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A growth-regulating substance produced by Pythium sylvaticum

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Abstract

Culture filtrates of *Pythium* spp. (mainly *P. sylvaticum*) were studied as such and after purification and separation by paper chromatography. A bio-assay was developed in which the elongation of seedling roots was used as a criterium. The following results were obtained from these bio-assays:
1. *P. sylvaticum* is able to produce auxin under laboratory conditions.
2. Culture filtrates of *P. sylvaticum* cause the same symptoms as IAA on roots of cucumber, flax and wheat seedlings.
3. The auxin concentration in culture filtrates of *P. sylvaticum* increases very rapidly.
4. A linear relationship was found between the logarithm of the dilution of the culture filtrate and the probit of the inhibition of root growth.
5. *P. irregulare* and *P. paroecandrum* produce probably also IAA. *P. ultimum*, *P. torulosum* and *Fusarium oxysporum* f.sp. *pisi* apparently are not able to produce auxins.

Introduction

Almost every soil sample in the Netherlands contains *Pythium sylvaticum* Campbell & Hendrix. This species sometimes constitutes over 80% of the total number of *Pythium* isolates. Tests, carried out in the laboratory, showed that *P. sylvaticum* is very pathogenic to seedlings of several plant species (Blok, 1970). The fungus causes lesions in roots of flax, especially near the root tip, total browning, reduction in longitudinal growth and swelling just behind the root tip. Sterile culture filtrates of the fungus cause the same symptoms, except browning of the roots. It was therefore suggested that toxins, secreted by the fungus, might play a role in the disease complex.

Later on it became clear that the observed symptoms are similar to those caused by solutions of 3-indoleacetic acid (IAA) (Hejnowicz, 1961). The aim of the investigations was therefore mainly to determine if auxin was present in cultutre filtrates of *P. sylvaticum*.

Materials and methods

Fungi. Several isolates of *P. sylvaticum* were tested. For comparison, some other *Pythium* spp. and *Fusarium oxysporum* f. sp. *pisi* were included in the test. All fungi were isolated from soils in the Netherlands. The fungi were cultured on cornmeal agar (CMA) at room temperature. For seeding cultures, 5 mm discs were taken from the margin of a two days old colony.

Culture filtrates. Culture filtrates were obtained from 8–12 days old shake cultures in cornmeal (CM) decoction or Czapek-Dox (CD) solution. Cultures were grown in

darkness at a temperature of about 25°C. The filtrate was passed through a Seitz-filter and stored at 2°C in darkness. In some tests the filtrate was divided into three aliquots. One was pasteurized (3 × 1 hour at 70°C), another autoclaved (45 min at 120°C), and the third was not treated. For a quantitative assesment of changes in auxin content in growing cultures, the fungi were grown in 100 ml Erlenmeyer flasks, each containing 45 ml CD solution and 2 discs of inoculum. After different periods of incubation the filtrate was passed through a Seitz-filter and stored.

Hosts. Flax (*Linum usitatissimum*, various varieties) was used in all tests, and in some tests wheat (*Triticum vulgare*, various varieties) and cucumber (*Cucumis sativus* 'Gele Tros') were used in addition. The seeds were disinfected for ten minutes with 0,2% $HgCl_2$, rinsed with sterile water, and germinated on wet blotter paper at 23–25°C. When the rootlet was 1–2 mm long (generally after one day), the seedlings were used for the bio-assay.

Bio-assays. The methods are based on those used by Luke and Wheeler (1955) and by Vanterpool and Truscott (1932).

A. Sterile filter paper discs (⌀ 5 cm) were placed in sterile petri dishes and wetted with 1 ml culture filtrate. Ten germinated flax seeds (or seven germinated wheat or cucumber seeds) were placed on each disc and incubated at room temperature for two days.

B. As A, but the filter paper was wetted with sterile water; two or three agar discs with inoculum were placed between the seeds.

C. As A, but the filter paper disc was replaced by a piece of a filter paper chromatogram of the culture filtrate, usually 2 × 2 cm, wetted with sterile water.

D. Germinated seeds were placed on the surface of 1% water agar, together with some drops of culture filtrate or some agar discs with mycelium.

