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Melampsora pinitorqua (Braun) Rostr. on progenies of *Pinus silvestris* L. and in relation to growth regulating substances

Melampsora pinitorqua (Braun) Rostr. på avkommor av Pinus silvestris och i förhållande till tillväxtreglerande ämnen

by

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Abstract

Various methods of recording pine twisting rust have been discussed, and attack frequencies on 75 progenies of *Pinus silvestris* plus trees have been noted. Mention has also been made to the possibility of resistance breeding.

Acid ether extracts of annual shoots of *Pinus silvestris* have been examined by various bioassays. Marked differences in the inhibitor effect between extracts from different pine clones have been established. A possible connection between inhibitor content and resistance has been discussed. Reduction in inhibitor content during axial extension and inhibitors in leachates from pine shoots has been connected with *Melampsora* occurrence.

Gel filtration combined with thin layer chromatography has been used for separating the extract components. Among the inhibitors a number of resin acids have been identified by chemical means.

Introduction

This work on *Melampsora pinitorqua* consists of two main parts. The first contains observations from field experiments and information regarding the occurrence of the fungus on different species of *Pinus*. It also deals with the spontaneous occurrence of pine twisting rust on 75 progenies of plus trees.

In the second part of the work a study is made of *Melampsora pinitorqua* on Scots pine in relation to growth regulating substances, in particular growth inhibitors in acid ether extracts of annual shoots. Various methods have been used to separate components in extracts of pine shoots which inhibit the fungus' basidio spore germination. Changes in quantity with regard to growth regulators in the annual shoots during the axial extension have been associated with the occurrence of the fungus. Biologically active substances in acid ether extracts have been identified by chemical means.

I. Melampsora pinitorqua on progenies of Pinus silvestris

1. Melampsora pinitorqua on Pinus

Melampsora pinitorqua occurs in Europe on a number of *Pinus* species; the major works are as follows:

Pinus silvestris, SYLVÉN (1917), BÖHNER (1952), REGLER (1957), KLINGSTRÖM (1963).

Pinus mugo, BIRAGHI (1954), REGLER (1957), GREMMEN (1963).

Pinus pinaster, BIRAGHI (1954, 1963), MORIONDO (1962 a), YOUNG (1963), GREMMEN (1963), ILLY (1966), DURRIEU (1967).

Pinus pinea, BIRAGHI (1954, 1963), MORIONDO (1954).

Pinus nigra, MORIONDO (1957, 1962 b), KISPATIĆ (1961).

Pinus nigra var. *laricio*, BIRAGHI (1963).

Pinus nigra var. *calabrica*, LONGO et al. (1967).

Pinus nigra var. *austriaca*, LONGO et al. (1967).

Pinus halepensis, PEACE (1962), MORIONDO (1962 b), BIRAGHI (1963).

Pinus murrayana, TROSCHANIN (1952).

Pinus strobus, BÖHNER (1952), TROSCHANIN (1952), BIRAGHI (1954), REGLER (1957).

In practice *Pinus contorta* has proved to be resistant in Sweden BERGMAN (1954), BJÖRKMAN (1963), a circumstance which should be considered in the light of reports of the occurrence on *Pinus murrayana* (*P. contorta* var. *latifolia*). The occurrence on *Pinus strobus* suggests that the fungus is not entirely confined to pitch pines, but can be assumed to occur on other hosts. *Melampsora pinitorqua* is also reported as occurring on *Pinus ponderosa* in North America ZILLER (1961, 1962). This report was later retracted ZILLER (1965).

Current research in Italy LONGO et al. (1967) and my own in Sweden KLINGSTRÖM (1967) have shown that *Melampsora pinitorqua* can attack other conifers, from among other places North America.

Thus both the Swedish and Italian experiments have shown that *Pinus ponderosa*, *P. resinosa* and *P. banksiana* are susceptible. Furthermore *P. attenuata* and *P. virginiana* were attacked in the Swedish experiments, and

no less than 13 conifers are reported as being susceptible in the Italian work: *P. lambertiana*, *P. echinata*, *P. taeda*, *P. contorta*, *P. radiata*, *P. elliottii*, *P. palustris*, *P. excelsa*, *P. canariensis*, *Larix decidua*, *L. occidentalis*, *L. leptolepis* and *Pseudotsuga menziesii*. Admittedly these are only preliminary results, but it is remarkable that *Melampsora pinitorqua* could attack other conifers than *Pinus*. It is necessary to take into consideration the existence of several *Melampsora* species under *Melampsora populina* (Pers.) Lév. and *Melampsora salicina* Lév. with *Larix* and *Abies* as a conifer host (GÄUMANN 1959).

The fungus' biology was described in the latter half of the 1800's. SYLVÉN (1917) has summarised this literature in a very meritorious way. For half a century hardly any work of a similar scope has been devoted to *Melampsora* on *Pinus*. The fungus' spermogones and acio spores appear on *Pinus* species towards the end of shooting. The acio stage is developed as a caeoma. Uredo spores and telio spores occur on the leaves of various Populus species. The occurrence of the fungus on *Populus* will not be treated of here; there is extensive literature on the subject and mention can be made of REGLER (1957). The telio spores spend the winter on the dead aspen leaves and in the spring they germinate with basidies, from which basidio spores again infect pines at the time of shooting. On certain species of *Populus* the fungus can spend the winter in buds; in this connection REGLER (1957) verifies earlier works by KLEBAHN (1938). Similar reports have been made by MORIONDO (1956, 1961).

The extensive presence of twist rust on pine during the early summer has usually been connected with damp weather conditions in the spring and early summer. This phenomenon has been discussed by SYLVÉN (1918); REGLER (1957) has described research into the question; KLINGSTRÖM (1963) studied the way in which the telio spores' ability to germinate changes under the influence of different climatic conditions. The behaviour of the fungus has been regarded as an example of so-called exposition resistance GÄUMANN (1951). The pines are susceptible to the fungus only for a limited time during shooting, and only if this critical period coincides with the dissemination of spores, which in its turn is influenced by climatic factors, does infection occur. Thus exposition resistance does not signify resistance in the ordinary meaning, but arises when the dissemination of spores does not take place at the time when the pine is receptive. The following will touch on several matters which indicate that climatic factors do not constitute a complete explanation of the fungus' occurrence. Despite the fact that *Melampsora pinitorqua* can be regarded as an almost classic subject in forest pathology, knowledge concerning the biology of the fungus is in many respects unsatisfactory.

Melampsora pinitorqua occurs in the whole of Europe and in adjacent parts of North Asia and may be growing in importance as a parasite.

Pine is susceptible to the disease at virtually all ages. KARDELL (1962, 1966) shows that pines more than 50 years old can also be attacked. The most important practical aspect of twist rust is the damage it inflicts on young plants, and the younger the pines are the greater the risk that the plants will die or suffer permanent injury. In the most favourable cases the wound formed during the fungus' acidio stage heals and only a small scar remains in the bark. In other cases a number of defects that lead to a lowering of quality result from the attack. The terminal leader can be bent and the damage can remain in the form of a deformed bottom log. Very often the terminal leader is bent and breaks off at the site of the wound caused by the fungus. The result can be the formation of several stems and unsuitable branch angles at the wound. The lower part of a broken terminal leader often lives for several years and results in a troublesome vertical branch.

The lasting damage is thus in the form of defects leading to inferior quality and a certain reduction in terminal growth. The latter can be particularly bothersome in those parts of Sweden where other parasites of the *Phacidium infestans* and *Scleroderris lagerbergii* type can damage the plants, more particularly before they have grown above snow level.

In connection with the discussion of ways to counteract damage from *Melampsora pinitorqua* attention has also been paid to the possibility of hereditary resistance. GAVRIS (1939) considered that he had discovered a correlation between the 1,000 grain weight and the *Melampsora* frequency, but this has been refuted by among others TROSCHANIN (1952).

RENNERFELT (1954) and BERGMAN (1954) have published reports indicating that different clones can have varying degrees of susceptibility to the disease. This material is limited and both works deal with the same clones. KLINGSTRÖM (1963) has also shown that there can be great differences between clones. The differences are in this instance similar both after spontaneous infection and following inoculation for several years. EKLUNDH EHRENBERG (1963) has found that differences also exist between progenies. NOSKOV (1958) has mentioned differences between provenances and between stands after sowing or planting.

SCHÜTT (1964, 1965) and HATTEMER (1965) have described differences between progenies after open pollination. In this case too both authors worked with the same progenies. The above comments concern *Pinus silvestris* only. ILLY (1966) has described differences between progenies of *Pinus pinaster*.

2. Melampsora pinitorqua on 75 progenies of Pinus silvestris plus trees

A. Pine material

Progenies were made available by the research stations at Brunsberg, about 180 miles west of Stockholm, and Kratte Masugn, about 96 miles north-west of Stockholm. The crossings had been carried out in 1958 and 1959 according to methods described by EKLUNDH EHRENBERG & SIMAK (1957) and BLOMQVIST (1961). The material was cultivated at the stations and in the spring of 1963 was taken over by the Department of Forest Botany for planting in a nursery near Södertälje, about 18 miles south-west of Stockholm. The material consists of approx. 7,000 plants and the crossing plan can be seen in tables 1 and 4.

It was decided to space the plants at 50 × 100 cm. The planting area measured 25 × 150 m and the material was divided into three blocks. Each block consisted of three 25-row parcels with 25—30 pines per row of each crossing. For technical reasons there was no randomization between the rows which of course is a drawback. Each plant that was not obviously malformed, physically damaged or stagnate, was recorded during the latter part of July 1964, 1965, and 1966 with regard to the number of caeomata on the terminal leaders and first whorls. A record was also kept of the length of the terminal leaders and in 1967 an attempt was made to assess the degree of permanent damage. This took into consideration only serious faults that could be regarded as having economic consequences, such as double top, vertical branches, marked crookedness, etc., and where in all probability *Melampsora* could be regarded as the cause.

B. Registration methods

It is uncertain how *Melampsora* frequency can best be measured as there are no generally accepted norms. The registration of the number of attacks per pine does not take into account the size of the trees or the fact that the number of shoots in the whorls varies. SYLVÉN (1917) gave a detailed account of *Melampsora pinitorqua* and to some extent number of attacks on terminal leaders and first whorl.

Attack per shoot does not take into account the length of the shoots or the physiological differences between leaders and first whorls. The per-

centage of pines without attack or the percentage of diseased pines per progeny recorded by SCHÜTT (1964) put a tree with negligible damage together with those that have suffered any number of attack. This has already been pointed out by HATTEMER (1965). ILLY (1966) has tried to assess the damage with the help of a scale of five grades.

The percentage of pines with permanent damage is from a practical aspect most interesting, although this way of recording pine twist rust has not been used. There are in general few works of a practical nature to cite (cf. KARDELL 1962, 1966). Attacks on terminal leaders is both more usual and of greater practical importance than attacks of first whorls. When assessing somewhat older pine plants the number of attacks per cm terminal leader can be a practical way of measuring pine twist rust. Different assessment norms have been included in table 1 for the sake of comparison. These are: attack per cm terminal leader; attack per leader; percentage healthy pines; percentage with permanent damage.

Furthermore it is generally unknown how the majority of environmental factors influence the occurrence of *Melampsora*. For the time being uniform requirements at least should be drawn up concerning the age and treatment of pine plants when testing the progenies. Certain information regarding the effect of soil conditions on *Melampsora*-damage on pine is contained in TROSCHANIN (1952). The greatest differences according to this work seem not to be in the attack frequency but possibly in the percentage distribution between damage with and without lasting effect.

C. Orientation from mean values

The progenies examined here were not produced with *Melampsora pinitorqua* in mind and the material need not be especially suitable for studying differences in attack frequency between progenies. The choice of parent trees was based on quality characteristics, ANDERSSON (1966). On the other hand a collation of attack frequencies concerning *Melampsora* on such a large number of progenies can be of some importance as a reference in future breeding work. This concerns in the first instance the 53 progenies from the Kratte Masugn Station (Table 1).

The material is summarized in tables showing the mean values. An attempt is also made to make a statistical analysis of a selection of the progenies. Concerning the mean values the figures are for: a) attack per terminal leader, b) attack per cm terminal leader, c) percentage unattacked pines. Thus the entire material is grouped as follows:

I. The whole of the Kratte Masugn Station material is recorded in table 1, which has been arranged according to the 1965 values for attack per cm leader.

Table 1. Summary of *Melampsora pinitorqua* attacks recorded according to different norms and concerning progenies from *Pinus silvestris* clones from the Kratte Masugn Station. The values are shown according to the 1965 grading of attacks per cm leader. Approx. 75—90 pines in each progeny. Material divided into three blocks.

Plus trees ♀ × ♂	Attacks per cm leader			Attacks per leader			% unattacked pines			Average length			Sowing	% with permanent injury	Remarks
	1964	1965	1966	1964	1965	1966	1964	1965	1966	1964	1965	1966			
X 2209 × X 2201	0,012	0,008	0,012	0,29	0,22	0,43	56	48	51	24,2	27,5	34,9	1959	50,7	
Z 4404 × X 2201	0,015	0,008	0,012	0,44	0,24	0,45	33	37	39	28,3	28,3	36,2	1959	42,2	
Z 1000 × Z 4404	0,014	0,008	0,015	0,37	0,25	0,51	42	42	37	25,8	32,0	34,2	1959	39,0	
X 4207 × W 2000	0,007	0,009	0,012	0,19	0,27	0,43	56	44	64	24,9	28,9	34,6	1959	43,2	only 54 pines
X 4207 × Z 4404	0,010	0,009	0,016	0,26	0,27	0,51	49	50	43	24,7	28,3	32,5	1959	38,4	
X 4207 × X 4203	0,013	0,009	0,024	0,32	0,28	0,87	37	45	25	25,2	31,8	35,8	1959	51,9	
X 2209 × Z 4404	0,019	0,011	0,016	0,47	0,28	0,54	39	39	23	24,0	25,9	33,5	1959	37,6	
Z 1000 × X 2201	0,009	0,011	0,017	0,22	0,36	0,58	61	43	35	26,1	32,3	33,5	1959	29,5	
Z 2017 × X 4400	0,005	0,011	0,033	0,13	0,38	1,12	61	26	39	25,5	33,7	41,4	1959	19,2	
Z 2016 × X 2201	0,021	0,012	0,017	0,52	0,35	0,59	39	37	26	24,5	29,5	33,6	1959	32,5	
Z 4400 × X 4300	0,026	0,013	0,029	0,71	0,46	1,14	23	24	44	27,7	35,1	39,4	1958	43,3	
X 4207 × X 2201	0,009	0,014	0,009	0,19	0,41	0,26	62	41	60	22,3	28,8	30,1	1959	55,1	only 54 pines
Z 2016 × X 4203	0,023	0,014	0,027	0,58	0,46	0,94	24	26	19	24,9	32,1	35,2	1959	34,6	
W 4637 × X 4203	0,005	0,015	0,008	0,10	0,43	0,29	72	35	78	18,7	29,2	35,5	1959	27,8	
X 2209 × X 4203	0,015	0,015	0,021	0,36	0,44	0,73	38	43	18	23,5	29,9	35,1	1959	34,5	
Y 4014 × X 4203	0,020	0,015	0,027	0,49	0,45	0,92	44	31	55	24,3	29,2	34,6	1959	31,8	
Z 4300 × X 4203	0,017	0,015	0,028	0,42	0,48	0,97	33	27	48	24,4	32,6	34,8	1959	39,9	
X 4201 × X 4206	0,016	0,016	0,032	0,33	0,51	1,29	49	29	45	20,8	32,2	46,6	1958	24,3	
X 4207 × X 4206	0,017	0,017	0,029	0,50	0,39	1,26	29	29	38	29,4	38,3	43,7	1958	38,6	
Z 2017 × W 2000	0,013	0,017	0,020	0,32	0,49	0,74	49	15	55	24,0	28,9	36,8	1959	60,7	only 55 pines
X 4201 × Y 4015	0,019	0,017	0,038	0,49	0,58	1,49	34	20	37	25.1	33,7	39,6	1958	25,5	
X 4207 × X 4501	0,019	0,017	0,027	0,55	0,63	1,22	23	18	13	28,8	36,7	45,7	1958	58,4	
Z 2017 × E 726	0,006	0,018	0,023	0,11	0,45	0,73	79	27	59	18,9	24,7	31,9	1959	21,2	
Z 2017 × X 4203	0,026	0,018	0,027	0,68	0,57	1,02	24	25	48	25,8	31,3	37,2	1959	48,9	
Z 1000 × X 4203	0,027	0,018	0,022	0,67	0,59	0,77	27	22	28	25,0	33,3	35,0	1959	33,7	
Z 2016 × X 4501	0,027	0,018	0,031	0,71	0,64	1,22	28	27	11	26,7	35,0	39,9	1958	46,6	
Z 4404 × X 4203	0,034	0,019	0,033	0,99	0,63	1,32	9	23	16	29,0	33,4	39,8	1959	67,5	only 52 pines
X 2207 × X 4501	0,011	0,019	0,030	0,29	0,65	1,30	42	32	36	26,2	35,2	42,8	1958	32,9	
Z 4300 × X 4501	0,023	0,020	0,036	0,63	0,73	1,46	31	14	44	27,0	35,6	40,4	1958	46,6	
Z 4400 × X 4501	0,030	0,020	0,041	0,90	0,75	1,72	15	22	28	29,8	37,2	41,9	1958	45,2	
X 4600 × X 4203	0,027	0,021	0,027	0,72	0,66	0,99	32	21	45	26,1	32,2	36,8	1959	51,8	
Z 1001 × X 4501	0,021	0,021	0,043	0,51	0,75	1,73	24	11	23	24,2	34,4	40,1	1958	56,5	
W 4637 × X 4206	0,018	0,021	0,029	0,48	0,81	1,29	30	22	38	26,7	38,7	44,3	1958	45,3	
X 4201 × X 4404	0,019	0,022	0,037	0,53	0,71	1,46	29	25	34	26,4	32,7	39,9	1958	23,6	
X 4207 × X 2222	0,017	0,022	0,027	0,45	0,82	1,08	45	18	47	27,0	37,4	40,6	1958	33,0	
Z 2017 × X 4206	0,028	0,022	0,036	0,80	0,84	1,59	17	22	34	28,6	38,8	44,2	1958	33,5	
X 4201 × X 4501	0,022	0,022	0,049	0,56	0,93	1,98	34	16	34	24,8	34,2	40,4	1958	28,1	
Z 4404 × X 4501	0,013	0,023	0,032	0,35	0,80	1,27	29	11	6	26,3	34,3	40,3	1958	44,4	
W 4642 × X 4206	0,020	0,023	0,034	0,52	0,85	1,47	30	17	37	25,6	36,6	43,4	1958	48,9	
X 4303 × X 4501	0,018	0,023	0,033	0,53	0,89	1,51	19	9	38	29,5	39,5	45,1	1958	42,6	
Z 1000 × X 4206	0,030	0,023	0,042	0,81	0,95	1,91	20	7	26	26,9	41,8	45,1	1958	33,8	
X 2208 × X 4501	0,024	0,024	0,032	0,72	1,02	1,39	28	18	46	30,5	42,3	43,8	1958	43,3	
X 2220 × Z 4404	0,023	0,025	0,042	0,56	0,76	1,49	22	8	41	24,2	30,5	35,4	1958	52,0	
Z 2017 × X 4501	0,029	0,025	0,043	0,75	0,88	1,76	27	16	29	26,3	35,8	41,1	1958	49,5	
X 4201 × X 4300	0,016	0,026	0,034	0,39	0,83	1,26	40	18	39	24,7	31,8	36,8	1958	30,4	
W 4636 × X 4501	0,020	0,026	0,048	0,61	0,96	1,98	27	16	29	30,4	36,8	40,9	1958	49,5	
X 4601 × X 4501	0,032	0,026	0,041	0,93	1,01	1,81	17	11	30	29,3	39,4	44,2	1958	35,9	
W 4604 × X 4501	0,023	0,027	0,041	0,67	0,98	1,73	23	19	24	27,9	36,9	41,8	1958	59,8	
Z 2017 × X 4300	0,021	0,028	0,033	0,65	0,92	1,30	26	20	45	30,0	35,1	40,1	1958	37,9	
W 4637 × Z 4404	0,020	0,029	0,025	0,59	0,94	1,00	32	19	47	28,4	31,7	39,2	1958	40,0	
W 4607 × X 4501	0,030	0,031	0,038	0,79	1,01	1,50	22	14	43	26,7	32,9	39,3	1958	46,5	
W 4642 × X 4501	0,034	0,036	0,044	0,97	1,26	1,79	22	10	37	29,0	35,1	40,0	1958	62,2	
X 2200 × X 4501	0,018	0,047	0,059	0,38	1,52	1,87	35	6	25	21,4	32,4	31,6	1958	70,1	only 54 pines

II. Two summaries comprising 3 male clones × 3 female clones have been compiled from table 1. These are shown in tables 2 and 3. These summaries are based entirely on the presence of the greatest possible number of male clones × female clones in the material in question. The progenies of these clones will for the most part be found in one half of the summarizing table 1, which makes the material less suitable if it is the intention to investigate whether there are any differences at all between progenies where the occurrence of *Melampsora pinitorqua* is concerned.

