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COMPOSITION AND INHIBITING ACTION OF A FRACTION OF SECONDARY METABOLITES FROM COTTON PLANT LEAVES

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The qualitative and quantitive compositions of fractions from the leaves of two self-pinching-out lines of cotton plant and of the standard variety 108-F have been determined in parallel by mass chromatography, and differences in their contents of the main metabolites have been determined. A fraction of the secondary metabolites (SMs) obtained from mature leaves of a cotton plant of the self-pinching-out line L-49 has shown a retardant activity in treatment of the growth points of plants of the Tashkent-1 variety.

One of the promising directions in the breeding of the cotton plant is obtaining lines with autonomous regulation of the growth of the main stem. The line (*G. hirsutum* L.) L-249 isolated in the Biolog Scientific Production Combine [1] possess the property of "self-pinching-out" — on the accumulation of 12-16 sympodal branches a blocking of the genes responsible for the growth process takes place, which leads to the cessation of the growth of the main stem, and the growth point completes its development by the formation of bolls. This line was obtained as a result of the hybridization of the ultraprecocious (100 days) low (60 cm) variety AN-Chelyaki-1 with the late-ripening (150-160 days) tall (180-200 cm) line L-598. The height of the main stem of L-249 is 70-90 cm, leaves small with 3-5 blades, weight of a boll 6-7 g, vegetation period 120-130 days, yield 3500-4500 kg/ha.

The peculiar nature of the ontogenesis of the line L-249 and its analogs that has been shown is attractive from the economic point of view, since it permits the elimination from cotton cultivation technology of the laborious and extremely expensive operation of mechanical pinching out and enables the bolls of the upper levels of the plant to be retained, which gives an increase in yield of of the order of 400-600 kg/ha.

In the light of the fact given above, it appeared to us to be of interest to isolate from plants of the L-249 line a fraction of the components responsible for their inhibiting activity and to test its exogenous action on a plant of the standard variety in a phase coinciding with the period of the cessation of the growth of L-249 and subsequently investigating the composition of this fraction. The starting point for the search was the experience that we had accumulated on the identification of the SMs in cottonplant leaves [2-6] and on establishing the differences in their quantitative levels and the dynamics of their accumulation in the phases of ontogenesis in materials from lines with different genetic [3] and selection [2] characteristics, and also the positive results of trials of the defoliant activity of SM fractions obtained from leaf-shedding lines of the cotton plant.

The material for studying the composition and extracting the active principle consisted of mature leaves of plants of the L-249 line. Part of the dry residue (fraction F-1) obtained as described in [2] was used for analysis in parallel with the total material obtained from obtained from the standard variety 108-F under identical conditions. Another part of F-1 was treated with 50% aqueous KOH and was then extracted with benzene [4]. The dry residue (fraction F-2) was used for obtaining a preparative form in field trials.

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TABLE 1. Change in Height and in the Percentage Inhibition of the Main Stem and in the Number of Bolls in Plants of the Tashkent-1 Variety in the 1991 Season

Variant of the treatment	Mean height of the stem						Inhibition, %		Number of bolls					
	01.08		15.08		30.08		15.08	30.08	15.08			10.09		
	cm	%	cm	%	cm	%			total opened		%	total opened		%
0.2 ml of 0.5% × 5, on Aug. 1, 2, 5, 7, and 9	92	100	93	101	94	102	14	20	28	5	18	35	15	43
0.6 ml of 0.5% on Aug. 1 and 2	89	100	103	116	111	125	1	2	27	2	7	43	13	30
Control	95	100	111	117	122	128	–	–	32	2	6	60	10	16

The trials were conducted on plants of the variety Tashkent-1 in August, 1991 and 1992. In 1991, two variants of the experiments were performed: five treatments of the growth points of a cotton plant with 0.2 ml, or two treatments with 0.6 ml, of a 0.5% aqueous emulsion of fraction F-2 from L-249 to which a few drops of OP-10 emulsifier had been added, with an interval of 1-2 days between the treatments. The three variants of the experiment in 1992 consisted in fivefold treatments with 0.2 ml of 0.5 and 1.5% solutions of the same composition and also with 0.1 ml of a solution of the latter concentration.

The results of the trials are given in Tables 1 and 2. Those of the trials in the two seasons were monotypical qualitatively but differed substantially in the quantitative respect, which was obviously due exclusively to the climatic fluctuation of the 1992 season, leading to serious shifts in the time of setting in of the ontogenetic phases and their duration.

