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Short-lived protection of pea plants against Mycosphaerella pinodes by prior inoculation with Pseudomonas phaseolicola

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Abstract

Pea plants, *Pisum sativum* cv. Eminent, were inoculated by spraying with a suspension (10^8 cells/ml) of *Pseudomonas phaseolicola*, a bacterium not pathogenic to pea. At intervals of 0, 2, 4, 6, or 8 days the same plants were challenged with a conidial suspension (10^6 conidia/ml) of the pea-pathogenic fungus *Mycosphaerella pinodes*. Periodically, leaf samples were examined for development of symptoms and phytoalexin content.

Up to 6 days after inoculation with the non-pathogenic bacterium the plants were protected against the pathogenic fungus. Pisatin, however, seemed not to be responsible for the observed protection.

Additional keywords: phytoalexins, pisatin, 6a-hydroxy-inermin.

Introduction

The role phytoalexins are supposed to play in several host-pathogen interactions has been the subject of many investigations (for recent reviews see, e.g. Van Etten and Pueppke, 1976; Cruickshank, 1977; Gross, 1977; Keen and Bruegger, 1977; Albersheim and Valent, 1978; Grisebach and Ebel, 1978). Most of these deal with phytoalexin production as induced by fungal infection, but in a few studies bacteria were the inducing agents (Stholasuta et al., 1971; Lyon, 1972; Lyon and Wood, 1975; Lyon et al., 1975; Gnanamanickam and Patil, 1977a, b). In these studies special attention has been paid to the antimicrobial activity of phytoalexins; however, little is known about their inhibitory effect on the establishment of infection in plant disease (Oku et al., 1975b, 1976, 1977; Shiraishi et al., 1977, 1978a, b).

In our laboratory fungal and bacterial pea pathogens and non-pathogens have been studied with regard to induction of pisatin synthesis and its metabolism in pea plants (De Wit-Elshove and Fuchs, 1971; Van 't Land et al., 1975; Bruin et al., 1977; Platero Sanz and Fuchs, 1978). The chemical structure of some of these metabolites has now been established and their fungitoxicity determined (Van Etten et al., 1975; Van 't Land et al., 1975; Fuchs et al., 1976, 1980a, b). The pathogenicity of most fungal pea pathogens seems to be associated with their ability to degrade pisatin to 6a-hydroxy-inermin or further metabolites, in such a way that degradation always proceeds 'beyond the level of fungitoxicity' for the pathogen concerned. Usually, non-

pathogens, on the other hand, are not able to decompose pisatin beyond that level (cf. Fuchs and Hijwegen, 1979).

It should be realized that under normal conditions of infection a pea pathogen generally will not only elicit the synthesis of pisatin, but also will immediately start its degradation. Low concentrations of pisatin might even be a prerequisite for degradation to occur. High concentrations, on the other hand, are often inhibitory to both non-pathogens and pathogens. Therefore, it was of interest to study the effect of pisatin, elicited by prior inoculation with a non-pathogenic organism, on the infection of pea plants by a pea pathogen and to see whether this offered a possibility of controlling the disease by activating the plant's natural mechanism for disease resistance (cf. Kuć, 1977). In our experiments, the bacterium *Pseudomonas phaseolicola* was used as the non-pathogen in attempts to protect pea plants against infection by the pea pathogen *Mycosphaerella pinodes*. The main results have been presented in a poster session at the 3rd International Congress of Plant Pathology, held in Munich (Germany), 1978. In this paper, a more comprehensive report is given.

Material and methods

Micro-organisms. Pseudomonas phaseolicola, strain 113, not pathogenic to pea, was kindly provided by Mr H. P. Maas Geesteranus of the Research Institute for Plant Protection, Wageningen. Bacteria were grown on nutrient agar (Oxoid) slants for 2 days at 25 °C. Bacterial cells were then suspended in distilled water (10^8 cells/ml) to be sprayed onto pea plants.

