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Bacterial induction of the accumulation of phaseollin, pisatin and rishitin and their antibacterial activity

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Abstract

Bean hypocotyls, pea pods and tomato fruits were tested for phaseollin, pisatin and rishitin production when challenged with the phytopathogenic bacteria *Erwinia carotovora, Pseudomonas phaseolicola, P. pisi* and *P. solanacearum,* and their isolated extracellular polysaccharides. All bacteria induced phytoalexin accumulation, whereas only phaseollin and pisatin, but not rishitin, were elicited by EPS. The inhibitory effect of these three phytoalexins on bacterial growth was studied in liquid medium; whereas phaseollin and pisatin strongly inhibited growth, only a slight inhibitory effect resulted from the presence of rishitin in the medium.

Additional keywords: Erwinia carotovora, Pseudomonas phaseolicola, P. pisi, P. solanacearum, Phaseolus vulgaris, Pisum sativum, Lycopersicum esculentum, extracellular polysaccharides (EPS), phytoalexins.

Introduction

The mechanism of induction of phytoalexin accumulation as well as their role in plant disease resistance have been studied in many different plant-fungus interactions, but only few cases related to bacterial induction of phytoalexins have been described (Stholasuta et al., 1971; Lyon and Wood, 1975; Gnanamanickam and Patil, 1977). Contrary to pathogenic fungi which usually not only induce the synthesis of phytoalexins in their hosts, but also are able to metabolize these substances to non-fungitoxic compounds (Fuchs et al., 1980a, 1980b) non-pathogenic fungi usually do not degrade phytoalexins (see, however, Fuchs and Hijwegen, 1979). This lack of capacity to metabolize phytoalexins is also attributed to bacteria, however, independently of their being pathogenic or non-pathogenic to plants (Bruin et al., 1977; Platero Sanz and Fuchs, 1978).

Similarly, since Keen et al. (1972) started research on elicitors, many of them have been isolated from varous fungi (Ayers et al., 1976; Henfling et al., 1980; De Wit and Kodde, 1981), but not a single one has been reported yet from bacteria.

In this paper, the induction, by some phytopathogenic bacteria, of phaseollin, pisatin and rishitin in bean, pea and tomato plants, respectively, will be dealt with, as well as the effect of each of these phytoalexins on the growth of the inducing micro-organisms.

Material and methods

Micro-organisms; cultural conditions. *Erwinia carotovora* var. *carotovora* NCPPB 312, *Pseudomonas solacacearum* NCPPB 1018, *Pseudomonas phaseolicola* 113 and *Pseudomonas pisi* 82, the two latter ones being kindly provided by Mr H.P. Maas Geesteranus of the Research Institute for Plant Protection, Wageningen, were grown on nutrient agar (Oxoid) slants at 25°C for two days. From time to time, they were transferred onto bean extract agar (Tejerina and Platero Sanz, 1976) to resuscitate their activity and virulence. Suspensions of bacterial cells in distilled water (10^8 cells.ml^{-1}) were used to inoculate vegetative tissue.

Plant material. The hypocotyls of seven-day-old bean seedlings, *Phaseolus vulgaris,* cv. Prelude, were removed from the plants and divided into two halves by a longitudinal cut. They were then placed on moist filter paper in covered Petri dishes and inoculated with 100-μl drops of the bacterial suspension to be assayed on each of the cut surfaces.

Pods, about 3 cm long, from pea plants, *Pisum sativum,* cv. Kelvedon Wonder, grown in a climate room at 25°C for six weeks, were divided into two halves. These were placed into Petri dishes, and their endocarp surfaces treated as described above for the bean hypocotyls.

Green tomato fruits, 3-4 cm in diameter, of *Lycopersicum esculentum,* cv. Sonato, were disinfected in 96% ethanol for 10 min. Thereafter, they were cut into four pieces and the seeds with the surrounding tissue removed. The inner surfaces were then inoculated with the bacterial suspensions as mentioned for bean hypocotyls and pea pods.