The length of the rootlets was measured (except in method D) after two days, and growth inhibition was calculated with the formula:

$$\text{growth inhibition} = \frac{(l_c - l_o) - (l_t - l_o)}{(l_c - l_o)} \times 100$$

in which:

l_o = root length of seedlings at the start of the test (estimated),

l_c = root length of control seedlings at the end of the test,

l_t = root length of treated seedlings at the end of the test.

Fractionation of the culture filtrate. Preparation of the chromatograms, and further chemical and physical methods are described by Posthumus (1973).

Results

The influence of medium, heat and light on the activity of the culture filtrate. CM decoction itself had a remarkable inhibiting effect on root growth, especially of flax. When CM decoction was used in the bio-assay, the root length of flax was only 6 mm and of wheat 53 mm, whereas with water the root length was 28 mm and 84 mm resp. CM decoction was therefore not used in later experiments. CD solution slightly

stimulated flax roots but retarded the growth of wheat roots. Modifications of CD solutions were also tested, but their influence on root growth was not less than that of CD solution itself.

Heat treatment of the culture filtrate, i.e. pasteurization or autoclaving, resulted in either none or only a slight reduction of activity in most experiments. The active component of the filtrate was slowly inactivated in light.

Some properties of the culture filtrate. The pH of shake cultures of *P. sylvaticum* in CM decoction decreased from initially pH 7 to about pH 5 in ten days.

The active compound was shown to be of low molecular size, because it passes freely through a dialysis membrane.

There was a good correlation between the dilution of the culture filtrate and the degree of inhibition of seedling root growth (Fig. 1). The relation between the logarithm of the dilution and the probit of the inhibition was linear.

With Salkowski reagent the culture filtrate showed a slight pink colour, which indicates the presence of IAA. Its presence was also demonstrated by the stimulating effect of the filtrate on rooting of petioles of *Phaseolus vulgaris*.

Comparison of culture filtrate with IAA solution. Several bio-assays were carried out comparing 10^{-4}, 10^{-5}, 10^{-6} and 5×10^{-7} M solutions of the K-salt of IAA in water with culture filtrates of *P. sylvaticum*. Root growth was reduced by pure IAA during the first day of incubation, especially by the higher concentrations, but after the first day the IAA was apparently broken down, probably by IAA-oxidase, and the rootlets started to grow rapidly. After two days differences with the control seedlings were small. This phenomenon did not or hardly occur with culture filtrates.

Fig. 1. The relation between the dilution of culture filtrate of *P. sylvaticum* and the inhibition of longitudinal growth of flax roots.

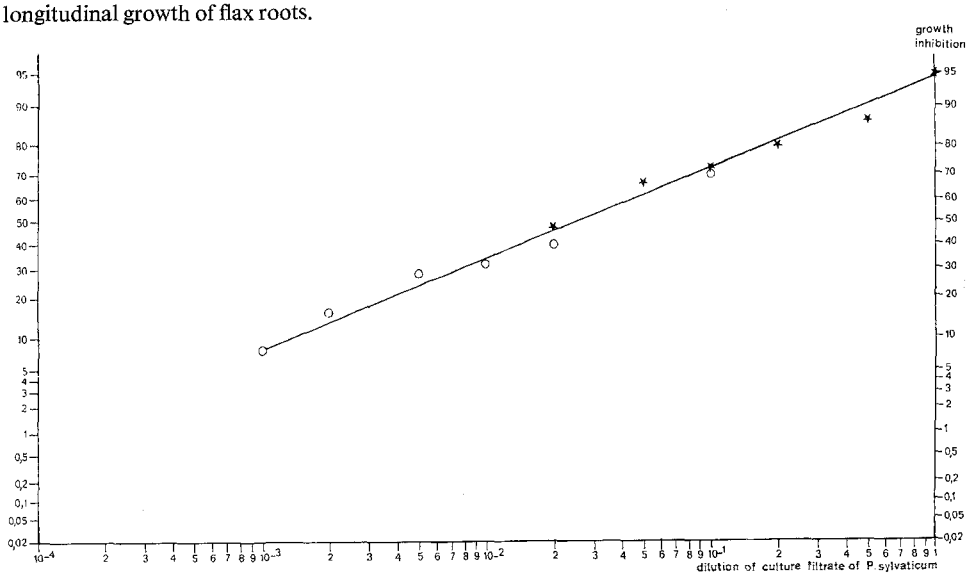

Fig. 1. De relatie tussen de verdunning van cultuurfiltraat van P. sylvaticum en de remming van de lengtegroei van vlaswortels.

