Effects of Bioregulators on the Terpenoid Aldehydes in Root-Knot Nematode Infected Cotton Plants†

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Four naturally occurring and two synthetic plant growth bioregulators were surveyed for their effects on terpenoid aldehydes (TAs) that have been associated with defense mechanisms against the root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood (RKN) in cotton (*Gossypium hirsutum* L.). Salicylic acid (2-hydroxybenzoic acid), Pix (BAS 083 = mepiquat chloride = 1,1-dimethylpiperidinium chloride), and Burst (a mixture of cytokinins) increased the terpenoid aldehyde content of cotton roots, whereas kinetin [6-(furfurylamino)purine], indole-3-acetic acid, and gibberellic acid (2,4a,7-trihydroxy-1-methyl-8-methylene-4b-gibb-3-ene-1,10-dicarboxylic acid 1,4a-lactone) had either no significant effect on the TAs or decreased them. If the plants were inoculated with RKN, additional amounts of root gossypol and hemigossypolone were induced by the treatments with salicylic acid, Pix, and Burst.

INTRODUCTION

The root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood (RKN) is a sedentary endoparasite that retards growth and development of cotton (*Gossypium hirsutum* L.) by attacking the root system, causing galling, stunting, and other adverse effects. Gossypol and related cotton plant terpenoid aldehydes (TAs) have been implicated as toxins to cotton insects (Giebel, 1974; Hedin et al., 1988a,b), to RKN (Hedin et al., 1984), and to *Fusarium* wilt (Bell, 1986). Plant growth regulators and related synthetic bioregulators have been shown to have some host plant resistance properties and to elicit increases in the content of various plant allelochemicals. Bioregulators are hormone-like compounds; they may be of either natural or synthetic origin and are applied directly to a target plant as a spray at extremely low concentrations. They can alter growth patterns and nutritional components and increase resistance to different kinds of stress, such as cold, heat, insect attack, and disease (Jung, 1985; Hedin, 1990).

The objective of this study was to examine whether some naturally occurring and synthetic plant growth regulators induced increases in TAs, namely gossypol and hemigossypolone, in RKN-inoculated and -noninoculated lines of glanded and glandless cotton roots.

In previous work, the bioregulators kinetin and Burst (a commercial preparation of naturally occurring cytokinins including zeatins), when applied as foliar sprays to cotton, increased the content of known allelochemicals and evidently also contributed to plant resistance to the tobacco budworm [*Heliothis virescens* (Fab.)] (Hedin and McCarty, 1991) because modest increases in yield were observed. The effects of the plant growth regulator mepiquat chloride [1,1-dimethylpiperidinium chloride (Pix)] on cotton, including its allelochemicals, have been widely studied and have been summarized in a recent review (Hedin, 1990). Bud gossypol was increased, while flavonoids and tannins were slightly decreased. Yields tended to be decreased, but the use of Pix may still be indicated because of enhanced maturity. Other bioregulators (BAS 109, BAS 110, and BAS 111) increased gossypol, tannins, and flavonoids in cotton leaves (Hedin et al., 1988a,b). A recent review on bioregulators (Hedin, 1990) describes their diverse scope and activities.

MATERIALS AND METHODS

Cultivars and Race Stocks. The susceptible (S) cotton lines Aub-201 (glandless) (gl) and Coker-201 (glanded) (GL) and resistant (R) lines 89-8275 (glandless) and M-120 RNR (glanded), were used this study. All of these lines are of the *G. hirsutum* species.

Seeds and Nematode Treatment. The procedures described below with regard to handling of the RKN are those of Shepherd (1979) with only minor variations. The seeds were shaken in 50 mL of ethanol–sterile water (95/5 v/v) for 3 min and then shaken for 10 min in 20 mL of aqueous 25% Clorox containing 0.05% Tween 20. Seeds were rinsed four times with sterile water and then germinated in a seed-pack growth pouch (Vaughan's Seed Co., Downers Grove, IL) containing 5 mL of Hoagland (Hoagland and Arnon, 1938) nutrient and 15 mL of water. Later, they were transferred to 250-cm^3 pots for the nematode tests.