The inoculated tissues were incubated, together with a control treated with distilled water, at 25°C during 1, 3, 5, 7 or 9 days. At these times, samples of each of the different assays were taken and stored in a freezer at −20°C, until being analyzed.

Extraction and quantitative determination of phytoalexins. The bean hypocotyls to be analyzed were homogenized in ethyl acetate and kept overnight at 4°C. After centrifugation the upper phase of the supernatant was taken to dryness and the residue redissolved in ethanol. The lower aqueous phase was extracted with petroleum ether (b.p. 40-60°C) and ethyl acetate. These extracts were also taken to dryness and dissolved in ethanol. The ethanol solutions were concentrated and applied onto silicagel t.l.c. plates (Kieselgel $60F_{254}$, 0.25 mm thickness, Merck) which were developed with chloroform/methanol (97 : 3) and/or with hexane/ethyl acetate/methanol (60 : 40 : 1) or (40 : 60 : 5). Phaseollin was identified under UV and quantitated spectrophotometrically taking an $O.D.\frac{1\ cm}{280\ nm}$ of 1.0 as being equivalent to 35.3 μg.ml^{-1}.

For pisatin the method described by Platero Sanz and Fuchs (1980) was used. After homogenization of the vegetative tissue, a series of extractions with petroleum ether and ethyl acetate and subsequent t.l.c. separation led to a spectrophotometrically pure compound, identical to pisatin. It was quantitated by taking an $O.D.\frac{1\ cm}{309\ nm}$ of 1.0 as being equivalent to 43.8 μg.ml^{-1}.

For the extraction and estimation of rishitin the method described by De Wit and Flach (1979) was used. The frozen tomato pieces were homogenized with methanol in a Virtis 45 mixer, and the homogenates filtered through a Büchner funnel. The

filtrates were taken to dryness, dissolved in 60% methanol and extracted with chloroform. The dried chloroform extract was redissolved in a small amount of acetone and then analyzed by t.l.c. The solvent systems used were chloroform/methanol (95 : 5) and, occasionally, cyclohexane/ethyl acetate (1 : 1). Developed t.l.c. plates were dried and sprayed with a conidial suspension of *Cladosporium cucumerinum* to detect fungitoxic activity (Homans and Fuchs, 1970) and to locate rishitin.

Gas-liquid chromatography (G.l.c.). G.l.c. was carried out according to the method of Price et al. (1976) as modified by De Wit and Flach (1979). Spots containing rishitin and other fungitoxic products were cut out from the t.l.c. plates and eluted with acetone. After complete evaporation of acetone, methyl stearate dissolved in cyclohexane/ethyl acetate (1 : 1, v/v) was added as an initial standard. Samples of 3 μl were analyzed on a Varian 3700 using a 152 cm × 4 mm glas column packed with 10% OV 210 on Diatomite CQ 100 to 120 mesh (Chrompack). Nitrogen was used as carrier gas at a flow rate of 40 ml.min^{-} and detection was by flame ionization. Operating temperatures were as follows: injection port 220 °C, detector 250 °C and column 200 to 210 °C. Rishitin was estimated quantitatively by relating the peak heights to those of the internal standard:

$$\frac{\text{peak height}_{rishitin}}{\text{peak height}_{standard}} \times \frac{\mu g_{standard}}{0.65} \times \mu g \text{ rishitin}$$

(0.65 is the average response ratio for rishitin). Pure rishitin generously supplied by Dr P.J.G.M. de Wit, was analyzed in an identical manner to be used as a reference.

Preparation of crude extracellular polysaccharide (EPS). The method of Forsberg et al. (1970) was used. The bacteria were grown in liquid medium according to Smith (1958) in a shaker at 25 °C for two days. The culture suspensions were centrifuged at 5000× g for 40 min. The pellets were washed three times with 0.01 M Tris-HCl buffer pH 8.1. The washed bacteria were resuspended in 0.03 M Tris-HCl buffer pH 8.1, plus 20% sucrose and 10^{-3} M EDTA (80 ml buffer per gram of bacterial cells). The suspensions were shaken at 180 r.p.m. at room temperature for 10 min, and then centrifuged at 13000× g for 10 min. After repeating the whole extraction procedure five times, the combined supernatants were concentrated by dialysis against a 10% polyethyleneglycol (Carbowax 4000) physiological solution. When the sample volume had been reduced to about a twentieth of the original one, dialysis was continued against distilled water, until sucrose was completely eliminated from the EPS fraction.

