Free Sugars in Root-Knot Nematode Susceptible and Resistant Cotton Plant Roots and Leaves†

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The sugars glucose, fructose, sucrose, and raffinose were present in both roots and leaves of root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood (RKN)] susceptible and resistant cotton (*Gossypium hirsutum* L.). Raffinose is reported in cotton roots and leaves for the first time, although it has previously been reported in cottonseed meal. In general, there was more glucose in leaves than in roots of both RKN resistant and susceptible cotton, but roots contained more sucrose. Leaf glucose was higher in RKN susceptible plants. The sharply increased glucose in susceptible leaves after inoculation may occur because the RKN has decreased the mass of the root system by feeding, thereby limiting opportunity for translocation of glucose to the root. Roots of resistant plants contained more raffinose than susceptible roots. Sucrose and raffinose were lower in roots after inoculation of resistant plants, and raffinose was also lower in roots after inoculation of susceptible plants.

INTRODUCTION

Root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood (RKN)] retards growth and reproduction of cotton (*Gossypium hirsutum* L.) by attacking the root system, causing galling of the roots and other debilitating effects. Since 1965, work to breed cotton genotypes resistant to RKN has resulted in the development and release of Auburn 623 RNR, Auburn 634 RNR, M-120 RNR, 89-8275, and several other RKN resistant strains (Shepherd et al., 1988). Resistant varieties can limit RKN reproduction to less than 1000 eggs per plant at 40 days following inoculation of seedling plants with 10 000 *M. incognita* eggs per plant (Shepherd et al., 1988).

In recent studies, oligosaccharides have been implicated in the stimulation of the biosynthesis of phytoalexins, which have been shown to contribute to plant defense (Albersheim and Darvill, 1990). The trisaccharide raffinose generally accumulates in storage organs such as seeds (Shiroya, 1962), but it is also found in roots and leaves in small quantities (Drey, 1990).

Raffinose occasionally occurs in leaves of some plants during the winter in concentrations as high as that of sucrose (Shiroya, 1962). Raffinose and other oligosaccharides may also serve as frost resistance factors in plants (Miller, 1973). Previously, it was shown that cotton plants contain significant levels of protein, sugars, and other nutrients (Hedin and McCarty, 1990). The goal of this study was to identify free sugars in RKN susceptible and resistant cotton and to investigate whether there was a relationship between host plant resistance and their concentrations. High-performance liquid chromatography (HPLC) and spectrophotometric procedures were used to analyze underivatized free sugars in roots and leaves.

MATERIALS AND METHODS

Cultivars and Race Stocks. The RKN susceptible (M-8) and resistant (Aub-634) cotton lines developed by Shepherd (1979) were used in this study.

Greenhouse Methods and Harvest of Samples for Analysis. The following procedures for harvesting RKN-inoculated tissues were developed by Shepherd (1979). Cotton seeds of the susceptible and resistant lines listed above were planted in a greenhouse in autoclaved soil that either had been inoculated or had not been inoculated with 10 000 *M. incognita* eggs. The seeds were planted in 250-cm^3 pots in triplicates with four plants in each replicate. Beginning 1 week after emergence, the roots and the tops were harvested, cleaned thoroughly with water to remove dirt and nematode eggs, and dried by blotting with tissue, and their approximate weights were determined. The roots and the leaves were freeze-dried separately as soon as possible after harvesting to limit enzymatic activity and then ground in a Wiley mill (40-mesh screen) to a fine powder and stored at −20 °C in sealed plastic bags. The samples were harvested at 5-day intervals during a 3-week period. These tests were replicated three times and subjected to the described statistical analysis.

Preparation of Samples and Standards for HPLC Analysis. Depending on availability, between 0.3 and 0.6 g of roots and between 0.2 and 0.4 g of leaf powders were extracted with 10 mL of 80% aqueous ethanol at 80 °C for 30 min. After cooling to room temperature, the extracts were filtered and the residues were extracted twice more. The extracts were combined and evaporated to dryness under reduced pressure in a rotary evaporator. The residues were redissolved in acetonitrile–water (60/40 v/v), cooled to 4 °C, and filtered through 0.45-μm Millipore membranes prior to HPLC analysis.

The standard sugars were obtained from Sigma Chemical Co., St. Louis, MO. Acetonitrile of analytical grade was freshly distilled. Stock solutions were prepared by dissolving glucose (0.6339 g), sucrose (0.6569 g), fructose (0.4596 g), and raffinose (1.1149 g) in 1 mL of water and then diluted to 25 mL in a volumetric flask with acetonitrile–water (60/40 v/v). A series of dilutions from the stock solutions were analyzed by HPLC to construct a calibration curve.