III. The progeny material from the Brunsberg Station, 7 male clones × 3 female clones is summarized in table 4. The Brunsberg material is included only as an orientating guide in current breeding programmes. Above all the choice of pollen origin gives an unusual geographic distribution on the side of the father (France—Siberia).

Table 1 indicates the scope in relation to pine twisting rust of a more comprehensive breeding material directly connected with current breeding work. The summaries in table 2—4 have been made to illustrate as simply as possible eventual relationship with parent trees. These should be regarded as an introduction to the statistical analysis below.

Table 2 and 3 show that

X 2201 is consistently positive male clone

X 4207 is consistently positive female clone

X 4203 is a negative male clone

Z 1000 is a negative female clone

Where the Brunsberg material is concerned it is more difficult to detect tendencies that can be traced to parent trees. The values change from year to year and there appears to be no palpable and constant connection with *Melampsora*.

D. A preliminary analysis of the material

In collaboration with the Department of Forest Biometry at the Royal College of Forestry an attempt was also made to analyse the two alternatives from the Kratten material and the entire Brunsberg material as randomized blocks. The formal requirements for the analysis—randomization of the treatments (= progenies) within blocks—were, as already pointed out, not met. For this reason significant values regarding treatments must be interpreted with great caution.

An analysis of variance of the number of attacks by pine twisting rust resulted in significance both for block effects and treatment effects. Further analysis suggests that some of the differences between treatments can depend on differences as regards the length of terminal leaders and the num-

Table 2. Summary of mean values from table 1 for 3 male clones × 3 female clones concerning the occurrence of *Melampsora pinitorqua*.

a = attacks/terminal leader, b = attacks/cm terminal leader, c = % unattacked pines
+ indicates lowest mean values for *Melampsora*-attack
— „ highest „ „ „ „ „

Alternative I

1964

♀ \ ♂	Z 4404	X 4203	X 2201	Mean value	Remarks
X 4207	0.26 0.010 49	0.31 0.013 37	0.19 0.009 62	0.25 + 0.011 + 49 +	a b c
Z 1000	0.37 0.014 42	0.67 0.027 27	0.22 0.009 61	0.42 — 0.017 — 43 —	a b c
X 2209	0.47 0.019 39	0.36 0.015 38	0.29 0.012 56	0.38 0.015 44	a b c
Mean value	0.37 0.014 43	0.45 — 0.018 — 34 —	0.23 + 0.010 + 59 +	0.35 0.014 45	a b c

1965

X 4207	0.27 0.009 50	0.28 0.009 45	0.41 0.014 41	0.32 0.011 + 45 +	a b c
Z 1000	0.25 0.008 42	0.59 0.018 22	0.36 0.011 43	0.40 — 0.012 — 36 —	a b c
X 2209	0.38 0.011 39	0.44 0.015 34	0.22 0.008 48	0.31 + 0.011 + 40	a b c
Mean value	0.27 + 0.009 + 44 +	0.44 — 0.014 — 34 —	0.33 0.011 44 +	0.34 0.011 40	a b c

1966

X 4207	0.51 0.016 43	0.87 0.024 25	0.26 0.009 60	0.55 + 0.016 + 43 +	a b c
Z 1000	0.51 0.015 37	0.77 0.022 28	0.58 0.017 35	0.62 — 0.018 — 33	a b c
X 2209	0.54 0.016 23	0.73 0.021 18	0.43 0.012 51	0.57 0.016 + 31 —	a b c
Mean value	0.52 0.016 34	0.79 — 0.022 — 24 —	0.42 + 0.013 + 48 +	0.58 0.017 35	a b c

Table 3. Summary of mean values from table 1 for 3 male clones × 3 female clones concerning the occurrence of *Melampsora pinitorqua*.

a = attacks/terminal leader, b = attacks/cm terminal leader, c = % unattacked pines
+ indicates lowest mean values for *Melampsora*-attack
— „ highest „ „ „ „ „

Alternative II

1964

♀ \ ♂	X 4501	X 4203	X 2201	Mean value	Remarks
Z 2016	0.71 0.027 28	0.58 0.023 24	0.52 0.021 39	0.60 — 0.024 — 30	a b c
X 4207	0.55 0.019 23	0.31 0.013 37	0.19 0.009 62	0.35 + 0.014 + 41 +	a b c
Z 4404	0.35 0.013 29	0.99 0.034 9	0.44 0.015 33	0.59 0.021 24 —	a b c
Mean value	0.54 0.020 27	0.63 — 0.023 — 23 —	0.38 + 0.015 + 45 +	0.51 0.019 32	a b c
1965					
Z 2016	0.64 0.018 27	0.46 0.014 26	0.35 0.012 37	0.48 0.015 30	a b c
X 4207	0.63 0.017 18	0.28 0.009 45	0.41 0.014 41	0.44 + 0.013 + 35 +	a b c
Z 4404	0.80 0.023 11	0.63 0.019 23	0.24 0.008 37	0.56 — 0.017 — 24 —	a b c
Mean value	0.69 — 0.019 — 19 —	0.45 0.014 31	0.33 + 0.011 + 38 +	0.49 0.015 29	a b c
1966					
Z 2016	1.22 0.031 11	0.94 0.027 19	0.59 0.017 25	0.92 0.025 18 —	a b c
X 4207	1.22 0.027 13	0.87 0.024 26	0.26 0.009 59	0.78 + 0.020 + 33 +	a b c
Z 4404	1.27 0.032 6	1.32 0.033 16	0.45 0.012 38	1.01 — 0.026 — 20	a b c
Mean value	1.24 — 0.030 — 10 —	1.05 0.028 20	0.43 + 0.013 + 41 +	0.90 0.024 24	a b c

Table 4. Summary of mean values for progenies from the Brunsberg Station, 7 male clones × 3 female clones concerning the occurrence of *Melampsora pinitorqua*.

a = attacks/terminal leader, b = attacks/cm terminal leader, c = % unattacked pines
+ indicates lowest mean values for *Melampsora*-attack
— „ highest „ „ „ „ „

♀ \ ♂	S 3098 Vägsjö-fors	Eo 27 Klöv-dala	Eo 16 Ydre-hammar	Up 16 Frank-rike	Up 17 Sibirien	Sm 60 Ljus-vattnet	S 3245 Höljes	Mean value	Re-marks
1964									
S 3006	0.38 0.021 49	0.20 0.010 57	0.27 0.016 63	0.31 0.017 57	0.40 0.022 51	0.35 0.023 53	0.30 0.020 50	0.32 — 0.018 — 54 —	a b c
S 3244	0.22 0.014 66	0.17 0.013 59	0.25 0.015 42	0.08* 0.005 58	0.15 0.009 63	0.29 0.017 43	0.15 0.010 56	0.18 + 0.012 + 55	a b c
S 6256	0.39 0.022 52	0.13 0.009 61	0.19 0.013 62	0.22 0.017 65	0.25 0.018 53	0.25 0.019 51	0.14 0.010 54	0.24 0.015 57 +	a b c
Mean value	0.33 — 0.019 56	0.17 + 0.011 + 59	0.24 0.015 56	0.20 0.013 60 +	0.27 0.016 56	0.30 0.020 — 49 —	0.20 0.013 53	0.25 0.015 55	a b c
1965									
S 3006	0.78 0.021 20	0.29 0.009 42	0.57 0.017 32	0.37 0.012 36	0.42 0.013 40	0.57 0.019 35	0.28 0.010 46	0.47 + 0.014 + 36 +	a b c
S 3244	0.59 0.021 32	0.30 0.011 40	0.38 0.012 39	0.75* 0.026 35	0.55 0.018 29	0.61 0.019 33	0.42 0.013 34	0.51 0.017 35	a b c
S 6256	0.64 0.018 20	0.45 0.015 45	0.53 0.019 29	0.55 0.021 32	0.50 0.019 30	0.64 0.023 30	0.49 0.017 41	0.54 — 0.019 — 32 —	a b c
Mean value	0.67 — 0.020 — 24 —	0.35 + 0.012 + 42 +	0.49 0.016 33	0.56 0.020 — 34	0.49 0.017 33	0.61 0.020 — 33	0.39 0.013 40	0.51 0.017 34	a b c
1966									
S 3006	0.99 0.023 21	0.39 0.010 38	0.41 0.012 40	0.46 0.013 47	0.78 0.020 34	0.78 0.022 36	0.53 0.015 34	0.62 0.016 + 35	a b c
S 3244	1.05 0.032 15	0.61 0.018 39	0.71 0.020 27	0.65* 0.019 43	1.09 0.028 16	1.09 0.027 21	0.86 0.022 13	0.87 — 0.024 — 25 —	a b c
S 6256	0.76 0.018 28	0.53 0.015 36	0.49 0.015 49	0.18 0.006 57	0.79 0.022 29	0.53 0.016 44	0.68 0.019 40	0.57 + 0.016 + 40 +	a b c
Mean value	0.93 — 0.024 — 21 —	0.51 0.014 38	0.54 0.016 38	0.43 + 0.012 + 49 +	0.87 0.023 26	0.80 0.022 33	0.69 0.019 29	0.68 0.019 33	a b c

* Total of only 46 plants, in other progenies 80—90 pines.

ber of terminal leaders. This analysis was made through analysis of covariance with terminal leader length and the number of pines in the progenies, inter alia as so-called concomitant variables. When the linear influence of terminal leader length and the number of pines has been eliminated a certain variation remains, which could possibly be of a genetic nature. Furthermore, the values for certain treatments have been divided into components, which correspond with the father and mother trees represented in the material.

The analysis, table 5, 6, and 7, should be considered together with the summaries in table 2, 3, and 4, which treat of the same pine material. *Melampsora* rust displays block effects, but also certain treatment effects and general combining ability.

3. Discussion

Practically all the information concerning resistance to *Melampsora* on pines in the literature cited above stems from more or less fortuitously recorded data on spontaneously infected pine. The information has often been intended for quite different purposes, e.g. genetic variation without regard to parasitic attack in the first place EKLUNDH EHRENBERG (1963), research into *Lophodermium* SCHÜTT (1964, 1965), HATTEMER (1965); the material referred to above, however, is intended for studying *Peridermium*. Moreover the information concerning the occurrence of *Melampsora* refers only to isolated years.

For a study of the hereditary transmission of resistance it is necessary to give *Melampsora* a central position in the collection of plant material and in making the desired crossings. Meanwhile, leads, suggestions and practical observations can be gained from other works. The material used here shows that information from one year can give convincing values, while those for another year taken from the same material can be highly contradictory. Thus the need for repeated experiments over a number of years is patent.

Studies of *Melampsora* on pine aimed at resistance breeding are therefore still in their embryonic stage. Both the way of recording the amount of *Melampsora* and methods of inoculation must be discussed. The material on which the above summary is based concerns progenies only and in this way distinguishes itself from previous studies of *Melampsora*. The pine material is consequently more clearly defined and the desired crossings can be repeated.

The material indicates, however, that there are certain differences in attack frequency between progenies, on which grounds there is every reason for

Table 5. Analysis of the Kratte Masugn Station material. Alternative I

Mother trees: X 4207, Z 1000, X 2209
Father trees: Z 4404, X 4203, X 2201

1964

Analysis of variables	Concomit. variables	Block-effect	Treatment (progeny)	Male	Female	♀×♂
x_1	—					
x_2	—					
x_3	—					
x_4	—	*		**		
x_5	—			*		
x_6	—		*	**		
y_1	—		**			*
y_2	—	*	*	*		
y_3	—		*			
y_4	—		*	**		
x_4	x_1	*		*		
x_5	x_2			*		
y_1	x_2		**			
y_1	x_2, x_5					
y_2	x_3	*	**	**		
y_2	x_3, x_6					

1965

Analysis of variables	Concomit. variables	Block-effect	Treatment (progeny)	Male	Female	♀×♂
x_1	—					
x_2	—		*			
x_3	—					
x_4	—	***				
x_5	—	***				
x_6	—	***				
y_1	—	***	*			
y_2	—	***	*	*	**	
y_3	—					
y_4	—	*	*			
x_4	x_1	**				
x_5	x_2	***				
y_1	x_2	***				
y_1	x_2, x_5	*				
y_2	x_3	***	*	**	**	
y_2	x_3, x_6	*		*	**	

1966

Analysis of variables	Concomit. variables	Block-effect	Treatment (progeny)	Male	Female	♀×♂
x_1	—		*			
x_2	—	*	**			
x_3	—		**	*	*	
x_4	—	***	*	*		
x_5	—	***	**			*
x_6	—	***	**			
y_1	—	***				
y_2	—	***	*	**	**	
y_3	—					
y_4	—					
x_4	x_1	***				
x_5	x_2	***	*			
y_1	x_2	***				
y_1	x_2, x_5					
y_2	x_3	***		*	*	
y_2	x_3, x_6				*	

Legend: *** strongly significant ($P < 0.001$)
** significant ($0.01 > P > 0.001$)
* almost significant ($0.05 > P > 0.01$)

Cf reservation in text
Significant values of treatments must be interpreted with great caution as the progenies are not randomized within blocks.

variabel x_1 number of pines
x_2 total length terminal leaders
x_3 number first whorl shoots
x_4 number completely unattacked pines
x_5 length in cm completely unattacked terminal leaders
x_6 number first whorl shoots on unattacked pines
y_1 number attacks, leaders
y_2 number attacks, first whorls
y_3 dead leaders
y_4 dead first whorl shoots

Table 6. Analysis of the Kratte Masugn Station material
Alternative II

Mother trees: Z 2016, X 4207, Z 4404
Father trees: X 4501, X 4203, X 2201

1964

Analysis of variables	Concomit. variables	Block-effect	Treatment (progeny)	Male	Female	♀×♂
x_1	—					
x_2	—		**			*
x_3	—					
x_4	—		**			
x_5	—		*			
x_6	—		**	*		
y_1	—		***			***
y_2	—	**	***			**
y_3	—			**		
y_4	—	**	***			**
x_4	x_1		*			
x_5	x_2					
y_1	x_2		**			**
y_1	x_2, x_5		**			**
y_2	x_3	**	***			**
y_2	x_3, x_6	*	**			***

1965

Analysis of variables	Concomit. variables	Block-effect	Treatment (progeny)	Male	Female	♀×♂
x_1	—					
x_2	—		*			
x_3	—					
x_4	—	**	*			
x_5	—	**				
x_6	—	**	*			
y_1	—	***	*	*		
y_2	—	***	*	**	*	
y_3	—			*	*	
y_4	—	***	*			
x_4	x_1	*	*			
x_5	x_2	*				
y_1	x_2	***	*			
y_1	x_2, x_5	**				
y_2	x_3	***	**	*	*	
y_2	x_3, x_6	*		*	*	

1966

Analysis of variables	Concomit. variables	Block-effect	Treatment (progeny)	Male	Female	♀×♂
x_1	—		*			
x_2	—		*			
x_3	—		**			**
x_4	—	***	***	**		
x_5	—	***	***	*		
x_6	—	***	***	*		
y_1	—	***	*	**		
y_2	—	***	**	*		
y_3	—					
y_4	—					
x_4	x_1	***	***	*		
x_5	x_2	***	**	*		
y_1	x_2	***		*		
y_1	x_2, x_5	*				
y_2	x_3	***	**	*		
y_2	x_3, x_6	***				

Legend: *** strongly significant ($P < 0.001$)
** significant ($0.01 > P > 0.001$)
* almost significant ($0.05 > P > 0.01$)

Cf reservation in text
Significant values of treatments must be interpreted with great caution as the progenies are not randomized within blocks.

variabel x_1 number of pines
x_2 total length terminal leaders
x_3 number first whorl shoots
x_4 number completely unattacked pines
x_5 length in cm completely unattacked terminal leaders
x_6 number first whorl shoots on unattacked pines
y_1 number attacks, leaders
y_2 number attacks, first whorls
y_3 dead leaders
y_4 dead first whorl shoots

Table 7. Analysis of the Brunsberg Station material

Mother trees: S 3006, S 3244, S 6256
Father trees: S 3098, Eo 27, Eo 16, Up 16, Up 17, Sm 60, S 3245

1964

Analysis of variables	Concomit. variables	Block-effect	Treatment (progeny)	Male	Female	♀×♂
x_1	—	*	***			
x_2	—		***			**
x_3	—		***			***
x_4	—	**				
x_5	—	*				*
x_6	—	**				
y_1	—		**		**	
y_2	—	*	*			*
y_3	—					
y_4	—				*	
x_4	x_1	*				
x_5	x_2	*				
y_1	x_2				*	
y_1	x_2, x_5		*		*	
y_2	x_3	**				
y_2	x_3, x_6					

1965

Analysis of variables	Concomit. variables	Block-effect	Treatment (progeny)	Male	Female	♀×♂
x_1	—		***			
x_2	—	**	***			***
x_3	—	**	***		**	***
x_4	—	***		**	*	
x_5	—	***	*		*	
x_6	—	***	*	*	**	
y_1	—	***	*			
y_2	—	***	**			**
y_3	—	**				
y_4	—	***				
x_4	x_1	***				
x_5	x_2	***		*		
y_1	x_2	***		*		
y_1	x_2, x_5	**				
y_2	x_3	***	*			
y_2	x_3, x_6	**				

1966

Analysis of variables	Concomit. variables	Block-effect	Treatment (progeny)	Male	Female	♀×♂
x_1	—		***			
x_2	—	*	***			***
x_3	—		***		**	***
x_4	—	***	***	**	***	
x_5	—	***	***	*	***	
x_6	—	***	***	**	***	*
y_1	—	***	***	*		
y_2	—	***	**			*
y_3	—					
y_4	—					
x_4	x_1	***	***	*	**	***
x_5	x_2	***	***	**	***	
y_1	x_2	***	**	*	**	
y_1	x_2, x_5					
y_2	x_3	***	***	**	***	***
y_2	x_3, x_6			**	***	

Legend: *** strongly significant ($P < 0.001$)
** significant ($0.01 > P > 0.001$)
* almost significant ($0.05 > P > 0.01$)

Cf reservation in text
Significant values of treatments must be interpreted with great caution as the progenies are not randomized within blocks.