Chromatographic separation, physical and chemical identification of the active compound. Ultimate paper chromatograms of culture filtrates of *P. sylvaticum* under U.V. illumination showed a clear fluorescent band corresponding in Rf with IAA, in addition to some other vague bands. Strips of the chromatograms, cut into 20 equal pieces corresponding to Rf values 0.00–0.05, 0.05–0.10 etc., were used in the bio-assay. Activity was present only on the place corresponding with that of IAA and in a small strip just above this place (Fig. 2). Slight inhibitory effects from pieces of other Rf values were rather erratic.

According to Posthumus (1973) the eluate of the active band contained a rather pure sample of IAA as shown by the fluorescence characteristics, the U.V. absorption spectrum and the colour reaction with the Salkowski reagent.

Reactions of the host plant. Roots of flax seedlings placed on 1 % water agar usually grew into the agar, and were smooth and hairless. Wherever the root tips came into contact with the growth inhibiting compound produced by *P. sylvaticum*, from either culture filtrates or living mycelium, the region right behind the root tip started to swell and often produced high numbers of root hairs (Fig. 3). The time needed for recovery of the root and further growth depended largely on the concentration of the

Fig. 2. The inhibition of longitudinal growth of flax roots caused by different components of culture filtrate of *P. sylvaticum* and by IAA (broken line), separated by paper chromatography.

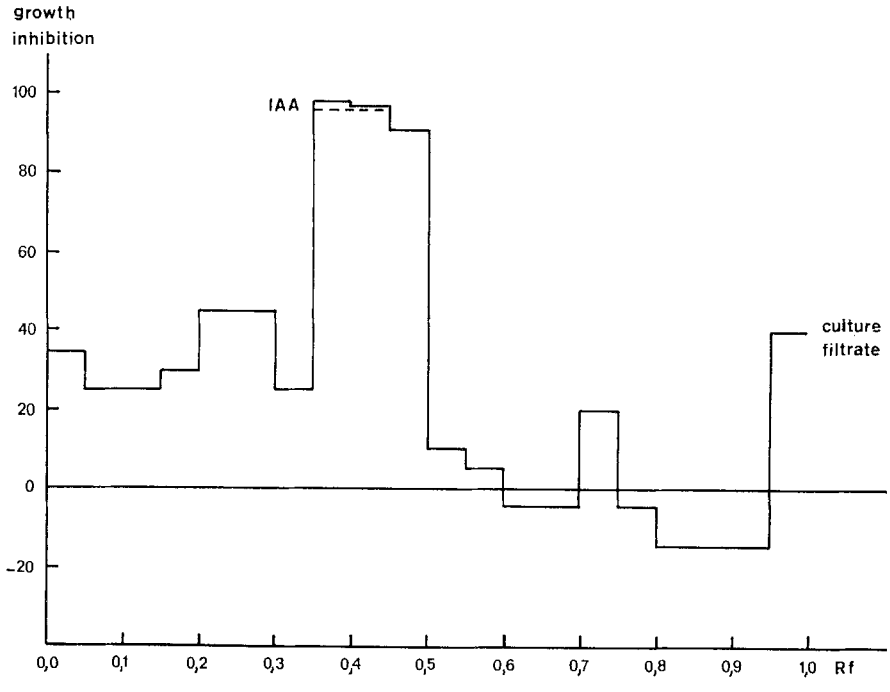

Fig. 2. De remming van de lengtegroei van vlaswortels veroorzaakt door verschillende componenten van cultuurfiltraat van P. sylvaticum en van een IAZ-oplossing (gebroken lijn), gescheiden door middel van papierchromatografie.