An isolate of *Mycosphaerella pinodes* was obtained from Dr T. Limonard of this Laboratory; its virulence was maintained by repeated inoculation into and reisolation from pea plants. The fungus was grown on oatmeal agar plates for 7 days at 30 °C. Then, the conidia were washed off with water and after centrifugation and filtration through cotton wool resuspended in Lyon and Bayliss (1975) GA solution (without glucose) to a final concentration of 10^6 conidia/ml.

Plant material. Seeds of *Pisum sativum* cv. Eminent were sown at ambient temperature and humidity in a glasshouse. Six-week-old plants were used in our experiments.

Experimental conditions (see also Fig. 1). First, the leaves were gently rubbed with wet cotton wool and then six lots of eight pots each (6 to 8 plants per pot) were immediately sprayed with a bacterial suspension of *Ps. phaseolicola*. Of two other lots one served as control and the other was inoculated with *M. pinodes*. Of the six lots inoculated with *Ps. phaseolicola*, five were once more sprayed with a conidial suspension of *M. pinodes* at different time intervals (0, 2, 4, 6, and 8 days) after spraying with *Ps. phaseolicola*. After being treated, all inoculated plants were covered with plastic sheeting for 24 h in order to facilitate penetration of micro-organisms into the leaves. At intervals (2, 4, 6, and 8 days after treatment) leaf samples were taken from each lot.

Symptom expression. From several leaves of each lot, sampled at random, discs were punched with a metal cork borer of 1 cm diameter and examined under a binocular microscope to measure and count the lesions present.

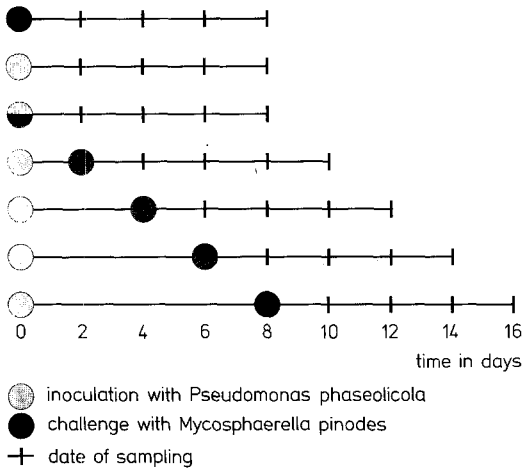

Fig. 1. Experimental set-up, to indicate times of inoculation with *Pseudomonas phaseolicola* and challenge with *Mycosphaerella pinodes*, respectively, and dates of sampling.

Fig. 1. Proefopzet, waarin het tijdstip in dagen is weergegeven van de inoculatie met Pseudomonas phaseolicola en Mycosphaerella pinodes, alsmede de dagen waarop de bladeren op aanwezigheid van pisatine en/of 6a-hydroxy-inermine werden geanalyseerd.

Determination of pisatin and 6a-hydroxy-inermin. Each leaf sample was weighed, frozen in liquid nitrogen, ground in a mortar to a powder and suspended in 70 % (v/v) ethanol. The homogenate was centrifuged and the pellet washed twice with 70 % ethanol. The combined supernatans were first extracted three times with equal volumes of petroleum ether and then with ethyl acetate in a separatory funnel. The upper layers were taken to dryness in a rotary evaporator. The residues were taken up in about 3 ml of 90 % (v/v) ethanol, transferred to small vials and dried under a stream of nitrogen. The newly obtained residues were redissolved in 100 µl of 90 % ethanol and applied onto silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}). The chromatograms were developed in chloroform/methanol 97 : 3. Pisatin (R_f 0.75) and 6a-hydroxy-inermin (R_f 0.32) were detected under UV light; subsequently, they were eluted with ethanol, purified by repeated chromatography, eluted again with ethanol and estimated quantitatively by UV spectrophotometry, taking an optical density of 1.0 at λ_{max}^{EtOH} 309 nm as equivalent to 43.8 µg/ml for both pisatin and 6a-hydroxy-inermin.

