Pisatin Production in Powdery Mildewed Pea Seedlings

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ABSTRACT

An antifungal principle isolated from powdery mildewed seedlings of pea was identified as pisatin by physico-chemical analyses. Pisatin was first detected 2 days after inoculation of leaves with the pathogenic powdery mildew fungus, *Erysiphe pisi* at a concentration of 3 μg/g fresh weight and then increased logarithmically, reaching 300 μg/g fresh weight by 4th day. When inoculated with a nonpathogenic fungus, *E. graminis hordei*, pisatin was detected much earlier, 15 hours after inoculation, although concentration was low (0.3 μg/g fresh weight). The conidia of the pea pathogen, *E. pisi* were 13 times more tolerant to pisatin than those of the nonpathogen, *E. graminis hordei*; the ED_{50} for conidia germination was 530 μg/ml for *E. pisi* and 40 μg/ml for *E. graminis hordei*.

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Although the role of phytoalexins in plant resistance against a pathogen has been extensively discussed and reviewed (4, 8, 11), little is known about the production and function of phytoalexins in diseases caused by obligate parasites. Cross-protection or acquired immunity was reported in several rust diseases but the mechanism was attributed to the release of toxic gaseous substance which is produced by the fungus (20, 21). Phytoalexins are assumed to be responsible for the host resistance in rust diseases (2, 12, 13), but demonstration of the antifungal activity by drop-diffusate technique using the *Zea mays-Puccinia graminis* system was unsuccessful (12).

We have recently found phytoalexin activity in exudates from powdery mildewed barley leaves and suggested that this antifungal activity may be of importance in development of the disease syndrome (14).

However, the isolation and characterization of a phytoalexin in an obligate parasite system have not been reported. This is probably due to the general concept that such a pseudosymbiotic association established between the host cells and haustoria of obligate parasites could not involve a phytoalexin.

In this paper evidence is provided showing that pisatin, previously isolated as a phytoalexin from pea plant (5, 15), is produced in response to the powdery mildew fungus of pea, *Erysiphe pisi* DC. The significance of this phytoalexin in the determination of host specificity of powdery mildew fungi is also discussed.

MATERIALS AND METHODS.—*Plant and fungal material.*—The seeds of *Pisum sativum* L. 'Alaska' were sown in vermiculite in plastic containers and grown in a growth chamber at 20 C for 15 to 20 days under a regime of 10 hours light (Toshiba Plantlux, 3,000 lux) and 14 hours dark.

The culture of *E. pisi* used in these studies was originally isolated from pea plants in the experimental field of Okayama University and has been maintained on pea plants in a growth chamber. The conidia formed on the freshly inoculated leaves were used as the compatible inoculum.

As an incompatible inoculum, conidia of *Erysiphe graminis* DC. f. sp. *hordei* Marchal, race Hr 74 were used. The fungus was cultured on leaves of barley cultivar, Russian 74, for 8 to 10 days in a growth chamber under 12 hours illumination per day at 20 C.

Whole leaves and stems of 15-day-old pea seedlings were densely inoculated with the conidia of powdery

