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Resin acids as the potential growth-affecting component of pine oleoresin

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Abstract:

The nonvolatile fraction of the oleoresin of *Pinus silvestris* L. was found to contain substances which inhibit growth of wheat ceoleoptile and oat mesocotyl sections in standard bioassays. The inhibition is mainly confined to the fraction of resin acids. Among the seven authentic resin acids tested, the effects of dehydroabietic and abietic acids were most sifgnificant. Palustric, pimaric and isopimaric acids were not effective in the wheat coleoptile section straight growth test. None of the substances, in the amounts tested, except for extremely high concentration, exerted an inhibitory effect on natural or IAA-induced elongation of pine hypocotyl sections. Neither was an inhibitory effect discovered in the microbiological test with the *Aspergillus niger* van Tiegh. The results obtained with pine hypocotyl sections, allow the conclusion that resin acids interfering with the results of standard bioassays are probably not effective as inhibitory factors in the regulation of pine tissue growth.

INTRODUCTION

The frequent occurrence of the metabolism bringing about the synthesis of resins in higher arborescent plants, and the evolution of specialized tissue systems connected with the production of resin, discussed recently by Langenheim (1969) in her excellent review concerning the origin of amber published in Science, leaves no doubt that the role of resinous terpenoids in the physiology of trees deserves special attention. Indeed, this opinion seems to be well founded on the fact that numerous compounds of the family of terpenoids were found to be growth regulators, like gibberellins and abscisic acid, or to play an important role in the metabolism of plants (Sandermann 1958). Surprisingly, the role of resins which are, generally speaking, mixtures of volatile mono- and sesquiterpenes, nonvolatile unsaturated carboxylic di- and triterpene acids, and other hydrocarbons of nonisoprenoid origin, alcohols, aldehydes and estres, has been only rarely investigated. In spite of scarse

experimental work, various theories have been advanced to explain the function of resins as fungicides and bactericides connected mostly with the ecology of different species (Tokin 1953) or with their protective role in wound healing. In the case of the oleoresin of conifers, it has been traditionally believed, to be a waste product of the substantial metabolism (Mutton 1962; Sandermann 1962). On the other hand, the paper of Sukhov (1956), who investigated incorporation of radioactive carbon into oleoresin of pine and discovered its complete disappearance from this substance a few days after the administration of $^{14}CO_2$ to foliage, threw a new light upon this problem.

Works concerning natural growth substances in conifers (Ogasawara 1961 a, b, 1962, 1966; Ogasawara, Kondo 1963; Clark, Bonga 1963; Wodzicki 1964) brought also to attention the inhibitory effect produced by the acidic ether fraction of extracts from tissues of conifer species interfering with bioassays of auxins and gibberellins (Michniewicz, Kopcewicz 1966). The inhibition which on the basis of the results of chromatography could be tentatively ascribed to the inhibitor β (Bennet-Clark, Kefford 1953; Kefford 1955), in fact, was suggested to result from the presence of resinous compounds (Wodzicki 1968). The technique of purification with the use of high vacuum for concentration of the extracts applied in these studies, excluced practically the effect of volatile substances. Nonvolatile resin acids, which constitute above 50% of the oleoresin of *Pinus silvestris* (Prosiński 1969) have been but little investigated as the potential factor effective in regulation of growth. To the author's knowledge only abietic acid has been tested in this respect, but the results were inconsistent (Avery, Sargent 1939; Klingström 1969). Therefore investigations on the growth effect produced by the fraction of resin acids from the oleoresin of pine were undertaken.

METHODS

Oleoresin droplets, appearing on the surface of trunks of *Pinus silvestris* L. tress when the bark is removed, were dissolved in absolute methanol and stored at —20°C. The procedure of fractionation of oleoresin (Fig. 1) was adopted from Jerzmanowska (1967). It allowed separation of a reasonably pure fraction of resin acids on the basis of UV examination (Fig. 2), (Loeblich, Lawrence 1958). The curve for the fraction of resin acids was found to be almost identical with that described by Klingström (1969) for his fraction 5 after gel filtration (Sephadex LH 20) of the ether fraction of extracts from the shoots of *Pinus silvestris*, in which several resin acids were identified by means of gas chromatography. The $\lambda_{max}=241$ established for this fraction corresponds to that of abietic acid (Joye, Lawrence 1967).