Results

As is evident from Fig. 2 *Pseudomonas phaseolicola*, strain 113, did not cause lesions in pea. The isolate of *Mycosphaerella pinodes*, on the other hand, was highly virulent to *Pisum sativum*, producing brown lesions in the leaves which increased with time in number (Fig. 2) as well as in size (Fig. 3).

Simultaneous or prior inoculation with *Ps. phaseolicola* greatly diminished the number of lesions caused by *M. pinodes*. This suppression of symptom development, however, decreased with time, so that a challenge with *M. pinodes* 8 days after inoculating the pea plants with *Ps. phaseolicola* resulted in lesion development which did not differ from that in plants previously not inoculated (Fig. 2). Effects on size of lesions were similar although less pronounced (Fig. 3).

Ps. phaseolicola differed from *M. pinodes* also in its effects on pisatin synthesis and breakdown (Figs 4 and 5). *Ps. phaseolicola* induced pisatin synthesis which reached a level of about 20 µg/g fresh weight after 2 days, to increase only slightly afterwards (Fig. 4). Since no 6a-hydroxy-inermin was detected, *Ps. phaseolicola*, like other bacteria,

Fig. 2. (left). Number of lesions in pea leaves in relation to time, after inoculation with *Pseudomonas phaseolicola* alone, *Mycosphaerella pinodes* alone, or *Pseudomonas phaseolicola* followed by *Mycosphaerella pinodes* after 0, 2, 4, 6, or 8 days, respectively.
Fig. 3. (right). Same as Fig. 2, size of lesions.

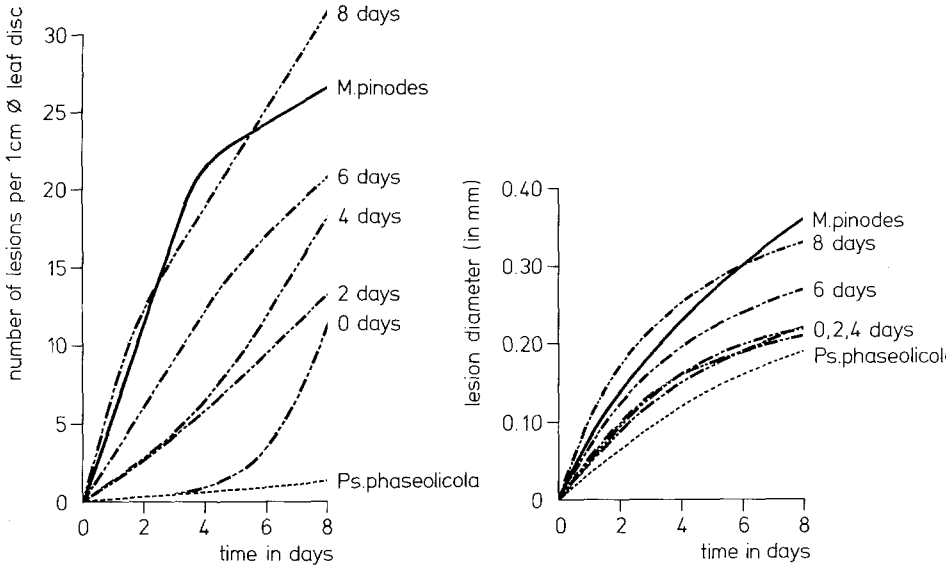

Fig. 2. (links). Aantal lesies in erwtebladeren in relatie tot de tijd, na inoculatie met, respectievelijk, Pseudomonas phaseolicola alleen, Mycosphaerella pinodes alleen en Pseudomonas phaseolicola, na 0, 2, 4, 6 of 8 dagen gevolgd door Mycosphaerella pinodes.
Fig. 3. (rechts). Als Fig. 2, grootte der lesies.