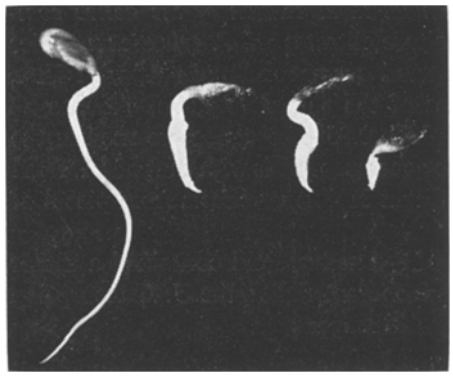

Fig. 3. The reaction of flax roots on the application of culture filtrate of *P. sylvaticum*. At left a non-treated seedling. The picture is taken two days after the beginning of the best.

Fig. 3. De reactie van vlaswortels op toediening van cultuurfiltraat van P. sylvaticum. Links een onbehandelde zaailing. De foto is genomen twee dagen na het inzetten van de proef.

active substance. The characteristic effect of swelling and root hair formation was observed exclusively in the elongation zone of the root.

Sections through the swollen parts of cucumber roots showed that the increase in root diameter was not caused by an increase in the number of cortical cell layers, but by lateral growth of the cells (Fig. 4).

Fig. 4. Longitudinal section through cucumber roots. Left, untreated; right, roots swollen as a result from application of culture filtrate of *P. sylvaticum*.

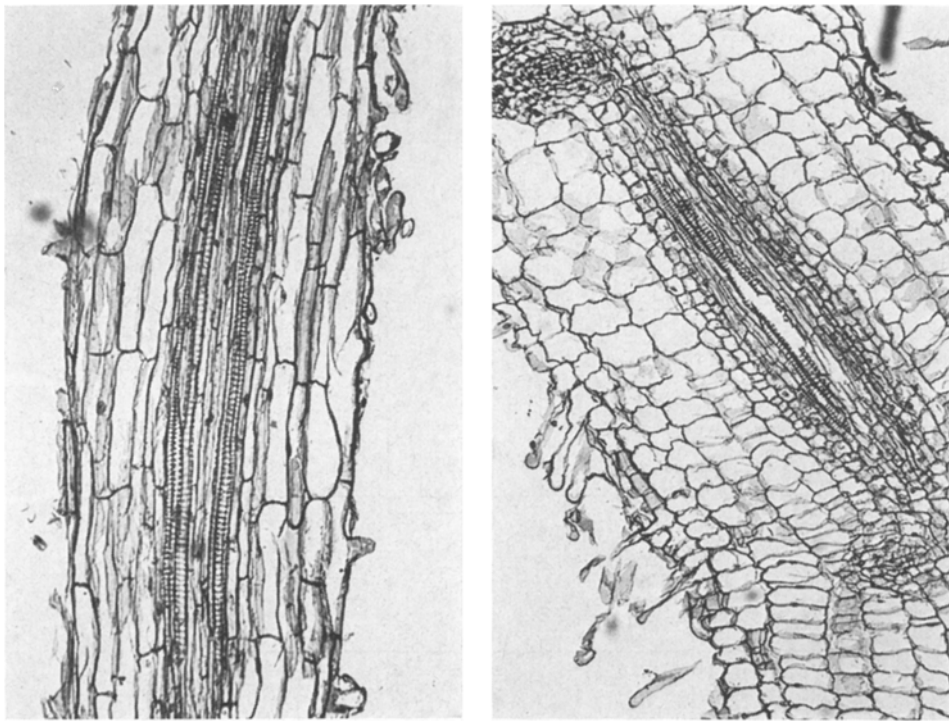

Fig. 4. Lengtedoorsnee door komkommerwortels. Links, onbehandeld; rechts, wortel opgezwollen onder invloed van cultuurfiltraat van P. sylvaticum.

External symptoms on roots of flax-, cucumber-, pea- and wheat seedlings were identically, although the symptoms on wheat roots were less evident. Roots of seedlings inoculated with living mycelium stopped growth, started to swell and subsequently were invaded by the mycelium. The root tip was often totally destroyed. Lateral roots were then often formed. Older parts of the roots were not attacked. A slight swelling of the root tip and subsequent root hair formation could also be incited by wounding the growing root tips with a needle.