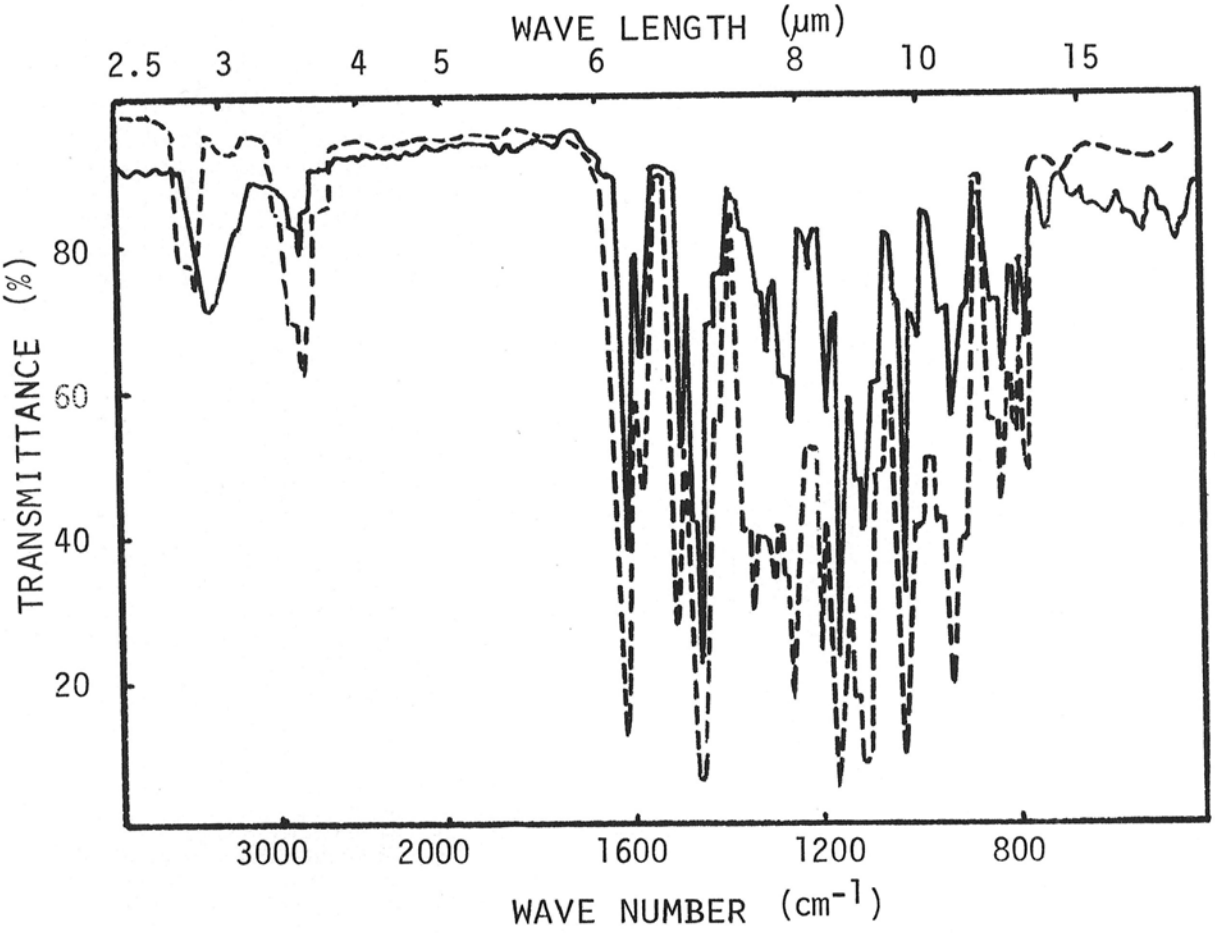

Fig. 1. Infrared spectra of the antifungal principle isolated from powdery-mildewed pea seedling and pisatin in KBr pellet. Solid line: antifungal principle isolated from powdery-mildewed pea seedling. Broken line: pisatin [Perrin and Bottomley, 1962 (15)].

mildew fungi employing a soft hair brush. The inoculated plants were incubated at 20 C and periodically subjected to the extraction and determination of pisatin described below.

Extraction, isolation, identification, and determination of pisatin.—Mildewed whole pea plants (15 days after inoculation) were homogenized with absolute ethanol (1:20, fresh weight:volume) in a Waring Blendor and the resulting suspension was filtered. The filtrate was concentrated in vacuo and the resultant aqueous solution was centrifuged at 10,000 *g* for 20 minutes at 4 C. The supernatant solution was extracted five times with equal volumes of light petroleum ether and the petroleum ether extract was evaporated in vacuo to dryness. The residue was dissolved in 0.2 ml of ethanol and subjected to thin layer chromatography on a silica gel GF 254 (Merck Co.,) plate in a solvent of (hexane:ethyl acetate:methanol = 60:40:1, v/v) (7). The silica gel on the areas corresponding to the R_f value of pisatin, that absorbed ultraviolet light, was scratched from the plates, and extracted with ethanol. The ethanol extract was concentrated to dryness in vacuo, and the resultant solid was recrystallized twice from light petroleum ether.

Identification and characterization of the compounds was carried out by infra-red and mass spectrometry.

Pisatin concentration was determined spectrophotometrically from the ethanol extract of silica gel plate, which was prepared by the same procedure as described above, using pure pisatin as a standard.

Determination of antifungal activity of pisatin.—Two milligrams of pisatin was dissolved in 0.2 ml of ethanol and then diluted with 1.8 ml of deionized water to make a 1 mg/ml solution. A series of 10% alcoholic aqueous solution containing known quantities of pisatin was prepared from the 1 mg/ml solution and incorporated in known concentrations into 80 mg agarose blocks. The conidia of the test organisms, *E. pisi* and *E. graminis hordei*, were placed on the agarose blocks and allowed to germinate for 20 hours at 20 C. The conidia of *Cochliobolus miyabeanus* (Ito et Kurib.) Drechsler were also used as a reference and incubated on the agarose blocks at 28 C. Germination of conidia was determined by microscopic observation and ED_{50} and ED_{90} values for conidial germination of each fungus were calculated.