among which *Pseudomonas pisi* (Bruin et al., 1977), apparently lacked the ability to degrade pisatin. Leaves of plants inoculated with *M. pinodes*, on the other hand, showed only small to trace amounts of pisatin (Fig. 4), but increasing concentrations of 6a-hydroxy-inermin (Fig. 5), the first conversion product of pisatin produced by various pea-pathogenic fungi (see e.g. Van Etten et al., 1975; Van 't Land et al., 1975). In experiments *in vitro* both compounds were rapidly degraded to non-toxic products (Fuchs and De Vries, unpublished results). Thus, in interpreting the data, it should be realized that the amounts of pisatin and 6a-hydroxy-inermin as observed in plants inoculated with *M. pinodes* were obviously the result of induced synthesis and simultaneous breakdown. In fact, the data only represent the actual amounts present without giving any information about the absolute rates of turnover of both compounds.

Simultaneous or successive inoculation with both micro-organisms affected the concentrations of the two compounds in a rather complex way (Figs 4 and 5). Leaves of plants to be inoculated with both organisms on the same day (Table 1, column A, upper row; see also Materials and methods) did not contain pisatin at the onset of the experiment (or at least not more than uninoculated control plants, where pisatin concentrations were always below the detection limit). Simultaneous inoculation then led to pisatin and 6a-hydroxy-inermin concentrations (Table 1, column A) which were intermediate between those found upon inoculation with either organism separately

Fig. 4. (left). Concentrations of pisatin (μg/g fresh weight) in pea leaves in relation to time, after inoculation with *Pseudomonas phaseolicola* alone, *Mycosphaerella pinodes* alone, or *Pseudomonas phaseolicola* followed by *Mycosphaerella pinodes* after 0, 2, 4, 6, of 8 days, respectively.
Fig. 5. (right). Same as Fig. 4, concentrations of 6a-hydroxy-inermin (μg/g fresh weight).

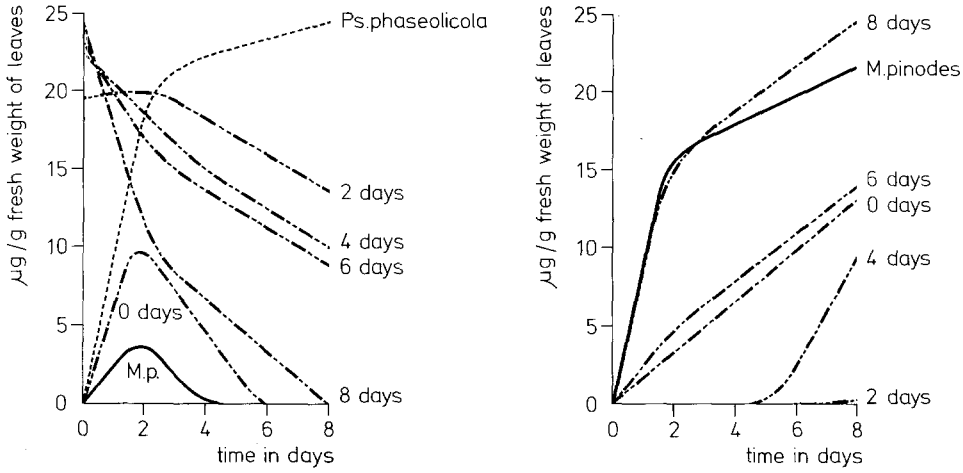

Fig. 4. (links). Pisatine concentraties (in μg/g vers gewicht) in erwtebladeren in relatie tot de tijd, na inoculatie met, respectievelijk, Pseudomonas phaseolicola alleen, Mycosphaerella pinodes alleen en Pseudomonas phaseolicola, na 0, 2, 4, 6 of 8 dagen gevolgd door Mycosphaerella pinodes.
Fig. 5. (rechts). Als Fig. 4, 6a-hydroxy-inermine concentraties (in μg/g vers gewicht).