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x_3 number first whorl shoots
x_4 number completely unattacked pines
x_5 length in cm completely unattacked terminal leaders
x_6 number first whorl shoots on unattacked pines
y_1 number attacks, leaders
y_2 number attacks, first whorls
y_3 dead leaders
y_4 dead first whorl shoots

continuing the work on resistance breeding. In the shorter term it is likely that studies of the effects of fertilization or systemic fungicides can give practical results.

If one could prove general combining ability according to the analysis of variance and covariance in conjunction with the occurrence of *Melampsora* this could be regarded as a good condition for genetic gain in pine seed orchards always based on a number of clones. It would also be indicative of a real connection between the parent tree and progeny.

The work has endeavoured to draw attention to the difficulty of recording the disease in an unexceptionable way. Taken overall, the plant height has increased from 0.5 to 2.0 m in the four years the work lasted. Irrespective of how the disease is recorded—attack per pine, attack per shoot, attack per cm of terminal leader, the percentage of healthy pines, dead pines, or pines that sustained lasting economic damage—there is a great amount of work involved in dealing with such large plants. If this not withstanding it was decided for instance to record attack per centimetre of terminal leader as constituting a reasonable criterion so far as the older plants are concerned, there remains the objection that the permanent defect —recorded for the Kratten material, table 1—does not appear to be entirely compatible with the number of attacks. It is after all this defect, in addition to a certain decline in height growth, that is of importance. It is therefore fitting to investigate whether progeny testing might not be carried out using 1 or 2 year old plants that have been inoculated under control. In this case the recording could be limited to concern attacked or unattacked pine plants. It would be possible in this way to avoid the bothersome recording of top and first whorls separately together with the corresponding attack frequencies. Early recording is preferable in every respect, but it is necessary to examine how such early recording correlates to later damage and permanent defects caused by twist rust after the regeneration stage.

It would also seem easier to influence several environmental factors in a quite different way if a drastic reduction could be made in the size of the test area. It is certainly possible, in a plastic greenhouse for instance, to bring about a uniform dissemination of spores from aspen leaves in combination with suitable irrigation.

The reason for the contradictory results of tests that have been repeated for several years may be sought partly in a complex variation of environmental factors and in irregular spontanous infection. One of the few measures that can be taken in experiments of large field size is to spread aspen leaves with telio spores on the ground so as to in this way increase the likelihood of a more uniform infection. Climatic factors will nevertheless be decisive in

studies of large pine material where it is impracticable to give each pine individual treatment.

The investigation described above has thus in general attempted to illustrate the *Melampsora* problem in conjunction with field tests. The next section will deal with questions of a physiological nature in an endeavour to gradate the interplay between host and parasite.

In connection with the field tests it might be mentioned that certain insect damage is more common in regenerations with a high *Melampsora* frequency, BÖHNER (1952), REGLER (1957). It can be added that insect larvae often choose to eat the actual fungus tissue in the developing caeoma. It has also been noticed that in very damp years a number of fungi appear in combination with *Melampsora*, and that in consequence the damage tends to be more extensive. In this connection genera such as *Fusarium, Alternaria, Botrytis, Südowia* (= *Sclerophoma*, cf. BUTIN 1963, 1964) are common.

No extensive attempt to assess the economical aspect of the damage caused by the fungus has been made. The fungus occurs in the whole of Europe and the adjacent parts of North Asia and its significance as a parasite varies. Concerning Swedish conditions KARDELL (1966) has tried to analyse the cost of certain measures, e.g. the correction of double top and similar defects in reforestation. The only counter measure that can be taken at the present time is to exterminate the alternate host, the aspen, which involves very considerable practical difficulties. *Pinus contorta* has to a certain extent replaced *Pinus silvestris* in some parts of the Norrland coastland. In the long term it may be necessary to resort to the breeding of pines, that are resistant to twist rust. There are no chemical fungicides with which to combat the fungus when it is present on pines or to use against teilo spore germination on aspen leaves on the ground.

II. Melampsora pinitorqua on Pinus silvestris and in relation to growth inhibitors

1. Approach to a study of Melampsora pinitorqua and growth regulating substances

It was noticed at an early stage that *Melampsora pinitorqua* is more common on terminal leaders than on first whorl, SYLVÉN (1917), KLINGSTRÖM (1963). The examination of progenies also shows almost without exception that the average length of terminal leaders on cankered pines is greater than that of terminal leaders of pines without pine twist rust within the progenies (Fig. 1). The material is taken from table 3. The relationship between the length of the fully-developed shoot and the processes that regulate the actual course of infection is not known. Similar details concerning the influence of plant height have been recorded by EKLUNDH EHRENBERG (1963), SCHÜTT (1964), KARDELL (1966); and ILLY (1966) contends that the more vigorous a plant is, the greater the likelihood that it will be attacked. It would appear easy to assume that growth regulating substances in *Pinus silvestris* affect the occurrence of *Melampsora pinitorqua*, either directly or indirectly. Moreover, clone material is available that for many years has been the subject of study with regard to pine twist rust, and that has consistently displayed a high and low attack frequency, BERGMAN (1954), RENNERFELT (1954), KLINGSTRÖM (1963). The above has formed the basis of research into *Melampsora pinitorqua* and growth regulating substances in *Pinus silvestris*.

There are very few works dealing with growth regulators in *Pinus silvestris*, and earlier works make practically no reference to the connection between resistance and growth regulators cf. e. g. GIERTYCH (1964), KOZLOWSKI (1964). On the other hand there is a wealth of literature on differences between healthy and diseased plant parts with reference to growth regulating substances, these have been summarised by SEQUEIRA (1962), or changed physiological processes, RICH (1963), TOMIYAMA (1963), SHAW (1967).

Earlier reports on differences in the amount of growth regulating substances in various parts of the crowns of pine species are contradictory. GIERTYCH and FORWARD (1966) describe a distinct gradient in growth re-

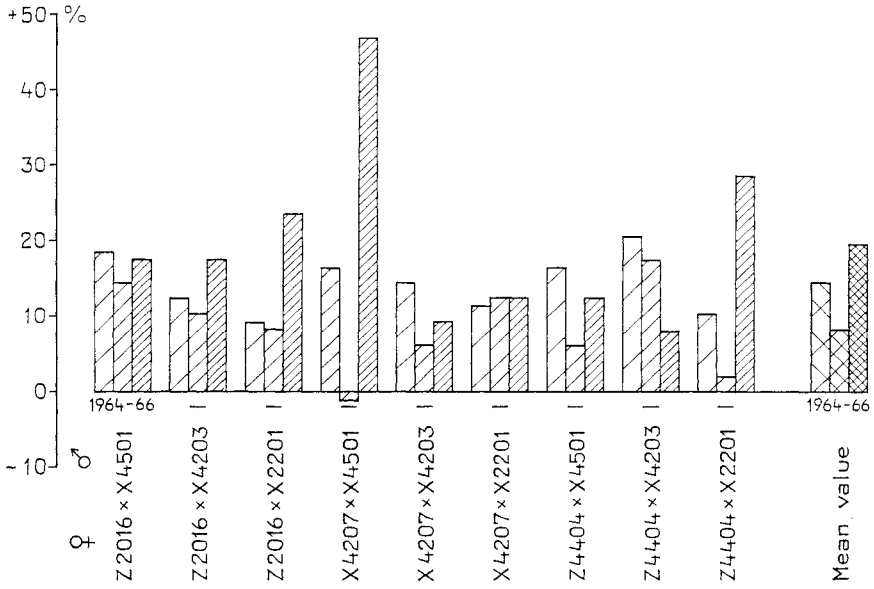


Fig. 1. Comparisons within progenies between the average length of terminal leaders in healthy pines and that of pines attacked by *Melampsora pinitorqua*.
The average length of terminal leaders in healthy pines in the diagram = 0.

gulator levels in *Pinus resinosa* showing the highest values in the lower part of the crown. Also differences between the top and the first whorl are described. This does not exclude the possibility that there is a connection between susceptibility to *Melampsora pinitorqua* and growth regulator content.

2. Materials and methods

The Avena coleoptile straightgrowth tests were carried out at the Department of Botany, University of Stockholm according to methods described by HEMBERG (1958). Oat seeds (var. Brighton) were swelled and germinated for 4 days; the first day the oat seeds are kept on moist filter paper at + 15° C in darkness, on the second day artificial light is added (incandescent light, 25 W—50—100 cm). The third to fourth days the coleoptiles are cultivated in water saturated vermiculite in total darkness at + 24—25°C and 80—90 % relative air humidity. (During the fourth day the coleoptiles are irradiated for three hours with red light, PF 704 E about 20 hours before use.)

Coleoptiles with lengths between 20—25 mm were selected. But in every special test the limits were narrower, 20.0—22.5 mm or 22.5—25.0 mm. One 5 mm section was cut from each coleoptile 3 mm below the tip. Different

compounds, strips of chromatograms, or fractions separated by gelfiltration were placed in small beakers and to these were added 4 ml citrate buffer (approx. pH 4.2)—0.248 g monopotassium citrate, 16 g glucose, and 1 g Tween 80 per litre. Ten coleoptile sections were placed in each beaker for approx. 20 hours in darkness and at + 25°C and approx. 70—80 % relative air humidity; the beakers were gently shaken during this time on a reciprocal shaker. The cutting was carried out in green safe-light (fluorescent tubes Philips TL 20 W/17—and filter—CEA 4 B). At the end of the test time the coleoptile sections were measured in a dissecting microscope to the nearest 0.1 mm. They were compared with the values from control beakers with all ingredients except the plant extract or other test compounds. The mean value of 10 sections from each beaker is compared with the total mean value of the controls as a percentage value. This can be found in all essentials in sundry publications.

The citrate buffer used has been studied in tests made at the Department of Botany, which have shown that inhibition is clearly registered in straight growth tests using the buffer in question. Stimulation, on the other hand, was not so well defined. A different buffer solution (phosphate buffer) is now in routine use in the Department, cf. ELIASSON (1969): K_2HPO_4 0.01 M, citric acid 0.005 M, glucose 1.6 per cent and with pH approx. 5.0. 2 ml of this solution is used in each test beaker. In this work the newer method has been used only in some of the final tests—cf. text. Further it has been used for checking the coleoptile reactions in table 9 and in a few tests with resin acids.

Melampsora basidio spore germination test. Aspen leaves with telio spores of *Melampsora* which were ready to germinate were used in these tests. The suitable time usually coincides with the shooting of Scots pine. KLINGSTRÖM (1963) has proved that the spores' ability to germinate can be poor if the aspen leaves are gathered too early. The dry aspen leaves can be stored for a long time at a low temperature and retain their ability to germinate. Cool storage rooms at + 4°C and freezers at — 25°C have both been used with good results.

It is plain that all telio spores on aspen leaves need not with one hundred per cent certainty belong to *Melampsora pinitorqua*, although the degree of probability in Swedish conditions can be regarded as being quite high.

The germination test was conducted in the following way. The aspen leaves were moistened in cold water (+ 4°C) for one night, after which they were put in ordinary Petri dishes at + 15—20°C. Care was taken to see that the leaves were neither allowed to dry out nor to become too moist. If the aspen leaves are gathered at the right moment the spores often germinate after only one hour—usually within 24 hours—and the basidies appear as a light velvety surface over the dark telio spore crusts. It is not possible to define the time more accurately, and it is necessary to determine the germination properties of the test material from year to year. This can be seen with the naked eye or with slight magnification. The telio spores seldom germinate uniformly over the entire leaf surface.

As soon as it was possible to establish that the telio spores had started to germinate, suitable pieces (approx. 1 cm^2) of the aspen leaves with abundant basidio spore formation were fastened with a drop of water to the underside of the cover of a Petri dish containing water agar (1 %), so that the basidio spores were able to fall onto the surface of the agar. After about 24 hours at

+ 15—20°C it was possible to establish that a very high percentage of the spores had germinated.

In all experiments spores were considered germinated when the length of the germ tube equalled or exceeded spore diameter.

In this work the germination tests were made in four-section Petri dishes of glass, each section containing 2 ml of water agar (1 %). The materials tested were dissolved in ether, which was introduced uniformly to the entire surface of the agar in the section concerned. The ether evaporated in a few seconds, after which the basidio spores were introduced in the manner described above.

The result of the spore germination test was noted as an eventual inhibition of spore germination due to the added test substances. No attention was paid to eventual stimulation effects. In many cases the result is given as one plus and one minus value, the intention being to ring in the interval where the minus value denotes the smallest quantity of a test substance that causes a definite inhibition of germination, while the corresponding plus value denotes the greatest quantity of the substance that has no effect.

The entire method is simple and is not very sensitive to changes in temperature and time during the test. The only exception is the actual fixing of the pieces of aspen leaves in the cover of the Petri dishes, as the basidies are sensitive to drying and higher temperatures. This phase must be done fairly quickly.

Certain variations in the spore germination test can well be considered. The substance introduced to the agar surface can occasionally form crystals or other structures that make it difficult to note the results. This can be avoided by setting the test substance in the agar.

An extract of Scots pine material was made according to FRANSSON (1953). Pine shoots from which the needles had been removed were ground in a turmix mill (approx. 10 sec.) together with a double amount by volume 96 % ethanol (+5°C). This means that for 25 g of pine material, for instance, 50 ml of ethanol was used. All details of fresh weight in relation to ethanol, chloroform and ether are to be interpreted on this basis throughout the entire description. The material was extracted for three hours (+15° C in darkness) and the ethanol was changed 3 times.

This extract was filtered on a Büchner funnel and evaporated at + 37°C in a rotary evaporator. The residue was then shaken for one hour with $CHCl_3$ (4 times fresh weight volume). This $CHCl_3$-solution was filtered and evaporated under vacuum as above at + 37°C. The residue was shaken for three hours with distilled water (4 times fresh weight). The water was filtered and stored overnight (+15°C). The following day a saturated $NaHCO_3$-solution was added to pH 8—9, after which the water was shaken 3 times with freshly distilled diethyl ether (double fresh weight quantity). The water was then made acid with HCl to pH 3.4 and again shaken 3 times with ether (double fresh weight quantity). The acid ether layer was in the first place intended for further experiment. The ether layer was chilled for two hours at — 30°C so that the water was removed from the ether by freezing, after which the ether was concentrated by evaporation, rotary evaporator as above. The finished extract was stored in nitrogen at — 20°C. After the taking of samples in the field the material was protected from daylight, and low temperatures were aimed at in the course of the work.

During 1966 and 1967 freshly distilled ether was used. During final and supplementary experiments in 1968 and subsequently ether with a stabiliser was used (MALLINCKRODT $(C_2H_5)_2O$ anhydrous analytical reagent). Differences which could be linked with the change in the quality of the ether could not be traced.

The method includes several phases which should be discussed if the work is to continue. An attempt was made to dispense with the ether shaking. The ether was replaced by chloroform, which is at least less explosive, but the yield of inhibitory substances was considerably lower (WÄRN, unpublished).

The method described above concerned only the preparation of small quantities of pine material, seldom over 100 g fresh weight. This results in the evaporation residue on the flask walls before and after shaking with chloroform and with water appearing to remain within tolerable limits. If on the other hand the intention is to prepare larger quantities, it is preferable to use a method that does not permit of an excessive residue, as this involves the risk of obtaining an inferior yield. A method has been tried on an experimental basis that is more in keeping with established practice in organic chemistry work. It can be outlined as follows: Extraction in ethanol, as above, and evaporation. The residue is shaken with a mixture of $CHCl_3$ and ethanol (3/1), which usually dissolves the entire evaporation residue. A viscous mass may still remain on the beaker wall, but this too can be dissolved if small quantities of water are introduced—although this simultaneously increases the risk of foam forming later in the process. The mixture is fractioned in a first step towards $NaHCO_3$-solution and in a second step towards 0.5 N NaOH, which aims at the preparation of organic acids and phenols respectively. It is possible to re-shake with $CHCl_3$. Thus it should be possible by using this method to avoid the basic ether shaking. Both fractions are acidulated with HCl to pH 3.0—3.5 and fractionated with ether (the $NaHCO_3$-fraction perhaps in a further step to pH 1). The acid ether preparation does not in this case contain the green components which have been interpreted as chlorophyll and related substances in comparison with the preparation sequence described first, provided that water is not introduced to the chloroform-ethanol. These substances remain in the chloroform-ethanol. Also other components are absent, e.g. several substances with a high Rf-value in accordance with the summarising table 9. On the other hand the yield of other substances is relatively better, for instance the components with a low Rf-value shown in the same table. Without having studied the biologial effects in detail, it is nevertheless safe to say that the bicarbonate layer, as an acid ether extract, has a clear inhibitory effect on the *Melampsora* basidio spore germination test. After preliminary chromatography it appears that the inhibition for the most part agrees with fraction 5 in table 9.

Chromatography. In conjunction with above all the coleoptile test material was chromatographed *on paper* (Whatman No. 1). Prior to chromatography the paper was washed for one day with distilled H_2O and for 2 to 3 days with the cromatographic solvent system for which the paper was intended. The washing was done in the same way as a descending chromatography.

Ascending chromatograms on 2.5 cm paper strips were used and the solvent front was allowed to rise 15 cm. (Line application). The chromatograms were run in tubes, one in each, at + 25°C in darkness in all essentials according to

NITSCH (1956) and HEMBERG (1958). Ethanol, 70 %, according to FRANSSON (1953, 1959) and isopropanol-ammonia (sp.gr. 0.91)-water (100/14/6 v/v) according to HEMBERG (1958) served as solvents in most of the orientating experiments. During 1966 the chromatograms were cut in 1 cm segments for the bioassays, and later on the chromatograms were cut in 10 segments corresponding to Rf-units.

For separation *on TLC plates*, only instant thin layer chromatography plates —TLC plates Silica Gel F_{254} (Merck) 20×20 cm were used. For the most part butanone(= ethylmetylketone)-hexane (30/70 v/v) was used for separation in combination with multiple development, which in this case means that each plate was chromatographed four times (2×6 and 2×12 cm). The plates were chromatographed in vessels of a suitable size (approx. 21×24×8 cm), which were carefully lined on the inside with filter paper so as to ensure saturation, and the gel coated side of the plate was always turned obliquely towards the vessel wall. The temperature was + 25°C, but this affects the Rf-values less for instance than slight variation in the degree of concentration of the chromatographic solvent system, personal dexterity, or applied amount. A special account of the work from which the above method was evolved will be given by KIM v. WEISSENBERG.

For the studying of chromatograms, sometimes in conjunction with photography, a Desava Uvis has been used (cf. STAHL 1967). It is possible with the same film to photograph partly in visible light and partly in UV light 366 mμ and 254 mμ. In this work Agfacolour CT 18 has been used, and the UV light used a filter combination of UV + CClOM + CClOG + CClOY and aperture 8 with an exposure time of between 15 and 25 seconds. For visible light the usual exposure was half a second with aperatures between 11 and 16. The type of filter combination depends to some extent on the type of indication given by UV light. A Hasselblad 500 C camera was used. The method was used following a discussion with Dr MATTI VARESMAA, Department of Food Chemistry, Helsinki 71, Finland, who worked out the filter combination.

For the purpose of this experiment Ehrlich's reagent is 4-dimethylaminobenzaldehyde. The reagent is normally used to show the presence of indole compounds but it also reacts to many other substances, in this experiment, for instance, to certain organic acids and phenol-type substances.

In accordance with normal practice in the Department 2 g dimethylaminobenzaldehyde was mixed with 80 ml ethanol, 3 ml H_2O and 17 ml concentrated HCl. The reagent was stored at + 4°C. The spray reagent differs somewhat from, for instance, STAHL (1967) Nos. 66 and 67. The above method usually gives very distinct colour reactions on TLC plates. Certain phases are sensitive however; when the plate has been sprayed completely matt with the reagent it should be warmed for 2 or 3 minutes at 60—70°C under supervision. If after heating the coated side is protected by taping fast a glass plate of a similar size, this will prevent the yellowing of the chromatogram.

