The effect of some endogenic substances contained in the cortical parenchyma of apple trees on growth *in vitro* of the fungus *Phytophthora cactorum* (Leb. of Cohn) Schroeter

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Abstract

Gel filtration of an alkaline extract of the cortical parenchyma yielded four fractions of which two had activity *in vitro* on the growth of *Phytophthora cactorum*. Fraction I was stimulatory and fraction IV inhibitory; one of the inhibitors in fraction IV was identified as phloridzin. When the extraction solvent contained sodium sulphite in addition to sodium hydroxide, fraction II showed inhibitory activity.

INTRODUCTION

Collar rot of apple trees caused by Phytophthora cactorum occurs in many European countries as well as in the United States. Field observations by the authors confirm that there is much variation in susceptibility to this disease. The cv. Cox's Orange Pippin is highly susceptible (Sewell, Wilson 1959) as are some vegetative rootstocks (Fischer 1966; McIntosh and MacSwan 1966). Changes in the susceptibility of particular apple varieties during the growing season have been noted (Braun, Nienhaus 1959). It has been suggested that the explanation of susceptibility may be largely mechanical — a property of the cortical tissues (Schwinn 1965a, 1965b). Based on studies by Luckwill, there have been attempts to explain the varieties in susceptibility as being concerned with variation in levels of growth substances present (Braun, Nienhaus 1959). However, Kuć and co-workers (1959), Holowczak and co-workers (1962), working with apples, reported increased resistance to Venturia inaequalis, the apple scab fungus, when D — and DL — phenylalanine were applied to susceptible varieties. They suggest that this resulted in an increase in phenolic substances in the plant, two of which, phloretin and phloretic acid (hydrolysis products of phloridzin) were identified. Further evidence of the activity of phloridzin in apple tree-parasite physiology has been reported by Goodman and co-workers (1967) in *Erwinia amylovora* infections.

Schwinn (1965a, 1965b), however, reported that, in vitro, phloridzin only affected the germination of zoospores and mycelial growth of *Phytophthora cactorum* at very high concentrations (0.1% and 1.0%).

Recently both stimulatory and inhibitory fractions affecting *P. cactorum* have been reported as occurring in the cortical parenchyma tissue of the apple (Borecki and co-workers 1970). A third active fraction has been revealed (Millikan, Glowinkowska 1970) following the addition of sodium sulphite to the extraction solvent. The current work was done to confirm these findings and to investigate these fractions more closely.

MATERIALS AND METHODS

One-year-old shoots of apple trees cv. Hibernal were used. The bark was removed and samples taken of the cortical parenchyma tissue. After storage at a low temperature, the tissue was dried. 5 g aliquots of the tissue were extracted at 4° C for 90 min using the following extraction solvents (A) 50 ml 0.01N NaOH, (B) 50 ml of 0.01N NaOH + Na₂SO₃, (C) 50 ml 0.01N NaOH +0.01% sodium diethyldithiocarbonate (DIECA). Both Na₂SO₃ and DIECA are inhibitors of oxidative enzymes. The extract was filtered through gauze and centrifuged at 16000 rev/min for 10 min using a Unipan type 317 centrifuge. The supernatant was chromatographed on Sephadex-G25; 35 ml was applied to a 4×40 cm column which was developed with 0.02M phosphate buffer pH 6.7, at a flow rate of 0.5 ml/min and the eluate collected in 10 ml fractions. The absorption of each fraction was measured at 254 nm and the contents of the appropriate tubes were combined to give fraction I, II, III and IV corresponding to the absorption peaks at 254 nm (Fig. 1).

The effect of the four fractions on the growth in vitro of P. cactorum was studied. Three kinds of 3% agar media were used; 8% brewer's wort agar; corn meal agar; and a mineral medium containing 24 g sucrose, 4 g NaNO₃, 4 g KH₂PO₄, 1 g KCl and 0.6 g MgSO₄ · 7H₂O. The tested fractions filtered through a bacteria-proof filter of pore size 0.22 μ m, and 3 ml was added to 3 ml of medium in 5 cm petri-dishes. Inoculum was added in uniform amounts, incubation was at 25°C, and all tests were replicated between 6 and 10 times. The diameter of the fungal colonies was determined after 3 to 5 days. The strain of P. cactorum used was originally obtained as isolate 15 from Columbia University, Missouri, U.S.A. The other cultures used in tests were obtained from diseased trees at the Institute of Pomology, Skierniewice.