All fractions obtained were tested initially in three different straight-growth bioassays: wheat coleoptile (Bentley, Housley 1954), oat mesocotyl (Nitsch, Nitsch 1956) and pine hypocotyl section tests (Witkowska-Żuk, Wodzicki 1970). The wheat variety "Opolska" (Wodzicki, Witkowska-Żuk 1964) and oat variety "Biały Mazur" (Kentzer, Rowicka 1963) were used for these tests.

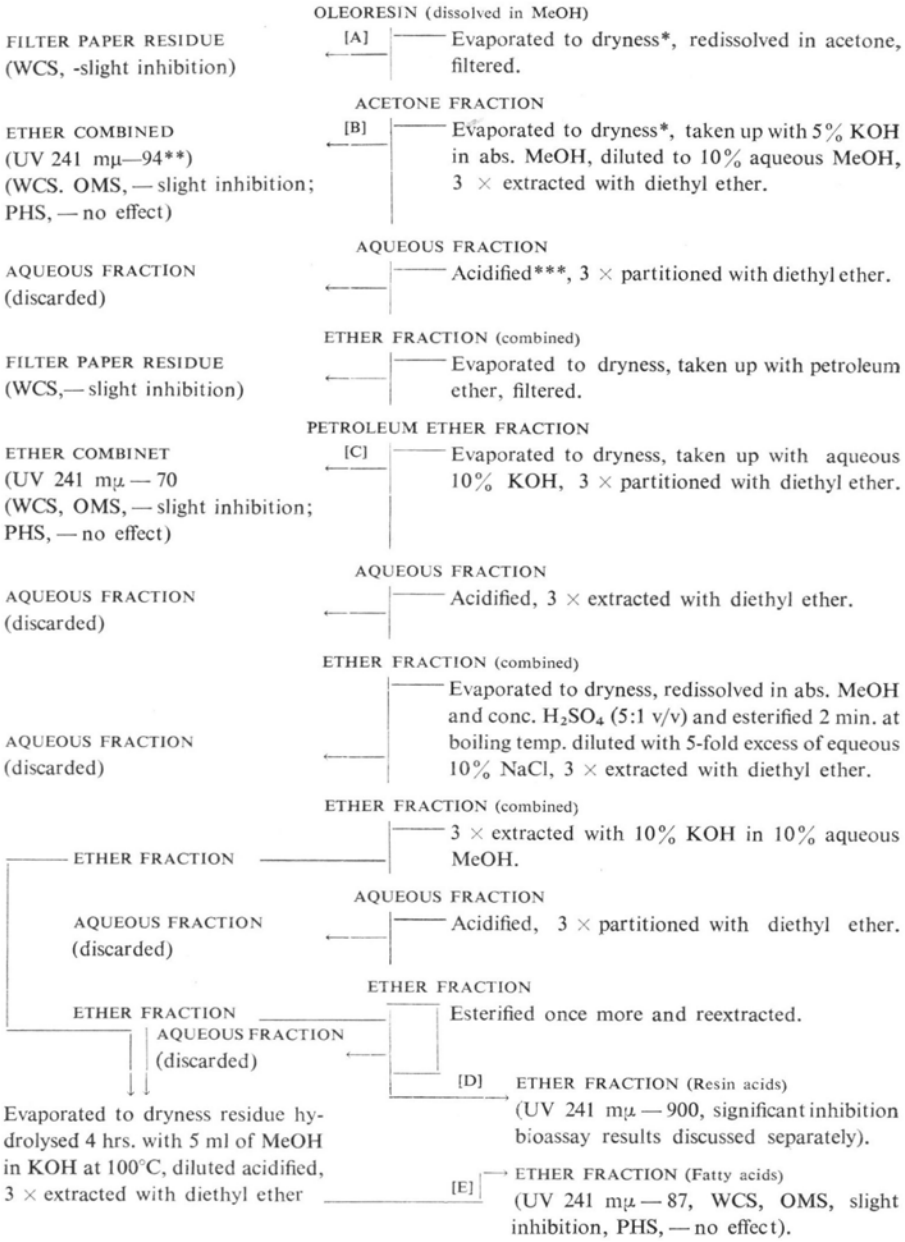

Fig. 1. Procedure of separation of resin acids from oleoresin of *Pinus silvestris*, and results of bioassays of substances separated from the oleoresin at various steps of purification procedure

* evaporation was accomplished in vacuo, at 0°C.