3. Orientating experiment

The original intention was to discover whether it was at all possible to produce an ether extract of pine shoots and to relate this work to earlier works. In this connection the first step was to study the possibility of using the *Avena* straight growth test as a bioassay, but it was considered important to try to use *Melampsora pinitorqua* as a test organism for the ether extract of pine. A method was devised for testing the effect of the extract on the basidio spore germination of the fungus. Both the *Avena* test and the basidio spore germination test, as also the production of the extract, are described in material and methods. The pine shoots were collected towards the end of the shooting period which coincided with the formation of the pine twist rust acio spores.

Linking up with FRANSSON's work (1953, 1959), an acid ether extract of *Pinus silvestris* terminal leaders was chromatographed on Whatman paper No. 1 with 70 % ethanol. These works were generally speaking all that could be referred to as regards *Pinus silvestris* when the present work started in 1966. The *Avena* straight growth test shows a distinct inhibition at Rf 0.8—0.9, and the same inhibition occurs if one uses the *Melampsora* basidio spore germination test as a bioassay and eluates strips of the paper chromatograms in ether, see fig. 2. In contrast to this, FRANSSON describes no inhibitor effect. The material used by him consisted partly of three-week old pine plants and partly of two and three-year old stem and branch parts, which limits the comparability. As far as can be determined, the method of producing the ether extract was the same as that used by FRANSSON (1953).

The same material was chromatographed with isopropanol-ammonia-water (100/14/6), a solvent system found to be very useful in studies of growth regulators in plant material, KEFFORD (1955), HEMBERG (1958). Both the *Avena* test and basidio spore germination indicate marked inhibition at lower Rf-values in the region of 0.4—0.6, see fig. 2. The inhibitors in ether extracts of *Pinus silvestris* correspond to the inhibitor β complex of BENNET-CLARK & KEFFORD (1953)—see also HEMBERG (1961) and MILBORROW (1967).

Eluations for the spore germination test cannot in this case, however, be made in ether direct but by an acid fractionation with ether, which presumably depends on the presence of NH_3 in the solvent system. The chromatograms were usually divided into 10 parts corresponding to Rf-units,

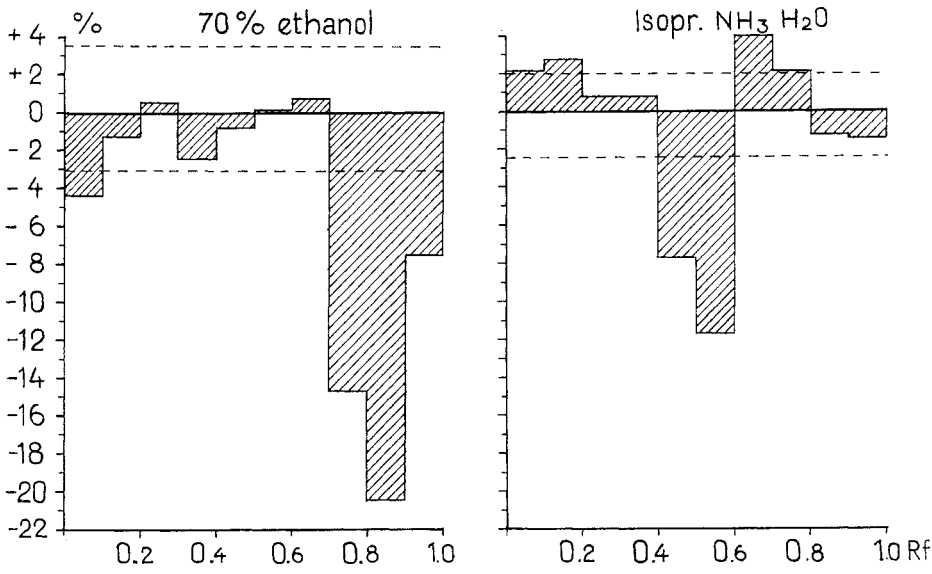


Fig. 2. *Avena* coleoptile assay of paper chromatogram sections of acid ether extract of *Pinus silvestris* terminal leaders. Progeny W 4642 × X 4501. Extract from 0.5 g fresh weight. Left: 70 % ethanol. Right: isopropanol-ammonia-water (100/14/6). Final length of *Avena* coleoptile segments in per cent of final length of control segments. Dotted lines = extremes of controls.

which were put in test tubes with about 1.5 ml diluted HCl (pH 2.5) and 1 ml ether. The tubes were shaken vigorously, and after a minute or so the ether could be removed with a pipette and the separation repeated once again. A similar procedure is described by MILBORROW (1967). The β inhibitor has previously been described in *Pinus resinosa* and *P. palustris*, GIERTYCH and FORWARD (1966), ALLEN (1960). The β-complex probably also occurs in other conifers, BONGA and CLARK (1965), COUVY (1962), OGASAWARA (1966).

In both these methods of chromatography the biologically active Rf-values give distinct colour reactions with Ehrlich's reagent, which suggests that among other things indole compounds may be present. The colours can vary in shades of yellow, blue, violet and green for the given Rf-values, which suggests unsatisfactory separation of an unknown number of substances.

Butanol-propanol-ammonia-water (2/6/1/2) was also tested on thin layer plates as a comparison with an extensive work by MILBORROW (1967). MILBORROW compares earlier reports on inhibitor β complex with the presence of (+)abscisin II and illustrates the possibility that the inhibitor effect can in

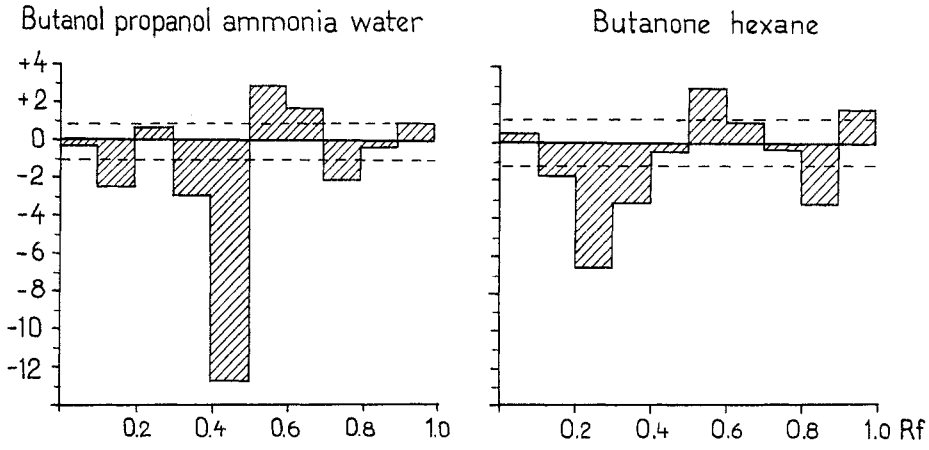


Fig. 3. *Avena* coleoptile assay of chromatograms on TLC-plates (silica gel F_{254} Merck) with acid ether extract of *Pinus silvestris* terminal leaders. Progeny W 4642 × X 4501. Extract from 0.5 g fresh weight. Left: butanol-propanol-ammonia-water (2/6/1/2). Right: Butanone-n-hexane (30/70) and multiple development (2 × 6 + 2 × 12 cm). Final length of *Avena* coleoptile segments in per cent of final length of control segments. Dotted lines = extremes of controls.

many cases have been caused by (+)abscisin II. In this case the same pine material gives a similar inhibition at Rf 0.4—0.5 in the *Avena* test. Moreover basidio spore germination is inhibited at higher Rf-values, which suggests better separation and also that more than one substance can be biologically active. Eluation for the spore germination test must, as in the previous case, be done via fractionation with acid ether. See fig. 3.

MILBORROW used silica gel GF_{254} (Merck). In the above tests the same gel was used in the form of instant TLC plates. Needless to say, no conclusions will be drawn at this early stage as to the eventual presence of (+)abscisin II.

It can be seen from the above that with the help of current methods it is possible to study the biological effects of an acid ether preparation of *Pinus silvestris* terminal leaders. However, these methods include several less favourable details and phases, such as a less distinct separation on paper than on TLC plates, slow separation when water is present in the solvent systems, the need to use an acid ether fractionation in connection with eluation of TLC plates and paper for further spore germination test when ammonia is present.

Developing with Ehrlich's reagent gives clear but complex colour reactions; further, preliminary tests with gel filtration indicate that the acid ether extract can contain several substances, KLINGSTRÖM (1967).

To make the picture more gradated a number of solvent systems were

tested, the aim being by using chromatography to obtain better separation. It transpired that butanone-hexane (30/70) and multiple development (2 × 6 cm + 2 × 12 cm) on TLC plates gave at least 16 bands that could be developed with Ehrlich's reagent and furthermore Rf-values that appeared in UV light were observed. (In this connection there has been valuable collaboration with Forest Officer KIM VON WEISSENBERG.) The method using multiple development has been described by among others STAHL (1967).

An acid ether extract of pine terminal leaders can in other words contain so many substances, that considerable difficulties can be expected when making a chromatographic separation. There are with certainty several substances in the biologically active Rf-values mentioned above.

The same pine material was chromatographed with butanone-hexane (30/70) for further orientating bioassay. It was discovered that it is possible to scrape off the silica gel and put this straight in the vessel used for *Avena* test, see fig. 3. The most marked inhibition occurs here at Rf 0.2—0.3. If there is doubt about the wisdom of adding the gel material to *Avena* test, bearing in mind the risk of eventual synergistic effects, KIRCHNER (1967), it was found that as a comparison it was possible instead to eluate the material in e.g. ether direct, without having to use the acid fractionating described earlier. This is an advantage in the spore germination test.

If the substances are eluated from the silica gel before the coleoptile assay the readings tend to be somewhat greater than in the diagram, fig. 3. It should also be noted that the Rf-values vary somewhat depending on the amount chromatographed when using this solvent system.

In the *Melampsora* basidio spore germination test inhibition occurs at the same Rf-values as for the *Avena* test but also at higher Rf, in the case of this pine preparation at 0.2—0.7. It is thus possible tentatively to assume that more than one substance is biologically active, and that the *Avena* test and the spore germination test do not react in exactly the same way to the same substances or concentrations.

The above tests concern only *one* acid ether preparation of pine using a uniform amount corresponding to 0.5 g fresh weight. Considerable variations occur between different pine materials, KLINGSTRÖM (1967). It has often been found suitable to start with tests concerning the total acid ether extract without prior chromatography.

A dilution series as given in fig. 4 shows exactly where the effect lies and also the amounts that can be suitable for e.g. chromatography. The figure also implies that the *Avena* test is in this case somewhat more sensitive than the spore germination test.

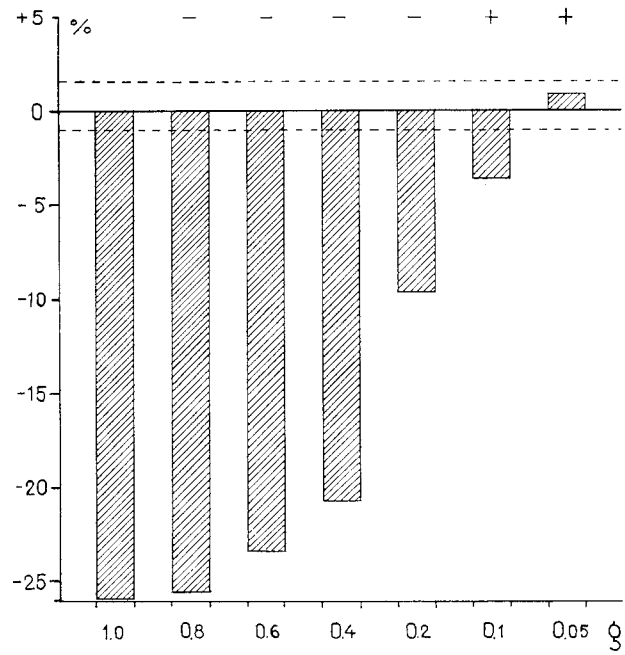


Fig. 4. *Avena* coleoptile assay of different amounts of total acid ether extract of *Pinus silvestris* terminal leaders. Progeny W 4642 × X 4501. Different amounts of extract corresponding to g fresh weight. Final length of *Avena* coleoptile segments in per cent of final length control segments. Dotted lines = extremes of controls. *Melampsora* basidio spore germination indicated by +, no germination by —.

The extract always contains varying amounts of impurities in the form of chlorophyll and related compounds. The colour of the extract, however, can seldom be related to its biological activity; this emphasizes the need for orientating dilution series. An orientating spectrophotometric test gives distinct readings at 660 and 400 mμ, which can be taken to indicate the presence of chlorophyll, and also in the UV band at 280 mμ, which is typical for many indole compounds, and a very distinct reading at 240 mμ.

Orientating experiments with the spore germination test were made with a number of other substances, for instance an acid ether extract from *Populus tremula*, which was made available by Dr. Eliasson, Department of Botany, University of Stockholm and also the substances listed in table 8. Tested as a total extract without prior chromatography, the *Populus* extract had a certain inhibiting effect.

In the same way it should be possible to use aecio spores or uredo spores of e.g. *Melampsora pinitorqua* for bioassay when studying the occurrence of the fungus on *Populus* instead of *Pinus*. In principle this should be easier than working with basidio spores. Taris (1965) has studied the germination of uredo spores.

Table 8. Orientating experiment with some organic compounds and *Melampsora* basidio spore germination test. Germination +. No germination —.

	1 mg	0,1 mg	0,01 mg
Pyrocatechol	—	+	+
Trans-cinnamic acid	—	—	+
Salicin	+	+	+
Salicylic acid	—	+	+
White coumarin	—	+	+
Indole-3-acetic acid	+	+	+
Indole-3-aldehyde	—	+	+
3 (β-hydroxyethyl) indol	—	+	+
Indole-3-glykoxylamide	+	+	+
Indole-3-acetamide	+	+	+
Indole-3-pyruvic acid	+	+	+
Indole-3-carbonic acid	+	+	+

4. Melampsora pinitorqua on pine clones in relation to inhibitors

During the first test year, 1966, samples were collected from clones that had previously been studied with regard to attack by *Melampsora*, viz. Z 66, Z 4013, Z 4005, KLINGSTRÖM (1963) and Z 25 and X 4207, BERGMAN (1954) and RENNERFELT (1954).

The material was available on Tenö, near Stockholm; the grafting material had previously been supplied by Svenska Cellulosa AB's pine seed orchard in Nedansjö. The samples were collected towards the end of axial extension when the fungus' caeomata was discernible. The acid ether extract was studied partly by straight growth test and partly by germination test. The material was chromatographed on paper with 70 % ethanol as described in the earlier works cited above.

Both clone groups showed that clones with a low *Melampsora* frequency give a stronger inhibitor effect than clones with a high *Melampsora* frequency. This holds good irrespective of whether *Avena* straight growth or *Melampsora* basidio spore germination is used as bioassay, see fig. 5 and 6.

If the total acid ether fraction is studied further in a spectrophotometer at 200—340 mμ it will be seen that the preparations from clones with a low *Melampsora* frequency have an appreciably greater absorbance over the whole wavelength scale than preparations from clones with a high attack frequency. The comparison concerns extract quantity per gram fresh weight. Thus the photometer curves indicate a greater absorbance for the extract from clone Z 66 with a low *Melampsora* frequency than for extracts from the

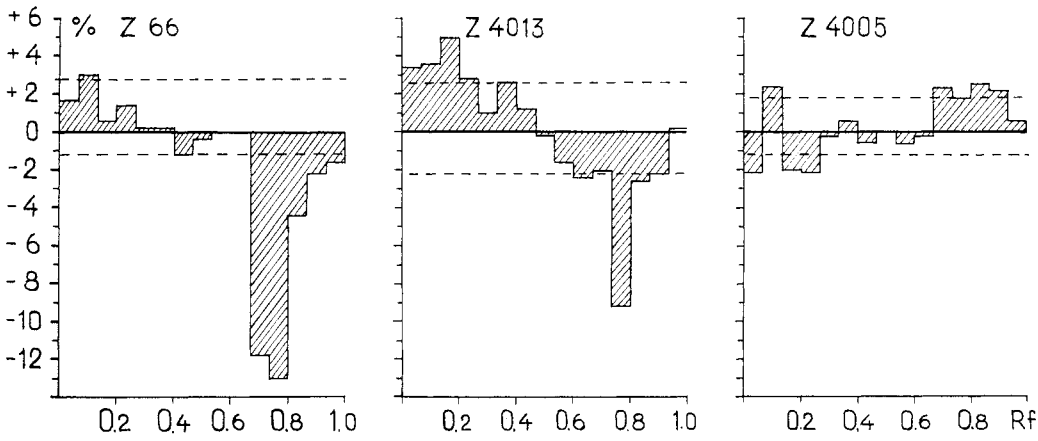


Fig. 5. *Avena* coleoptile assay of paper chromatogram sections of acid ether extract of *Pinus silvestris* terminal leaders from different clones. Extract from 0.1 g fresh weight. 70 % ethanol as solvent system. Final length of *Avena* coleoptile segments in per cent of final length of control segments. Dotted lines = extremes of controls.

Clone	1952* Number of		1957* Caeomata per terminal leader	Acid ether extract corresponding to gram fresh weight					
	grafts	caeomata		2.0	1.0	0.5	0.1	0.05	0.01
Z 66	38	0	0.9	—	—	—	—	—	+
Z 4013	29	3	0.9	—	—	—	—	+	+
Z 4005	41	16	3.3	—	+	+	+	+	+
Z 4009	27	34	9.0						
X 4207	10	5	—	—	—	—	—	+	+
Z 25	10	> 30	—	—	—	+	+	+	+

* From Bergman 1954 and Klingström 1963.

Fig. 6. Bioassay of acid ether fractions of *Pinus silvestris* terminal leaders of different clones. Germination of *Melampsora pinitorqua* basidio spores on water agar with different amounts of ether extract chromatographed with 70 % ethanol, Rf 0.66—0.86. Critical interval determinated by two or more replicates. Material collected at the end of axial extension. Germination +, no germination —.

other clones. The picture is not in proportion to the attack frequency in the field according to the collation fig. 6. But the clones have an interrelationship that is compatible with the *Melampsora* frequency.

If there is a connection at all between *Melampsora* frequency and the components in the acid ether extract, the result can be interpreted in such a way that only some of the substances that affect absorbancy have any connection with *Melampsora* frequency.

The experiments made in the first year indicate partly that there can be big differences in preparations from different clones, and partly that there

can be a connection between strong resistance to *Melampsora* and a high content of extractive substances (alternatively inhibitors) in the acid ether layer. This assumption is regarded as a new way of tackling a practical forestry problem and for this reason it was decided to repeat the experiments in 1967.

Continued experiments in 1967. During 1967 work was concentrated on clones Z 66, Z 4013, Z 4005 and Z 4009, which partly display great differences in susceptibility to pine twisting rust, and partly appear to be more reliable after being studied for several years. As before, the terminal leaders were collected towards the end of the shooting period. This coincided with the development of the first visible symptoms. Two acid ether extracts were made from each clone, and these were studied partly through *Avena* section test, partly through basidio spore germination and partly photometrically. See fig. 7, 8, and 9.

The pairwise preparations agree well with each other.

The clones retain the same internal order as in 1966.

The assumption remains that the extractive amount correlates to the occurrence of *Melampsora*, and that during shooting the clones can contain widely different amounts of inhibitors.

The chromatographic technique used above, and which is based on earlier works dealing with *Pinus silvestris*, involves an incomplete separation, but by way of recompense a coherent inhibitor effect. With the aim of gradating the picture the acid ether extract was also chromatographed with isopropanol-ammonia-water (100/14/6) as solvent system for a further *Avena* test using a phosphate buffer. As was mentioned in Materials and methods, this buffer emphasizes the stimulation effects.