RESULTS

Sephadex chromatography of the extracts obtained using the three different extraction solvents revealed the existence of four peaks of ultraviolet light absorption (Fig. 1). Two biologically active fractions

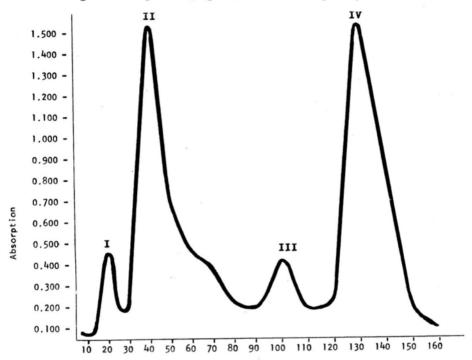


Fig. 1. Sephadex chromatography of NaOH extracts of cortical parenchyma tissue of 'Hibernal' variety of apple. From left to right peaks numbers I to IV

Table 1 The influence of Fraction groups I, II, and IV^α obtained from the cortical parenchyma of the wood of apple cv. Hibernal on the growth of *Phytophthora cactorum* after 3 days at $25^{\circ}C$

	Diameter of colonies (mm) ^b on agar			
Fraction	Corn meal	Mineral medium		
I	37.5	36.6		
II	15.6	15.0		
IV	15.0	12.2		
Distilled				
water	34.9	26.6		

a Fractionation was on Sephadex-G25 of an extract obtained using 0.1N NaOH + Na₂SO₃.

b Means values for 6 to 10 replicates in each case.

 $$T\,a\,b$$ The influence of fraction groups I, II, and IV^α obtained from the cortical paren on brewer's wort

Fraction	Phytophthora cactorum 15	Phytophthora cactorum 9	Phytophthora parasitica
I	33.0°	32.0c	43.5c
II	2.25	no growth	no growth
IV Distilled	23.0	20.4	22.2
water	28.7	29.7	44.5

a Fractionation was on Sephadex G-25 of an extract obtained using solvent (B) con-Mean diameters (mm) of 6 to 10 replicates measured after 24 h(b), 72 h(c), and 8 days(d).

were found in both the NaOH (A) and the NaOH + DIECA (C) extracts. Fraction I exhibited a stimulatory effect on growth in vitro in P. cactorum in contradistinction to fraction IV which was inhibitory. Following the use of NaOH + Na₂SO₃ (B) as an extraction solvent, fraction II became markedly inhibitory to the fungus (Table 1).

Studies of the effect of active fractions on the growth of other pathogenic fungi revealed considerable similarity of action on fungi in the *Pythiaceae* (Table 2). An exception was an unidentified species of *Pythium* sp. (No. 1).

Molisch tests indicated the presence of sugar in fractions I, II and IV. Fehling's test revealed reducing sugars in fraction II. The use of solvent (B), NaOH+ Na₂SO₃, as will be mentioned in the discussion, allowed the detection of sulphur in fraction II. It was also demonstrated that sodium sulphite and DIECA used directly in the culture medium inhibited the growth of *P. cactorum*.

When fraction IV was kept at 4° C for 24 h, a substance crystallized out. After filtering and drying this material had an inhibitory effect on the growth of P. cactorum at a dilution of 100 ppm. Tests showed that this substance contained sugar (Molisch test) and its colour reaction with ferric chloride indicated the presence of a phenolic compound. Since a major phenolic component of apples is the glycoside phloridzin (Podstolski and Lewak1970), the properties of phloridzin and the crystalline material from fraction IV were compared. The two compounds had identical R_f values after paper chromatography in each of three different solvent systems and in addition gave identical absorption maxima when their spectra were measured at three different pH values (Table 3). Furthermore synthetic phloridzin was found to have an inhi-

le 2 chyma of the wood of apple cv. Hibernal on the growth of various parasitic fungi agar at $25^{\circ}\mathrm{C}$

Pythium ultimum	Pythium sp. 1	Pythium sp. 2	Nectria galligena	Leucostoma cincta
39.3 ^b	21.5 ^b	52.5 ^b	30.0^{d}	28.5°
6.5	22.7	no growth	13.5	22.8
10.0	29.0	38.3	29.2	30.5
39.8	37.8	53.2	30.3	32.5

taining NaOH + Na2SO3.

