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IDENTIFICATION OF SOME ENDOGENOUS METABOLITES IN THE LEAVES OF DWARF AND TALL LINES OF THE COTTON PLANT

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The components of cottonplant leaves have been studied. A GC-MS analysis has been made of the secondary metabolites (SMs) in the total extractives of the leaves of cotton plants with different phenotypic characteristics. It has been found that a change in the genotype leads to a change in the composition of the SMs.

At the present time, the regulation of the growth of plants is considered in close connection with the hormonal regulation and functions of the genetic apparatus. Here an important role belongs to natural growth regulators. The synthetic plant growth regulators used in the majority of cases change the balance of endogenous regulators and may be toxic for it.

In view of this, there is definite interest in the investigation of the components of the leaves of cotton plants with different genetic characteristics (tallness, dwarfism) in which the mechanism of the self-regulation of both the growth and the development of the plants is genetically based.

We have investigated plant material, beginning from the cotyledon stage, when no difference is observed between any of the lines in the change in height of the shoots; in the flowering–budding phase with the most substantial differences in the growth of the stems; and in the ripening period. On the basis of literature reports of a high level of inhibitors in dwarf varieties of peas, beans, and other crops [1], we decided first of all to make a search for them among dwarf and short lines of the cotton plant.

Extracts of the leaves of cotton plants of the varieties L-501, L-650 (dwarf mutants) and L-463 (tall control) (the lines were obtained by M. F. Abzalov, Tashkent State University) were separated by preparative TLC into 10 zones, each of which was subjected to biotesting separately on wheat coleoptiles and cotton explants [2]. The results obtained revealed three growth-inhibiting zones — 6, 7, and 10 — for L-501, and one zone — 8 — for L-463. Zones 6, 7, 8, and 10 for L-463, and 8, 6, 7, 10 for L-501, were studied mass-spectrometrically in order to compare their compositions. The results are presented in Table 1.

In the case of the dwarf line, three zones possessed an inhibiting action, even though each line contained different compounds. The most active in inhibition proved to be zone 10, in which such groups of compounds as tocopherols, sterols, and the triterpenoid amyrin were detected mass-spectrometrically. It must be mentioned that we are the first to have detected α-tocopherol (α-T) in cottonplant leaves [3]. It appeared of interest to determine its amount in the dwarf lines L-501 and L-650 and in the tall lines 108-F and L-463 in various phases of their development.

Since the active zones included components of the total unsaponifiable substances, we made quantitative measurements of them by the ion-current integration method [4]. The results obtained are given in Table 2. The levels of α-T were different in the two dwarf lines, in one case being higher and in the other lower than in the tall line. To obtain a complete picture of the composition of the components and a semiquantitative estimate, we used the GC-MS of the initial total materials and of

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TABLE 1. Compositions and Inhibitory Activities of the Chromatographic Zones

Zone	R_f	Inhibition of growth, %		Mol. mass, a.m.u.	Characteristic fragmentary ions	Elementary comp. of the mol. ions	Class
		wheat coleoptiles	cotton explants				
6	0.71	20**	32**	286	257,237	$C_{15}H_{10}O_6$	Flavan
				302	273,153	$C_{15}H_{10}O_7$	
				304	–	$C_{15}H_{12}O_7$	Flavan-one
7	0.85	37**	39**	300	285	$C_{20}H_{28}O_2$	Diter-penes
				314	299	$C_{21}H_{30}O_2$	
8	0.93	42*	45*	388	180,167	$C_{21}H_{24}O_7$	
				416	–	$C_{21}H_{20}O_9$	
				418	210,137	$C_{22}H_{26}O_8$	
				420	–	$C_{22}H_{28}O_8$	
10	1.0	47**	65**	416	–	$C_{28}H_{48}O_2$	Isopre-noids
				430	165	$C_{29}H_{50}O_2$	
				446	165	$C_{29}H_{50}O_3$	
				426	218	$C_{30}H_{50}O$	
				414	–	$C_{29}H_{50}O$	
				412	–	$C_{29}H_{48}O$	
				400	–	$C_{28}H_{48}O$	
				386	–	$C_{27}H_{46}O$	

*L-463.
**L-501.

TABLE 2. Proportion of α-Tocopherol in the Total Unsaponifiable Substances of Cottonplant Leaves

Line of cotton plant	Phase	Yield of unsaponifiable substances, g	α-Tocopherol content, % of the total
L–463	Cotyledons	0.034	0.500
L–501	Cotyledons	0.034	0.720
L–463	Maturation	0.095	1.200
L–501	Maturation	0.102	1.800
108–F	Cotyledons	0.077	0.730
L–650	Cotyledons	0.061	0.570
108–F	Maturation	0.096	0.890
L–650	Maturation	0.076	0.750

TABLE 3. Mass Numbers of the M^+ Ions and Nature of the Components of the Total Unsaponifiable Substances from L-650