Plant challenge with EPS. All plant tissues described above were treated with 10, 25 or 50 μl of aqueous solution (1 mg.ml^{-1} in glucose equivalents) of each of the EPS assayed. Incubation periods, extraction and determination of phytoalexins were as mentioned above for bacteria.

Effect of phytoalexins on bacterial growth. To assay the effect of phytoalexins on growth, bacteria were suspended in liquid peptone medium (Oxoid bacteriological peptone), in shaken reagent tubes, especially adapted to be periodically read in a Klett-Summerson colorimeter (cf. Platero Sanz and Fuchs, 1978). To this end,

measured quantities of each phytoalexin in ethanol were pipetted in these tubes. After evaporation of the ethanol, sterile liquid medium was added to obtain the phytoalexin concentration required (100 $\mu g.ml^{-1}$). The tubes were then inoculated with 0.1 ml of suspensions (10^8 cells.ml^{-1}) of the bacteria tested and incubated at 25 °C for 48 hours. Control tubes without phytoalexin were included. At intervals, growth was measured turbidimetrically and expressed in arbitrary units. Non-inoculated control tubes served as reference.

Results

Induction of phytoalexin accumulation. The legume pathogens *P. phaseolicola* and *P. pisi* are the most effective inducers of phaseollin and pisatin, but the least efficient as far as induction of rishitin is concerned (Table 1). The third *Pseudomonas* species tested, viz. *P. solanacearum,* and *E. carotovora,* both typical pathogens of solanaceous species, induced rishitin to levels comparable to those of the legume phytoalexins.

Accumulation of phaseollin was accompanied by the appearance of other isoflavonoid phytoalexins in bean hypocotyls treated with *E. carotovora* and *P. phaseolicola,* but not with the other two species; however, the latter phytoalexins have not been assayed thoroughly.

The data given for pisatin should be considered with some caution, since with all bacteria except *P. solanacearum,* in addition to pisatin increasing amounts of 6a-hydroxy-inermin (6a-HI) were found, with a maximum of c. 20 $\mu g.g^{-1}$ for *E. carotovora* and *P. pisi* and of 9 $\mu g.g^{-1}$ for *P. phaseolicola* after 5 days (Table 2). Most probably, 6a-HI has arisen as a degradation product of pisatin (Fuchs et al., 1980b). Alternatively, 6a-HI might have been formed as a plant metabolite of pisatin or as a phytoalexin per sé, as has been found in *Lathyrus* species (A. Fuchs and F.W. de Vries, pers. comm.).

In tomato tissue in some cases, especially with *P. solanacearum,* in addition to rishitin, up to four fungitoxic products were detected in t.l.c. bioassays. Two of them remained very close to the origin and showed weak fungitoxic activity. The other two, provisionally indicated as A and Z, appeared at Rf 0.49, respectively, in chloroform/methanol (95 : 5), in which solvent rishitin had a Rf-value of 0.45. Their fungitoxicity seemed to be comparable to that of rishitin. As shown by g.l.c., A decreased after 5 days, whereas Z continued to increase until the end of the experiment (9 days) (Fig. 1). Their exact structure awaits further elucidation.

The efficiency of EPS as an 'elicitor' of phytoalexins. The EPS of three bacterial species were quite efficient in eliciting the phytoalexins of their own host plant, but not in eliciting those of others. An exception was the EPS from *P. solanacearum* which, though being able to induce traces of phaseollin and pisatin, like the other species did not induce any rishitin. This means that the isolated EPS induced only isoflavonoid phytoalexins. Contrary to what was found upon treatment with bacteria, the concentration of phytoalexin obtained at the third day of treatment with 50 μl EPS remained essentially constant during the whole experiment (Table 3). This quantity did not increase after treatment with a higher dose of elicitor (100 μl). Lower doses (10 and 25 μl) only induced traces of phytoalexin.