 ${
m Table~3}$ R_{j} values and the effect of pH on the UV extinction for synthetic phloridzine and the crystalline material from fraction IV

Material	Solvents *		max (mμ) at pH			
	A	В	С	2.0	6.5	12.0
Phloridzin Compound from	0.41	0.72	0.17	283	283	323
Fraction IV	0.41	0.72	0.17	284	284	323

* R_f values, using Whatman No. 2 filter paper. Solvent details:

A - Isopropanol: ammonium | hydroxide: water, 80:5:15.

B - Butanol : acetic acid : water, 6:1:2.

C - Butanol: ammonium hydroxide: water, 8:1:1.

bitory effect on the growth of *P. cactorum* which increased proportionally with increasing concentration (Table 4).

During March 1971, inoculation experiments were done on one year old shoots of 'Hibernal' and another variety 'Antonovka'. The latter variety is the less susceptible of the two to *P. cactorum* infection and contains more phloridzin (Table 5).

DISCUSSION

The examination of cortical parenchyma tissue extracts has revealed the existence of several biologically active fractions influencing the *in vitro* growth of *Phytophthora cactorum* and some other bark and xylem pathogens. Similar results were obtained in 1970 by Millikan and Glowinkowska (1970) in their work on extracts from the 'Grimes'

Table 4

The influence of different concentrations of phloridzin on growth of *P. cactorum*

Concen- tration		Diameter of fungus colonies in mm	
0.0	ppm	34.1	
100	ppm	32.5	
250	ppm	31.3	
500	ppm	29.1	
1000	ppm	26.1	

Table 5

Susceptibility of cv. Hibernal and cv. Antonovka of apple to *P. cactorum* infection and the phloridzin levels in the cortical parenchyma tissues of these varieties

Apple Cultivar	Length of necro- sis on one-year old shoots in mm after 8 days	Mg phloridzin per g of cortical parenchyma tissue, dry weight
Hibernal	41.04	19.00 ^b
Antono- vka	24.7	40.18

- a Mean of 10 replicates.
- b Note that the data do not represent the total phloridzin contents in subcortical tissue.

and 'Starking' cultivars of apple. In our work, the ninhydrin, xanthoproteic and biuret reactions were negative indicating that there was no protein in the active fractions. This result is not, however, consistent with the suggestion of Millikan and Glowinkowska (l.c.) that their active fractions are protein. The sulphur detected in fraction II when the extracting solvent (B), NaOH + Na₂SO₃ was used suggests the formation of a sugar-sodium sulphite complex that might be responsible for the inhibitory action of the fraction II. No sulphur, and no biological activity, in this second fraction was detected when the (C) extraction NaOH + DIECA was used.

These results contradict the statement (Millikan and Glowin-kowska 1970) that fraction II contains a natural endogenous inhibitor of *Phytophthora cactorum*.

The substance contained in fraction IV was identified as phloridzin. Podstolski and Lewak (1970) have drawn attention to the role played by this substance in the physiological processes connected with growth and development of apple trees. Barnes and Williams (1961) and Goodman et al. (1967) have shown that phloridzin and its components influence the susceptibility of apple trees to infection by apple scab. These authors also noted the antibacterial activity of phloretin (the aglucone of phloridzin) against *Erwinia amylovora*.

In the light of the results presented, the statement made by Schwinn (1965a, b) that phenolic substances, including phloridzin do not play any part in the mechanism of the resistance of apple trees to $P.\ cactorum$ would appear to be incorrect.

The general occurrence and considerable concentration of phloridzir. in different tissues of apple trees (Podstolski and Lewak 1970), and the greater content of this substance in the bark of a variety less susceptible to *P. cactorum*, gives considerable ground for considering phloridzin as one of the factors influencing the resistance of apple trees to ring rot of the bottom of the trunk.