Peak No.	M^+ of the ion	Compound
1	256	$C_{13}H_{27}COOC_2H_5$
2	284	$C_{15}H_{31}COOC_2H_5$
3	298	$C_{16}H_{33}COOC_2H_5$
4	296	$C_{20}H_{40}O$
5	306	$C_{17}H_{29}COOC_2H_5$
6	312	$C_{17}H_{35}COOC_2H_5$
7	340	$C_{19}H_{39}COOC_2H_5$
8	368	$C_{21}H_{43}COOC_2H_5$
9	382	$C_{22}H_{45}COOC_2H_5$
10	396	$C_{23}H_{47}COOC_2H_5$
11	410	$C_{30}H_{50}$
12	408	$n-C_{29}H_{60}$
13	430	$C_{29}H_{50}O_2$
14	414	$C_{29}H_{50}O$

these materials after their treatment with silylating reagents. Analysis of the mass spectra obtained at the apices of chromatographic peaks 1-14 showed that the main components of the mixture were fatty acid ethyl esters. The ethylation of

TABLE 4. Mass Numbers (*m/z*) of the M^+ Ions, Nature of the Components of the Total Unsaponifiable Substances from L-463 and L-501 Leaves, and Contributions of the Corresponding Spectra to the Total Ion Current

Peak No.	M^+ ion	Compound	Contribution to the total ion current	
			L–463	L–501
1	226	TMS ether of a monoterpene alcohol	0.5	0.7
2	256	$C_{13}H_{27}COOC_2H_5$	0.1	0.2
3	300	$C_{13}H_{27}COOSi(CH_3)_3$	0.7	0.4
4	284	$C_{15}H_{31}COOC_2H_5$	12.6	9.5
5	328	$C_{15}H_{31}COOSi(CH_3)_3$	18.5	11.3
6	306	$C_{17}H_{29}COOC_2H_5$	10.6	6.6
7	368	$C_{20}H_{40}OSi(CH_3)_3$	16.1	20.3
8	352	$C_{17}H_{31}COOSi(CH_3)_3$	0.3	–
9	350	$C_{17}H_{29}COOSi(CH_3)_3$	4.3	6.8
10	354	$C_{17}H_{33}COOSi(CH_3)_3$	2.4	6.7
11	356	$C_{17}H_{35}COOSi(CH_3)_3$	3.9	2.2
12	324	n- $C_{23}H_{48}OSi(CH_3)_3$	1.1	–
13	340	$C_{17}H_{35}CHOSi(CH_3)_3$	0.9	1.1
14	338	n- $C_{24}H_{50}$	0.8	2.0
15	384	$C_{19}H_{39}COOSi(CH_3)_3$	–	0.8
16	352	n- $C_{25}H_{52}$	0.8	1.9
17	366	n- $C_{26}H_{54}$	0.5	–
18	410	$C_{30}H_{50}$	1.5	5.3
19	440	$C_{23}H_{47}COOSi(CH_3)_3$	–	–
20	408	n- $C_{29}H_{60}$	–	1.2
21	302	$C_{29}H_{54}OOSi(CH_3)_3$	5.2	1.4
22	482	$C_{27}H_{55}CH_2OSi(CH_3)_3$	0.2	0.5
23	484	$C_{29}H_{47}OSi(CH_3)_3$	0.2	0.5
24	486	$C_{29}H_{49}OSi(CH_3)_3$	9.6	6.3
25	484–510	$C_{29}H_{47}OSi(CH_3)_3$–$C_{30}H_{61}OSi(CH_3)_3$	1.3	2.9
26	498	$C_{30}H_{49}OSi(CH_3)_3$	30.6	2.9

the fatty acids took place during the extraction of the leaves with ethanol. Table 3 gives the *m/z* values of the M^+ ions appearing in the spectra of all the chromatographic peaks. The most widely distributed components were ethyl palmitate (peak 2) and ethyl linolenate (peak 5). Ethyl esters of other acids were represented by weak peaks (1, 3, 6-10). Peaks of medium height corresponded to the diterpene alcohol phytol (peak 4) and to squalene (peak 11). The two latter compounds are intermediates in the biosynthesis of a number of very important classes of compounds present in all plants — triterpenoids, steroids, chlorophylls, and tocopherols [5]. The mass spectrum of peak 13 corresponded to free α-tocopherol, and that of peak 14 to sitosterol.

The unsatisfactory separation of the sterol components compelled us to study their TMS derivatives. Since the synoptic mass spectra showed that there were no qualitative differences between the lines, the study of the TMS derivatives was carried out on silylated samples from L-501 and L-463, as examples. There were 30 peaks with identical respective retention times, 26 of which were identified from their mass spectra. The overwhelming part of the ion currents of the chromatograms related to the ethyl and TMS esters of the fatty acids that were present in the unsilylated material and to the TMS ether of phytol. Among the acids, palmitic predominated. In both samples, the areas under peaks 4 and 5 (ethyl and TMS esters, respectively, of palmitic acid) exceeded the area under peak 7 of the TMS ether of phytol. The areas under the peaks corresponding to linolenic acid (peaks 6 and 9), although still large, were smaller than that for palmitic acid. By computer processing it was possible to obtain information on the presence of the TMS derivatives of three C18 acids in an unresolved triplet of peaks (8 — 18:0; 9 — 18:3; 10 — 18:1). Peak 11, of the TMS ester of stearic acid, issued separately. The central part of the chromatogram contained a series of small peaks (12-17, 19, 20) representing higher fatty acids TMS esters and *n*-paraffins (Table 4). In this part of each of the two chromatograms peak 18, corresponding to squalene, stood out. The areas of the peak (21) for the TMS ether of α-tocopherol differed considerably in the two chromatograms. The contribution of the ion current of this spectrum amounted to 5.2% of the total ion current in the case of the material from L-463 and to only 1.4% in the case of L-501.