Inoculum was obtained from previously inoculated susceptible cotton roots. When these plants were 40 days old, the roots were harvested and the soil was rinsed away thoroughly with low-pressure water spray. They were then placed in sealable plastic containers and 25 mL of 20% Clorox. Clorox was then added to each root, and they were shaken for 3 min. The Clorox–egg solution was then poured onto a screen (200-mesh over 500-mesh screen), and the Clorox was washed off with water. The *M. incognita* eggs that had been suspended in water were then collected in a small beaker. The number of *M. incognita* eggs per milliliter was determined by counting them under a light microscope. The *M. incognita* eggs hatched over a period of 2 days in aerated water. Approximately 3 days later, 10 000 *M. incognita* juveniles were applied with a medicine dropper to half of the transplanted plants (in 250-cm^3 pots); the other half of the plants were not inoculated and were used as controls. The concentrations as shown in Table I were based on those used as a foliar application in field tests (Hedin et al., 1988a).

Four days after inoculation, six bioregulators were applied to leaves, stems, and roots at two levels to drip off (low and high; see Table I for concentrations) together with two sets of controls, which were also either inoculated or not inoculated with *M. incognita*. The bioregulators were applied at the same levels a

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Table I. Concentrations of Bioregulators Applied to Drip-Off

	g/L	
	low level	high level
IAA	1.0×10^{-2}	1.8×10^{-2}
Pix	2.9×10^{-2}	4.5×10^{-2}
salicylic acid	8.4×10^{-3}	1.7×10^{-2}
Burst	4.5×10^{-2}	9.1×10^{-2}
kinetin	9.1×10^{-3}	3.9×10^{-2}
gibberellic acid	9.1×10^{-3}	1.5×10^{-2}

second time after 1 week. Fifteen days after inoculation, the roots were harvested, rinsed with distilled water, dried by blotting with tissue, weighed, freeze-dried, ground in a Wiley mill (40-mesh screen), and stored at −20 °C in sealed plastic bags.

HPLC Methods. The extracting and HPLC solvents were filtered through a 0.45-μm filter. Extractions were conducted in subdued light. Samples were analyzed with a Waters system which included a 6000A pump, a variable autoinjector Model 712, and a UV–vis detector 490E. The separation of terpenoid aldehydes was performed with a 4.6 mm × 25 cm Hypersil ODS column (Alltech Associates, Inc., Deerfield, IL). The mobile phase of ethanol–methanol–isopropyl alcohol (IPA)–acetonitrile (ACN)–water–ethyl acetate (EtOAc)–dimethylformamide–phosphoric acid (16.70:6.50:12.10:23.75:31.95:3.80:5.10:0.10) (Stipanovic et al., 1988) was monitored at 272 nm at a flow rate of 1 mL/min. All samples were analyzed as replicates of two or more injections of 100 μL. Depending on availability, between 10 and 50 mg of roots was shaken in capped specimen bottles with 15 mL of glass beads (regular 140/170), 10 mL of hexane–EtOAc (3:1, solvent 1), and 100 μL of 10% acetic acid (HOAc) for 1 h. The solution was filtered through Whatman No. 1 filter paper into a 50-mL round-bottom flask, and the residue was rinsed three times with solvent 1. The solvent was evaporated, and the flask was washed with solvent 1 (5 × 1 mL) and transferred to a silica Sep-Pak (Fisher Scientific, Fair Lawn, NJ). The Sep-Pak was dried with nitrogen gas, and the terpenoid aldehydes were eluted with 5 mL of IPA–ACN–H_2O–EtOAc (35:21:39:5), and then 100-μL aliquots were analyzed by HPLC.

Procurement and Preparation of the Standards. Gossypol was provided by the U.S. Department of Agriculture (USDA), Southern Regional Research Center, New Orleans, LA, and hemigossypolone was provided by Dr. Robert Stipanovic (USDA, College Station, TX). Stock solutions were prepared by dissolving 6.9 mg of gossypol and 3 mg of hemigossypolone in 1 mL of methanol, which was then diluted to 50 mL with the HPLC mobile phase. A series of dilutions were made from the stock solution for the calibration curve. Aliquots were analyzed by HPLC.