Of the two treatment variants in 1991, the fivefold treatment with the smaller amount of emulsion gave an effect overwhelmingly greater than the twofold treatment with 0.6 ml — on the 30th day after the first treatment the mean height of the stems and increased by only 2% (as compared with 25% in the second variant and 28% in the control) (see Table 1). Therefore, in the planning of the 1992 experiments we took as a basis the variant involving five treatments with the 0.5% emulsion, supplemented by two variants with an increased content of the active principle (see Table 2). On the 27th day after the first treatment (September 2) the mean heights of the stems had increased by 18, 14,and 11%, respectively, as compared with 29% in the control. Thus, the increases in the growth of the control plants in the period recorded were comparable for the two seasons. At the same time, the effect of treatment with an emulsion of a fraction of the extractive substances from the L-249 leaves proved to be coniderably smaller in the 1992 season. A clear dependence of the change in height on the concentrate existed and was also traced in the later observations (September 22).

We also used the results of these experiments for calculating the percentage inhibition of the height of the main stem (see Tables 1 and 2). In the experiments with a fivefold treatment of the growth point, the inhibition in 1991 was approximately twice as great as in the analogous experiment of 1992 and reached 20% at the third determination. The increase in the amount of active principle in the 1992 experiments enhanced inhibition by 3-5%.

On August 15 and September 10 in the 1991 experiments, larger numbers of bolls, including opened ones, were counted on all the plants (Table 1). On these dates, the percentages of opened bolls in the fivefold variant of the treatment were 3 and 2.7 times higher, respectively, than in the control. At the same time, in the control during the period between the two observations the total number of bolls increased by a factor of 1.9, while in the treated plants this index was 1.25 and 1.6, respectively. Unfortunately, the analogous calculations could not be performed for the 1992 experiments because of the extremely small number of bolls formed in the period of observation (an average of 3-4 per plant) and because of damage to the control plants by the red spider mite at the beginning of September. We may mention that 95% of the treated plants had remained undamaged on September 22.

In the choice of the procedure for the analysis of the active fractions, we started from their multicomponent nature, the absolute unpredictability of the structure of the compounds forming the "growth-inhibiting factors", and the low probability of finding and identifying any individual compound whatever that was responsible for the inhibiting activity. Information on

TABLE 2. Change in the Height and Percentage Inhibition of the Main Stem of Plants of the Tashkent-1 Variety in the 1992 Season

Variant of the treatment on August 6, 7, 10, 12, and 14	Mean height of the stem								Inhibition, %		
	06.08		20.08		02.09		22.09		20.08	02.09	22.09
	cm	%	cm	%	cm	%	cm	%			
0.2 ml of 1.5% ×5	79	100	86	109	88	111	89	113	8	14	16
0.1 ml of 1.5% ×5	70	100	76	108	80	114	80	114	9	12	16
0.2 ml of 0.5% ×5	75	100	82	110	88	118	90	120	7	9	11
Control	69	100	82	119	89	129	93	135	-	-	-

the main and some minor components of the total extractive substances of cottonplant leaves [2, 5, 6] and the parallel investigation of fractions obtained from different materials under identical conditions should have provided some simplification of the solution to the problem posed.

Thus, the qualitative and quantitative compositions of the F-1 fraction from leavs of the L-249 line were determined in parallel with those of the fractions obtained by the same method from leaves of the standard variety L-4 [1] gathered in the same phase. In addition, a comparison was made of the compositions of fractions F-1 and F-2 of the L-249 line. In all cases we used the mass-spectrometric method, as the most sensitive. Here, in place of the method of multipeak monitoring that we had developed previously [7], we employed the method of mass chromatography, which is more highly productive and informative because of the use of a system of computer data processing. The method consists of measuring and comparing the total heights of the peaks of any key ion recorded with the repeated rapid scanning of a selected range of mass numbers over a wide temperature interval after the passage of a strictly defined amount of sample through a direct injection system.

The variant of the mass-chromatographic method that was used possesses the additional advantage that it is possible from the time of appearance of the maxima on the ion-current curve to determine the nature of the fragments, while repeated scanning (up to 300 scans in the experiments under consideration) permits the recording of the peaks of the minor components. These features are extremely important for the analysis of multicomponent systems the majority of the ingredients of which are not desorbed from a chromatographic phase in an unmodified state.