This comparative test shows that clones with low *Melampsora* frequency, Z 66 and Z 4013, give an acid ether extract with a considerable inhibitor effect partly at Rf 0.4—0.6, which can be said to correspond to the classic β inhibitor, and partly at Rf 0.7—0.9. Stimulation effects are absent or are concealed by the strong inhibition. The concealed IAA effect is described by Wodzicki (1968).

Clones with high *Melampsora* frequency, Z 4005 and Z 4009, in general have some inhibitor β effect, but against this no conspicuous inhibition at higher Rf values. In this instance it is also possible to detect a certain stimulation partly at Rf 0.3—0.4, which can be thought to be IAA, and partly at Rf 0.0—0.2 fig. 10. The number of clones and the number of parallel tests is still very limited, and the result of the coleoptile test can of course be generalized to reflect the *Melampsora-Pinus* relationship only with the utmost caution. *Avena* tests and spore germination tests, however, show considerable differences between ether extracts from clones with different *Melampsora* frequency.

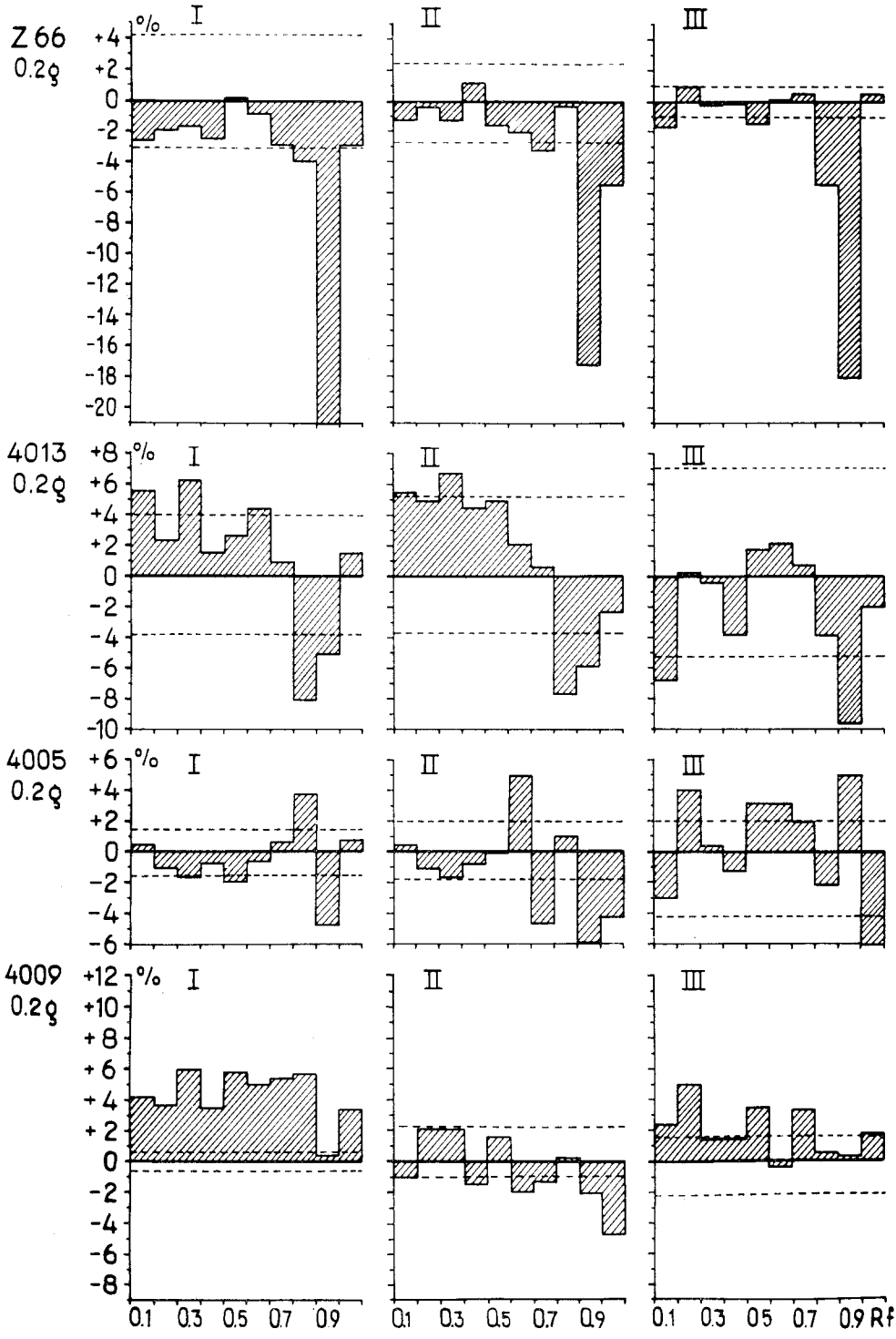


Fig. 7. *Avena* coleoptile assay of paper chromatograms of acid ether extract of *Pinus silvestris* terminal leaders from different clones. Extract from 0.2 g fresh weight. 70 % ethanol as solvent system. Two preparations were made from the same pine material—I and II from the first, III from the second preparation.
Final length of *Avena* coleoptile segments in per cent of final length of control segments. Dotted lines = extremes of controls.

Clone	Acid ether extract corresponding to gram fresh weight										
Preparation I	2.0	1.5	1.0	0.8	0.6	0.4	0.2	0.1	0.05	0.025	0.01 g
Z 66	—	—	—	—	—	—	—	—	—	—	+
Z 4013	—	—	—	—	—	—	+	+	+	+	+
Z 4005	—	—	+	+	+	+	+	+	+	+	+
Z 4009	—	—	—	+	+	+	+	+	+	+	+
Preparation II											
Z 66	—	—	—	—	—	—	—	—	—	—	+
Z 4013	—	—	—	—	—	+	+	+	+	+	+
Z 4005	—	—	—	+	+	+	+	+	+	+	+
Z 4009	—	—	+	+	+	+	+	+	+	+	+

Fig. 8. Bioassay of total acid ether fractions of *Pinus silvestris* terminal leaders from different clones. Germination of *Melampsora pinitorqua* basidio spores on water agar with different amounts of acid ether extract. Germination +, no germination —.
Critical intervals determinated by two or more replicates.

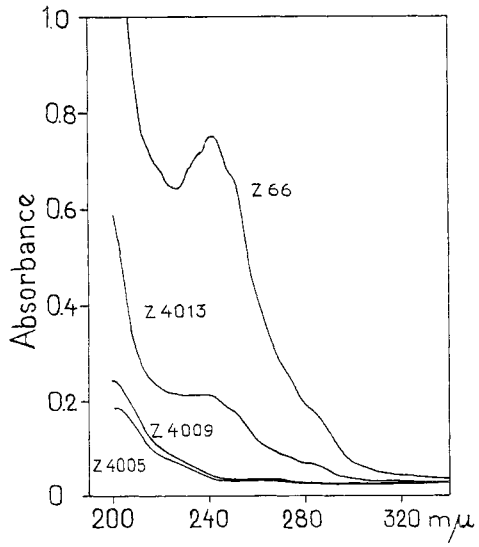


Fig. 9. UV absorption spectra of total acid ether extract of *Pinus silvestris* terminal leaders from different clones. Extract from 0.01 g fresh weight per ml ethanol.

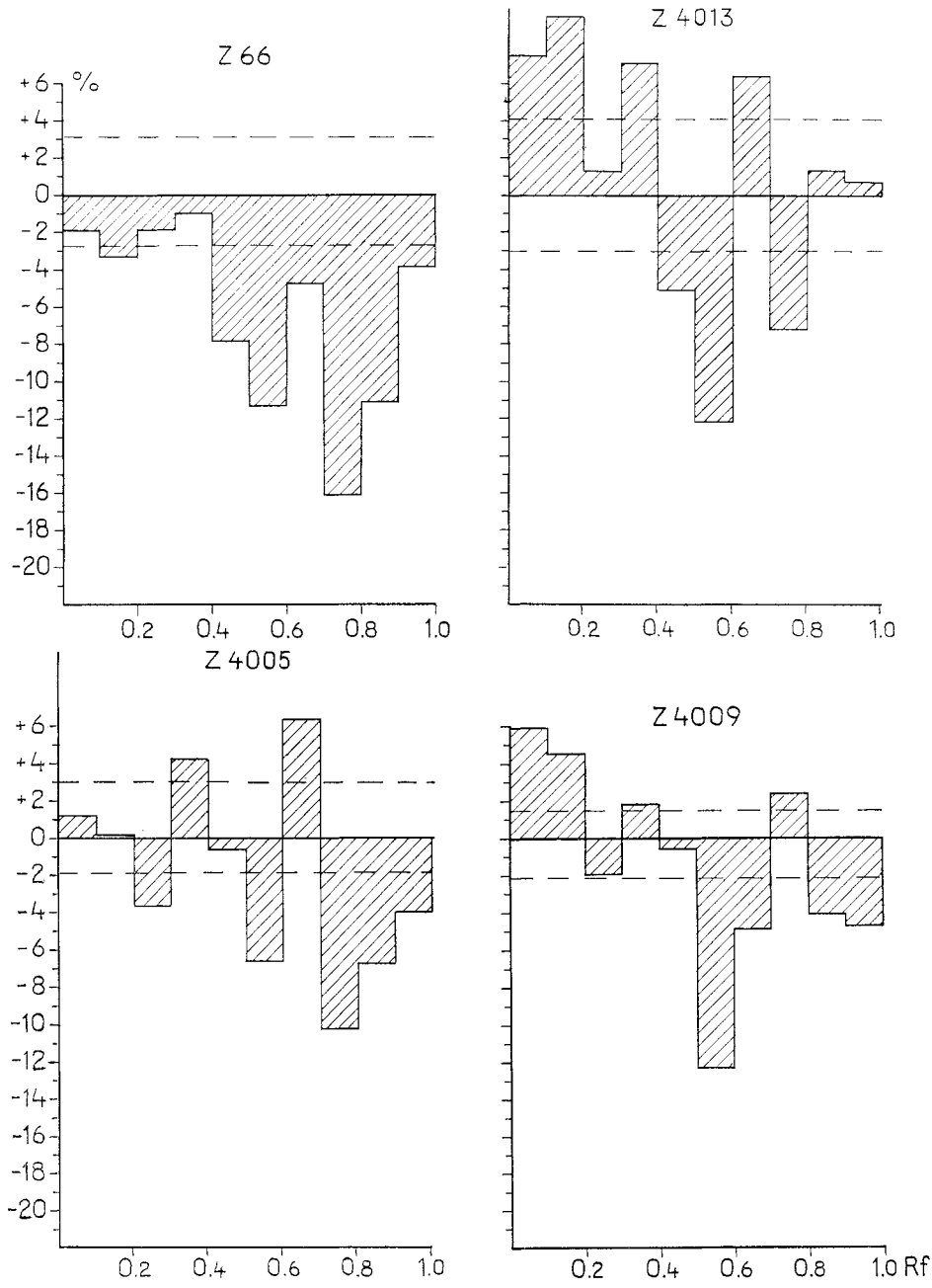


Fig. 10. *Avena* coleoptile assay of paper chromatograms of acid ether extracts of *Pinus silvestris* terminal leader from different clones. Extract from 0.2 g fresh weight. Isopropanol-ammonia-water (100/14/6) as solvent system.
Pine material as in I and II fig. 7.
Avena phosphate buffer.
Final length of *Avena* coleoptile segments in per cent of final length of control segments. Dotted lines = extremes of controls.

5. Inhibitors during axial extension

As was mentioned in the first part of the section dealing with *Melampsora* on *Pinus*, it is a generally held opinion that exposition resistance plays a decisive part in the occurrence of *Melampsora* on pine shoots. Climatic factors have without doubt a lot to do with the great differences in frequency that occur from year to year. On the other hand other explanations must be sought for the fact that for instance one clone is consistently freer from attack than another. The following tests intend to illustrate eventual changes of the acid ether extract during shooting, which can be important for instance in deciding when to take samples. It was considered advantageous to use clone material in the experiments and the choice fell to T 3057 and T 2009, which each embraced 60-odd grafts. Fifteen terminal leaders of each clone were collected on four occasions:

1 Buds near breaking—17/5 1967
2 Beginning of axial extension—5/6 1967
3 Rapid extension, needles more than half developed—21/6 1967
4 Shoot and needles fully developed—12/7 1967.

Pine twist rust attacks for a short time between two and three.

In keeping with the experiment in general, needles were removed to the extent that these formed.

Bioassay through *Avena* straight growth and *Melampsora* basidio spore germination show that the amount of inhibitors undergoes a considerable change during shooting. The inhibitor effect is first strong, and then falls very considerably before it increases once again. This pattern is the same for both the clones used in the test. The indication is somewhat greater for T 3057. The test is described in fig. 11 and 12.

The total acid ether fraction was also studied photometrically. From the absorbtion in 230—250 mμ it can be seen that the amount of extract substances in relation to fresh weight falls by about 40 times between the first and second samplings in the case of clone T 3057 in 1967. The difference in water content of the leader tissue during shooting is less than 10 % and cannot have been responsible for the result. The same is true of the fact that successively fewer leaders were used in the preparations as the weight of the leaders increased.

A change in the amount of inhibitors in conjunction with shooting has been demonstrated in the case of other plants, as summarised by among

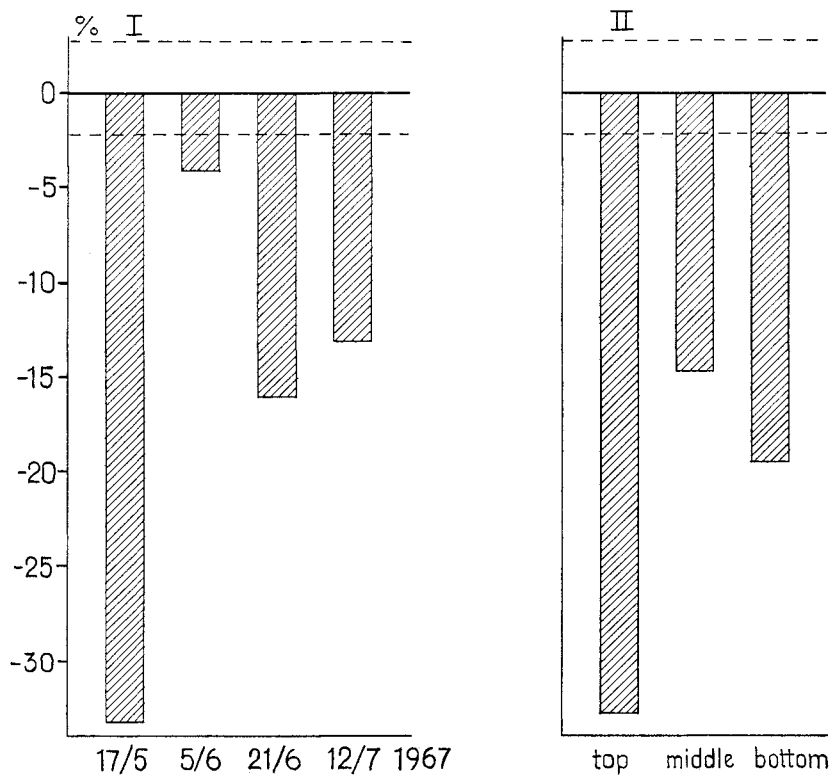


Fig. 11. *Avena* coleoptile assay of total acid ether extracts of *Pinus silvestris* terminal leaders, clone T 3057.
I. During axial extension May 17, June 5, 21, July 12.
II. July 12, preparations from top, middle, and bottom parts of shoots.
Extracts from 0.1 g fresh weight.
Final length of *Avena* coleoptile segments in per cent of final length of the control segments. Dotted lines = extremes of controls.

others HEMBERG (1961) and GIERTYCH (1964), and regarding conifers also by ALLEN (1960), COUVY (1962), OGASAWARA (1966), KOPCEWICZ et al. (1967). As regards the occurrence of *Melampsora pinitorqua* the above experiment supplements the previous one-sided theory concerning exposition resistance. Greater attention should be paid to growth regulators, at least in connection with the behaviour of *Melampsora pinitorqua.*

Clone T 3057 was also used for preparations from the third and fourth samplings, in that the terminal leaders were divided into top, middle and bottom thirds. The ether preparations were tested in the same way as in the previous test. See fig. 11 and 12. The top third gives both the highest absorbtion and in bioassay the greatest inhibitor effect. The middle section gave the lowest reading. The differences are greater for the fourth sampling

During axial extension

T 3057	Acid ether extract corresponding to gram fresh weight							
	0.5	0.4	0.3	0.2	0.1	0.05	0.025	0.01 g
17/5 1967	—	—	—	—	—	—	—	+
5/6	—	+	+	+	+	+	+	+
21/6	—	—	—	—	—	—	+	+
12/7	—	—	—	—	—	—	+	+
T 2009								
17/5	—	—	—	—	—	—	+	+
5/6	—	—	—	—	—	+	+	+
21/6	—	—	—	+	+	+	+	+
12/7	—	—	—	—	—	+	+	+

Different part of top leaders.

Clone T 3057 21/6 1967	0.5	0.4	0.3	0.2	0.1	0.05	0.025	0.01 g
top	—	—	—	—	—	+	+	+
middle	—	—	—	—	+	+	+	+
bottom part	—	—	—	—	—	+	+	+
12/7 1967								
top	—	—	—	—	—	—	—	+
middle	—	—	—	—	+	+	+	+
bottom part of terminal leaders	—	—	—	—	+	+	+	+

Fig. 12. Bioassay of total acid ether fractions of *Pinus silvestris* terminal leaders, clones T 3057 and T 2009. Germination of *Melampsora pinitorqua* basidio spores on water agar with different amounts of acid ether extract. Germination +, no germination —.
Critical interval determined by two or more replicates.

than for the third. Compare with Couvy (1964), who examined various tissues and parts of leaders from *Pinus pinaster* and discovered varying amounts of stimulating and inhibiting substances. See also the classification of the biologically active fractions page 58.

By way of comparison it can be mentioned that Giertych and Forward (1966) worked throughout with 0.02 g fresh weight of ether preparations of pine buds. At least the first sample, taken 17 May, should be comparable with this experiment and the inhibitory effect of the extract is of comparable strength.

The test was repeated in a somewhat modified way in 1968. The material was collected from clone T 3057 at eight sampling times during the entire

vegetation period, although there was a certain concentration in conjunction with the actual shooting. Pine shoots were also gathered from both terminals and first whorl. The difficulty in making a meaningful comparison between plant parts collected during such widely different stages of development has already been implied (cf. COUVY 1962). The buds from the first samplings are less differentiated than the shoots from later samplings, with wood, cambium and primary bark. Preparations from such tissues can individually give rise to different biological effects if a comparison is made with COUVY (1962, 1964).

Two preparations of terminal leaders and first whorls were made from each sampling. In the ensuing tests the parallel preparations showed only minor differences between each other.

The test is described in the form of a coleoptile test (fig. 13) and spore germination test (fig. 14) without previous chromatography, and partly visually as photographs of TLC plates fig. 16 and the actual pine shoots at different stages of development fig. 15. What has been reported to be taking place in French experiments in March will occur in Stockholm in the beginning of June, which is why the pictures showing pine shoots at various stages of development have been included.

The spore germination test made in 1968 in general confirms the results from previous years with the same reduction in inhibitor effect in conjunction with shooting. It is also possible that a slightly greater inhibitor effect is shown for the ether extract of first whorls than from terminal leaders. The spore germination test is summarised in fig. 14.

