a histochemical reagent.

RESULTS.—The $SbCl_3$ reagent produced a bright red colored complex in vitro with gossypol and hemigossypol. Apogossypol (1,1′-6,6′,7,7′-hexahydroxy-5,5′-diisopropyl, 3,3′-dimethyl, 2,2′-binaphthalene), synthesized by the method of Clark (12), however, produced no colored product with $SbCl_3$. Apogossypol lacks only the aldehyde group of the various groups present in gossypol and hemigossypol. Aromatic aldehydes with ortho hydroxyl groups readily form chelates (11). Chelation of Sb between the aldehyde group and the 7-hydroxyl group of gossypol and related terpenoids probably is the reaction mechanism for formation of the red-colored complex.

Thin-layer chromatography of extracts from 1-wk-old seedling roots revealed five red- or orange-colored products after application of either $SbCl_3$ reagent. An identical pattern of orange- or magenta-colored spots indicative of aromatic aldehydes such as gossypol and related terpenoids, was noted after application of the DNP (Fig. 1) or phloroglucinol reagents, respectively, to TLC plates. Control TLC plates sprayed with only 60% $HClO_4$ revealed the same pattern of five compounds as the $SbCl_3$ reagent, but the colors were yellow to orange. This formation of colored products induced by $HClO_4$ alone may be caused in part by the dehydration of the terpenoids similar to that reported for gossypol in concentrated H_2SO_4 (1). No color developed from the compounds on plates sprayed with the 2N or 6N HCl.

Mass, NMR, and infrared spectra demonstrated three of the compounds to be the triterpenoid aldehydes gossypol (8,8′-dicarboxaldehyde, 1,1′, 6,6′, 7,7′-hexahydroxy, 5,5′-diisopropyl, 3,3′-dimethyl, 2,2′-binaphthalene), 6-methoxygossypol, and 6,6′-dimethoxygossypol; the other compounds were the sesquiterpenoid aldehydes hemigossypol (1,6,7-trihydroxy,5-isopropyl,3-methyl,8-naphthaldehyde) and 6-methoxyhemigossypol. The R_f's (×100) of gossypol, 6-methoxygossypol, 6,6′-dimethoxygossypol, hemigossypol, and 6-methoxyhemigossypol [separated by two-dimensional chromatography on polyamide with chloroform:acetone:formic acid (95:4:1) × benzene:methanol:formic acid (194:6:1) as solvents] were 70 × 36, 72 × 47, 73 × 57, 33 × 9, and 48 × 19, respectively. Data on the chemical characteristics of the individual compounds is published elsewhere (9, 23).

Gossypol and related terpenoids were not detected histochemically in the apical 3-cm of the tap root. They first occurred in the epidermis approx. 3 cm back of the tip as red and orange pigments after treatment with $SbCl_3$ and DNP reagents, respectively (Fig. 2 and 3). The terpenoids in root hairs (first root hairs occurred approx. 1 cm from the root tip) first occurred approx. 4 cm from the root tip (Fig. 2). The epidermal localization of the terpenoids persisted throughout the proximal portion of the root. The concn appeared to increase, as determined by the intensity of the red- or orange-colored products, with increasing distance from the apex. This increase in the intensity of the red or orange color was most evident in the root hairs which persisted to the top of the tap root. Control root sections treated with 60% $HClO_4$ or 2N HCl failed to give the red- or orange-colored products during a 10-min incubation period.

Gossypol and related terpenoids initially occurred in the tap root cortex in scattered parenchyma cells approx. 6 cm from the root tip. Terpenoids in these cells were detected as red and orange pigments after treatment with the DNP (Fig. 4) and $SbCl_3$ (Fig. 5) reagents, respectively. The Sb chelates often are detected as very fine particles (Fig. 5). No precise information on the size and shape of these particles could be obtained from the fresh freehand sections. This pattern of cortical localization persisted throughout the proximal portion of the tap root. No lysigenous terpenoid-containing glands were detected in the cortex or other root tissues.

Although detailed studies of lateral roots were not made, the same sequence of terpenoid occurrence described for the tap root was also found in 3-day-old lateral roots of 1-wk-old seedlings treated with the $SbCl_3$ and DNP reagents. Terpenoid zones occurred at much shorter distances back of the root tip than in the larger tap root.