Table 1. Concentrations of pisatin (pis) and 6a-hydroxy-inermin (6a-HI) (in μg/g fresh weight) in pea leaves, cv. Eminent, inoculated with *Pseudomonas phaseolicola* and after 0, 2, 4, 6, or 8 days challenged with *Mycosphaerella pinodes*; leaves analysed for pisatin and 6a-hydroxy-inermin 2, 4, 6, and 8 days after challenging with *M. pinodes*.

Time of analysis	Time of inoculation with *M. pinodes*									
	0 days (column A)		2 days (column B)		4 days (column C)		6 days (column D)		8 days (column E)	
	pis	6a-HI	pis	6a-Hi	pis	6a-HI	pis	6a-Hi	pis	6a-HI
0 days	(0.0)*	–	(19.5)*	–	(22.4)*	–	(23.1)*	–	(24.5)*	–
2 days	9.7	3.0	20.0	0.0	27.9	0.0	14.4	4.8	7.9	14.8
4 days	4.6	7.0	17.1	0.0	14.9	0.0	13.6	7.0	6.1	19.2
6 days	0.0	10.2	16.1	0.0	13.7	2.5	12.6	9.4	3.2	21.4
8 days	0.0	13.1	13.6	trace	10.0	9.4	8.9	14.0	0.0	24.7

*Concentrations of pisatin in leaves, 0, 2, 4, 6, and 8 days after inoculation with *Ps. phaseolicola*, and on these days challenged with *M. pinodes*. Leaves only inoculated with *M. pinodes* showed the following concentrations of pisatin and 6a-HI (compare Figs 4 and 5): pisatin: 2 days: 3.7; 4 days: trace; 6 and 8 days: 0.0; 6a-HI: 2 days: 15.7; 4 days: 18.0; 6 days: 19.2; 8 days: 21.3

Table 1. Concentraties van pisatine (pis) en 6a-hydroxy-inermine (6a-HI) (in μg/g vers gewicht) in erwtebladeren, cv. Eminent, geïnoculeerd met Pseudomonas phaseolicola en na 0, 2, 4, 6 of 8 dagen opnieuw geïnoculeerd met Mycosphaerella pinodes; bladeren op 2, 4, 6 en 8 dagen na deze laatste inoculatie onderzocht op aanwezigheid van pisatine en 6a-hydroxy-inermine.

(Fig. 1, 0 days). Leaves of plants challenged with *M. pinodes* 2, 4, 6, and 8 days after inoculating the plants with *Ps. phaseolicola* can be assumed to have contained pisatin on the day of challenge in concentrations ranging from about 20 to 25 µg/g fresh weight (as indicated in brackets in Table 1, columns B–E; see also Fig. 4). Growth-dependent degradation of pisatin by *M. pinodes* caused these concentrations to decrease and those of 6a-hydroxy-inermin to increase; both changes proceeded more rapidly as the time interval between inoculation with *Ps. phaseolicola* and challenge with *M. pinodes* was increased (Table 1). However, for a correct interpretation it must be emphasized again that the actual data are the resultant of two opposite processes: pisatin synthesis as induced by both micro-organisms and its degradation by *M. pinodes*. The data in Table 1, columns B and C, suggest that pisatin can be degraded without concomitant accumulation of 6a-hydroxy-inermin. Accumulation of 6a-hydroxy-inermin at a longer time interval (4–8 days) between inoculation and challenge (columns C–E) might then only be due to enhanced induction and subsequent degradation of pisatin, with 6a-hydroxy-inermin most probably only transitorily being accumulated.

Discussion

Leaf surfaces are known to support a bacterial flora which might interact in diverse ways with fungal and other plant pathogens. According to Blakeman and Brodie (1976) most epiphyte-pathogen interactions can be accounted for on the basis of one or more several mechanisms among which stimulation of the host's defence reactions might be one. Such a protective response might be associated with accumulation of inhibitory substances, such as phytoalexins. Increasing 'knowledge of the nature of such interactions may, in the future, (even) allow manipulation of the epiphytic bacterial flora to maximize their potential for disease control' (cited from Blakeman and Brodie, 1976). Prior inoculation of plants with non-pathogenic micro-organisms might be another method to activate mechanisms for disease resistance in plants, and thus to protect plants against disease (cf. Kuć, 1977).