REFERENCES

- Barnes E. M. and Williams E. B., 1961, The role of phloridzin in the host-parasite physiology of the apple scab disease, Canadian J. Microbiol. 7: 525 534.
- Borecki Z., Ross J. A. and Millikan D. F., 1970, Endogenous factors in apple bark which stimulate and inhibit the growth of *Phytophthora cactorum*, Phytopathology 60: 173-174.
- Braun N. and Nienhaus F., 1959, Fortgeführte Untersuchungen über die Kragenfäule des Apfels (*Phytophthora cactorum*), Phytopath. Z. 36: 169 208.
- Fischer D. V., 1966, High-density orchards for British Columbia conditions, Research Station Summerland, B. C. Canada Department of Agriculture: 6.
- Goodman R. N., Kiraly Z. and Zaitlin M., 1967, The Biochemistry and Physiology of Infectious Plant Disease, New York: 196.
- Holowczak J., Kuc J. and Williams E. B., 1962, Metabolism of DL- and L-phenylalanine in *Malus* related to susceptibility and resistance *Venturia* inaequalis, Phytopathology 52: 699-703.
- Kuc J., Barnes E., Daftsios A. and Williams E. B., 1959, The effect of amino acids on susceptibility of apple varieties to scab., Phytopathology 49: 313-315.
- McIntosh D. L. and MacSwan I. C., 1966, The occurrence of collar rot caused by *Phytophthora cactorum* in a planting of apple trees aged 1 to 7 years, Plant Dis. Rep. 50(4): 267-270.
- Millikan D. F. and Glowinkowska A., 1970, Izolation and some properties of biologically active substances from apple bark, International Congress of Plant Pathology Budapest, Hungary (unpublished data).
- Podstolski A., Lewak S., 1970, Specific phloridzin glucosidases from seeds and leaves of apple tree, Phytochemistry 9: 289 296.
- Sewell G. W. and Wilson J. F., 1959, Resistance trials of some apple rootstock varieties to *Phytophthora cactorum*, J. Hort. Sci. 34: 51-58.
- Schwinn F. J., 1965, Untersuchungen zum Wirt-Parasit-Verhältnis bei der Krægenfäule des Apfelbaumes (*Phytophthora cactorum*) and ihrer Bekampfung II Zur Rolle der Indolwuchostoffe für den Erreger, Phytopath. Z. 54: 162-184.
- Schwinn F. J., 1965, Untersuchungen zum Wirt-Parasit-Verhältnis bei der Kragenfäule des Apfelbaumes *Phytophthora cactorum* and ihrer Bekampfung. I Versuche zur Frage der Wundinfektion und zur Grundlage der Sortenresistenz, Phytopath. Z. 54: 1 30.

Wpływ substancji endogennych, zawartych w tkance miękiszu korowego jabłoni na wzrost *in vitro* grzyba *Phytophthora cactorum* (Leb. of Cohn)

Schroeter

Streszczenie

Dwie frakcje zawarte w ekstrakcie uzyskanym z tkanki miękiszu korowego jabłoni odmiany 'Hibernal', wykazywały biologiczne działanie na wzrost *in vitro* grzyba *Phytophthora cactorum*. Frakcja I działała jako stymulator a frakcja IV jako inhibitor.

Stosowanie roztworu ${\rm NaOH+Na_2SO_3}$ do ekstrakcji spowodowało powstanie właściwości inhibicyjnych we frakcji II. Inhibicyjne działanie drugiej frakcji może być spowodowane wytworzeniem się kompleksu cukrowo-siarczyno-sodowego.

Substancja działająca jako inhibitor we frakcji IV została zidentyfikowana jako florydzyna. Porównując zawartość florydzyny w tkance miękiszu korowego dwóch odmian jabłoni: 'Hibernal' i 'Antonówka' stwierdzono zależność pomiędzy podatnością odmiany na porażenie przez grzyb *P. cactorum* a ilością florydzyny w tkance.

Przeprowadzono także doświadczenia nad wpływem różnych frakcji na wzrost in vitro różnych gatunków grzybów patogenów kory i drewna. Grzyby z rodziny Pythiaceae, z wyjątkiem jednej formy Pythium sp., reagowały na inhibitor zawarty we frakcji II, a w mniejszym stopniu na inhibitor zawarty we frakcji IV.