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The role of pisatin in the resistance of pea plants – some further experiments on the breakdown of pisatin

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Abstract

Pisatin, added to a culture of a pea-pathogenic fungus and incubated during 1 week, could not be recovered in the nutrient broth or in the mycelium of this fungus, while pisatin, added to a fungal culture of a non-pathogen of the pea plant, could be recovered for the greater part after 1 week incubation. These experiments were done with 4 pathogens and 8 non-pathogens of the pea plant. Experiments with labeled pisatin show that pea-pathogens can break down pisatin to a product that is only sparingly soluble in petroleum ether.

Introduction

In an article in Japanese, Uehara (1964) mentioned the detoxication of pea-phytoalexin by some pea pathogens in tests in vitro. Independently of this observation, in a previous communication de Wit-Elshove (1968) demonstrated pisatin to be broken down by some fungi, pathogenic to *Pisum sativum*, as shown by the disappearance of characteristic peaks in the UV absorption spectrum. These experiments have been extended to some other fungi, pathogenic and non-pathogenic to peas. Also some strains of pea pathogens, which appeared to be non-pathogenic on pea plants, have been included in the experiments. In order to further elucidate the mechanism of pisatin breakdown, mycelium was incubated with labeled pisatin for several days, and afterwards culture fluids and mycelia were fractionated and the radioactivity of fractions obtained was measured.

Materials and methods

In the experiments described, pathogenic as well as non-pathogenic strains of fungi have been used. The term "pathogenic strains" merely described those strains which are pathogenic to some pea varieties; "non-pathogenic strains" are not pathogenic to any pea variety studied.
The strains of *Fusarium solani*, used in these experiments, were obtained from Mr J. H. van Emden of the Institute of Phytopathological Research at Wageningen; the strains were isolated from soils, infested with *Fusarium solani*. From soils, on which pea plants showed severe symptoms of the disease, the pathogenic strain could be isolated, while from soils, on which the pea plants looked healthy, the non-pathogenic strain was isolated.

The pathogenic strain of *Ascochyta pisi* was isolated from diseased plants. The non-pathogenic strain of this fungus was obtained from Dr L. A. Hadwiger of the Department of Plant Pathology in Washington: apparently due to prolonged culturing on artificial media this strain does no longer induce symptoms on pea plants.
The experiments with non-labeled pisatin have been carried out as described before (de Wit-Elshove, 1968). The following minor modifications were made: to obtain the pisatin from the supernatants, these were extracted four times with an equal volume of light petroleum (B.P. 40 °–60 °C). After the combined petroleum extracts were taken to dryness, the residual pisatin was dissolved in 10 ml of distilled ethanol. UV absorption spectra of the resulting solutions were measured in the range of 250–350 mμ. Pisatin is characterized by peaks in the absorption spectra at 286 and 309 mμ (Cruickshank and Perrin, 1960). To prevent chemical conversion of pisatin to anhydropisatin at lower pH, during the experiments the pH was kept as constant as possible; in those cases, where the pH in the broth decreased during incubation, it was adjusted to pH 6.6 before supplying the pisatin; thereafter the pH remained above 5 until the end of the experiment.
The experiments with labeled pisatin have been performed in the same way as those with non-labeled pisatin; labeled pisatin was made according to Hadwiger (1967). Phenylalanine-U-^{14}C with a specific activity of 393 mC/m mole was used as a precursor for pisatin; the pisatin extracted from the peas had a specific activity of 1.7×10^{-6} mC/m mole. The breakdown of labeled pisatin was studied, using Skrip ink bottles with a well inside (see Wolcott and Ross, 1966). Known amounts of pisatin, dissolved in ethanol, were pipetted in the main compartments of the bottles. After evaporation of the ethanol, fungal cultures were added, which had been grown as shake cultures at 24 °C for 1 week; uninoculated nutrient media were used as controls. In the wells 0.3 ml of ethanolamine and methylcellosolve were pipetted, to trap the CO_2 produced by the fungal cultures. After incubation of the cultures during a number of days, the contents of the wells were counted in a Nuclear Chicago 720 series liquid scintillation counter. The contents of the main compartments were fractionated as follows: after centrifugation at 18,000 g for 30 min, aliquots of the supernatants were extracted four times with an equal volume of petroleum ether; the petroleum ether soluble extracts were taken to dryness and redissolved in distilled ethanol. The supernatant fraction left after petroleum ether extraction is indicated as the aqueous phase. The pellets, mainly containing the mycelia, were extracted in distilled ethanol, giving the so-called ethanol soluble extracts: the ethanol insoluble residues were digested in perchloric acid and hydrogen peroxyde (Mahin and Lofberg, 1966). Known amounts of each fraction were counted in the liquid scintillation counter.