** UV absorption—extinction: concentration of substances equivalent to 100 μg of oleoresin per 1 ml MeOH.

*** the fraction was acidified to pH 2.5 with 10% aqueous H_2SO_4. WCS — wheat coleoptile sections astraight growth test. OMS — oat mesocotyl sections straight growth test. PHS — pine hypocotyl sectons straight growth test.

The original lengths of the coleoptile sections were 10 mm and 4.5 mm, both for the mesocotyl and hypocotyl sections. Methanolic solutions of the substances tested were placed on 25×55 mm strips of Whatman No. 3 paper. The strips were dried, put into 10-ml glass tubes and soaked in 2 ml of 2% sucrose dissolved in citrate-phosphate buffer pH 5.6 or pH 5.0 for wheat coleoptiles and the others tests, respectively. The tubes were constantly rotated in darkness at 26°C during 20 hours of incubation.

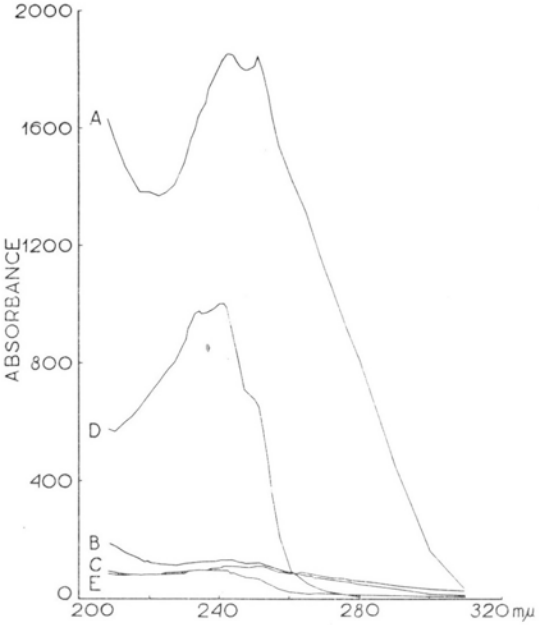

Fig. 2. UV spectra of pine oleoresin and five fractions obtained by its alkaline and acidic extraction For description of fractions see Fig. 1. Equivalent to 100 μg of oleoresin per 1 ml

The presence of paper strips in the tubes during incubation was found to be essential for the results of bioassay. It seems probable that the substances practically insoluble in water were absorbed by the tissue directly from the moist filter paper while rotated. No effect of the substances could be detected if only the eluates were tested. Owing to this difficulty, the amounts of the substances tested, or the equivalents of the amount of oleoresin, are given per tube, and not in concentration units.

No promotion of growth in the fractions listed in Fig. 1 was found in the preliminary coleoptile and mesocotyl section tests, on the contrary inhibition of various intensity was observed. The strongest inhibitory effect, found in the fraction of resin acids (fraction D) was subsequently studied in detail. In addition the effect of this fraction and of all the authentic resin acids studied upon germination of spores and the growth of mycelium of *Aspergillus niger* van Tiegh. was tested in malt-agar medium. The results were determined after two weeks of incubation at

30°C and 85–90% of air humidity according to the method of Jones (1968), and modified by Ważny and Rudniewski (1972).

The samples of standard resin acids were generously supplied as a gift by Dr. Ray V. Lawrence, Naval Stores Lab., Olustee Fla., U.S.A., to whom the authors are greately indebted. Abietic acid was synthesized from levopimaric acid just before the tests according to Schuller et. al. (1967).

RESULTS

The resin acids fraction from pine oleoresin as well as unpurified oleoresin, produced a visible inhibition of natural or IAA-stimulated elongation of wheat coleoptile, or oat mesocotyl sections. On the contrary, only a slight inhibition (if any) could be detected in the pine hypocotyl section test (Table 2).

