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The Effect of Some Antibiotic Substances on the Germination of the Conidia of *Polyporus annosus* Fr.

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The spruce (*Picea abies* Karst.) is subject to many diseases. One of the most important of these is root rot, caused by the root rot fungus, *Polyporus annosus* Fr. The losses to our forestry due to this fungus amounts to several million kronor a year. A throrough study of its biology and biochemistry is accordingly a matter of great urgency, and Professor Erdtman and the author have therefore initiated an investigation which it is hoped will, among other things, contribute to our knowledge of the effects on the fungus of antibiotic agents occurring in the soil.

Before entering upon that question, a brief review of the biology and manner of dispersion of the infecting fungus will be necessary. The principal host of the fungus is the spruce, and it is attacked in most parts of the country. Scots pine (*Pinus silvestris* L), is as a rule resistant to the disease, but in the southernmost counties this tree is also attacked. In the spruce the fungus is generally a saprophyte, and the disease is chronic. The mycelium produces a central rot in the heartwood, spreading slowly to the sapwood. In the pine, on the other hand, the disease is of acute character. In this tree the fungus appears as a parasite, kills the cambium and the outer sapwood, and causes the standing tree to dry very quickly. It does not, however, attack the heartwood.

The means available for the spreading of the fungus are its mycelium, its basidiospores, and conidia which are formed asexually on the mycelium. The infection enters the spruce through wounded or dead roots. It is not yet quite clear how the mycelium reaches the root system, at least two possibilities are conceivable. One is that an affected root establishes contact with an injured, but not yet attacked root of another root system. Such root contacts, actually, have been demonstrated on many occasions, and infection has been experimentally produced in the same manner [1–4].

It is also conceivable that the mycelium may spread through the soil from infected roots to the roots of unaffected trees, an opinion which is held by several authors [5–7]. This, however, is not easily demonstrated, as the root rot mycelium cannot be cultivated by spreading out a soil sample on malt agar in a Petri dish. In such experiments numerous mould fungi, bacteria, *etc.* will grow out instead, smothering any relatively slow growing *annosus* mycelium that may be present. Nor can *annosus* mycelium be induced to grow in unsterilized samples of humus or litter in a flask under laboratory conditions. However, it will grow readily if the material is sterilized. This is usually taken to mean that other micro-organisms in the samples actively prevent the *annosus* mycelium from growing [8, 9].

A close study of the distribution of root rot in Sweden shows that this disease is not evenly spread over the whole country. In some districts spruce is very severely attacked, *e. g.* in Silurian districts and on calcareous soil in general. In the northern spruce district of Sweden, which is situated on primary rock, hardly any root rot is found. Other rot fungi occur there instead [3].

Root rot may be very unevenly distributed even in small, restricted areas. In the Tönnersjöheden Experimental Park in Halland it is, for instance, common in spruce forest planted on the old moors, while the beautiful spruce stands growing on what used to be beechforest ground are practically free from root rot.

The composition of the microflora differs in different soils. In soil where root rot occurs sparingly, or not at all, antibiotic fungi and bacteria counteracting the *annosus* mycelium may be richly represented. This is difficult to demonstrate by analysing the microflora of the soil, a procedure which is, in any event, very time-consuming. A beginning has been made by Björkman, who has isolated from some soils fungi of antagonistic effects on *P. annosus* [7]. Other investigations indicate that a good many soil fungi — mainly moulds, but also bacteria — are able to check the growth of *annosus* mycelium on malt agar and on nutritive solutions [10, 11].

In the present investigation we have examined the effects of a number of antibiotic agents on the germination of *annosus* conidia. These conidia, which form on the mycelium, can easily be obtained in sufficient quantities for laboratory examinations, and are accordingly suitable for investigating the inhibiting effects of antibiotic agents on germination. The biology of germination of the conidia has been more closely examined in another connexion [11]. Here we will only note that the best germination was obtained in a 0.5 % solution of malt extract. Between 50 and 60 % of the conidia germinated in this substratum after being kept one day in a moist chamber at 22° C. The

Table 1. Inhibiting effect of various fungicides (in p. p. m.) on the germination of annosus conidia.