HPLC Analyses. HPLC was conducted with a Waters system, which included a 6000A pump, a variable loop autoinjector Model 712, and a UV–vis detector 490E at 195 nm. The separation of sugars was performed with a 4.6 mm × 25 cm Spherisorb NH_2 column (Isco, Inc., Lincoln, NE) using acetonitrile–water (80/20 v/v).

Quantitative Analysis of Total Sugars by the Anthrone Method. Depending on availability, between 0.05 and 0.10 g of ground root samples was suspended in 5 mL of 80% aqueous ethanol and heated for 30 min at 80 °C in a water bath. The solutions were then cooled to room temperature and filtered through Whatman No. 1 filter paper with rinsing. The extraction

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Table 1. Sugars (Milligrams per Gram) in Roots of Susceptible and Resistant Cotton 4–20 Days after Inoculation with (+) or without (−) RKN As Determined by HPLC

days after inoc	M-8		Aub-634	
	−	+	−	+
	Glucose			
4	1.2	27.0	1.9	16.3
8	12.2	19.2	4.3	14.2
12	19.7	9.6	14.3	30.0
16	42.2	119.4	38.4	35.4
20	22.6	50.7	17.9	13.6
lsd 0.05	2.3	3.6	2.8	3.9
av	19.6	45.2	15.4	21.9
	Sucrose			
4	163.9	326.5	217.7	167.3
8	102.9	136.5	251.9	157.4
12	137.3	186.8	110.2	109.8
16	362.8	226.3	190.5	137.9
20	233.2	203.0	267.2	71.0
lsd 0.05	15.0	17.9	15.4	13.9
av	200.0	215.8	207.5	128.7
	Raffinose			
4	30.3	4.6	66.7	23.5
8	0.1	1.4	16.8	3.0
12	1.0	0.3	3.9	10.3
16	5.8	3.0	62.0	7.6
20	5.2	0.0	12.2	3.0
lsd 0.05	1.4	1.0	4.7	2.0
av	8.5	1.9	32.3	9.5
total sugars, av	228.1	262.9	255.2	160.1

Table 2. Sugars (Milligrams per Gram) in Leaves of Susceptible and Resistant Cotton 4–20 Days after Inoculation with (+) or without (−) RKN As Determined by HPLC

days after inoc	M-8		Aub-634	
	−	+	−	+
	Glucose			
4	151.1	225.2	141.7	97.3
8	335.5	601.2	362.2	163.6
12	258.3	514.8	446.1	221.1
16	161.0	569.3	100.3	119.9
20	239.5	316.2	158.5	119.8
lsd 0.05	12.8	22.2	10.6	9.4
av	229.1	445.3	241.8	144.3
	Sucrose			
4	10.5	9.7	9.2	7.7
8	8.2	21.8	0.1	6.1
12	5.6	0.1	3.9	14.9
16	14.1	8.6	8.3	31.5
20	0.1	4.9	5.7	6.1
lsd 0.05	1.1	1.2	0.9	1.9
av	7.7	9.0	5.4	13.3
	Raffinose			
4	5.0	7.6	1.5	12.7
8	25.3	13.1	26.6	20.4
12	3.7	17.7	6.7	9.2
16	3.8	2.7	5.5	2.9
20	1.0	6.4	2.6	1.9
lsd 0.05	1.2	1.5	2.0	2.0
av	7.8	9.5	8.6	9.4
total sugars, av	244.6	463.8	255.8	167.0

was repeated twice more, and the extracts were then combined, concentrated to a minimum, and then made up to 10 mL with 80% aqueous ethanol. At the same time, a stock solution was prepared by dissolving 0.1943 g of glucose in 1 mL of water and made up to 50 mL with 80% aqueous ethanol in a volumetric flask. A series of dilutions were made from the stock solution, and 10 mL of anthrone solution was added to each sample and standard. The solutions were then heated for 10 min at 80 °C, after which time they were cooled and the absorbancies determined at 620 nm with a Perkin-Elmer Lambda 4B UV–vis spectrophotometer for subsequent calculation of percent total sugars.

Statistical Analysis. RKN susceptible cotton line M-8 (glanded) and RKN resistant line Aub-634 (glanded) were planted in a randomized complete block design with three replicates in time. Data obtained from various analyses and measurements were subjected to an analysis of variance, and least significant difference (lsd) values were calculated according to SAS (1985).

RESULTS AND DISCUSSION

The HPLC analyses for both roots and leaves gave four major peaks. By comparison with standards, they were found to be glucose (3.1 min), fructose (3.7 min), sucrose (4.6 min), and raffinose (7.4 min), in order of elution. All peaks were assigned by spiking the sample with the pure standard. They were also consistent with the retention times and the order of elution reported by the column fabricator (Isco). Finally, silica gel TLC in which the solvent system 1-propanol–acetic acid–water (100/1/1) was employed supported the identification of raffinose. Data obtained from analyses of the sugars glucose, sucrose, and raffinose and statistical analyses are presented in Tables 1–3. Data for fructose, present in very low amounts (about 10% or less of glucose) in all treatments, are not included. As previously described, the roots were thoroughly washed and rinsed to remove the nematode eggs prior to analysis. However, the contribution of the nematode eggs to sugar content would have been negligible at greatest, given that they do not initially exceed 1% of the root mass.