Table 1. Phaseollin, pisatin and rishitin concentrations (μg per g fresh weight) in bean hypocotyls, pea pods and tomato fruits, respectively, 1, 3, 5, 7 and 9 days after inoculation with *Erwinia carotovora (E.c.), Pseudomonas phaseolicola (P.ph.), P. pisi (P.p.)* and *P. solanacearum (P.s.).*

Days	Phaseollin				Pisatin				Rishitin			
	E.c.	*P.ph.*	*P.p.*	*P.s.*	*E.c.*	*P.ph.*	*P.p.*	*p.s.*	*E.c.*	*P.ph.*	*P.p.*	*P. s.*
1	12.16[1]	31.30	29.73	10.80	14.74	66.37	36.71	22.19	4.36	14.36	4.69	11.77
3	26.20	81.15	62.35	27.76	15.27	125.35	100.00	37.90	31.62	18.01	9.63	40.38
5	37.18	80.00	55.86	44.85	26.48	146.99	81.59	48.98	32.72	10.31	6.60	42.16
7	33.63	67.14	50.68	41.27	38.84	149.38	63.23	47.09	31.47	7.95	3.66	25.19
9	30.28	52.16	47.75	39.12	34.23	62.23	57.26	29.02	10.39	3.69	3.41	24.74

[1] Average data from several experiments; no phytoalexin was found in any of the assays with distilled water-treated controls.

Tabel 1. Concentraties van faseolline, pisatine en rishitine (μg per g vers gewicht) in bonehypocotylen, erwtepeulen en tomatevruchten, 1, 3, 5, 7 en 9 dagen na inoculatie met Erwinia carotovora (E.c.), Pseudomonas phaseolicola (P.ph.), P. pisi (P.p.) en P. solanacearum (P.s.).

Table 2. 6a-Hydroxy-inermin concentrations (in μg per g fresh weight) in pea pods 1, 3, 5, 7 and 9 days after inoculation with different phytopathogenic bacteria.

Days	*E. carotovora*	*P. phaseolicola*	*P. pisi*	*P. solanacearum*
1	7.60[1]	–[2]	9.00	–
3	13.53	7.32	17.50	
5	20.13	9.11	19.19	–
7	19.17	8.03	18.91	–
9	15.45	–	16.07	–

[1] See Table 1.
[2] – = not detected.

Tabel 2. 6a-hydroxy-inermine concentraties (μg per vers gewicht) in erwtepeulen, 1, 3, 5, 7, en 9 dagen na inoculatie met verschillende fytopathogene bacteriën.

Fig. 1. G.l.c. runs of tomato fruit extracts, 1, 3, 5, 7 and 9 days after inoculation with *Pseudomonas solanacearum,* showing relative concentrations of rishitin (R) and non-identified fungitoxic compounds (A, Z) (S = internal standard).