The most interesting proved to be the high-temperature section of each chromatogram, containing four peaks of different intensities. The weak peak 23 corresponded to the mass spectrum of a compound with *m/z* M^+ 484, which, judging from the presence of the peak of an ion with *m/z* 75 and (M − 90) was the TMS derivative of a compound with a mol. mass of 412. A direct comparison of the spectra with those in an atlas [6] pointed to the TMS ether of stigmasterol (stigmasta-5,22-dien-3-ol). The most characteristic peaks for this compound, with *m/z* 129, 255, and 343, were present with high intensities in spectrum 23.

The mass spectrum of the strongest peak in this region — peak 24 — showed M^+ 486. From all its characteristic it corresponded to the TMS ether of sitosterol. In the mass spectrum of peak 25 there were indications of the presence of two compounds. By special computer treatment of this section of the chromatogram the mass spectra of the two substances were actually obtained. The first had the peak M^+ 484 and fragmentary ions with *m/z* 386 (100), 296 (83), 281 (40), and 257 (22). This spectrum was closest to the spectrum of stigmasta-5,24(28)-dien-3-ol (fucosterol or isofucosterol), described for the first time in [7, 8]. The formation of the maximum ion in this spectrum with *m/z* 386 is the result of cleavage of the C-22-C-23 bond with migration of hydrogen to the fragment split out. The subsequent elimination of $(CH_3)_3SiOH$ led to the ion with *m/z* 296. It must be mentioned that the existence of a sterol with a π-bond at C-24 was predicted from an analysis of the synoptic mass spectra of the total extractive substances on the basis of the appearance in them of the peak of a *m/z* 314 ion, which is characteristic for such compounds [8]. However, the molecular mass of this compound was not determined because of the low intensity of the M^+ peak. The results obtained permitted the conclusion that it was 412 for the unsilylated sterol. The structure of this compound corresponds to 24-ethylidenecholesterol since the orientation of the substituent at C-24 was not established.

In the region of high mass numbers the second compound from the spectrum of chromatographic peak 25 had an intense peak of an ion with *m/z* 495. The corresponding spectrum was similar to the spectrum of peak 22, containing an intense peak of an ion with *m/z* 467 and identified as the TMS ether of octacosanol. On this basis, the compound corresponding to peak 25 was the TMS ether of triacontanol with a molecular mass of 510 — an aliphatic alcohol widely distributed in plants [9]. The ion with *m/z* 495 corresponded to $(M-15)^+$, which is characteristic of TMS ethers.

Finally, the spectrum of peak 26 with M^+ 498 and the maximum peak with *m/z* 218 unambiguously showed that it was the TMS ether of olean-12-en-3-ol, β-amyrin.

Thus the natures of all 26 chromatographic peaks have been determined (Table 4).

EXPERIMENTAL

The synoptic spectra were taken on an Mkh 1310 instrument with SVP-5 direct sample injection, at a temperature of 120°C.

Elementary compositions were measured and MD spectra were obtained as described in [10].

GC-MS spectra were obtained on a Finnigan 8200 chromato-mass spectrometer (USA) with a Varian 3700 chromatograph.

The capillary column had a diameter of 0.2 mm and a thickness of the cross-linked phase of 0.3 μm, the carrier gas being helium. Programming of the sample temperature: I: 170°C/2 10°C 290°C/4 (isothermal at 170°C/2, then rising to 290°C/4 at the rate of 10°C/min, isothermal at 290°C/4); sample II: 160°C/2 6°C 290°C/15.

The silylation of the L-463 and the L-501 totals was carried out with M TFA mixture at 60°C for 20 min.

The total extractive substances were isolated as in [10, 11]. The total material extracted was separated on 20 × 20 Silufol UV-254 plates. Since the plates contained a number of compounds interfering with separation and fluorescing in UV light, before use they were washed with 96% ethanol.

We found that the best charge for a clear separation of the zones was 0.03 g per plate. Isopropanol–ammonia–water system. Time of migration of the solvents at 20-25°C was 1 h 40 min to 2 h. After evaporation of the solvents, the plates were examined in UV light. A clear separation of colored bands, making 10 zones, was achieved. Each zone was scraped off and extracted with methanol; the solvent was evaporated off and the residues were biotested separately by the method described in [12].

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