There are, indeed, many examples of prior treatment of plants with heat-killed or attenuated bacteria leading to protection against infection with virulent bacteria (for literature, see Blakeman and Brodie, 1976). Whether an epiphytic resident bacterial flora or prior inoculation with bacteria can also induce protection against fungal diseases seems, however, less certain.

One of the prerequisites for the activation of the host's resistance mechanisms and thus for successful disease control is the occurrence of large populations of epiphytic bacteria or inoculation with dense bacterial suspensions in order to achieve a greater likelihood of sufficient bacteria entering the plant's natural openings (cf. Blakeman and Brodie, 1976). Unlike intact bean leaves, which readily respond to bacterial inoculation by accumulating phaseollin and related compounds (Stholasuta et al., 1971; Lyon and Wood, 1975; Gnanamanickam and Patil, 1977a, b), intact pea leaves were found to accumulate at most insignificant levels of pisatin (Stholasuta et al., 1971), even if dense bacterial suspensions (of *Pseudomonas phaseolicola*; 10^8 cells/ml) were used and inoculation was carried out by spraying under pressure or by introducing bacterial suspensions into the leaves with a hypodermic syringe.

The same was true when non-pathogenic fungi (Mansfield et al., 1975) or heavy metals (Robinson and Wood, 1976) were used as the inducing agents. However,

detached whole pea leaves and especially leaf discs accumulated distinct amounts of pisatin either upon treatment with heavy metals (Mansfield et al., 1975) or upon inoculation with non-pathogens (Bailey, 1969). The much larger production of pisatin by pea leaf discs might reflect the greater ease with which an inducing solution or suspension enters the leaf through the cut edges of a leaf disc than through the intact leaf surface (Mansfield et al., 1975). On the other hand, also physiological age of the pea leaves used (Bailey, 1969) strongly influenced the levels of pisatin attained. Unlike non-pathogens, pathogenic fungi, such as *Erysiphe pisi* and *Mycosphaerella pinodes*, induced high concentrations of pisatin in pea leaves (Oku et al., 1975a; Shiraishi et al., 1976, 1977); however, in the case of *E. pisi* pisatin appeared entirely confined to the epidermis.

In our experiments on the effect of prior inoculation with *Ps. phaseolicola* on protection of pea plants against *M. pinodes*, we always used whole plants – and thus intact leaves – and bacterial suspensions containing at least 10^8 cells/ml. Under our experimental conditions use of such dense suspensions invariably led to accumulation of up to 25 μg pisatin/g fresh weight of pea leaves. Most probably, the actual concentrations were even considerably higher, since thin-layer chromatographic separation and purification might have caused substantial losses of pisatin (cf. Gnanamanickam and Patil 1977a, b) who found an extraction efficiency of approximately 45 %). Assuming such an extraction efficiency in our experiments, we found concentrations of the same order of magnitude as those usually observed with other pea tissues, such as pod endocarp (cf., for instance, Cruickshank and Perrin, 1961).

Concomitantly with pisatin accumulation in pea leaves we observed a marked, though short-lived protection against infection by *M. pinodes*. However, the available data show no correlation at all between the concentrations of pisatin found in the pea leaves at the time of their being challenged with *M. pinodes* (Table 1) and the rate of subsequent symptom development (Fig. 2). Lesions developed at a rate which was independent of the initial pisatin concentrations present and which increased as the time interval between inoculation with *Ps. phaseolicola* and challenge wit *M. pinodes* became larger. A higher rate of disease development was paralleled by a more rapid overall decline of pisatin and a comparable rise of 6a-hydroxy-inermin concentrations. Thus, rather than a rate of disease development which reflected pisatin concentrations, the reverse was found: the rate of pisatin breakdown rather seemed the result of disease development. It means that phytoalexins are not responsible for the distinct, though short-lived protection of pea plants against *M. pinodes* by prior inoculation with *Ps. phaseolicola*.