Procurement of Bioregulators. Pix (mepiquat chloride) was provided by BASF, Ludwigshafen, Germany. Burst (a mixture of cytokinins) was provided by Burst Agritech, Overland Park, KS. Kinetin, salicylic acid, indole-3-acetic acid, and gibberellic acid were procured from Sigma Chemical Co., St. Louis, MO.

Statistical Analysis. Four cultivars of *G. hirsutum* cotton were planted in the greenhouse in a randomized complete block design with three replicates. Data obtained from various analyses and measurements were subjected to the analysis of variance, and least significant difference (LSD) was calculated using SAS (Spatz and Johnston, 1984; SAS, 1985; DiIorio, 1991).

Table II. Root Gossypol of RKN Susceptible and Resistant Cotton Plants 1 Week after Inoculation (Percent of Dry Weight)[a]

			%				
	level	inoc[b]	Coker-201 (GL)	Aub-201 (gl)	M-120 RNR (GL)	89-8275 (gl)	effect of bioregulator,[c] %
no bioregulator		−	0.29	0.30	0.23	0.22	100.0
		+	0.22	0.20	0.11	0.20	(70.2)
IAA	low	−	0.59	0.13	0.19	0.53	138.5
		+	0.23	0.61	0.11	0.30	120.2
	high	−	0.45	0.05	0.22	0.40	107.7
		+	0.40	0.62	0.89	0.22	204.8
		av	0.42	0.35	0.35	0.36	142.8[d]
Pix	low	−	0.46	0.06	0.28	0.11	87.5
		+	0.71	0.83	1.09	0.15	267.3
	high	−	0.58	0.35	0.33	0.44	163.5
		+	0.64	1.12	0.55	0.52	272.1
		av	0.60	0.59	0.56	0.31	197.6
salicylic acid	low	−	0.39	0.30	0.40	0.31	134.6
		+	0.51	0.59	0.57	0.42	201.0
	high	−	0.52	0.18	0.55	0.28	147.1
		+	1.00	0.78	0.81	0.33	280.8
		av	0.61	0.46	0.58	0.34	190.9
Burst	low	−	0.36	0.16	0.40	0.09	97.1
		+		0.67	0.57	0.14	184.0
	high	−	0.40	0.66	0.14	0.78	190.4
		+	0.83	0.50	0.13	0.60	198.1
		av	0.53	0.50	0.31	0.40	167.4
kinetin	low	−	0.37	0.47	0.31	0.16	126.0
		+	0.17	0.18	0.20	0.29	80.8
	high	−	0.42	0.06	0.17	0.04	66.3
		+	0.28	0.23	0.35	0.23	104.8
		av	0.31	0.24	0.26	0.18	94.5
GA	low	−	0.33	0.03	0.26	0.26	84.6
		+	0	0.08	0.55	0.01	85.3
	high	−	0.09	0.29	0.09	0.11	55.8
		+	0.02	0.08	0.04	0.01	14.4
		av	0.11	0.12	0.24	0.10	60.0

[a] Gossypol LSD 0.05 values (%): varieties (comparison between varieties within treatments, i.e., inoculation, level) = 0.06; bioregulator levels (comparison of low and high levels within treatments) = 0.05; RKN inoculation (comparison of bioregulators within a variety at the same level) = 0.04; bioregulators (comparison within a variety in same inoculation state and treatment level) = 0.08. [b] RKN inoculated = +. [c] Average of all varieties; percent of noninoculated control. [d] Average of average.

Table III. Hemigossypolone in Roots of RKN Susceptible and Resistant Cotton Plants 1 Week after Inoculation (Percent of Dry Weight)[a]