The reaction of the roots took place only when the substance could reach the root tip (e.g. by diffusion through the agar). In water agar the active compound, produced by living mycelium, is able to diffuse ahead of the mycelium. Downward transport in the seedlings could not be demonstrated.

Comparison of culture filtrates of several Pythium spp. and Fusarium oxysporum f.sp. pisi. Culture filtrates were obtained from *P. sylvaticum* (6 isolates), *P. irregulare*, *P. ultimum* (2 isolates each), *P. paroecandrum*, *P. torulosum* and *Fusarium oxysporum* f.sp. *pisi* (1 isolate each). Bio-assay methods A and B were used. The results are shown in Fig. 5.

P. sylvaticum, *P. irregulare* and *P. paroecandrum* caused the characteristic swelling

Fig. 5. The inhibition of longitudinal growth of flax roots caused by culture filtrates and agar discs with mycelium of some *Pythium* spp. and *Fusarium oxysporum* f.sp. *pisi*.

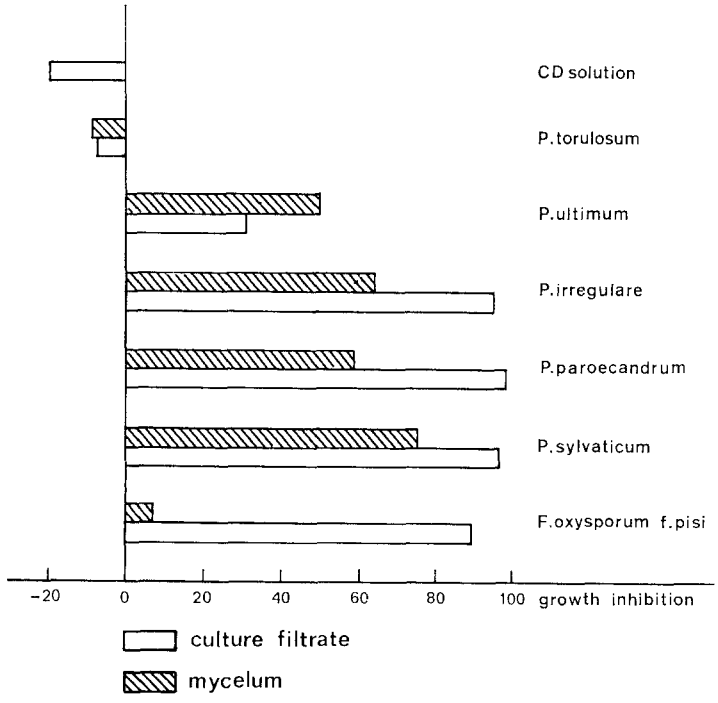

Fig. 5. De remming van de lengtegroei van vlaswortels veroorzaakt door cultuurfiltraten en ponsjes met mycelium van enkele Pythium spp. en Fusarium oxysporum f.sp. pisi.

Fig. 6. The influence of culture filtrates (upper row) and agar discs with mycelium (lower row) of *P. ultimum, P. irregulare* and *P. sylvaticum* on flax roots. At left the untreated control.