In their study on the antagonism of *Alternaria tenuissima* against *Alternaria zinniae* on dwarf bean leaves Van den Heuvel et al. (1978) likewise arrived at the conclusion that phytoalexins were not involved in the protection against the pathogen. However, in their case prior inoculation of bean leaves with the antagonist, *A. tenuissima*, did not induce phytoalexin synthesis at all, but still reduced the number of lesions caused by subsequent inoculation with *A. zinniae*. Also Deverall et al. (1979) failed to implicate antifungal substances in the cross-protection of wheat against take-all caused by *Gaeumannomyces graminis* var. *tritici* by prior inoculation with *G. graminis* var. *graminis*.

Of special interest with reference to our study are the observations on the role of pisatin in powdery mildew (*E. pisi*) and *M. pinodes*-infected pea plants by Oku et al.

(1977) and Shiraishi et al. (1978a, b). They concluded that the major role of pisatin in disease resistance is to be found in its ability to inhibit the establishment of fungal infection, thus preventing non-pathogens from invading the host plant. Pathogens, such as *M. pinodes*, on the other hand, were found to produce a peptide of low molecular weight which suppressed the induction of pisatin, thus enabling the fungus to successfully invade the host plant. As soon as infection was established the suppression was apparently abolished and pisatin started to accumulate. At this stage, pisatin, however, did not affect the fungus any longer, since mycelial growth of *M. pinodes* was highly tolerant to pisatin. This insensitivity of *M. pinodes* is readily explained by its before-mentioned ability to rapidly degrade the phytoalexin to non-toxic products (Fuchs and De Vries, unpublished results).

The results of our experiments are not readily reconcilable with those reported by Oku et al. (1977) and Shiraishi et al. (1978a, b). Although pisatin concentrations in pea leaves in our experiments remained virtually the same from 2–8 days after inoculation with *Ps. phaseolicola*, the rate of development of *M. pinodes* symptoms progressively increased with an increasing interval between inoculation with *Ps. phaseolicola* and challenge with *M. pinodes*. According to the latter authors pisatin concentrations of c. 25 µg/g fresh weight should have been sufficient to prevent infection by the fungus. However, in comparing both sets of data it should be realized that their data concern epidermis and stripped mesophyll cells, whereas ours constitute 'averages' based on intact whole leaves. Possibly, the concentrations of pisatin in the *Ps. phaseolicola*-inoculated leaves vary widely from cell to cell, thus providing the challenge organism some sites to infect successfully. Even then, however, the time-dependent protection by *Ps. phaseolicola* might be more readily ascribed to a direct, but rapidly evanescing effect of the bacterium itself than to an indirect one mediated by the host plant.

Samenvatting

Kortdurende bescherming van erwteplanten tegen Mycosphaerella pinodes door gelijktijdige of voorafgaande inoculatie met Pseudomonas phaseolicola

Erwteplanten, cv. Eminent, werden bespoten met een suspensie (10^8 bacteriën/ml) van *Pseudomonas phaseolicola*, een bacterie die niet pathogeen is voor erwt. Na 0, 2, 4, 6, of 8 dagen werden dezelfde planten opnieuw geïnoculeerd, nu echter met een conidiënsuspensie (10^6 conidiën/ml) van de voor erwt pathogene schimmel *Mycosphaerella pinodes*. Periodiek werden bladmonsters beoordeeld naar ontwikkeling van symptomen en aanwezigheid van fytoalexinen.

Tot 6 dagen na de inoculatie met de niet-pathogene bacterie bleken de planten – in afnemende mate – beschermd tegen aantasting door de pathogene schimmel. Aangezien er geen positieve correlatie bleek te bestaan tussen de pisatineconcentratie en de mate van bescherming, leek pisatine voor deze bescherming niet verantwoordelijk.

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