In the mass spectra of the individual scans of all the fractions analyzed, characteristic peaks witnessed the presence of phytosterols (sitosterol, stigmasterol, campesterol, 24-ethylidenecholesterol), tocopherols, and polyprenols. The sterols mentioned and also α-tocopherol and undeca- and dodecaprenols were determined quantitatively. In the spectra of fraction F-1 and the spectra of the analogous total materials from the 108-F and L-4 leaves we detected the free fatty acids from 16:0 to 30:0, amyrin, esters of fatty acids with sitosterol, with amyrin, and with phytol, and triacylglycerides. There were also traces of unidentified polyisoprenoids. The origins of some of the ions have remained unestablished, even though their elemental compositions have been measured. The amount of all such components in fraction F-2 was an order of magnitude less than in F-1, as can be seen from the results of quantitative analysis (Table 3). The total amount of the four main components in F-1 averaged about 17%, while in F-2 it exceeded 97%. The remainder of F-2 consisted chiefly of other steroids, as follows from Table 5.

On the saponification of fraction F-1, the amount of tocopherols and polyprenols in the air-dry mass (a.d.m.) of the leaves did not change appreciably (Table 3). It follows from this that the bound forms of these groups did not make an appreciable contribution to the the total F-1 fraction. On the other hand, the amount of free sitosterol in F-2 rose fourfold in the a.d.m. and more than 20-fold in the total SMs (see Table 3), which was a consequence of the saponification of esters of sitosterol with fatty acids. An analogous conclusion follows from an analysis of the shape of the curves of the ion current of the m/z 396 fragment, which is the $(M - H_2O)^+$ ion for free sitosterol and gives a low-temperature maximum on the curve, in contrast to the $(M - RCOOH)^+$ ion from bound sitosterol, revealed in the form of a high-temperature maximum.

Information on the directions of the changes in the amounts of the main and some minor components in the total F-2 as compared with the total F-1 of the L-249 line and the analogous total of the L-4 line is given in Table 4, which shows the contributions of characteristic fragments to the total ion current. These results do not reflect the true quantitative relationships

TABLE 3. Amounts of the Main SMs in Fractions F-1 and F-2 from the L-249 Line

Compound	Amount in the total, %		Amount in the a.d.m.* of the leaves, mg/g	
	F-1	F-2	F-1	F-2
α-Tocopherol	0.07 ± 0.01	0.24 ± 0.04	0.10 ± 0.015	0.07 ± 0.01
Sitosterol	0.70 ± 0.10	14.9 ± 2.2	1.05 ± 0.16	4.30 ± 0.60
Undecaprenol	12.0 ± 2.0	56.8 ± 8.5	17.6 ± 2.6	16.4 ± 2.5
Dodecaprenol	4.2 ± 0.6	24.6 ± 3.7	6.3 ± 1.0	7.0 ± 1.0

*Air dried mass.

TABLE 4. Contributions of the Characteristic Ions to the Total Ion Current Calculated from Mass Chromatograms of Fractions from L-249 and L-4 (10 μg, direct injection), %

Mass numbers, m/z	Compound	Fractions		
		F-1 (L-249)	L-4 (analog of F-1)	F-2 (L-249)
414	Sitosterol	0.95	0.73	1.9
412	Stigmasterol	0.25	0.16	0.30
400	Campesterol	0.20	0.16	0.26
314	24-Ethylidene-cholesterol	0.70	0.40	0.90
396, 397	Sitosterol esters	1.06(0.8)*	1.1(0.8)*	1.16(0.1)*
534, 562, 590	Phytol esters	0.06	0.07	0.07
430	α-Tocopherol	1.1	1.0	0.3
416	β- and γ-Tocopherols	0.55	0.66	0.27
426	Amyrin	0.25	0.25	0.25

*Allowing for the contribution of the $(M - H_2O)^+$ ion of unbound sitosterol.

between the components because of the different sensitivities of the ion detector to particles of different natures. Nevertheless, an increase in the contribution of free sterols in F-2 with a simultaneous decrease in the contribution of the m/z 396 and 397 ions (sitosterol esters) can be clearly seen. The contributions of the molecular ions of phytol esters and amyrin changed little.

On passing from F-1 to F-2, the contribution of the tocopherol ions with m/z 430 and 416 decreased. Simultaneously there was a relative increase in the areas under the diffuse high-temperature maxima of the ion-current curves and a decrease in the area under the low-temperature maximum corresponding to the liberation of free tocopherols. In our opinion, this shows that on alkaline saponification there was a partial loss of nonbound tocopherols and an accumulation of their esters decomposing at a high temperature under EI, the result of which was the appearance of the fragmentary ions with m/z 430 and 416.