Fungicides	Per cent germination				
	0	0–5	5–20	20–50	50–60
Corrosive sublimate	20	10	5	1	0.05
Copper sulfate	100	50		20	10
Phenol	10 000	5 000	2 000	1 000	100
Pentachlorophenol-Na	1 000	500	200		100

Table 2. Inhibiting effect of various fungal antibiotics (in p. p. m.) on the germination of annosus conidia.

Antibiotics	Per cent germination 0	0—5	5—20	20—50	50—60
Patulin	50	25	10	5	2
Enniatin		500	50	25	5
Griseofulvin				500	10
Penicillin	1 000	500	250	25	5
Spinulosin	250		100	50	10
Streptomycin	1 000	500	100	25	5

antibiotic agents were therefore dissolved in 0.5 % malt, and the same solution was used for all dilutions, and control tests. Its pH was about 5.0.

Germination was first examined in some common fungicides (Table 1). Of these, sublimate had strongest effect. It stopped germination completely at 20 p. p. m. and did not allow the conidia to germinate as well as in the control tests until at 0.05 p. p. m. Phenol had but little effect, and a relatively high concentration was required to inhibit germination of the spores even in sodium pentachlorophenolate.

Some agents antibiotic to fungi have been examined in Table 2. Of these, patulin was the most effective. Spinulosin also proved fairly effective. Enniatin, which is isolated from some species of *Fusarium* and is very toxic to tubercle bacteria [12, 13], had no great effect on *annosus* conidia. The same applies to both penicillin and streptomycin. Penicillin was also examined at pH 3.6 and streptomycin at pH 7.2, but their antibiotic effect was no greater at these pH-values.

Brian and his collaborators [14, 15] isolated from *Penicillium Janczewskii* a substance called griseofulvin. At low concentrations (1.0—0.2 p. p. m.) this substance produced peculiar curls and ramifications in the germ tubes of *Botrytis allii*. It had no similar effect on the germ tubes of *annosus* spores, nor was the germination of these appreciably inhibited. More than 30 % of the conidia germinated with normal germ tubes even at 500 p. p. m.

The effect of most antibiotic agents is bacteriostatic or fungistatic, and only a small number of them are bactericides or fungicides. Comparatively little is known of how they affect the bacteria and fungi, but presumably they interfere at one vital stage or another of their metabolism. Waksman [16] has pointed out many possible ways in which this might be done. It has for instance been shown that the pH of the solvent greatly influences the efficacy [17, 18]. As several of these agents are either acids or bases, the influence

Table 3. Inhibiting effect of various fungal antibiotics (in p. p. m.) on the germination of annosus conidia.

Antibiotics	pH	Per cent germination 0	0–5	5–20	20–50	50–60
Alternaria acid	3.6	1000	500	200	50	25
	5.0	1000	500	200	100	25
Citrinin	3.6	25	10	5	2	1
	5.0	250		100	50	10
Gladiolic acid	3.6	10	5	0.05	0.005	0.0005
	5.0	50	20		10	0.2
Gliotoxin	3.6	5	2	0.5	0.25	0.1
	5.0	25		10	5	2
Glutinosin	3.6	5	2	0.2	0.05	0.01
	5.0	5	2	0.05	0.02	0.01
Mycophenolic acid	3.6	1	0.2	0.02	0.005	0.0005
	5.0	500	250	100	10	5
Penicillic acid	3.6	250	100	50	2	0.1
	5.0	500	100	50	25	10
Puberulic acid	3.6	10	2	0.05	0.02	0.005
	5.0	250		100	50	25
Puberulonic acid	3.6	100	50	25	2	0.1
	5.0	250		100	25	5
'Red pigment'	3.6	5	2	0.5	0.2	1
	5.0	5	2	0.5	0.2	1
Stipitatic acid	3.6	100	50	25	5	2
	5.0	250	100		50	25
Viridin	3.6	5	2	1	0.1	0.02
	5.0	0.2	0.2	0.1	0.02	0.005

of pH is probably connected with the degree of dissociation of the antibiotic substances.