Results and discussion

The experiments show that pisatin could not be recovered in any of the extracts or in the supernatants of the pea pathogens, while in those of the non-pathogens of the pea the greater part of the pisatin was still present (Table 1). Striking is the difference in behaviour towards pisatin between the pathogenic and non-pathogenic strains of *Fusarium solani* and *Ascochyta pisi*.
The results of the experiments with labeled pisatin not only confirmed the former experiments, but also provided some data on the breakdown mechanism. Table 2

Table 1. Percentage of recovery of pisatin after 1 week incubation in cultures of fungi, pathogenic or non-pathogenic to peas. Each figure is the average of 6 experiments.

Fungi	*% recovery*
Fusarium oxysporum f. sp. pisi race 1 (pathogenic)	0
Fusarium solani f. pisi (pathogenic to peas)	0
Fusarium solani (non-pathogenic to peas)	78
Ascochyta pisi (pathogenic)	0
Ascochyta pisi (non-pathogenic)	85
Mycosphaerella pinodes (pathogenic)	0
Glomerella cingulata	85
Botrytis fabae	60
Colletotrichum lindemuthianum	90
Cladosporium cucumerinum	87
Monilia fructigena	87
Aspergillus fumigatus	80

Tabel 1. Percentage pisatine, dat teruggevonden wordt na incubatie gedurende een week in cultures van schimmels die pathogeen en die niet pathogeen zijn op de erwt. Ieder getal is het gemiddelde van 6 proeven.

Table 2. Distribution of radioactivity in different fractions obtained from fungal cultures pregrown in a liquid medium and after 7 days supplied with labeled pisatin, expressed as percentages of total radioactivity recovered after 3 days (total recovery was 90–100%).
The figures between parentheses indicate percentages of radioactivity in the petroleum ether fraction and the aqueous phase, in relation to total radioactivity in the supernatants.

Fungi	*Wells*	*Supernatants*		*Ethanol soluble extracts of the pellets*	*Digested mycelium residue*
		aqueous phase	*P.E. phase*		
Uninoculated control	–	11.8 (13)	80.0 (87)	8.2	–
	–	19.6 (20)	78.4 (80)	2.0	–
	–	21.0 (21)	77.3 (79)	1.7	–
Fusarium	6.9	60.0 (85)	11.0 (15)	14.5	7.5
oxysporum	2.0	71.2 (91)	6.6 (9)	16.6	3.6
f. sp. pisi*	2.5	71.2 (93)	5.4 (7)	16.8	4.1
Glomerella	1.2	12.6 (17)	61.2 (83)	21.5	3.5
cingulata	0	17.5 (22)	61.8 (78)	17.0	3.7
	1.0	18.6 (24)	58.7 (76)	17.4	4.3
Ascochyta	2.9	69.0 (90)	7.4 (10)	16.4	4.3
pisi*	3.1	71.9 (91)	7.3 (9)	11.0	6.7
Ascochyta	0	17.5 (22)	63.6 (78)	14.6	4.3
pisi (non-pathogenic)	0	18.0 (21)	67.9 (79)	10.2	3.9

* Pathogenic to pea plants

Tabel 2. Verdeling van de radioactiviteit in de verschillende fracties, verkregen van schimmelcultures, die zijn gekweekt in een vloeibaar medium, waaraan na 7 dagen gelabelde pisatine is toegevoegd, uitgedrukt als percentages van de totale radioactiviteit die na 3 dagen wordt teruggevonden (totaal werd 90–100% teruggevonden). De getallen tussen haakjes geven aan: die percentages radioactiviteit van de supernatants, die na uitschudden met petroleumether in de petroleumether-fractie terecht zijn gekomen of in de waterfase zijn gebleven.