The determination of the nature of the minor components will be illustrated for the case of the ion with m/z 548 appearing in the general spectra of all the fractions in the form of a peak of medium intensity. In the mass chromatograms of the F-1 fraction (L-249) and L-4 its relatively narrow maximum appeared at a temperature of 150°C (Fig. 1) and coincided with the appearance time of the ion-current maximum for an ion with m/z 313. In these fractions the latter had the composition

TABLE 5. Amounts of SMs in the Leaves of L-249, L-4, and 108-F Cotton Plants, mg/g a.d.m

Compound	L-249	L-4	108-F
α-Tocopherol	0.10	0.14	0.06
Sitosterol	1.05	0.96	0.97
Stigmasterol	0.30	0.23	0.30
Campesterol	0.06	0.05	0.07
24-Ethylidenecholesterol	0.30	0.20	0.37
Undecaprenol	17.6	19.5	7.2
Dodecaprenol	6.3	8.7	3.0

$C_{19}H_{37}O_3$, which is characteristic for fragments of di- and triacylglycerides [8]. The elementary composition of the ion with m/z 548 in these fractions was $C_{35}H_{64}O_4$, which may correspond to the $M^{+\cdot}$ ion of a diacylated propylene glycol, passing into the ion with m/z 313 by the loss of the acyl residue of a 16:2 acid.

On the ion-current curves of fraction F-2 (see Fig. 1), all the maxima are shifted into the higher-temperature region. The curve of the m/z 313 ion shows two maxima, the first of which appears at 200°C and coincides in its appearance time with with the ion-current maxima of the $M^{+\cdot}$ ions of sterols. At this temperature it is a doublet with the composition $C_{23}H_{37}$ + $C_{22}H_{33}O$, both components of which are formed on the breakdown of the molecular ions of sterols. The second maximum appears at 250-400°C, and the composition of the m/z 313 ion under these conditions is the same as in the F-1 fraction, i.e., $C_{19}H_{37}O_3$. The curve of the ionic current of the m/z 548 ion forms a maximum in the same temperature interval. In the high-resolution regime it was represented as a doublet with the composition $C_{34}H_{60}O_5^+$ + $C_{35}H_{64}O_4$ in a ratio of 2:1. The major component may have been a diacylglycerol with a different set of acylating acids.

A comparison of the amounts of the main components of fractions F-1 (L-249), L-4, and 108-F is given in Table 5. It can readily be seen that the levels of α-tocopherol and all the free sterols that were determined changed little from sample to sample. However, the amounts of undeca- and dodecaprenols in the leaves of the self-defoliating lines were 2.1-2.9 times greater than in the 108-F leaves. In view of the fact that in the F-2 fraction used for field trials the polyprenol content was ~80% of the total (Table 3), it may be assumed that it is just these compounds that indirectly determine its inhibiting activity. However, it is difficult to suggest a mechanism of their influence, since up to the present time polyprenols have been considered only as membrane-active participants in the transport of hydrophobic particles in the biosynthesis of polysaccharides [9].

It is also impossible not to take into account the fact that the remainder of the total material presumably consists of free sterols, a change in the balance of which accompanies such signs of aging of the cotton plant as leaf shedding [2, 6]. According to our results, a similar effect was given by the treatment of the growth point with mixtures close in composition to F-2 and in comparable amounts. However, these experiments were carried out in a later phase of ontogenesis and the changes in the lengths of the stem during them were not recorded. Consequently the idea of the susceptibility of the plant to exogeneous treatment of the growth point with total material of the type of F-2 as a signal for ontogenetic changes requires further checking. It is obvious that retardation of growth did not take place as the result of a pathogenic action, as was also shown by the increased resistance of the treated plants to the red spider mite.

EXPERIMENTAL

Production of Fractions F-1. Fractions F-1 (for 108-F, L-249, and L-4) were obtained as for the analogous experiment in [2]. The total yields of extractives were 185, 150, and 135 mg from 1 g of air-dry material, respectively.

Production of Fractions F-2. Air-dry and comminuted leaves (100 g) of L-249 and 108-F cotton plants were extracted by the method of [4]. The yields of unsaponifiable fractions were 2.90 and 2.71 g, respectively.