Preliminary treatment of fresh sections of tap roots with the aldehyde-reducing agent, $NaBH_4$, prevented the subsequent reaction of gossypol and related terpenoids in the epidermis and cortex with the $SbCl_3$ and DNP reagents. The reaction of the five root terpenoid aldehydes on TLC plates with DNP was also prevented by preliminary spraying of the plates with the $NaBH_4$ solution.

Intensification of the histochemical reaction for gossypol and related terpenoids was noted routinely in tap roots at the point of rupture of the epidermis by lateral roots (Fig. 6). Cross sections revealed these zones to be restricted to the epidermis.

DISCUSSION.—Gossypol has been identified in healthy cotton roots (20, 22), but the four additional gossypol-related compounds identified in our studies have not been identified previously in mature or seedling roots. Synthesis of hemigossypol, 6-methoxy-hemigossypol, and gossypol is induced by infection of the stele of cotton stems by *Verticillium dahliae* (6, 8, 24, 25), but 6-methoxygossypol and 6,6′-dimethoxygossypol found in the epidermis and cortex of healthy Acala 4-42 roots are not reported to occur in the stele of infected stems. No published data exists on the presence of these five terpenoids in *Verticillium*-infected root stelar tissue. The presence of these five terpenoids in the healthy root, the organ initially penetrated by *V. dahliae*, suggests that they may play a role as preformed fungitoxins.

Prevention of the reaction of gossypol and related terpenoids in fresh root sections with the $SbCl_3$ and DNP reagents by preliminary treatment with the aldehyde-reducing reagent, $NaBH_4$, indicates the in vivo existence of these terpenoids in the aldehyde form.

The confinement of gossypol and related terpenoids to lysigenous glands in the foliar portions of Acala 4-42

seedlings and their localization in epidermal and cortical parenchyma cells of the seedling root constitutes a major difference in the two plant parts. Our histochemical studies of the root stelar tissue and other studies of the stem stelar tissue (6, 7, 25) indicate that healthy stelar tissue of both stem and root are free of gossypol and related terpenoids.

The apparent localization of gossypol and related terpenoids in particles in Acala 4-42 root epidermal and cortical cells resembles the localization of gossypol in the lysigenous glands of cotton seeds (19). A phenolic amine, dopamine (3-hydroxytyramine), occurs in similar discrete cytoplasmic particles in the parenchyma cells of banana roots (5, 17). It is of special interest that dopamine and the closely related phenolic amine, 5-hydroxytryptamine, occur in sheep thyroid gland (2) as cytoplasmic particles much like those containing dopamine in banana roots.

The absence of gossypol and related terpenoids in the first 3-cm of healthy root tips may preclude their contact with *V. dahliae* at the time of initial root cap penetration. The root cap zone appears to be the primary point of infection of Acala 4-42 roots by *V. dahliae* (13). The histochemical localization of gossypol and related terpenoids in 3-day-old Acala 4-42 seedling tap roots comparable to those studied by Garber and Houston (13) was essentially like that described for 1-wk-old seedlings (M. E. Mace, *unpublished*). Once the fungus moves up the xylem vessels approx. 6 cm to the region of cortical localization of gossypol and related terpenoids, permeability of cortical tissues might be altered by the stelar fungal infection and permit the terpenoids to diffuse into the xylem and exert a toxic action on the fungus. The ability of *V. dahliae* to induce additional synthesis of all or part of the five preformed root terpenoids in infected cotton roots needs further study.

Stimulation of the synthesis of gossypol and related terpenoids in the ruptured epidermal tissue immediately around the sites of lateral root emergence may be associated with the reported failure (13) of these areas to serve as significant infection sites for *V. dahliae*.

Minton (18) reported that the root knot nematode (*Meloidogyne incognita acrita*) penetrated the roots of Auburn 56 and Rowden (*Gossypium hirsutum* L.) cotton seedlings principally within the apical 2 cm of the tap root. Histochemical data (M. E. Mace, *unpublished*) similar to that obtained for Acala 4-42 indicates that, as was true for Acala 4-42, the apical 2-3 cm of the tap root in 1-wk-old seedlings of Auburn 56 and Rowden cotton cultivars is free of gossypol and related terpenoids. This suggests that the absence of these terpenoids in the root tip zone may be causally related to the susceptibility of this zone to nematode penetration.

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