The effect of the pH of the solvent on some antibiotic agents produced by fungi is quite distinct (Table 3). In an acid solution (0.5 % malt extract + 0.001 *M* citric acid, pH = 3.6) a lower concentration was usually, *e. g.* in the case of citrinin, gladiolic acid, puberulic acid, *etc.*, required to inhibit the germination of conidia than in malt extract with a pH of about 5.0. In mycophenolic acid the difference was very large. Germination was completely inhibited by 1 p. p. m. at pH 3.6, while 500 p. p. m. were required in malt extract.

Some of these antibiotic agents are exceedingly effective on the *annosus* conidia. This statement applies to the 'Red pigment' — produced from *Penicillium nigricans-Janczewskii* [15] — gliotoxin, and viridin. The two last-

Table 4. Inhibiting effect of various bacterial antibiotics (in p. p. m.) on the germination of annosus conidia.

Antibiotics	pH	Per cent germination				
		0	0–5	5–20	20–50	50–60
Gramicidin	3.6			200	100	25
	5.0			200	100	25
Gramicidin S	3.6	100	50	10	2	1
	5.0	100	50	10	2	1
Pyocyaninchloride	3.6	200			100	25
	5.0	500			200	100
Tyrocidinchloride	3.6	100	50	25	5	2
	5.0	100	50	25	5	2

named substances, which are produced by *Trichoderma viride*, are of particular interest in this connexion. This fungus is very common in the soil, and may possibly counteract the *annosus* mycelium. Weindling, who among others has been examining the antibiotic properties of *Trichoderma*, found that this fungus was most active against *Rhizoctonia solani* in acid soils [19]. Rishbeth [4] points out that the *annosus* mycelium is possibly counteracted in the soil by *Trichoderma viride*, which in some parts of Great Britain is more common in acid than in alkaline soils.

Some bacteria-produced antibiotic agents have also been examined (Table 4). Gramicidin and tyrocidine are two closely related high-molecular polypeptides obtained from *Bac. brevis* and other soil bacteria. Of the two gramicidin preparations, the Russian 'Gramicidin S' was appreciably more effective than the British one. Tyrocidine chloride was also rather active, while pyocyanin, the blue pigment of *Pseudomonas aeruginosa*, had little effect.

These experiments show that the *annosus* conidia are sometimes inhibited by very small quantities of antibiotic substances. Since its mycelium will, as a rule, hardly tolerate them in appreciable concentrations [20], the spreading of the fungus in the soil might possibly be prevented — or at least made more difficult — by the presence in the soil of suitable organisms producing antibiotic agents. The mycelium would then be dispersed mainly via the root systems, in which moulds and bacteria cannot penetrate as easily as a fungus mycelium with cellulose-decomposing properties [3, 4, 9]. It must be borne in mind, however, that conditions are much more complicated in nature than in pure laboratory experiments. There are many other organisms in the soil that are not affected by these substances, but are perhaps instead able to break them down. At least some of these antibiotic agents are not very stable, and

Table 5. Inhibiting effect of various heartwood constituents (in p. p. m.) on the germination of annosus conidia.

Heartwood substances	Per cent germination				
	0	0–5	5–20	20–50	50–60
Pinosylvin	50	25	10	2	0.5
Pinosylvin monomethyl ether	50	25	5	1	0.5
Dihydropinosylvin *	100		50	25	5
Conidendrin				500 ***	100
Pinoresinol **			500 ***	100	50

* Not found in nature.

** Constituent of pine and spruce gum resin.

*** Saturated solution.

are easily inactivated in the presence of organic substances, *e. g.* sugars and proteins, at unfavourable pH-values, *etc.*

Assuming, however, that the *annosus* mycelium has successfully reached a root, despite all dangers in the soil, it must still penetrate into the host plant. Pine heartwood contains the strongly fungicide pinosylvin phenols [20, 21], which has a strong inhibiting effect on the germination of *annosus* conidia (Table 5). Spruce heartwood, on the other hand, contains hardly any fungicide substances. Saturated solutions of conidendrin or sulphite liquors lactone have only an insignificantly inhibiting effect on the germination of *annosus* conidia, and so has pinoresinol, a constituent of spruce gum resin, *etc.* (Table 5).