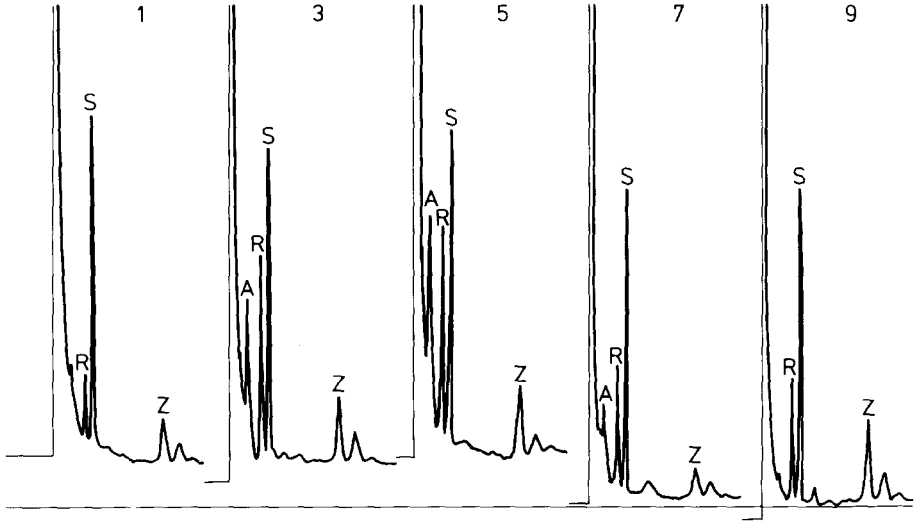

Fig. 1. Gas-vloeistof chromatografisch spectrum van extracten van tomatevruchten, 1, 3, 5, 7 en 9 dagen na inoculatie met Pseudomonas solanacearum; de pieken duiden de relatieve concentraties van rishitine (R) en twee niet-geïdentificeerde fungitoxische verbindingen (A, Z) aan; S = interne standaard).

Table 3. Phaseollin and pisatin concentrations (μg per g fresh weight) in bean hypocotyls and pea pods, 1, 3, 5, 7 and 9 days after treatment with 50 μl of solutions of extracellular polysaccharides (EPS) from *Erwinia carotovora (E.c.), Pseudomonas phaseolicola (P.ph.)* and *P. pisi (P.p.),* respectively.

Days	Phaseollin (EPS)			Pisatin (EPS)		
	E.c.	*P.ph.*	*P.p.*	*E.c.*	*P.ph.*	*P.p.*
1	–[1]	–	–	–	–	–
3	18.20[2]	37.40	17.21	20.63	15.19	39.16
5	20.12	40.25	19.42	22.15	17.77	43.21
7	21.87	41.68	21.85	25.99	19.35	45.95
9	20.06	39.10	20.00	23.13	17.43	41.57

[1], [2] See Tables 1 and 2.

Tabel 3. Faseolline en pisatine concentraties (μg per g vers gewicht) in bonehypocotylen en erwtepeulen, 1, 3, 5, 7 en 9 dagen na behandeling met 50 μl oplossingen van extracellulaire polysacchariden (EPS) van Erwinia carotovora (E.c.), Pseudomonas phaseolicola (P.ph.) en P. pisi (P.p.).

Fig. 2. Effect of phaseollin, pisatin and rishitin (100 $\mu g.ml^{-1}$) on the growth of:
a. *Erwinia carotovora;* b. *Pseudomonas phaseolicola;* c. *P. pisi;* d. *P. solanacearum.*