As regards the repetition in 1968 of the *Avena* straight growth test as bioassay a phosphate buffer as described in Materials and methods was used first. The citrate buffer is now seldom used at the Department of Botany. The acid ether extract gave consistent inhibition for all samplings, but the striking change seen the year before in conjunction with axial extension could not be shown. This concerns total extract without previous chromatography. The experiment was carried out twice.

In an attempt to determine the reason for the conflicting results between the 1967 and 1968 samplings the test was repeated with citrate buffer. With this buffer the considerable reduction in inhibitor effect during shooting previously reported appeared again. The experiment is summarised in fig. 13.

To further illustrate the question of the change of the acid ether layer in conjunction with shooting some *Avena* tests using chromatographed acid ether extract were carried out. These are summarised in fig. 17. Isopropanol-ammonia-water (100/14/6) was used as a solvent system and phosphate buffer for the *Avena* test. Here the extract of pine material just before shooting shows both inhibition and stimulation clearly. A marked fall in inhibition

occurs in conjunction with shooting. It can be assumed preliminarily that the effects cancel each other out in the above test using non-chromatographed material.

The chromatogram (fig. 16) shows that the extractive amount of the first two samplings is high. It undergoes a sudden reduction when shooting commences, which can be seen from the sampling on June 6th. The next two samplings, June 10th and 20th, which also fall within the sensitive period for *Melampsora*, show low extractive amounts but with a gradual return to higher values. The final three samplings with long intervals between them can only be regarded as random samples from the remainder of the vegetation period, which moreover because of lignification etc. are not exactly comparable with the earlier samplings. It is possible that chromatograms also show a slightly higher content of extractive substances in first whorls than in terminal leaders. The coleoptile test reflects consistently greater inhibition for first whorls than for terminal leaders. This applies to all samplings, including those taken June 6th, 10th and 20th during the sensitive period for *Melampsora*. The results should not, however, be emphasized; the connection terminal leader first whorl refers only for the 1968 experiment, and the experiment is based only on double samples. Furthermore, an effort was made in making the extracts to use as similar fresh weight amounts as possible. This entailed that in the preparations there are consistently more shoots of first whorl than terminal leaders for each sampling. With reservations of this kind in mind there still remains the assertion that the amount of extract, alternatively the amount of inhibitors, is somewhat greater in first whorls than in therminal leaders. This too fits in the picture as a partial explanation of why *Melampsora* is more common in terminal leaders than in first whorls.

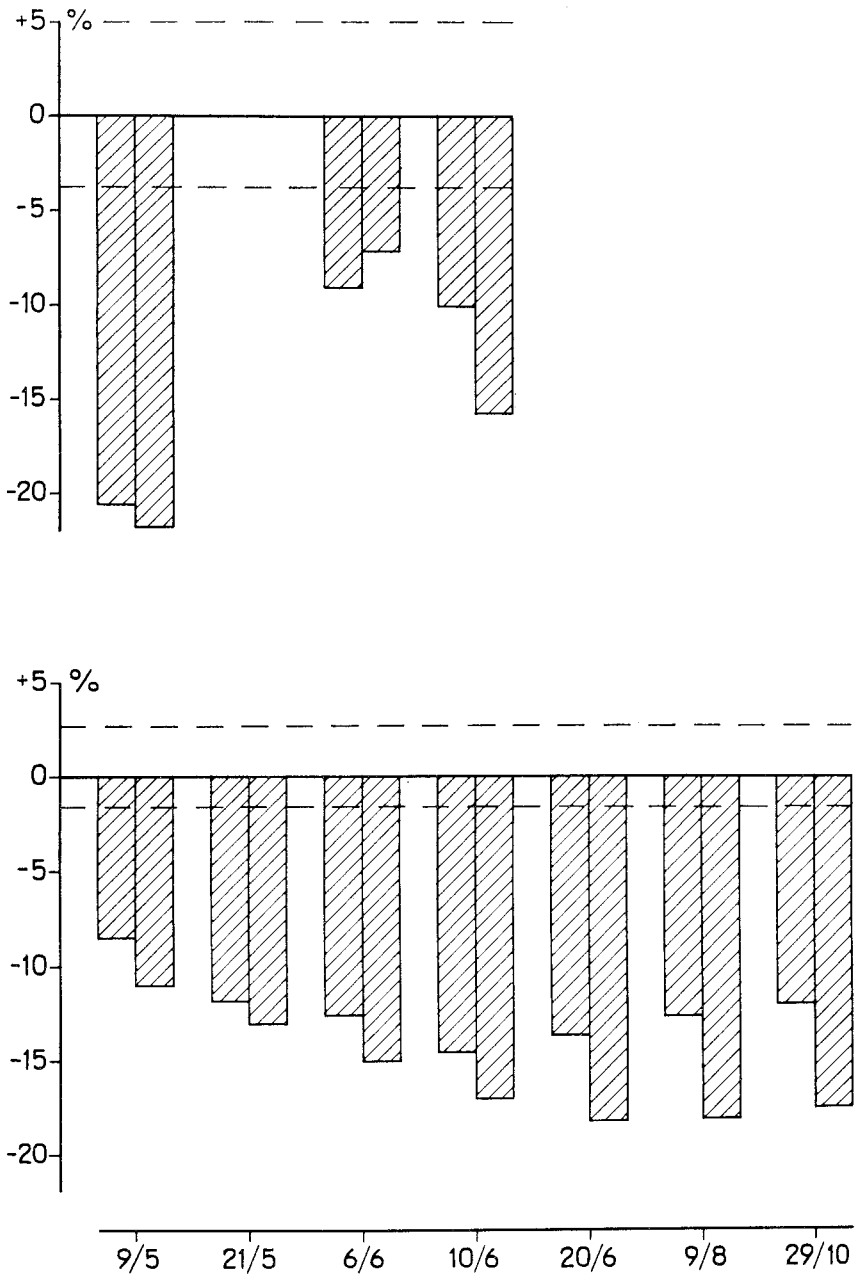


Fig. 13. *Avena* coleoptile assay of total acid ether extracts of *Pinus silvestris* annua shoots. Left bar = terminal leader, right bar = first whorl, in every pair.
Clone T 3057. Collection of material during summer 1968, dates in diagram.
Top. Avena-test with citrate buffer.
Bottom. Avena-test with phosphate buffer.
Extracts from 0.1 g fresh weight.
Final length of *Avena* coleoptile segments in per cent of final length of the control segments. Dotted lines = extremes of controls.

Terminal leader	Acid ether extract corresponding to gram fresh weight 0.5	0.4	0.3	0.2	0.1	0.075	0.050	0.025	0.010 g
9/5 1968	—	—	—	—	—	—	—	—	+
21/5	—	—	—	—	—	—	—	—	—
6/6	—	—	—	—	+	+	+	+	—
10/6	—	—	—	—	—	+	+	+	+
20/6	—	—	—	—	—	—	+	+	+
9/8	—	—	—	—	—	—	—	+	+
29/10	—	—	—	—	—	—	+	+	+
First whorl									
9/5 1968	—	—	—	—	—	—	—	—	+
21/5	—	—	—	—	—		—	—	—
6/6	—	—	—	—	+	—	+	+	—
10/6	—	—	—	—	—	—	+	+	—
20/6	—	—	—	—	—	—	—	+	+
9/8	—	—	—	—	—	—	—	—	—
29/10	—	—	—	—	—	—	—	+	+

Fig. 14. Bioassay of total acid ether fractions of *Pinus silvestris* terminal leaders and shoots of first whorl, clone T 3057. Germination of *Melampsora pinitorqua* basidio spores on water agar with different amounts of acid ether extract. Germination +, no germination —.
Critical interval determined by two or more replicates.

Fig. 15. Terminal leaders from clone T 3057 from different samplings May 9th and 21st 1968 have the same appearance and are represented by the first to the left, followed by June 6th, June 10th, June 20th and finally August 9th and October 29th of very similar appearance (the material was partially deep-frozen).

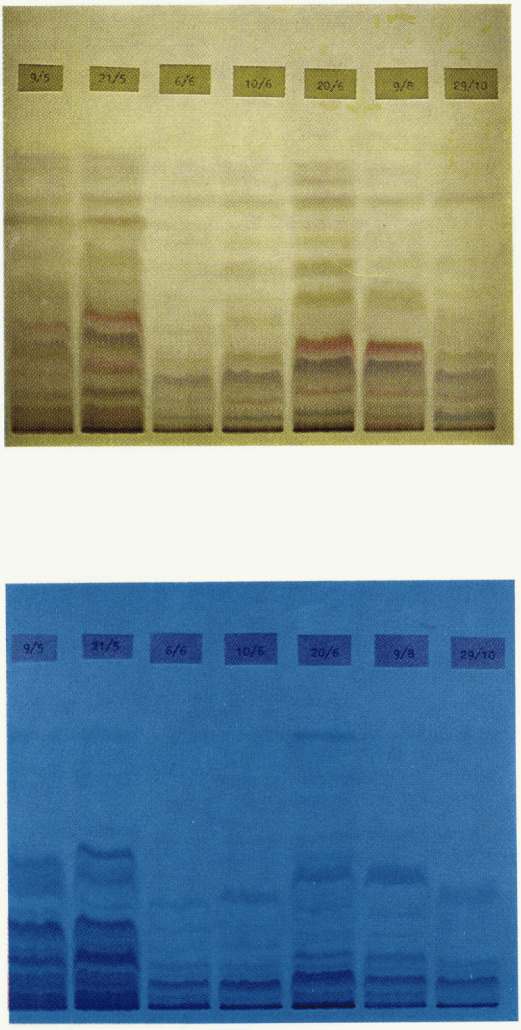


Fig. 16. Chromatogram of acid ether extract of *Pinus silvestris* terminal leader on TLC-plate silica gel F 254 (Merck) with butanone-hexane (30/70) and multiple development (2 × 6 + 2 × 12 cm).
Top. In visible light.
Bottom. In UV 254 mμ.
Collection time and pine material as in fig. 15.

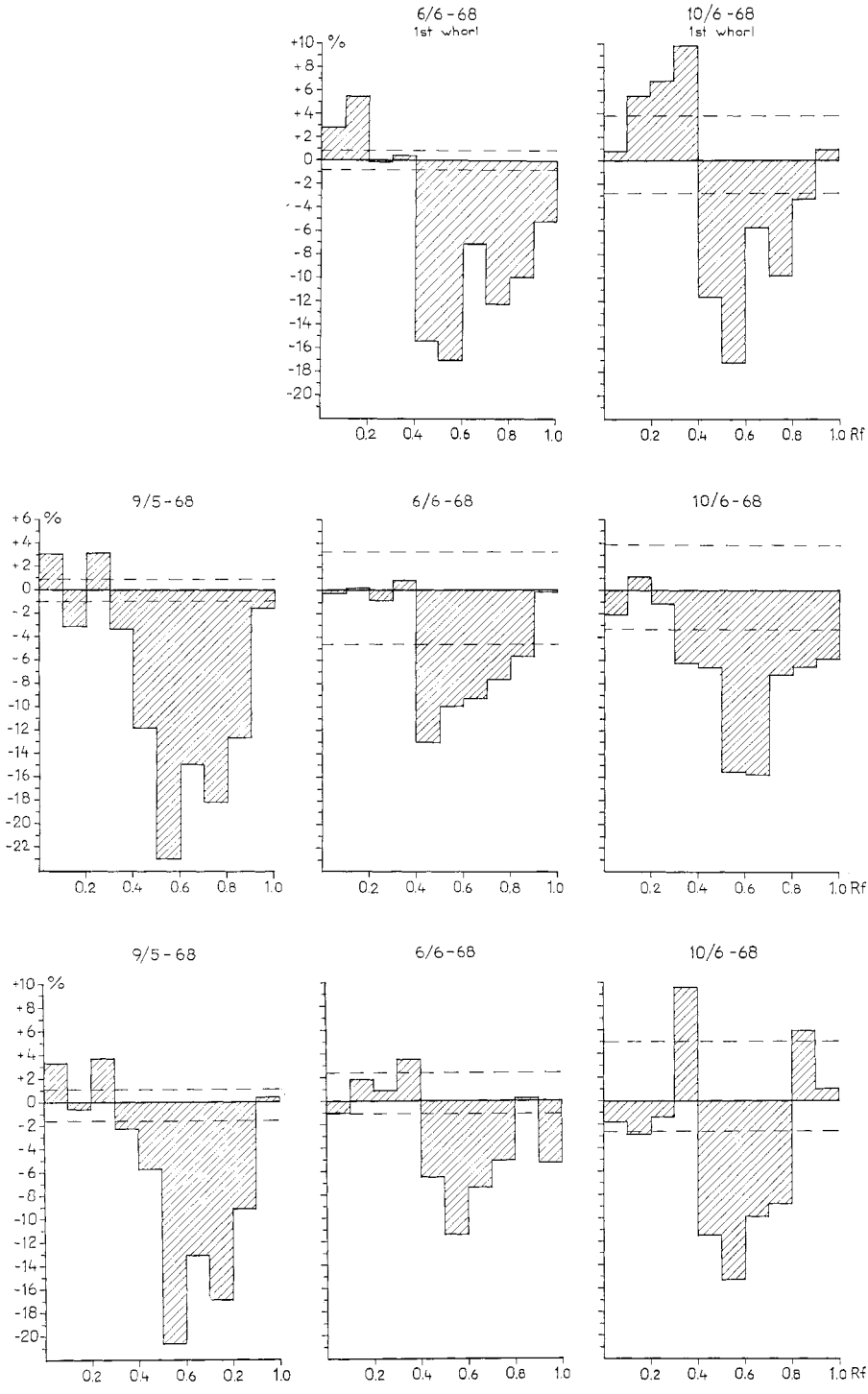


Fig. 17. *Avena* coleoptile assay of paper cromatograms of acid ether extracts of *Pinus silvestris* annual shoots. Clone T 3057. Second and third row from terminal leader. Extract from 0.2 g fresh weight. Isopropanol-ammonia-water (100/14/6). *Avena* phosphate buffer.
Collection time and pine material as in fig. 15.
Final length of *Avena* coleoptile segments in per cent of final length of the control segments. Dotted lines = extremes of controls.

6. Leaching and inhibitors in the leachates

The connection between the abundant occurrence of *Melampsora pinitorqua* on pine and damp weather in the spring was mentioned in the introduction. It has been taken for granted that this is due to the weather, in that the long periods of high air humidity give the fungus an opportunity to infect. It must be assumed that the fungus' basidio spores come into contact with drops of rain, dew or mist on the pine shoots. It is well known that epidermis is permeable in both directions for a number of substances, both inorganic and organic. Drops of water on the pine shoots will contain substances that may affect pine twist rust basidio spores. This hypothesis somewhat increases the extent of the problem of the part played by the weather in the spreading of the fungus.

The problem of exosmos or leaching has been dealt with in basic works by ARENS (1934) and LAUSBERG (1935). TAMM (1950, 1951, 1953) has studied the removal of plant nutrients from tree crowns by rain under Swedish conditions. In a great number of works TUKEY has studied the leaching of numerous substances from above-ground plant parts. He has also suggested the possibility of a connection with plant pathology (TUKEY 1959, 1966 and pers. comm.). SUCHORUKOW (1958) gives examples of how substances in an infection droplet can affect resistance. The present author has been able to prove in earlier works (KLINGSTRÖM unpublished) that water droplets from pine shoots have a slightly acid reaction, that the presence of organic acids can be proved, further that inorganic substances such as K, Ca, and Mg are easily identifiable in the droplets, and that radioactive ortophosphate P^{32} taken up by the roots of 2/1 pine can in a short time be traced to the droplets on the shoots and needles. Furthermore, it was possible with the help of conductivity measurements to prove that by far the greatest quantity of the substances in question was separated in a brief initial phase in the first hour of each protracted period of irrigation.

The following test was made in an attempt to establish to what extent biologically active substances occur in water droplets on pine shoots during sprouting, and which can possibly affect the germination of basidio spores. Twenty-odd pines from the progeny material in table 1 were bent so that the top shoots could be dipped into a test tube of a suitable size filled with distilled water. After 60 minutes the water was collected and vacuum frozen to dryness. The residue was shaken with a bicarbonate solution and

fractionated against ether. The pH was then set at 3.4 with the help of HCl and the solution again shaken against ether. The acid and basic ether extract obtained was examined with the help of the *Melampsora* basidio spore germination test. It was discovered that 5 to 6 % of the acid extract inhibited spore germination, and that 4 to 5 % of the basic extract also inhibits spore germination. Both extracts have a pronounced inhibiting effect if tested with *Avena* straight growth as bioassay.

If the extract is chromatographed with butanone-hexane (30/70) and multiple development, it is possible with Ehrlich's reagent to identify colourable substances in the acid extract with low Rf-values, 0.0—0.1. The basic extract gives no distinct Ehrlich stains, but possibly a high Rf-value, which can be identified better with UV at 0.8—0.9. If the chromatogram is examined with the spore germination test as a bioassay the acid extract shows an inhibitory effect at Rf 0.0—0.2 and a slight effect also at somewhat higher values. The basic extract shows inhibition at Rf 0.8—0.9. These figures do not agree with the values given in table 9, or the substances in question do not occur in such concentrations that these have been noticed. Preliminary *Avena* straight growth bioassays give corresponding inhibiting effects together with inhibiting effects concerning the *Avena* test only. The *Avena* test also give stimulating effects.

It is obvious that the experiment outlined above differs from natural conditions, but it cannot be precluded that the infection drops themselves contain growth regulating substances which can affect spore germination. TUKEY (1966) shows that water droplets from other plant material can contain, for instance, amino acids, nucleic acids and growth regulators.

The entire question must be examined from the start as an element in the study of the infection biology of *Melampsora pinitorqua*. Leaching may condition the host to be more susceptible to invasion by disease organisms during heavy rainfalls. Leaching can eventually give rise to a surface deposit which inhibits spore germination in combination with light rainfalls.

7. Gel filtration and thin layer chromatography

Growth regulating substances in plants have been studied with growing interest since the 1920's. Standard procedure has been studied in orientating experiments above. The following investigation is an attempt to try other ways of separating the components in the extracts. The work was initiated by promising results of gel filtration, KLINGSTRÖM (1967) (cf. DETERMANN 1967).

Methods: For the gel filtration Sephadex LH-20 was used, which is intended for organic solvents. Laboratory columns SR 25/45 and 25/100, solvent resistant (Pharmacia Fine Chemicals, Uppsala, Sweden), 5 ml siphon and a fraction collector (LKB Radi Rac) were used and a pump to give a constant flow rate of 0.5 ml/min.

The spectrophotometer in this investigation has been a Beckman DB, and for registration a Beckman Linear and Log Potentiometric Recorder was used.
Slit: narrow program
Scanning speed: 40 mμ/min.
Chart speed: 1″/min.

A. Preliminary experiments

In a first group of experiments the effect of ethanol concentration and pH was studied. The filtrates from the column have been identified photometrically in the UV range. The same acid pine extract was filtrated in pure ethanol and in water mixtures 80 : 20, 60 : 40, 50 : 50. In conjunction with 60 % ethanol the effect of the following pH-values was studied pH 6.5, 5.5, 4.5, and 4.0 which were set with the help of HCl. A retarding effect can be established in the case of lower pH.

The ethanol concentration has a pronounced effect upon the quantity in which an applied sample is filtrated from the column. Separation in 60 % ethanol makes possible a clear photometric division of the beginning and end of a number of fractions. In these experiments a short column (SR 25/45) was used and bed volume approx. 155 ml, flow rate 0.6 ml/min., and sample in two ml.