MS 25 RF chromato-mass spectrometer (Kratos, United Kingdom) with a DS 90 data-processing system. Combined EI/CI ion source in the EI regime, accelerating voltage 4 kV, ionizing voltage 75 V, collector current 50 μA, chamber temperature 300°C. Direct sample injection, the temperature of the bulb being shown at the top of Fig. 1; rate of scanning the

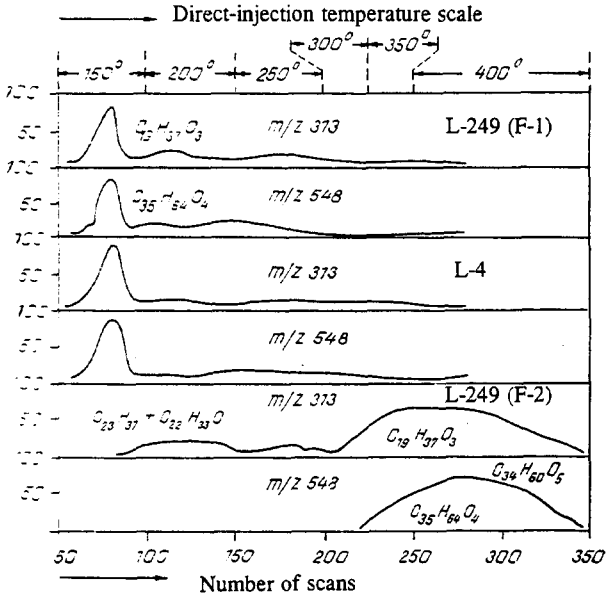

Fig. 1. Mass spectrograms (ion-current curves) of the ions with m/z 548 and 313 in the mass spectra of fractions of L-249 and L-4.

spectra 1 sec/mass decade. The ion-current curves of the individual ions were obtained by reconstructing the curve of the total ion current with the aid of the DS 90.

Sterols and α-tocopherol were determined quantitatively with the aid of an internal standard — cholesterol: for this, the relative sensitivities of the ion detector for all the substance to be determined and the standard had been measured beforehand by injecting mixtures of calibrated solutions in chloroform with different ratios between the substances to be analyzed. Then aliquots of solutions obtained by dissolving weighed amounts of the fractions to be analyzed, with the addition of a cholesterol solution, were injected. Relative sensitivities were determined from the formula:

$$Sr = \frac{I_{st} \cdot g_{ch}}{I_{ch} \cdot g_{st}},$$

where Sr is the sensitivity of the ion current detector to the M^+ ion of the substance in relation to the M^+ ion of cholesterol;

I_{st} nd I_{ch} are the total ion currents of the M^+ ions of the standard and cholesterol, respectively; and

g_{ch} and g_{st} are the amounts of cholesterol and standard substance, respectively, in the mixture injected.

Calculation by this equation gave the following results: cholesterol — 1; sitosterol — 0.41; stigmasterol — 0.34; campesterol — 0.77; 24-ethylidenecholesterol — 0.82; and α-tocopherol — 14.5.

The levels of sterols and α-tocopherol were calculated from the equation

$$C = \frac{I_p \cdot g_{ch} \cdot Y_0 \cdot 10}{I_{ch} \cdot Y_1 \cdot Sr \cdot M},$$

where C is the amount of SMs in the a.d.m. of the leaves, mg/g;

I_p is the total ion current of the M^+ ion of the sterol or the α-tocopherol in the total;

I_{ch} is the total ion current of the M^+ ion of cholesterol;

g_{ch} is the amount of cholesterol, as standard, added to the total mixture, μg;

Y_0 is the initial volume of the solution of the fraction, ml;

Y_1 is the volume of the solution of the fraction that was injected, μl;

Sr is the relative sensitivity, in relation to cholesterol, of the ion detector to the compound to be detected;

10 is a dilution factor; and

M is the amount of dry leaves extracted, g.

The polyprenols were determined by the successive injection of a standard mixture of undecaprenol and dodecaprenol and aliquots of solutions of the samples to be analyzed. Their amount was determined from the formula:

$$C = \frac{I_p \cdot g_{st} \cdot Y_0}{I_{st} \cdot Y_1 \cdot M} ,$$

where C is the amount of one of the polyprenols in the a.d.m., mg/g;

I_p is the total ion current of the M^+ ions of the undeca- or dodecaprenols;

I_{st} is the total ion current of the M^+ ion of the undeca- or dodecaprenol standard;

g_{st} is the amount of the undeca- or dodecaprenol standard, μg;

Y_0 is the volume of the solution of the fraction, ml;

Y_1 is the volume of the solution of the fraction that was injected into the mass spectrometer, μl; and

M is the weight of dry leaves, g.

All the experiments were performed in triplicate. The relative error of the determiantions was $\pm 15\%$.

The elemental compositions of the ions were determiend on a MKh 1310 instrument.

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