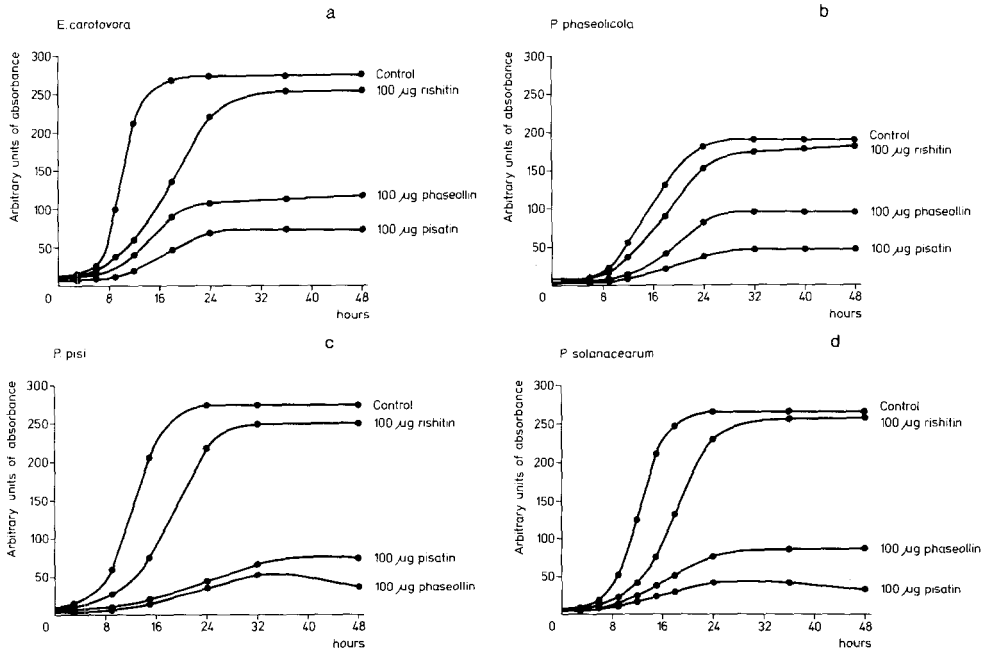

Fig. 2. De invloed van faseolline, pisatine en rishitine (100 $\mu g.ml^{-1}$) op de groei van:
a. Erwinia carotovora; b. Pseudomonas phaseolicola; c. P. pisi; d. P. solanacearum.

Effect of phytoalexins on bacterial growth. The present results support those, published previously on pisatin (Platero Sanz and Fuchs, 1978). The inhibition of growth of *E. carotovora, P. phaseolicola* and *P. pisi* by pisatin was about 75%, whereas it reached 90% for *P. solanacearum.* The results with phaseollin were similar to those with pisatin. *P. pisi* was affected most strongly (c. 85% growth inhibition). Growth of *E. carotovora, P. phaseolicola* and *P. solanacearum* was diminished to about 50-65%. Rishitin appeared to be less effective in its inhibitory action than phaseollin or pisatin towards any of the tested bacteria (Fig. 2).

Discussion

So far, little is known on phytoalexin induction by bacteria, whereas the eliciting activity of extracellular bacterial polysaccharides has, to our knowledge, not been investigated at all. In earlier investigations, Cruickshank and Perrin (1963) and Stholasuta et al. (1971) did not succeed in inducing the formation of pisatin in pea tissues by bacteria. The first-mentioned authors used also endocarps of detached pea pods, but the bacterial concentrations applied were lower (10^6 cells. ml^{-1}) than ours (10^8 cells.ml^{-1}). Stholasuta et al. (1971), in their assays, employed pea leaves, which produce only small quantities of pisatin ($\pm$ 27 μg per g fresh weight) as compared to pea pods, which accumulate up to 450 μg per g fresh weight (preliminary observations by Platero Sanz and Fuchs (unpublished) and Bruin et al., 1977). The much larger accumulation of pisatin by endocarp tissue treated with *E. carotovora, P. pisi* and other bacteria observed in the earlier experiments as compared with the amounts reported here might be due to the fact that different pea varieties (cvs Gloire de Quimper versus Kelvedon Wonder) were tested. In the earlier papers, no breakdown of pisatin by bacteria was observed. Our present results, on the other hand, show that significant quantities of 6a-hydroxy-inermin (the first metabolite of pisatin) were found in pea pods treated with *E. carotovora* and *P. pisi.* However, the 'in vivo' conditions differed considerably from those 'in vitro' referred to before.

The presence of phaseollin in bean leaves inoculated with *Pseudomonas* spp. and *P. phaseolicola* has been reported before (Lyon and Wood, 1975; Gnanamanickam and Patil, 1977). Our results with bean hypocotyls conform these earlier data.

Though rishitin is known to accumulate in potato tubers inoculated with *Erwinia carotovora* (Lyon, 1972), rishitin induction by bacteria in tomato plants has, to our knowledge, not been reported so far. Rishitin appears to accumulate to higher concentrations in our experiments than in those described by De Wit and Flach (1979) for tomato plants treated with *Cladosporium fulvum;* however, they are comparable to those given by Elgersma (1980) working with *Verticillium albo-atrum* in tomato stem segments. In agreement with these authors, we detected another four antimicrobial compounds, only two of which proved to be stable during storage; it was possible to prove their occurrence by t.l.c. and g.l.c. The other two, less fungitoxic compounds could be rishitin metabolites as described by Ishiguri et al. (1978). In general, the accumulation of the two tomato phytoalexins reached its maximum between the 3rd and the 5th day after inoculation, and started to decline on the 7th day, as has been shown in other host-parasite combinations before (De Wit and Flach, 1979; Elgersma, 1980).