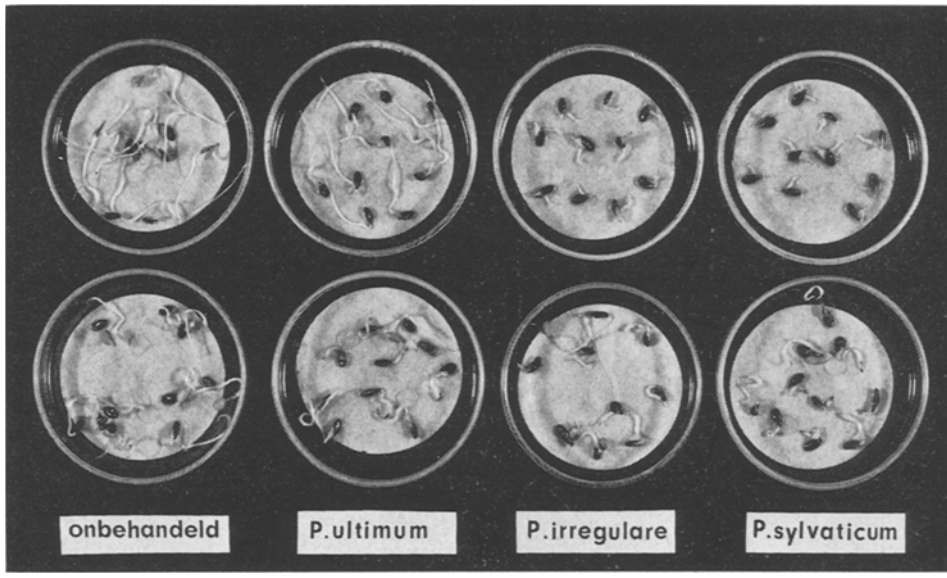

Fig. 6. De invloed van cultuurfiltraten (bovenste rij) en ponsjes met mycelium (onderste rij) van P. ultimum, P. irregulare en P. sylvaticum op vlaswortels. Links onbehandeld.

of the root-tip and a reduction of root growth. This was stronger with culture filtrates than with mycelium. Culture filtrate of *P. ultimum* caused a reduction of root growth of only 32% and did not show the characteristic swelling. Mycelium of all four species caused lesions on the roots (Fig. 6). *P. torulosum* caused no symptoms, either with mycelium or culture filtrate. While mycelium of *F. oxysporum* f.sp. *pisi* hardly influenced the roots, culture filtrate gave a 90% reduction in growth, without causing swelling of the root tip.

The speed of auxin production. A bio-assay to determine the speed of auxin production was carried out for all the filtrates at the same time. Two isolates of *P. sylvaticum* were used. A five hours old shake culture of one of the isolates gave already a growth inhibition of 34%. Maximum inhibition with both isolates was reached within 48 hours. The results of the test are shown in Fig. 7.

Discussion

The influence of metabolic products of *Pythium* spp. was investigated. To avoid side-effects of chemical substances occurring in natural soils and auxins produced by bacteria, epiphytic on non-sterile seedlings, or native in seeds, a bio-assay was chosen in soil-free, synthetic media with sterile seedlings. Root length was chosen as a criterium because the weights of swollen rootlets of treated plants do not differ much from those of the control rootlets.

Fig. 7. The inhibition of longitudinal growth of flax roots caused by filtrates of shake cultures of different age (hours) of two strains (♀ and ♂) of *P. sylvaticum*.

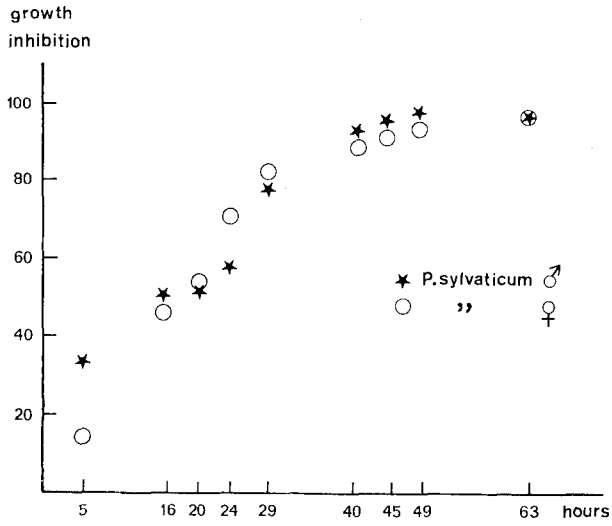

Fig. 7. De remming van de lengtegroei van vlaswortels veroorzaakt door filtraten van schudcultures van verschillende ouderdom (uren) van twee stammen (♀ en ♂) van P. sylvaticum.