The dead heartwood cannot provide any active, physiological protection against penetrating organisms, as do for example the rustresisting cereals, constituents of whose living protoplasm can actively counteract the growth of rust fungi. Once the mycelium has penetrated into the heartwood of spruce, the host is defenceless against the root rot fungus. To be resistant to root rot, spruce heartwood would have to contain toxic substances able to prevent the growth of the fungal mycelium, and — as we have just said — none such has been found there.

Conditions are different in the pine. The pinosylvin phenols have a strongly inhibiting effect not only on the germination of the *annosus* conidia but also on its mycelium [20]. In malt agar their inhibiting limit is at about 0.02 %, or 200 p. p. m., of pinosylvin. The root rot mycelium has obviously no chance of penetrating into the heartwood of pine, since the pinosylvin content is as a rule largest (1—2 %) in its peripheral parts [22, 23]. Nor has any rot due to *P. annosus* been observed in the heartwood of pine trees; in this tree the root rot mycelium apparently never grows except in the sapwood.

SUMMARY

The effects of antibiotic agents from fungi and bacteria on the germination of the conidia of the root rot fungus, *Polyporus annosus* Fr., have been investigated. Some of these agents, *e. g.* gladiolic acid, mycophenolic acid, and viridin, are very effective against the conidia of *P. annosus*, surpassing sublimate in this respect. In most cases the effect is greater at pH 3.6 than at pH 5.0. Germination is also effectively inhibited by pinosylvin. No substance possessing similar properties has, however, been found in spruce wood.

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REFERENCES

1. von Hopffgarten, E. H. *Phytopath. Z.* 6 (1935) 1.
2. Jörgensen, C. A., Lund, A., and Treschow, C. *Kgl. Vetr. & Landbohöjskoles Aarskr.* 71 (1939).
3. Rennerfelt, E. *Medd. Statens Skogsforskn.-inst.* 35 (1946) no. 8.
4. Rishbeth, J. *Forestry* 22 (1948) 174.
5. Lagerberg, T. *Sv. Skogsvårdsföreningens Tidskr.* 34 (1936) 396.
6. Roll-Hansen, F. *Medd. f. d. Norske Skogforsöksv.* 24 (1940) 1.
7. Björkman, E. *Physiologia Plantarum* 2 (1949) 1.
8. Hiley, W. E. *The fungal diseases of the common larch.* Oxford (1919).
9. Treschow, C. *Zentr. Bakt., Parasitenk.* Abt. II 104 (1941) 186.
10. Enebo, L. *Physiologia Plantarum* 2 (1949) 56.
11. Rennerfelt, E. *Oikos* 1 (1949) 65.
12. Plattner, Pl. A., and Nager, U. *Experientia* 3 (1947) 325.
13. Plattner, Pl. A., Nager, U., and Boller, A. *Helv. Chim. Acta* 31 (1948) 594.
14. Brian, P. W., Curtis, P. J., and Hemming, H. G. *Trans. Brit. Myc. Soc.* 29 (1946) 173.
15. Grove, J. F., and McGowan, J. C. *Nature* 160 (1947) 574.
16. Waksman, S. A. *Microbial antagonisms and antibiotic substances.* New York (1947).
17. Abraham, E. P., and Duthie, E. S. *Lancet* 250 (1946) 455.
18. McGowan, J. C. *Chemistry and Industry* 16 (1947) 205.
19. Weindling, R. *Phytopathology* 22 (1932) 837; 24 (1934) 1153.
20. Rennerfelt, E. *Svensk Botan. Tid.* 39 (1945) 311.
21. Erdtman, H. *Ann.* 539 (1939) 116.
22. Erdtman, H., and Rennerfelt, E. *Svensk Papperstidn.* 47 (1944).
23. Rennerfelt, E. *Medd. Statens Skogsforskn.-inst.* 36 (1947) no. 9.

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