shows the distribution of radioactivity in the different fractions expressed as percentages of activity recovered after 3 days (total recovery was 90–100%). Most of the label appeared in the supernatants of both pathogens and non-pathogens. However, there appeared to be a marked difference between pathogens and non-pathogens in the distribution of radioactivity in aqueous phases, resp. petroleum ether fractions of these supernatants. Whereas in the cultures containing the non-pathogens and in the controls, containing pisatin and nutrient medium only, about 4/5 of the radioactivity was present in the petroleum ether fraction and about 1/5 in the aqueous phase, in those containing the pathogens only about 1/10 appeared in the petroleum ether fraction, while 9/10 remained in the aqueous phase. Spectrophotometric determination showed pisatin to be the only labeled compound present in the petroleum ether fraction. Thus, whereas hardly any breakdown of pisatin seems to take place in cultures of non-pathogens, the pathogens appear to degrade pisatin to products which are no longer soluble in petroleum ether. A small percentage of the label appeared in the wells, but the quantities are too small to allow a definite conclusion. The ethanolic extracts of the mycelium and the ethanol insoluble residues together contained about 20% of the radioactivity. Spectrophotometric measurements showed the ethanolic extracts of non-pathogens to contain pisatin, while those of the pathogens did not.
In Table 3 the results are given of an experiment with *Fusarium oxysporum* f. sp. *pisi* – a pathogen of peas – in which the breakdown of pisatin has been studied in relation to time. From this table it is evident, that the label incorporated in the mycelium, both extractable and non-extractable with alcohol, increased with time, in the supernatants the percentage of radioactivity decreased with time, but the distribution of the radioactivity between the petroleum ether phase and the water phase remained constant during the experiment.

Table 3. Distribution of radioactivity in different fractions obtained at various times from cultures of *Fusarium oxysporum* f. sp. *pisi*, pregrown in a liquid medium and after 7 days supplied with labeled pisatin, expressed as percentages of total radioactivity recovered.
The figures between parentheses indicate percentages of radioactivity in the petroleum ether fraction and the aqueous phase, in relation to total radioactivity in the supernatants.

Number of days after supplying the pisatin	*Wells*	*Supernatants*		*Ethanol soluble mycelium entracts*	*Digested mycelium residue*
		aqueous phase	*P.E.-phase*		
1 (1)*	0	68.0 (85)	12.0 (15)	16.8	3.2
3 (3)	3.8	67.5 (90)	7.3 (10)	16.0	5.4
7 (4)	3.5	57.3 (87)	8.2 (13)	24.2	6.8
10 (2)	2.2	50.9 (87)	7.5 (13)	32.9	6.5
14 (2)	5.2	33.0 (85)	6.0 (15)	46.5	9.3

* Between brackets: number of expefiments

Tabel 3. Verdeling van de radioactiviteit in de verschillende fracties, verkregen na een verschillend aantal dagen uit cultures van Fusarium oxysporum f. sp. pisi, die zijn gekweekt in een vloeibaar medium, waaraan na 7 dagen gelabelde pisatine is toegevoegd, uitgedrukt als percentages van de totale radioactiviteit die wordt teruggevonden. De getallen tussen haakjes geven aan: die percentages radioactiviteit van de supernatants, die na uitschudden met petroleumether in de petroleumether-fractie terecht zijn gekomen of in de waterfase zijn gebleven.

From these experiments the conclusion can be drawn that pea pathogens are able to metabolize pisatin into a product, which does not show the peaks in the UV absorption spectrum characteristic for pisatin, nor the same "distribution coefficient" between petroleum ether and water as pisatin. The distribution coefficient for pisatin between light petroleum (B.P. 55°–60°C) and water is 2.3:1 (Cruickshank and Perrin, 1961). The distribution coefficient for pisatin found in our experiments is different due to the fact that extraction was carried out four times.
The role of pisatin in resistance of pea plants to fungal pathogens has been ascribed to the rate of formation of pisatin by the plant and the differential sensitivity to pisatin among fungal strains (Cruickshank and Perrin, 1965). Our results may be considered to indicate that also the ability of the fungal strain to break down pisatin may be responsible for pathogenicity or non-pathogenicity to occur.

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Samenvatting

De rol van pisatine in de resistentie van erwteplanten – enkele proeven over de afbraak van pisatine

Pisatine, toegevoegd aan een voedingsmedium, geïnoculeerd met een pathogene schimmel van de erwt, is na incubatie gedurende 1 week niet meer terug te vinden in het voedingsmedium of in het schimmelextract. Pisatine, toegevoegd aan een voedingsmedium geïnoculeerd met een niet-pathogene schimmel van de erwt, kon na incubatie gedurende 1 week voor het grootste deel worden teruggevonden. Deze proeven zijn gedaan met 4 pathogenen en 8 niet-pathogenen (Tabel 1). Proeven met radioactief gemerkte pisatine tonen aan dat de erwtepathogenen de pisatine omzetten in een product dat slechts weinig oplosbaar is in petroleumether.

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