A preliminary examination of biological activity was also made with the *Avena* section test and *Melampsora* basidio spore germination in conjunction with the above mentioned test with gel filtration and different ethanol concentrations. It was considered advisable to check as far as possible that the material's biological activity was not lost. The material was tested at a

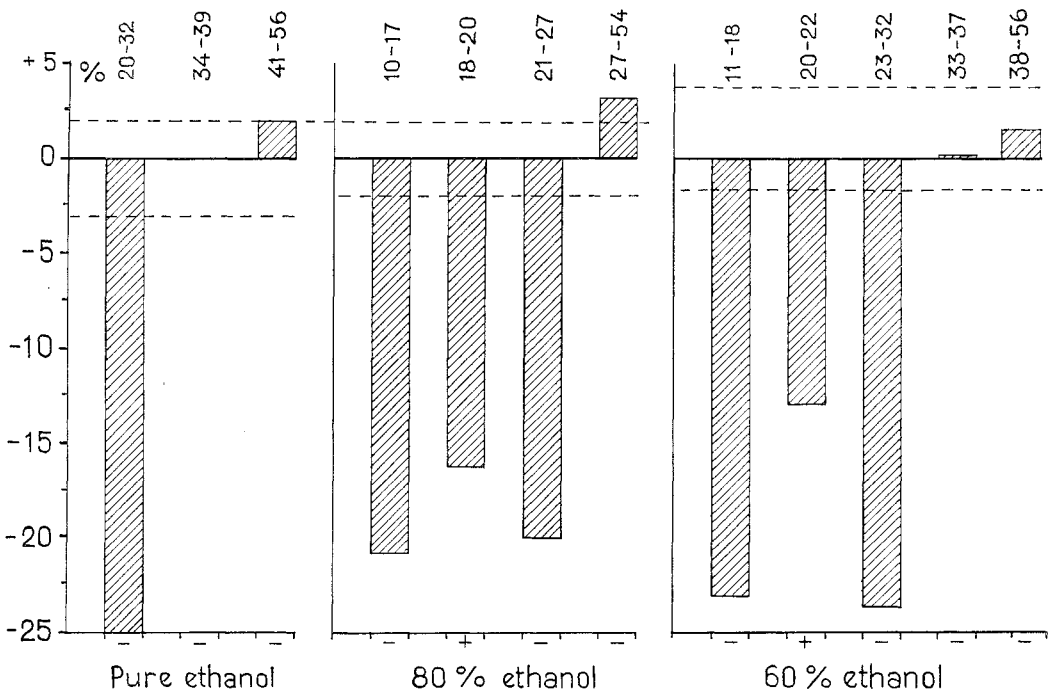


Fig. 18. *Avena* coleoptile assay of gel filtered acid ether extract of *Pinus silvestris* terminal leaders, clone Z 66. Gel filtration with different ethanol concentrations.
Figures = numerical order of tubes from fraction collector.
Amount of extract from each fraction corresponding to 0.2 g fresh weight.
Gel = Pharmacia LH 20.
Final length of *Avena* coleoptile segments in per cent of final length of the control segments. Dotted lines = extremes of controls.
Germination in *Melampsora* basidio spore germination test indicated by +, no germination by —.

level corresponding to 0.2 g fresh weight and is shown in fig. 18. When separated in 60 % ethanol the material was divided into 5 fractions (Nos. 1 and 2 can be found under the corresponding fractions in summarising table 9, No. 3 corresponds partially to 3 and 4 in the table). The figure shows that three of the fractions have a distinct inhibitory effect on the *Avena* test and four affect the spore germination test.

It can thus not be precluded that this material contains several inhibitors, or combinations of substances with an inhibitory effect, which can be shown with the help of bioassay even after the gel filtration of the material.

The test also shows that the straight growth test and the basidio spore germination test do not agree in sensitivity for all fractions. If the test quantity used in the experiment is doubled, both types of bioassay indicate a distinct inhibition for all five fractions. The above test used clone Z 66, which is characterised by a high extractive content.

B. Combined gel filtration—thin layer chromatography

For further work involving gel filtration an acid ether preparation was made from the terminal leaders of *Pinus silvestris*, which could be assumed to have less specific characteristics. At a later stage a comparison should be made between such a preparation and ether extracts from resistant and non-resistant clones. The extract is made from a random sample of all the 75 progenies examined in table 1. Moreover, during the course of the work a further column became available, SR 25/100, which allows more than twice as much gel as SR 25/45. The preparation in question initially represented one kilogram of terminal leaders. The finished ether phase was divided into 40 equal portions, which for gel filtration were applied to the column in 2 ml 60 % ethanol.

The division into fractions in the opening experiments was based only on distinct extinction curves in the UV-range. As a comparison with this photometric characteristic an attempt was also made to make a division based on Ehrlich staining. Instead of UV-curves, an examination was made of small ring chromatograms on TLC-plates. This showed that there is a very rich Sephadex-fraction which lacks a striking UV-curve and which for the most part has only end absorbtion. This constituted fraction 3 in the summarising table 9. In this attempt at summarising the material has been divided into seven fractions, with the intention that the fractions would contain a more suitable number of substances for further chromatographic separation than the total extract.

The material from several gel separations was brought together and each of the seven amalgamated fractions was further refined by a second passage through the column. The intention was then to study the fractions chromatographically and at least to start describing the inhibiting effects on *Melampsora* basidio spore germination test. An attempt to collate the data is made in table 9, which needless to say can be regarded only as an incomplete review of a number of unknown substances of unknown concentration and mixture, where moreover the Rf-values can vary within wide limits depending on the quantity. In addition it is probable that the fractions contain substances which were not discovered by the methods described here. To identify a single substance chromatographically involves a great amount of work with a number of solvent systems. In this connection there is reason to suspect the existence of about 50 substances, perhaps more. The table also shows the UV-curves which served as guide marks for demarcating the Sephadex-fractions. The actual amount of acid ether extract chromatographed corresponds to 5 g fresh weight. Further, instant TLC-plates with silica gel F 254 (Merck) were used, and as solvent system butanone-hexane (30/70) and multiple development ($2 \times 6 + 2 \times 12$ cm).

An initial test showed that fractions 2, 3, 4 and 5 had a distinct inhibitory effect on spore germination; this refers to the total fractions prior to further separation by chromatography. Quantities of a magnitude up to 10 g fresh weight were studied. Further work was concerned in the first instance with these fractions and the components that influence the spore germination test. Consequently no attention was paid to the discovery of e.g. IAA — if there is any it will occur in fraction 7.

It has already been mentioned that the Rf-values for certain of the substances in table 9 vary on account of the amount used. It can be pointed out that the concentration gradient within a given Rf-value for one and the same substance can give rise to varying shades of colour or even to different colours with Ehrlich's reagent, which in the preliminary table can have resulted in the same substance being recorded under two Rf-values in close proximity to each other—this may be so in e.g. fraction 3, Rf 0.44 and 0.46. Furthermore, the colour reactions depend on the developing temperature after the reagent has been sprayed on the TLC-plate, and the colours change during the first 24 hours after development with certain Rf-values appearing slowly. Preparative chromatography which aims at ringing in most active inhibitors in the *Melampsora* basidio spore germination test is recorded also in table 9.

Fraction 2.

The active compound is to be found immediately above the substance that served as a guide value for the entire Sephadex-fraction. The corresponding Rf-value appears relatively faintly at UV 254 mμ on the TLC-plate, but it has a distinct violet Ehrlich-stain. Rf varies with the quantity and reasonable values lie between Rf 0.1 and 0.25. Preliminarily no Rf-values give a very marked reading in the *Avena* straight growth test other than a slight inhibition for the spore-active value.

Fraction 3.

Also in this case the active compound is found just above the very distinct dark grey-blue Ehrlich-stain that dominates the whole fraction. The biologically active compound shows as a distinct yellow-brown Ehrlich-stain, which in the beginning can be reddish. Reasonable Rf-values can, depending on the amount applied, lie between 0.15 and 0.45, which is further complicated by the presence of other substances in the fraction that are only slightly affected by the amount. In this instance the distinct reading for the spore test coincides with a pronounced inhibition in the straight growth test, which moreover indicates inhibition for all lower Rf-values. Fraction 3 is in other words rich in content, having at least one spore-active Rf-value and several straight growth active values.

Fraction 4.

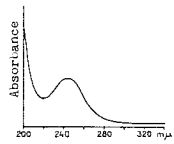
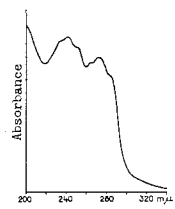
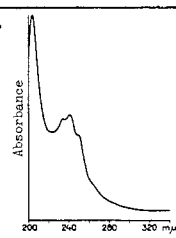
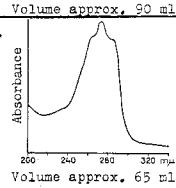
The substances which gave the two UV-curves with corresponding Rf-values, and which are the guide values for the entire fraction, did not display any activity in the spore germination test. On the other hand, an Rf-value of about 0.7 in the summarising table with corresponding substance(s) has in several experiments shown inhibition of the spore germination test. This value has no corresponding Ehrlich-stain or UV-indication in the sample quantities that were tested. For the Rf-area in question, however, it is possible to discern a certain coleoptile stimulation. Furthermore, there is a distinct inhibition of the *Avena* test in the region of Rf-value 0.27, which is one of the guide values for the fraction. Also this Rf-value varies with the amount applied.

It is thus possible with the spore germination test to illustrate an inhibiting Rf-value at 0.7. The difficulty of identifying this Rf-value in any other way was the cause of extensive research. It has already been explained that an acid ether extract from the top, middle and basal thirds of shoots has an inhibitory effect of varying strength in the *Avena* test. With the ordinary chromatography procedure used in this study it was possible with the help of Ehrlich's reagent to show an Rf-value at 0.7 of the extract from the apex section. The colour is faintly reddish and appears slowly, becoming clearest after several days. The same Rf-value can also be identified in extracts from material before shooting, which in fact consists only of terminal buds. With the *Melampsora* basidio spore germination test as a bioassay it could be established that the Rf-value in question has an inhibiting effect.

Is it possible that this Rf-value is the one sought-after in Sephadex-fraction 4? The amounts examined could have been too small to give colour reactions. It therefore remains to examine in which fraction the substance is to be found after gel filtration. An extract from the apex section of pine shoots was chromatographed on a TLC-plate and the Rf-area at 0.7 was eluated and added to the normal ether extract previously described. After the gel filtration of the extract concentrated regarding Rf 0.7 the applied substance appears in fraction 4 at the Rf-value that was originally biologically proven. In other words, it is possible that the inhibitory Rf-value at 0.7 originates in a substance that occurs primarily in terminal buds.

A further Rf-value at 0.79 has an inhibitory effect in the spore germination test. It appears in both fraction 4 and 5 and is not affected by the amount applied.

Table 9. Orientating summary of components in acid ether extract of *Pinus silvestris* terminal leaders at the end on axial extension. The extract was divided into fractions by gel filtration (Sephadex LH 20) with 60 % ethanol. The fractions were chromatographed on instant TLC plates silica gel F 254 (Merck) with butanone-hexane (30/70) and multiple development, see text.

SEPHADEX-FRACTION Flow rate 0.5 ml/min. Bed volume 407 ml Void volume approx. 125 ml Sample in 2 ml 60 % ethanol	Rf UV 254 mμ	Rf UV 366 mμ	Rf Ehrlich	Extinction-maximum	Biological effect: Melampsora basidio spore germination	Biological effect: Avena section test	Remarks
1. Void + all material coming before UV curve in fraction 2			0.21 reddish (weak)				Whole fraction has phenylacetic acid odour
			0.16 red				
	0.11						
			0.09 reddish				
	0.06						
			0.05 bluish				
Volume approx. 140 ml	0.04						10 % tot. weight
	0.00	0.00	0.00 brown				
2. 			0.40 (weak)		-	-	
	0.26		0.26 brownish				
			0.23 purple		inhib.	inhib.?	
	0.20		0.20 grey-brown	245 mμ (250 mμ in H_2O, 230 mμ in ether)	-	-	(Ehrlich stain occurs slowly)
			0.13 purple		-	-	dominating
	0.11				-	-	
			0.05 brownish		-	-	
Volume approx. 55 ml	0.02				-	-	10 % tot. weight
	0.00	0.00	0.00 brown				
3. Complex fraction - all material coming between UV curves in fractions 2 and 4			0.79 red-purple				
			0.71 blue-purple				
			0.60 reddish		-	-	cf. text
	0.51				-	-	
			0.46 (weak)				
			0.44 yellow-brown		inhib.	inhib.	
			0.40 red-purple				
			0.35 dark grey-blue		-	inhib.	dominating
	0.26						
			0.23 redbrown		-	inhib.	
	0.21		0.21 greyish		-		
			0.12 blue		-		
			0.09 grey-brown		-	inhib.	
Volume approx. 45 ml	0.03	0.03	0.03 grey-blue		-	-	21 % tot. weight
	0.00	0.00	0.00 brown		-	-	
4. 	0.79		0.79 grey-blue	235 mμ	-	stim.?	cf. fraction 5
			0.70 (weak)		inhib.	Rf 0.6-1.0	0.7 cf. text
			0.64 greyish		-		
			0.59 (weak)		-		
	0.40		0.40 grey-red (weak)		-		
			0.36 reddish		-	inhib.?	
	0.27		0.27 grey-brown	242 mμ	-	inhib.	dominating
			0.22 red-purple		-	inhib.?	
	0.17				-		
			0.02 blue		-		
			0.01 reddish	274 mμ (Rf 0.0-0.05)	-		
Volume approx. 65 ml	0.00	0.00	0.00 brown		-		42 % tot. weight
5. 	0.84		0.84 grey-purple		-	-	cf. fraction 6
	0.79		0.79 grey-blue		inhib.?	-	
	0.62		0.62 greyish	242 mμ	inhib.	inhib.	dominating
	0.27		0.27 (weak)		-	-	cf. fraction 4
	0.22		0.22 (weak)		-	-	cf. fraction 4
	0.15		0.15 (weak)		-	-	
	0.00	(0.00)	0.00 brown (weak)		-	-	
Volume approx. 90 ml							11 % tot. weight
6. 	0.84		0.84 grey-purple				
			0.70 reddish				
			0.65 greyish (weak)				
	0.53		0.53 grey-purple	274 mμ			dominating
			0.49 purple (weak)				
			(0.40 very weak)				
			(0.22 very weak)				
Volume approx. 65 ml	0.00	(0.00)	0.00 brown (weak)				2 % tot. weight
7. Rest-fraction (i.a. chlorophyll and related compounds)			0.88 reddish				
	0.61	0.61					
	0.52	0.52	0.52 green	660+400 mμ			
	0.44	0.44	0.44 yellow-green				
	0.15						
Volume approx. 100 ml	0.03	0.03	0.03 green				(possibly 2)
	0.00	0.00	0.00 brown				4 % tot. weight

Fraction 5.

The biologically active compound corresponds to the distinct value that appears in UV 254 mμ and after staining with Ehrlich's reagent and which dominates the fraction. Rf varies also in this case with the chromatographed amount, with common values between 0.4 and 0.65. Inhibition in the spore test and the *Avena* test occurs at this same Rf-area. Relatively small amounts of this fraction affect the spore germination test. A chromatogram according to the method described presents an uncomplicated picture, in which the active Rf-value can be studied after preparative chromatography with less interference by adjacent substances than in the other fractions.

Comparison between "normal pine" according to the orientating summarising table and clone material with low Melampsora frequency. It was considered of interest to compare an acid ether extract from a strongly resistant clone, Z 66, with the ether extract used in working out the general table 9. This comparison should be regarded only as an introduction to examinations of extracts from clones with high and low *Melampsora* frequency.

An acid ether extract from terminal leaders of clone Z 66 was gel filtrated and chromatographed in the same way as described above for the material in table 9. It was established that the extract from 3 to 4 g fresh weight from clone Z 66 gave similar absorbance values for the orientating UV curves as the extract from approx. 25 g fresh weight of the pine material that forms the basis for the general table.

Chromatographic examination of the seven Sephadex-fractions from Z 66 shows several deviations in comparison to the general table. Thus two components appear in fraction 2 at Rf 0.45—0.50 incompletely separated; the one with a slightly higher Rf appears with blue-green Ehrlich staining, the other in UV 254 mμ.

In fraction 3 several minor deviations from the figures given in the general table can be found. The most important seems to be the strongly emerging Rf-value 0.60, which in the earlier general collation was of secondary importance. Furthermore, this component in bioassay had a distinct inhibitor effect on spore germination which it has previously not been possible to demonstrate.

In fraction 4 there is a strong development at Rf 0.7 of the component discussed in detail in an earlier section, and which in this case is of comparatively wider extent. No big differences have been discovered in the other fractions at this stage.

Thus even a preliminary examination of only one resistant clone in the form of only one ether extract can point the way to the discovery of yet another crude product with an inhibitor effect on spore germination. Determining inhibitor levels with regard to spore germination inhibition for different plus tree clones, and as far as possible identifying the components, is clearly the natural continuation of this work.

8. Chemical identification of biologically active fractions

The chemical composition of some of the biologically active fractions obtained from Sephadex column chromatography separation described above has been determined. Fraction 5 (Table 9) showed striking inhibitory effect at *Melampsora* basidio spore germination test. Further separation with preparative thin layer chromatography on instant TLC-plates silica gel F 254 (Merck) (butanone-hexane (30/70)) and multiple development gave a biologically active crude product (Rf-value 0.62).

Mass spectrometry of the product showed a well-defined peak at m/e 302 which was suspected to be a molecular peak. High resolution mass spectrometry (Atlas SM 1) of m/e 302 gave the composition $C_{20}H_{30}O_2$.

The IR-spectrum of the crude product showed strong bands at 1700 cm^{-1} (C = 0), 2400—3400 cm^{-1} (OH hydrogen banded) and 2880—3050 cm^{-1} (various CH stretching bands).

On treatment with diazomethane the OH-bands in the IR-spectrum disappeared and the carbonyl band was shifted to 1733 cm^{-1} indicating that the biologically active product consisted of a mixture of carboxylic acids. Gas chromatographic investigation of the methylated product gave several peaks (Fig. 19). Combined gas chromatography and mass spectrometry (LKB 9000) showed that the crude product was a mixture of several methyl esters of resin acids, i.e. abietic acid, dehydroabietic acid, isopimaric acid, and smaller amounts of pimaric acid and/or cryptopimaric acid, neoabietic acid (traces), and a group of unidentified resin acids Fig. 19. (C.f. Bruun et al. 1958, Bruun & Gåsland 1960, Norin & Westfelt 1963).

The UV-spectrum of fraction 5 from the Sephadex column chromatography separation was almost identical with that of abietic acid. The dehydro abietic acid found should be regarded as an artefact, as the crude resin acid fraction undergoes secondary changes under the isolation procedure.