Data concerning the nature of the active substance, including low molecular size, acidity of the culture filtrate, root promoting activity and symptoms caused to young rootlets, gave a strong indication that an auxin was involved. The presence of IAA in culture filtrates was demonstrated by chemical and physical analysis by Posthumus (1973). A difference between pure IAA solution and unpurified culture filtrate is the stability of the active compound in the filtrate. In culture filtrate it was heat stable and it was not easily broken down in the bio-assay. Investigations to find an explanation for this phenomenon were unsuccessful. Growth inhibition, a swelling of the root tip densely covered with root hairs and transversal growth of cortical cells are the same reactions as Hejnowicz (1961) found when he applied IAA to wheat roots. The development of root hairs has been shown to result from retardation in vertical elongation of root epidermal cells (Cormack, 1949 and 1962). In addition to IAA, other indole compounds are known to have similar effects on plant roots (Slankis, 1951; Hejnowicz and Erickson, 1968).

Growth hormones often play a role in plant diseases. An increased level of IAA found in diseased host tissue as compared to healthy tissue, however, may be produced by the plant itself, as a reaction to adverse circumstances, e.g. wounding or penetration by a pathogen (Sequeira, 1963). This was shown in our experiments when growing root tips were wounded with a needle.

A substance with growth promoting properties, produced by fungi, was first demonstrated by Nielsen (1928), and later by several other investigators (Gruen, 1959). Other compounds between tryptophane and IAA may also be found. In our experiments no other compounds with root growth inhibiting properties could be demonstrated in culture filtrate of *P. sylvaticum*.

Hutzinger and Kosuge (1968) found in some strains of *Pseudomonas savastanoi* a conjugate of IAA with lysine, strongly resembling pure IAA. A conjugate of this type might explain the more stable character of the active compound in our culture filtrates. However, this could not be demonstrated.

Only few data are available about auxin production by *Pythium* spp. Ronsdorf (1935) found that *P. mamillatum* and *P. intermedium* produced auxin in a synthetic medium, but *P. splendens* and *P. debaryanum* did not. Yoshii and Hagedorn (1971) found that *P. debaryanum* produced IAA on potato dextrose broth. The symptoms described by Vanterpool (1938), caused by a complex of *Pythium* spp. on wheat roots, might have resulted from the presence of growth-regulating substances.

The active substance in culture filtrates of *P. sylvaticum* is produced very rapidly. Although it was produced on a relatively rich medium, one might assume that in soil IAA is likewise produced, probably in lesser amounts. In preliminary tests in soil I found symptoms on roots of several seedlings similar to those caused by IAA.

Brandenburg (1948 and 1950), Bell (1951) and Martin (1964) found a non-specific toxin, produced by *P. irregulare*, which was transported through the plant and caused leaf necrosis and wilting of several plant species. They did not use the culture filtrate directly but treated it to 'purify' the filtrate. The symptoms they found with this treated filtrates may have been caused by artefacts. In tests with our unpurified culture filtrates of P. irregulare these symptoms could not be reproduced.

It is uncertain whether the production of growth-regulating substances has some relation with the attack of the roots by *Pythium* spp. Most *Pythium* spp. attack plant roots only in the seedling stage or juvenile roots of older plants. Kraft et al. (1967), Mellano et al. (1970), Nemec (1971) and Royle and Hickman (1964) found that penetration of various *Pythium* spp. in undamaged seedling roots usually took place in the zones of elongation and maturation, and never occurred in the meristem or in the differentiation zone. Zoospores and mycelium were attracted to the first mentioned zones. The root tip and meristem attracted zoospores only if wounded. Kraft et al. (1967) and Nemec (1971) observed that root hair invasion is very often a major means of entry into the host tissue. If the presence of root hairs is an adventage in that they serve as infection courts, we may assume that one of the mechanisms in plant disease development is the production of IAA by an organism.

Acknowledgments

I wish to thank especially Dr A. C. Posthumus for carrying out physical and chemical analysis, and Dr F. F. Hendrix Jr. for critical reading and correcting the English text.