Several authentic resin acids, listed in Table 1, were tested subsequently with wheat coleoptiles. It is seen from Fig. 3 that the most active inhibitor was dehydroabietic acid. Moreover, inhibition was produced by levopimaric, neoabietic and abietic acids. The least amounts of the substance required to produce significant inhibition varied from 50 to 100 µg per tube (equivalent to 25–50 ppm). Palustric, pimaric and isopimaric acids did not inhibit growth of wheat coleoptile sections at any of the tested concentration equivalents. Resin acids inhibited also natural and IAA-induced growth of oat mesocotyl sections. In spite of the higher equivalent of concentration used (amount of 1 mg/tube), levopimaric acid appeared less effective

Table 1

Effect of resin acids upon elongation of pine hypocotyl and oat mesocotyl sections. Averages of three replicate tests
Final length of sections in millimeters

	Substance tested	Oat mesocotyl			Pine hypocotyl			
		IAA µg/ml						
		0	0.01	0.1	0	0.01	0.1	1.0
1 mg per tube	Control	4.9	5.7	6.8	5.9	6.3	6.8	7.0
	Abietic acid	4.7**	4.9**	5.7**	5.9	6.4	6.7	7.0
	Dehydroabietic acid	4.7**	5.0**	5.3**	5.8	6.1	7.0	7.0
	Neoabieitc acid	5.0	5.3**	6.4	5.9	6.5	7.2	7.2
	Palustric acid	4.9	5.3**	6.3**	6.1	6.5	7.2	7.3
	Levopimaric acid	4.9	5.5	6.4	6.1	6.3	6.9	7.2
	Pimaric acid	4.9	5.5	6.6	5.9	6.3	6.9	6.9
	Isopimaric acid	4.9	5.6	6.2**	5.9	6.4	6.9	7.2
10 mg per tube	Abietic acid	—	—	—	5.7	5.8**	6.0**	5.9**
	Levopimaric acid	—	—	—	5.9	6.4	6.7	7.1

** Results significantly different from control at 99 per cent level of confidence.

Table 2

Effect of oleoresin and its resin acid fraction upon elongation of wheat coleoptile, pine hypocotyl and oat mesocotyl sections.
Averages from 3 replicate tests.
Final length of section in millimeters

IAA mg/l	Wheat coleoptile					Pine hypocotyl					Oat mesocotyl		
	Control	Oleoresin		Resin acids fraction		Control	Oleoresin		Resin acids fraction		Control	Oleoresin	Resin acids fraction
		mg/tube*					mg/tube*					mg/tube*	
		2	18	2	18		2	18	2	18		2	2
0	21.1	19.3**	17.3**	19.9**	18.0**	5.7	5.5	5.5	5.8	5.6	4.8	4.5**	4.7
0.01	—	—	—	—	—	6.4	6.0	6.0	6.2	5.9**	5.3	4.8**	4.8**
0.1	21.7	19.9**	18.4**	20.5**	18.8**	6.6	6.4	6.3	6.4	6.1**	5.8	5.3**	4.9**
1.0	23.8	21.3**	20.6**	22.6	20.5**	6.7	6.9	6.4	6.9	6.4	—	—	—

*) Equivalents of milligrams of oleoresin used for fractionation.
** Results significantly different from control at 99 per cent level of confidence.

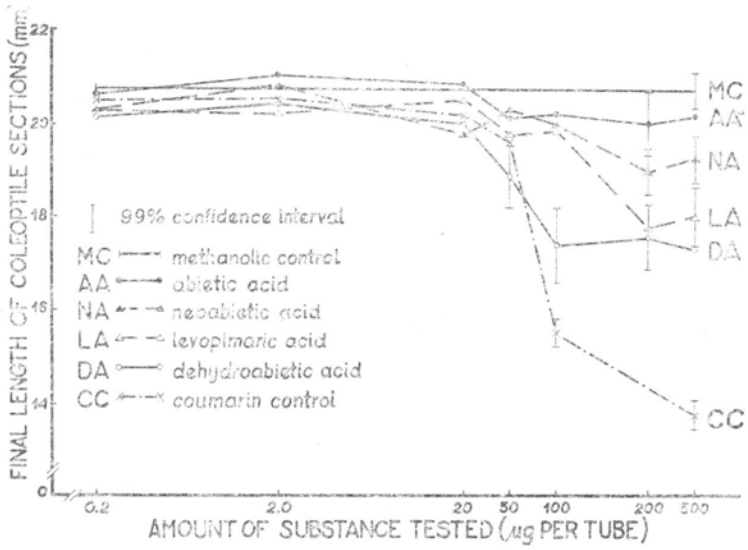

Fig. 3. Effect of resin acids one longation of wheat coleptile sections

and inhibition due to abietic acid was stronger. Moreover, the inhibitory effects of palustric and occasionally isopimaric acids were detected. However, abietic acid (unlike levopimaric acid) arrested the IAA-stimulated elongation of pine hypocotyl sections if given in 10 times greater amount (10 mg per tube, or equivalent of 5000 ppm). The slight stimulation, or synergism with IAA of palustric acid observed in the latter test was found insignificant after further investigation (results omitted).