The different abilities of the four bacterial EPS preparations to elicit phytoalexins

could reflect a basic difference in the composition of these polysaccharides. However, an exact determination of the sugar composition of each EPS is needed to fully comprehend their role as phytoalexin elicitors in the plant. Until now, EPS of some bacteria have been studied in relation to phytotoxicity (Goodman et al., 1974), expression of virulence (Anderson and Jasalavich, 1979) or induction of water-soaking (El-Banoby and Rudolph, 1980), but never in relation to phytoalexin induction, in spite of the fact that several fungal elicitors with a main sugar moiety in their molecule have been described (Albersheim and Valent, 1978; De Wit and Roseboom, 1980). The inability of any of the EPS tested to induce rishitin is in agreement with the suggestion of De Wit and Roseboom (1980) that a small peptide fraction in the elicitor molecule seems to be necessary to elicit rishitin accumulation. Therefore, glycoproteins could be involved in the bacterial elicitation of tomato phytoalexins.

Regarding the antibacterial activity of the three phytoalexins, the results confirm and enlarge those presented in an earlier paper (Platero Sanz and Fuchs, 1978). Phaseollin and pisatin share a remarkable inhibitory effect towards all bacterial species tested, at a concentration of 100 $\mu g.ml^{-1}$. Similar effects were found by Wyman and Van Etten (1978) as opposed to the results reported by Cruickshank (1962) and Stholasuta et al. (1971), who did not detect any inhibitory activity. As mentioned earlier (Platero Sanz and Fuchs, 1978) the composition of the media used to assay these effects as well as the assay conditions differed considerably. Likewise, Gnanamanickam and Smith (1980) pointed out a lack of activity of phaseollin and kievitone on Gram-negative bacteria. The highest dose used was 50 $\mu g.ml^{-1}$. With the double dose used in the present study bacterial growth was distinctly inhibited by phaseollin and pisatin. Rishitin exerted only a moderate inhibition of bacterial growth. At higher concentrations of rishitin (above 250 $\mu g.ml^{-1}$) growth of *E. atroseptica, E. carotovora, P. solanacearum* and other bacteria was also clearly impaired (Lyon and Bayliss, 1975).

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Samenvatting

De rol van fytoalexinen in door bacteriën geïnfecteerde planten

Bonehypocotylen, erwtepeulen en tomatevruchten werden onderzocht op hun vermogen tot vorming van respectievelijk faseolline, pisatine en rishitine, na inoculatie met de fytopathogene bacteriën *Erwinia carotovora, Pseudomonas phaseolicola, P. pisi* en *P. solanacearum* en na behandeling met oplossingen van hun extracellulaire polysacchariden (EPS). Alle bacteriesoorten induceerden fytoalexinevorming, terwijl hun EPS wel faseolline- en pisatine- , maar geen rishitinevorming induceerden. Faseolline en pisatine remden de groei van de bacteriën in vloeibaar medium sterk; rishitine daarentegen had slechts een geringe groeiremming tengevolge.

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Book review

K. Maramorosch and K. F. Harris (Eds), 1979. Leafhopper vectors and plant disease agents. Acad. Press, New York, San Francisco, London. XIV + 654 pp of text including many tables and illustrations (numbered per contribution), references at end of each contribution, and 28 pp of general index; bound; price US $ 39.

Plant diseases caused by viruses and virus-like agents are studied increasingly in their ecological context. Vectors with complicated ecologies as such, the pathogens concerned, and the complex interactions between both constitute a fascinating field of study, the results of which are essential for improving disease control.

The present book is the second in a multivolume series on vectors, vector-borne disease agents, and plant disease spread edited by K.F. Harris and K. Maramorosch. For a review of