Samenvatting

Een door Pythium sylvaticum geproduceerde groei-regulerende stof

Cultuurfiltraten van *Pythium* spp. (voornamelijk *P. sylvaticum*) werden onderzocht als zodanig en na extractie en scheiding door papierchromatografie. Een bio-toets werd ontwikkeld waarbij één dag oude zaailingen in contact gebracht werden met cultuurfiltraten of levend mycelium van de schimmel. Na een incubatietijd van twee

dagen werden de wortellengtes gemeten en de mate van groeiremming vastgesteld. De symptomen die cultuurfiltraten van *P. sylvaticum* in deze bio-toets veroorzaken op wortels van jonge zaailingen zijn: groeiremming, een zwelling dicht achter de top en een dichte bezetting van deze zwelling met wortelharen (Fig. 3). De volgende resultaten werden verkregen uit de bio-toets:

1. *P. sylvaticum* is in staat onder laboratorium omstandigheden groeistof te vormen.
2. De symptomen die cultuurfiltraten van *P. sylvaticum* veroorzaken op de wortels van komkommer-, vlas- en tarwezaailingen zijn gelijk aan die veroorzaakt door indolylazijnzuur (IAZ).
3. De groeistofconcentratie in cultuurfiltraten van *P. sylvaticum* wordt zeer snel opgebouwd (Fig. 7).
4. Er werd een lineaire correlatie gevonden tussen de logarithme van de verdunning van het cultuurfiltraat en de probit van de groeiremming van de wortel (Fig. 1).
5. *P. irregulare* en *P. paroecandrum* produceren waarschijnlijk eveneens IAZ. *P. ultimum*, *P. torulosum* en *Fusarium oxysporum* f.sp. *pisi* zijn blijkbaar niet in staat groeistof te vormen (Fig. 5).

Of de vorming van IAZ door *P. sylvaticum* een rol speelt bij de wortelaantasting van zaailingen is niet zeker. Mede door interpretatie van gegevens uit de literatuur mag worden aangenomen dat dit inderdaad het geval kan zijn.

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Book review

Domsch, K. H. (Editor): Umweltschutz in Land- und Forstwirtschaft. No 1. Naturhaushalt. No 2. Pflanzliche Produktion. No 3. Tierische Produktion. (Environmental protection in agriculture and forestry. No 1. Influences of agriculture and forestry on natural ecosystems. No 2. Plant production. No 3. Animal production). Berichte über Landwirtschaft 50 (1972), Paul Parey, Hamburg/Berlin. Also obtainable from Bundesministerium für Ernährung, Landwirtschaft und Forsten, Abt. II A 1, Bonn-Duisdorf. P. 1–208, 209–509, 517–783; 17, 25, 23 contributions, DM 48.30, 70.60, 63.50, paperback.

The German Ministry of Food, Agriculture and Forests sponsored the preparation by experts from different institutes of a comprehensive review on environmental protection under the careful editorship of K. H. Domsch. Each paper contains a survey of contents, English and French summaries and a list of references. Each part has an index. Plantpathologists will find the following contributions of particular interest: in No 1: Plant protection and landscape maintenance (Th. Eggers); Effects of industrial emissions on agricultural and horticultural products (A. Kloke); in No 2: Economic aspects of environmental protection in plant protection (K. Meinhold et al.); Mycotoxins and their originators in agricultural products (H. K. Frank); Potential contamination of waters by agricultural waste (K. Scherb); Decomposition products of pesticides as environmental contaminants (W. Ebing and I. Schuphan); Amounts and types of pesticides retained in foods and feedstuffs (H. Maier-Bode); Systemic application to help reduce environmental pollution with pesticides (H. Kohsiek); Evaluating the toxicity of pesticide residues (K. Hansen); Influence of pesticides on microbial processes and ecologic interrelations in the soil (K. H. Domsch); How pesticides affect wildlife (P. Blaszyk); Biological pest control (J. M. Franz); Integrated control-pest management (H. Wilbert); Breeding plants for resistance to diseases and pests (W. H. Fuchs and J. Ullrich); Present state of legislation, organization, and prospects in plant protection (L. Quantz); papers in No 3 concern mainly the influences of expanded livestock keeping (bio-industry) on the environment and include: Chlorinated insecticides, fasciolicides, and antibiotics in milk (A. Tolle et al.).

It is impossible to assess the individual contributions critically here. There are numerous cases where stringent regulations and changes in current practices are urgently needed for the sake of environmental hygiene. Many good proposals are given in the text. It is hoped that this review will reach the responsible authorities. The papers will certainly help those concerned about chemical control to form a balanced opinion.

W. Gams