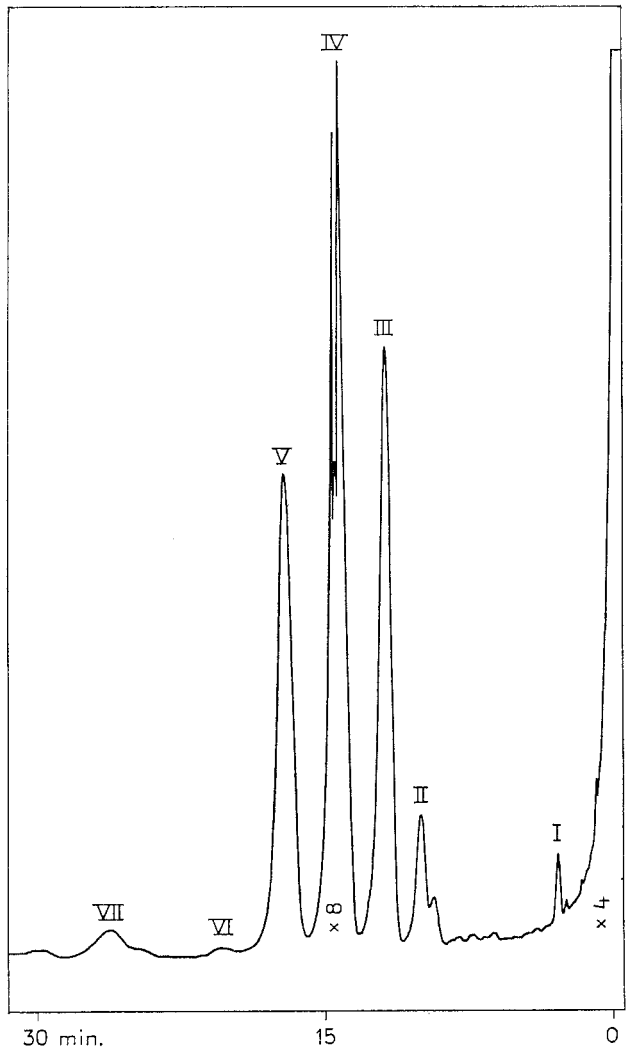


Fig. 19. Gas chromatogram of the methylated product of Sephadex-fraction 5, Rf-value 0.62 (cf. table 9).
1 % E 301—Gas Chrom. P—100—200 mesh—temp. 175°C—12 inch/h
I. Unidentified
II. Methyl esters of pimaric acid and/or cryptopimaric acid
III. Methyl ester of isopimaric acid
IV. „ „ „ dehydroabietic acid
V. „ „ „ abietic acid
VI. „ „ „ neoabietic acid (?)
VII. „ „ „ unidentified resin acids

With the intention of comparing with the current discussion on inhibitors in botanical material, fraction 5 was also chromatographed on paper with isopropanol-ammonia-water (100/14/6) for further *Avena* straight growth test. This gives a distinct inhibition at Rf 0.4—0.6, and it is therefore necessary to include a number of resin acids in the inhibitor β complex. An early statement concerning the biological activity of abietic acid was made by AVERY and SARGENT (1939), but the purity of the product is not mentioned. Furthermore, the work referred to used entirely different biological methods. The abietic acid is said to have a growth promoting effect, which is the opposite of the result mentioned above for the mixture of resin acids including abietic acid.

Different resin products are available as bi-products of the pulp industry. In a few cases the composition of the resin acids is known, but the product is usually to be regarded as technical (pract.). The following is an account of an experiment with *Melampsora* basidio spore germination test and a few resin acid mixtures, an ester form and a Na-salt. The spore test was modified in that the resin acid was first dissolved in a small quantity of ether and then set in 1 % water agar; i.e. the test substance was not introduced to the surface of the agar. It can be mentioned that, with the exception of the Na-salt, the solubility of the product in water is low. At least in the higher concentrations used in the test there is clear evidence of dropwise deposition in the agar. The test shows univocally that spore germination is inhibited with the exception of the ester form; in the *Avena* straight growth test, however, also the ester form has an inhibitory effect (Fig. 20).

	1000	100	10	1	0.1	0.01 ppm
Abietic acid I	—	—	+	+	+	+
Abietic acid II	—	—	+	+	+	+
Abietic acid III	—	—	+	+	+	+
Ester from No. III	+	+	+	+	+	+
Na-salt from No. III	—	—	—	+	+	+

Abietic acid I = tallharts HA (Bergvik & Ala)
Resin acids:

tetrahydroabietic acid	1.8 %	dehydroabietic acid	42.4 %
pimaric acid	4.6	abietic acid	29.0
palustric acid	7.8	unidentified	1.0
isopimaric acid	13.4		

Abietic acid II = melting point 155—165°C pract. (source unknown)
Abietic acid III = recrystallized from No. I
Ester and Na-salt = from No. III.

Fig. 20. Bioassay of different amounts of abietic acid and a corresponding ester and Na-salt in 1 % water agar. Germination of *Melampsora pinitorqua* basidio spores indicated by +, no germination by —.

The continuous work aims at the isolation and structure determination of remaining biologically active fractions from the acidic ether extract mentioned above. The Sephadex-fraction 2 (table 9) shows appreciable activity and preparative thin layer chromatography (butanone-hexane (30/70)) gives a crude product with Rf-value 0.23.

Mass spectrum of the product does not give any satisfactory molecular peak. The IR-spectrum shows strong bands at 1700 cm^{-1} (C = 0), 2400—3400 cm^{-1} (OH hydrogen banded) and 2880—3050 cm^{-1} (various CH stretching bands). On treatment with diazomethane the OH band disappeared and the carbonyl band was shifted to 1733 cm^{-1}. The product evidently consisted of a mixture of carboxylic acids.

The results obtained from the GLC-MS investigation cannot be easily interpreted.

The Sephadex-fractions 3 and 4 also show distinct inhibiting activity concerning basidio spore germination. Preparative thin layer chromatography (butanone-hexane (30/70)), fraction 3, gives a crude product with Rf-value 0.44 (table 9). GLC-MS investigation gives a number of peaks some of which have been identified as aliphatic fatty acids of different kinds. The continuous work, to be published separately, must establish which components are responsible for the inhibitory effect. (c.f. HYPPEL 1969).

Sephadex-fraction 4 with inhibiting crude product of Rf-value 0.7 and fraction with inhibiting crude product of Rf-value of 0.79 have not been further investigated because of scarcity of material.

9. Discussion

It would be beyond the scope of a paper on *Melampsora pinitorqua* to try to summarise known facts concerning resistance to parasitic fungi. The multiformity of the subject is perhaps made most clear in works as for instance by GÄUMANN (1951), BARNETT (1959), RICH (1963), SHAW (1963), TOMIYAMA (1963), GERHOLD et al. (1966). Even if the problem is confined to obligate parasites, the extent of the problem is clearly expressed in e.g. SHAW's work (1967). The individual researcher is prone to exaggerating the significance of for instance phenols, inhibitors, antibiotics or enzymes when appraising the cause of resistance. To get back to the subject under discussion in this paper, it is certain that a great number of factors affect the resistance during the different stages of development of a parasite—from germination on a pine shoot to the final aecio stage. It is necessary to consider factors having a passive effect, such as the nature

of the cell walls, the absence of essential vitamins and similar compounds, and the presence of inhibiting substances. Further, there is a string of reactions, triggered-off by the combination of parasite—host, which can be regarded as an active type of resistance-reaction. To this belongs the production of fungitoxic substances etc. following infection, and metamorphosis in a large number of physiological processes, SHAW (1967). All these passive or active reactions can moreover be influenced by environmental factors, and the whole can be linked to a discussion as to how genetic and breeding work fits into the picture. Attention can also be drawn to the existence of paradoxically active causes; a highly susceptible host can in practice escape being cankered by a parasite if only a few cells collapse at the actual point of attack, thereby rejecting the parasite before it has had time to establish itself. Another host, having a certain active resistance that is overcome by the parasite, could succumb to a similar attack.

Furthermore both the host plant and the parasite possess a degree of variation within certain genetic limits; different races of the parasite are able to combine with different varieties of the host plant. Much of the knowledge in this sphere rests on findings from examinations of the uredial stage of *Puccinia graminis tritici* ERIKS. and HENN., which have been the subject of meticulous study and accounted for in hundreds of papers. In theory it is possible that the parasite possesses one (or several) specific gene(s) which enable it to overcome the corresponding resistance barrier in the host plant. A number of corresponding genes would thus dictate the degree of resistance. PERSON (1967) has summed up the problem. So far as *Melampsora* is concerned, nothing is known about the genetic variability of the parasite in combination with the pine as host.

In addition it is necessary in this investigation to consider the old question of how to test biologically active substances (cf. e.g. NITSCH & NITSCH 1955). The coleoptile test is a standard method, but an effect on *Avena* coleoptile need not necessarily mean anything regarding an eventual connection between, for instance, *Pinus* and *Melampsora*. To be able better to assess the effect of the substances that were prepared from pine terminal leaders, a simple method was devised to use basidio spore germination as a bioassay. In this way just the spores that normally germinate on pine shoots were used to get as close to the problem of pine twist rust as can reasonably be expected of a laboratory test. Even so there is a gap between the laboratory test and the course of infection in the field, and this is among the least-known aspects of the biology of *Melampsora pinitorqua*. It is worthwhile following the Finnish work that is in progress regarding telio spore and basidio spore germination (KURKELA, personal comm.). The spore germination test does not seem to be affected by germination inhibitors from the

spores, which is usually a common complication when studying spore germination (cf. SUSSMANN and HALVORSON 1966 and PRITCHARD and BELL 1967).

Where *Melampsora pinitorqua* is concerned, the basidio spores are sensitive to drying, which normally limits the dispersion range between aspen leaves on the ground and the pine shoots. It is likely, although difficult to prove with certainty, that the basidio spores germinate in droplets on the surface of the shoots formed by dew, rain and mist. It is a well-known fact, even though it is seldom considered when studying the course of infection that a great number of substances, inorganic and organic compounds, can be separated or absorbed by epidermis. Water droplets on the surface of pine shoots contain both acid and basic components which, according to the experiment described above, inhibit basidio spore germination. In other words, also leaching can be thought to affect the course of infection.

The entire work was started as a result of observations concerning the occurrence of pine twist rust in *Pinus silvestris*, for example the localisation of the disease to the top of the pine, a somewhat higher frequency on the terminal leader than on the first whorl, and in the study of progenies, where there is a consistently higher frequency of disease in the longer terminal leaders than the shorter. This led to interest being directed towards the growth regulating substances in pine, a subject which it transpired has not been satisfactorily examined.

It is also conceivable that the presence of the fungus stimulates the shoot to attain a greater length by a production of growth stimulating substances. No proof for this theory has been found (WÄRN, unpublished). But it may be too early to reject the possibility as other host-parasite references favour the hypothesis (e.g. BUXTON 1964, DEVERALL 1964).

A theoretic explanation for the differences in twist rust frequency between long and short terminal leaders in the examined progenies might be traced to a time factor. It is possible that the longer leaders shoot for a longer time than the shorter ones. Such speculation on apparantly simple questions is, however, based on a weak factual foundation.

Both growth regulation and the *Melampsora* problem are each very extensive subjects. Initially an attempt was made to prepare an ether extract from pine shoots and to refer to older works. Various methods of separating the extract components were also tried. The existence of an inhibitor β complex in *Pinus silvestris* could be established, but the usual method of separating growth regulating substances—paper chromatography—was considered as being unsatisfactory for a preparative separation.

The method used in preparing the ether extract is discussed under Materials and Methods. A limitation for the time being is that only the acid ether

preparation has been studied. This, furthermore, because of the method of preparation used, would appear to consist for the most part of acids; in addition the output depends on the water solubility.

Also the basic ether layer is biologically active, and it is unknown whether also in the basic ether layer it is possible to find a connection with the occurrence of *Melampsora*. On account of the preparation method used, this layer should, among others, embrace phenols.

Very few works concerned with *Pinus silvestris* and growth regulation were available when the present work was started. FRANSSON's works from 1953 and 1959 have already been mentioned. Several Polish researchers are now active in this sphere—MICHNIEWICZ and KOPCEWICZ (1966), MICHALSKI (1967), KOPCEWICZ et al. (1967). Among other things the presence of gibberellines and inhibitors has been established. In these works, however, the inhibitors are described as gibberelline inhibitors and not as β inhibitor, and in any case without effect on the *Avena* straight growth test. The work described here differs from those cited both as regards chromatography and the methods of preparation. I think it is likely that we have nevertheless studied the same inhibitors to some extent. Furthermore, WODZICKI (1968) has studied the occurrence of IAA in *Pinus silvestris*. This subject has also been studied in a Swedish investigation by ALDÉN and ELIASSON (1969).

Certain pine clones can year after year show constant differences in their behavior to *Melampsora pinitorqua* in field experiments. It also seems that there are progenies with a difference regarding *Melampsora*-frequency (tables 1—7). Clones with a low *Melampsora*-frequency exhibit after repeated preparations, and for at least two consecutive years, a considerably larger amount of extract than other clones with a higher *Melampsora*-frequency. The acid ether layer of the extract has been studied and the inhibitory effect of the extract—both *Avena* straight growth test and the *Melampsora* spore germination test—correlate with the *Melampsora*-frequency of the original clones.

The Melampsora basidio spore germination test is best suited for indicating an inhibition of spore germination. Should there be a stimulation of the germination process during the infection it must be assumed that this easily escapes attention because of the nature of the spore germination test. Normally the spore germination test has been used to study the effects of total extract, which has not been chromatographed or separated in any other way. The extracts contain a number of substances some of which have inhibitory or stimulating effects. One of the risks when studying total extract can be that the effects so to say cancel out each other, as has been proved when using the *Avena* test. In addition the inhibitor effect in the comparative coleoptile test is exaggerated on account of the buffer solution

used in the *Avena* test. Using another type of buffer solution it could be demonstrated that susceptible clones give an acid ether extract which may give a stimulation of the *Avena* test. A warning should once more be given, however, about transferring the interpretation of the result from the *Avena* test to the occurrence of *Melampsora* on pine.

The acid ether extract has been studied before, during and after axial extension. Bioassay of the material shows two years in succession a brief but marked reduction of the inhibitory effect during the short extension time of the shoot, when infection by *Melampsora* also takes place. This circumstance can perhaps provide a new aspect of the fungus' behaviour. Furthermore, it appears that the amount of inhibitor is consistently somewhat higher in the first whorl than in the terminal leader. Also this circumstance is compatible with the behaviour of the fungus. It would appear desirable also to study, for instance, clone material with varying susceptibility to twist rust by taking daily samples during the shooting period in an attempt to discover whether the decline in inhibitors is uniform, or whether the decline is more or less and lasts for a longer or shorter period.

It is thus possible to form the hypothethis that substances with inhibitory effect that are found in the acid ether extract of pine shoots might play a part in the occurrence of *Melampsora pinitorqua* on pine.

Melampsora pinitorqua is only one of many species of rust fungi, with a basidio spore stage that spreads to a host plant, that develops new growth parts at the beginning of a vegetation season. The common genera of *Chrysomyxa*, *Pucciniastrum* and *Gymnosporangium* in Swedish forestry may be mentioned. It would be advantageous when assessing the *Melampsora* problem if parallel examinations were made of other rust fungi and host plants.

It is not a generally proven or accepted circumstance that the interplay between host and obligate parasite is to some critical extent triggered or mediated by substances already present, which before the infection were present in the plant and formed a foundation for resistance or receptivity. This is emphasized also in SHAW's work of 1967.

Attempts were also made to test new ways of separating the components of the ether extract. Thus an attempt was made using gel filtration of the ether extract, dissolved in ethanol, in combination with chromatography on TLC-plates utilizing multiple development. In this way it could be established that an acid ether extract of pine shoots can consist of a great number of components, which can be described as the Rf-value, and demonstrable with the help of UV light, Ehrlich staining, or bioassay. After having complicated the picture of an acid ether extract to embrace a multitude of substances, it should however be pointed out that not all the substances that

are to be found in the extract need necessarily have any real function in pine—or for that matter even exist in pine. To some extent it must be expected that decomposition products and similar substances can be formed during the course of the work.

The examined acid ether extract is made from a pine material that does not display extreme reactions to *Melampsora pinitorqua*. A future task must be to collect and study in detail pine material with very high and low attack frequency.

The basic aim was to separate the substances with effect on the *Melampsora* basidio spore germination test, although several other compounds in the acid ether extract of *Pinus silvestris* have pronounced effect in *Avena* test. After gel filtration the material could be divided into seven Sephadex-fractions, of which four affected the spore germination test prior to further separation by chromatography. These four fractions were subjected to further study by chromatography and several biologically active bands could be noted as shown in table 9. In a few cases the work could be followed up with an exact chemical identification. Thus six resin acids could be identified; this group of substances showed itself to have a distinct spore germination inhibiting effect, and occurs in the *Avena* straight growth test in the β inhibitor position.

Summary

The introductory chapter contained a summary of information about the occurrence of pine twisting rust on different pine species and in relation to climate and other factors according to generally known facts on the subject. Mention is also made of the start that has been made on resistance breeding.

Various methods of recording pine twisting rust have been discussed, and during a three-year period attack frequency on 75 progenies of *Pinus silvestris* plus trees has been noted. It has been possible to demonstrate that there are differences in attack frequency between progenies. Part of the material has been treated as randomized block.

The localisation of *Melampsora* rust to the actual pine leaders and the relationship between plant height or the length of terminal leader and the disease frequency were made the starting point for research into growth regulating substances in pine as being a possible factor affecting the occurrence of pine twisting rust.

Acid ether extracts of annual shoots of *Pinus silvestris* have been examined by *Avena* straight growth test and *Melampsora* basidio spore germination test, that is to say the spores that infect the pine have been used for bioassay. The extracts contain a number of substances—inhibitors—that inhibit spore germination. Marked differences in the inhibitor effect between extracts from different pine clones have been established. The possible connection between a high inhibitor content and resistance to pine twisting rust has been discussed.

During the axial extension a brief reduction in the inhibitor content in the annual shoots of pine has been proved. Also this has been related to the occurrence of the fungus on pine.

Further it has been shown that inhibitors can be leached into water droplets on the surface of pine shoots. This too can be considered as having an effect on the occurrence of the fungus.

Gel filtration combined with thin layer chromatography was used for separating the extract components. An acid ether extract of pine annual shoots contains a great number of substances. These were divided into seven groups through gel filtration and then studied through preparative chromatography. The presence of a number of crude substances, or combinations of substances, having an inhibitor effect could be proved. Of these it was possible to identify a number of resin acids by chemical means.

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Sammanfattning

Inledningsvis har sammanfattats uppgifter om knäckesjukans uppträdande på olika tallarter och i förhållande till klimat och andra faktorer i enlighet med allmänt kända fakta inom området. Vidare har berörts den ansats som finns till en resistensförädling.

Olika metoder att registrera knäckesjuka har diskuterats, och under en treårsperiod har angreppsfrekvens noterats på 75 avkommor av plusträd. Skillnad i angreppsfrekvens mellan avkommor har kunnat påvisas. En del av materialet har behandlats som randomized block.

Knäckesjukans lokalisering till själva talltoppen och samband mellan planthöjd eller toppskottslängd och sjukdomsfrekvens har tagits till utgångspunkt för en undersökning av tillväxtreglerande ämnen hos tallen som en tänkbar faktor för knäckesjukans uppträdande.

Sura eterextrakt av tallskott har undersökts genom *Avena* straight growth test och basidiesporgroningstest — de sporer som infekterar tallen har alltså använts för bioassay. Eterextrakten innehåller ett flertal ämnen — inhibitorer — med sporgroningshämmande effekt. Påtagliga skillnader i hämmande effekt mellan extrakt från olika tallkloner har konstaterats. Ett tänkbart samband mellan hög halt av inhibitorer och resistens mot knäckesjuka har diskuterats.

Under tallens skottskjutning har en kortvarig minskning av halten av inhibitorer kunnat påvisas i årsskott av tall. Även detta har satts i samband med svampens uppträdande på tallen.

Vidare har påvisats att ämnen med sporgroningshämmande effekt kan exuderas till vattendroppar på ytan av tallskott, ett förhållande som kan tänkas påverka svampens uppträdande.

Gelfiltrering i kombination med tunnskiktskromatografi har använts för separation av i extrakten ingående komponenter. Ett surt eterextrakt av årsskott av tall innehåller en stor mängd ämnen. Genom gelfiltreringen indelades dessa i sju grupper, vilka därefter studerades genom preparativ kromatografi. Ett antal ämnen — eller ämneskombinationer — med inhibitoreffekt kunde påvisas. Av dessa kunde ett antal hartssyror identifieras kemiskt.