			%				
	level	inoc[b]	Coker-201 (GL)	Aub-201 (gl)	M-120 RNR (GL)	89-8275 (gl)	effect of bioregulator,[c] %
no bioregulator		−	0.04	0.08	0.07	0.03	100.0
		+	0.03	0.03	0.02	0.04	(54.5)
IAA	low	−	0.13	0.07	0.02	0.05	122.7
		+	0.05	0.12	0.03	0.04	109.1
	high	−	0.06	0.00	0.02	0.02	45.5
		+	0.06	0.05	0.05	0.02	81.8
		av	0.08	0.06	0.03	0.03	89.8[d]
Pix	low	−	0.06	0.03	0.06	0.02	77.3
		+	0.22	0.14	0.28	0.05	313.6
	high	−	0.13	0.05	0.03	0.04	113.6
		+	0.09	0.11	0.08	0.04	145.5
		av	0.13	0.08	0.11	0.04	162.5
salicylic acid	low	−	0.06	0.12	0.05	0.11	154.5
		+	0.07	0.11	0.24	0.05	213.6
	high	−	0.07	0.03	0.04	0.02	72.7
		+	0.20	0.10	0.17	0.02	222.7
		av	0.10	0.09	0.13	0.05	165.9
Burst	low	−	0.08	0.04	0.06	0.03	95.5
		+		0.11	0.09	0.02	122.2
	high	−	0.11	0.04	0.04	0.01	90.9
		+	0.15	0.09	0.02	0.04	136.4
		av	0.11	0.07	0.05	0.03	111.3
kinetin	low	−	0.04	0.06	0.04	0.01	68.2
		+	0.04	0.03	0.03	0.02	54.5
	high	−	0.03	0.02	0.05	0.01	50.0
		+	0.05	0.03	0.02	0.02	54.5
		av	0.04	0.04	0.04	0.02	56.8
GA	low	−	0.05	0.01	0.02	0.03	50.0
		+	0.01	0.01	0.13	0.02	77.3
	high	−	0.02	0.07	0.02	0.02	59.1
		+	0.03	0.02	0.02	0.01	36.4
		av	0.03	0.03	0.05	0.02	55.7

[a] Hemigossypolone LSD 0.05 values (%): varieties (comparison between varieties within treatments, i.e., inoculation, level) = 0.01; bioregulator levels (comparison of low and high levels within treatments) = 0.01; RKN inoculation (comparison of bioregulators within a variety at the same level) = 0.01; bioregulators (comparison within a variety in same inoculation state and treatment level) = 0.02. [b] RKN inoculated = +. [c] Average of all varieties; percent of noninoculated control. [d] Average of average.

RESULTS AND DISCUSSION

The roots from noninoculated plants, both resistant and susceptible, appeared to be silky white, whereas those of inoculated plants had a large number of galls on their surfaces. These observations, together with results from previous tests, clearly indicate that the juveniles penetrated the roots of both resistant and susceptible plants. However, as previously reported (Shepherd, 1979), only a very few juveniles in the resistant roots became mature and developed into later stages, but practically all of those juveniles entering susceptible roots became mature and developed into reproducing females.

HPLC of the terpenoid aldehyde mixture (see Materials and Methods) afforded six maxima. In addition to the relatively large maxima at 3.05 min for the hemigossypolone and at 12.10 min for gossypol, four other small maxima appear. On the basis of previous work, the peak at 4.12 min is hemigossypol, and those at 7.50 and 8.06 min are gossypolone and gossypol lactone, respectively (Phillips and Hedin, 1990; Hedin et al., 1991). Finally, there was an unidentified maximum with a slightly longer retention time than gossypol. Heliocides, though prominent in leaves (Stipanovic et al., 1988), have not been reported to be present in roots. They were searched for, but were not found in this study.

The percents gossypol and hemigossypolone in roots from cotton plants treated two times with two levels of six bioregulators (see Materials and Methods) and controls are presented in Tables II and III. The genotypes included glanded-susceptible (Coker-201), glandless-susceptible (Aub-201), glanded-resistant (M-120 RNR), and glandless-resistant (89–8275).

Statistical information is also given in Tables II and III. LSD 0.05 values are given with explanations about permitted comparisons as footnotes in the tables.

While leaf and bud gossypol and hemigossypolone in glandless lines are very low (Hedin and McCarty, 1990), significant quantities of these TAs are known to be present in roots (Hedin et al., 1984), and this was confirmed in the present study (Tables II and III, line one).