Surprisingly, the fraction of resin acids from oleoresin and of authentic resin acids exerted no effect on germination and growth of *Aspergillus niger* van Tiegh. when analogous concentrations of the substances in filter paper (equiv. 10 mg oleoresin, or 1 mg per tube of resin acids), were tested.

DISCUSSION

Oleoresin of pine was found to contain substances which inhibited natural or IAA-stimulated growth of wheat coleoptile and oat mesocotyl sections in standard bioassays. The substances were not the inhibitors known from the literature, found in extracts from the tissues of *Pinus silvestris* (Michniewicz 1967; Bonnet-Masimbert 1969), which, if present, would be separated during the purification procedure applied. The inhibition was restricted predominantly to the fraction of resin acids. The results of analogous bioassays performed with several pure resin acids confirm the supposition that the inhibitory effect produced by the fraction

tested was due to the presence of analogous compounds. Resin acids of abietic acid type, especially dehydroabietic acid, were found to be most effective as growth inhibitors. This finding supports the previous data published by Klingström (1969) concerning the inhibitory effect produced by a mixture of resin acids including abietic acid.

Most interestingly the growth of hypocotyl section of pine seedlings was not inhibited by resin acids or the resin acids fraction of oleoresin, except when extremely high concentrations of some of these substances were used. Considering, that the amount of substances tested exceeded many times the concentrations at which known plant growth regulators are active, there is little probability that any of the resin acids studied could play same role as inhibitor effective in regulation of pine growth. However, the interference of resin acids with the results of standard bioassays must not be underestimated, especially when extracts from tissues of conifers are tested. The sterically hindered carboxyl group in the molecule of resin acids, makes it difficult to include all these substances into a single organic fraction by the commonly used various adaptations of Larsen's (1955) procedure. Besides, all the resin acids discussed in the present paper were found to migrate to R_f 0.65–0.9 if chromatographed with isopropanol, ammonia, water (10:1:1 v/v), the solvent commonly used in paper chromatography of growth substances.

Negative results of the test performed with *Aspergillus niger* van Tiegh. do not preclude the possibility of the effect upon the growth of other microorganisms. Such an inhibitory effect of abietic acid upon *Melampsora pinitorqua* (Braun) Rostr. has already been demonstrated by Klingström (1969). However, according to Vanin (1939) and Bavendamm (1936), results of Zeller's (1916) experiments are known in which even extremely high concentrations of conifer resin in the medium (up to 50%) did not inhibit the growth of some species of fungi.

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Wpływ kwasów żywicznych i żywicy sosnowej na wzrost tkanek roślinnych

Streszczenie

Nielotna frakcja żywicy *Pinus silvestris* L. zawiera substancje hamujące wzrost odcinków koleotile pszenicy lub mesokotylu owsa w powszechnie stosowanych testach biologicznych. Inhibicję tę powodują głównie substancje dające się wyodrębnić we frakcji kwasów żywicznych. Spośród siedmiu oczyszczonych kwasów żywicznych które zbadano, najsilniejszą inhibicję powodowały kwasy dehydroabietynowy i abietynowy. Kwasy palustrowy, pimarowy i izopimarowy nie powodowały inhibicji w teście koleoptile pszenicy. Żadna z substancji w badanych ilościach, z wyjątkiem skrajnie wysokiej koncentracji, nie powodowała zahamowania naturalnego lub stymulowanego-IAA wydłużania odcinków hypokotylu sosny. Nie wykryto także wpływu hamującego w teście mikrobiologicznym z *Aspergillus niger* van Tiegh. Brak efektu wzrostowego w teście hypokotylu sosny, pomimo, że badane ilości substancji wielokrotnie przewyższały równoważniki wymagane dla uzyskania efektu znanych regulatorów wzrostu roślin, pozwalają wyciągnąć wniosek, że interferujące w testach standardowych kwasy żywiczne nie są prawdopodobnie włączone w system inhibitorów wzrostu biorących udział w regulacji procesów wzrostowych u sosny.