Table 3. Total Sugars (Milligrams per Gram of Dry Weight; Cumulative HPLC and Anthrone Procedures) in Roots and Leaves of RKN Susceptible and Resistant Cottons 4–28 Days after (+, −) Inoculation with RKN

procedure/sample[a,b]	M-8		Aub-634	
	−	+	−	+
	Roots			
HPLC/days 4–20	228.1	262.9	255.2	160.1
anthrone/days 8–16	249.6	257.0		228.0
anthrone/day 28	173.0	174.5	166.5	152.0
	Leaves			
HPLC/days 4–20	244.6	463.8	255.8	167.0

[a] HPLC data transcribed from Tables 1 and 2; anthrone data are an average of analyses in triplicate. [b] −, noninoculated; +, inoculated.

Enzymatic conversion of sucrose and other sugars to monomers was minimized by prompt processing and freeze-drying of the roots.

Sucrose was not significantly different in RKN susceptible and resistant cotton plant roots before inoculation (200.0 and 207.5 mg/g). Glucose was not significantly different in RKN susceptible and resistant cotton leaves before inoculation (229.1 and 241.8 mg/g). Glucose was low and also not significantly different in susceptible and resistant roots before inoculation (19.6 and 15.4 mg/g), and sucrose was likewise not significantly different in susceptible and resistant leaves before inoculation (7.7 and 5.4 mg/g).

There was more glucose in leaves than in roots (265.1 vs 25.5 mg/g; average of all treatments), and there was more sucrose in roots than in leaves (188.3 vs 8.9 mg/g; average of all treatments). The glucose was increased in roots of susceptible plants after inoculation (19.6 vs 45.2 mg/g) but was not significantly increased in resistant roots after inoculation (15.4 vs 21.9 mg/g). Glucose was much increased in leaves of susceptible plants after inoculation (229.1 vs 445.3 mg/g) but markedly decreased in leaves after inoculation of resistant plants (241.8 vs 144.3 mg/g).

Sucrose was not significantly different in roots of susceptible plants after inoculation (200.0 vs 215.8 mg/g) but markedly decreased in roots of resistant plants after inoculation (207.5 vs 128.7 mg/g). The sucrose content in leaves was too low (range 5.4–13.3 mg/g) in all treatments for any trends to be evident.

Raffinose was higher in resistant than susceptible roots, both before and after inoculation. Its decrease after inoculation of resistant plants paralleled that of sucrose (S non = 8.5, S inoc = 1.9, R non = 32.3, R inoc = 9.5 mg/g). The content of raffinose in leaves of both susceptible and resistant plants, before and after inoculation, was essentially the same (range 7.8–9.5 mg/g).

Total sugars as analyzed by the HPLC and anthrone procedures gave generally similar results (Table 3) with values ranging from 15 to 46% of dry weight. These data are similar but somewhat higher than that for cotton anthers (12.3%) but of the same order of magnitude (Hedin and McCarty, 1990) except for sugars in leaves of susceptible plants after inoculation (4–20 days). This increase may signify a plant in stress. This increased sugar content in leaves of susceptible inoculated plants may occur because the RKN have decreased the root system by feeding, thereby limiting opportunity for translocation of sugars to the roots. As noted in Tables 1 and 2, the contributions of glucose and sucrose in roots and leaves are inversely related. The somewhat lower content of sugars present in inoculated Aub-634 roots and leaves is supported by the differences noted in Tables 1 and 2 for individual sugars.

A review of the literature did not reveal any report concerning the presence of raffinose in roots and leaves of cotton plants, although it has been reported in cottonseed meal. In general, total sugar concentrations obtained by HPLC were higher than those obtained by the anthrone method. The present inability to rear this nematode apart from the intact root limits the capability to directly evaluate the effects of sugars on growth and development.

Raffinose is found in storage organs of seeds (Albersheim and Darvill, 1990), and it was found for the first time in both roots and leaves of the cotton cultivars studied above. Sucrose and raffinose were lower in roots after inoculation of resistant plants, and raffinose was also lower in roots after inoculation of susceptible plants. This suggests that the RKN may be utilizing these storage sugars for growth and reproduction. They could also have a function other than as a carbohydrate reserve because oligosaccharides are strongly implicated as major regulatory molecules and chemical messengers in plants (Drey, 1990).

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