To simplify the assessment of the effects of the bioregulators on the TA concentrations as presented in Tables II and III, the data for all varieties were summed and normalized to percent of the noninoculated control (line one), which is the overall experimental baseline. The determined effects of the bioregulators would actually be about 30% greater than shown if the decreases in TA concentrations upon inoculation had been considered in the calculation. The normalized data show that gossypol was increased by Pix (197.6% of the control), salicylic acid (190.9%), Burst (167.4%), and IAA (142.8%), while kinetin (94.5%) had no effect and GA (60.0%) had a negative effect (Table II). Upon treatment with Pix, salicylic acid, Burst, and IAA, the average increase in gossypol was from about 0.26% for the noninoculated control to 0.54% (Coker-201; GL-S), 0.48% (Aub-201; gl-

S), 0.45% (M-120 RNR; GL-R), and 0.35% (89-8275; gl-R). Thus, gossypol was increased somewhat more in susceptible varieties by the four bioregulators. The gossypol also tended to be increased somewhat more in the glanded than in the glandless varieties.

For confirmation, the overall effects of bioregulators on the TAs of each line were evaluated by summing and averaging (Tables II and III) the analyses of inoculated and noninoculated roots treated at both the low and high levels. As expected, the root TA contents of glanded lines, both susceptible and resistant, were generally higher than in their related glandless lines (isolines).

The effects of the bioregulators on hemigossypolone were generally similar to that of gossypol, although not as large. Hemigossypolone was increased by Burst (167.4% of the control), salicylic acid (165.9%), and Pix (162.5%), while kinetin (94.5%) and IAA (89.8%) had no effect and GA (60.0%) had a negative effect. Upon treatment with Pix, salicylic acid, and Burst, the average hemigossypolone content was 1.1% (Coker 201; GL-S), 0.8% (Aub-201; gl-S), 1.0% (M-120 RNR; GL-R), and 0.4% (89-8275; gl-R). These contents are somewhat higher than those of the noninoculated controls (0.03–0.08%). The susceptible noninoculated varieties tended to be higher than the resistant varieties in hemigossypolone.

The lower level of Pix did not increase gossypol above the noninoculated control level, but gossypol increased by about 60% at the higher level. With inoculated plants, the gossypol content was about 270% of the control at both levels. The pattern with salicylic acid was similar in that both the low and high levels of treatment increased the gossypol content to about 140% of that in noninoculated plants, but to over 200% of the control with inoculated plants. We did not find any report on the effect of salicylic acid on the allelochemicals in cotton or any other crop plant. The pattern of Burst was more similar to that of Pix in that gossypol was not increased in noninoculated plants at the lower level but was doubled at the higher level and at both levels in the inoculated plants (Table II). Generally, higher levels of hemigossypolone were observed with the higher levels of bioregulators Pix, salicylic acid, and Burst, both with inoculated and with noninoculated plants (Table III).

In leaves, hemigossypolone and the heliocides H_1, H_2, H_3, and H_4 are major TAs, with gossypol present in lesser concentrations (Stipanovic et al., 1988; Hedin et al., 1991). However, the major terpenoid aldehyde in roots is gossypol, with the other terpenoids present as minor constituents (Hedin et al., 1984; Stipanovic et al., 1988). Only traces of heliocides have been found in the roots of cotton plants (Hedin et al., 1984). In previous work, kinetin, Burst, BAS 109, BAS 110, and BAS 111 induced increases in the gossypol content of cotton leaves and buds, but the increases, though statistically significant, generally were not greater than 20% (Hedin et al., 1988a,b, 1991).

Salicylic acid is a metabolite located in the phloem and is found at higher concentrations in leaves infected with a necrotizing pathogen (Kuc, 1982; Gaudin et al., 1990). It has been suggested that salicylic acid can act as an endogenous signal in the transmission of systemic acquired resistance (Malamy et al., 1990). Also, application of exogenous salicylic acid to some cultivars of tobacco significantly increased resistance of the treated areas to TMV and to some other viruses (Malamy et al., 1990).

This study showed that Pix, Burst, and salicylic acid elicited increases of gossypol and hemigossypolone in two glanded and two nonglanded lines (one each of these susceptible and resistant). If the plants were inoculated with RKN, additional amounts of gossypol and hemigossypolone were induced.

These results provide some hope for developing crop strategies in which bioregulators may be applied in fieldtests as a spray to the plants, resulting in increased resistance against pathogens and other pests, thereby improving yield and crop quality. There is evidence from this work that bioregulators elicit the biosynthesis of allelochemicals such as gossypol and hemigossypolone in the cotton plant.

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