RESULTS.—*Identification of pisatin.*—The crystalline compound isolated from a powdery mildewed pea plant was identified as pisatin according to its melting point (72 C) and its infra-red spectrum (Fig. 1). The mass spectrum of the compound showed a molecular ion peak at m/e 314 and the base peak (M^+-H_2O) at m/e 296. These values correspond to the molecular weight of pisatin and anhydropisatin respectively.

The yield of pure pisatin was about 6 mg from 40 g fresh weight of powdery mildewed pea seedlings.

Pisatin induction by pathogenic and nonpathogenic powdery mildew fungi.—The amount of pisatin induced by pathogenic and nonpathogenic powdery mildew fungi was determined after inoculation of leaves of pea seedlings with *E. pisi* and *E. graminis hordei* (Table 1).

It is apparent that a detectable amount of pisatin was induced much earlier (15 hours post inoculation) in the leaves inoculated with *E. graminis hordei* compared with those infected with *E. pisi*. In leaves inoculated with *E. pisi*, pisatin could not be detected 36 hours after inoculation, but was detectable by 48 hours after inoculation. The concentration of pisatin in leaves inoculated with *E. pisi* increased logarithmically after 48 hours, and reached more than 400 μg/g fresh weight, at the time seedlings withered.

TABLE 1. Accumulation of pisatin in pea seedlings inoculated with a pathogenic (*Erysiphe pisi*) or a nonpathogenic (*Erysiphe graminis hordei*) powdery mildew fungus

Time after inoculation (hr)	Conc. of pisatin (μg/g fresh weight)[a]	
	Inoculated with:	
	E. pisi	*E. graminis hordei*
8	0	0
12	0	0
14	0	0
15	0	0.3
16	0	0.3
20	0	1.2
24	0	2.0
36	0 or trace	1.0
48	3	0.5
72	36	1.5
96	300	1.5

[a]Data are averages of three replications.

TABLE 2. Antifungal activity of pisatin against pathogenic (*Erysiphe pisi*) and nonpathogenic (*Erysiphe graminis hordei*) powdery mildew fungi

Fungal species tested	Antifungal activity for conidial germination	
	ED_{50} (μg/ml)	ED_{90} (μg/ml)
Erysiphe pisi	530	>700
E. graminis hordei (race Hr 74)	40	120
Cochliobolus miyabeanus[a]	480	590

[a]Tested as a reference because this fungus is generally tolerant to many toxicants.

Antifungal activity of pisatin.—The antifungal activity of pisatin against pathogenic and nonpathogenic fungi is shown in Table 2. The conidia of *E. pisi* was 13 times more tolerant than those of *E. graminis hordei*, as assessed by ED_{50} vaues. The conidia of *C. miyabeanus* which is in general very tolerant to many toxicants were more sensitive to pisatin than those of *E. pisi* as reflected by ED_{90} values.

DISCUSSION.—Whereas many phytoalexins were characterized from diseased plants of nonobligate parasitic in nature, the direct demonstration of phytoalexin production has not been successful in obligate parasitic diseases, with the exception of phaseollin in *Phaseolus vulgaris* inoculated with *Uromyces appendiculatus* (1).

Evidence in this paper explicitly demonstrates the production of a phytoalexin, pisatin, in a typical obligate parasitic disease, powdery mildew of pea plants. The antifungal principle in the extract from diseased tissue was identified as pisatin by physico-chemical procedures. The nonpathogenic powdery mildew fungus, *E. graminis*

hordei, elicites pisatin production at a much earlier stage (15 hours) than the pathogenic fungus, *E. pisi*, as have been encountered in the induction of many other phytoalexins. Pea plants, therefore, responded to the nonpathogen with great rapidity.

Tolerance of *E. pisi* to pisatin seems to account for the colonization of the fungus on pisatin-containing tissue. The ED_{50} of pisatin for conidial germination indicates that conidia of *E. pisi* were 13 times more tolerant to pisatin than that of *E. graminis hordei*. Thus it is proposed that the nonpathogenic *E. graminis hordei* induced the synthesis of pisatin to which the fungus is extremely sensitive, thus terminating the association as an unsuccessful infection. Although the exact concentration of pisatin at the infection site has not been determined, preliminary results indicate that the concentration in the leaf tissue under the colony was extremely high compared to those in the surrounding tissue and healthy leaves of infected plants (Oku et al., *unpublished*).

The results therefore suggest that phytoalexin may play an important role in the determination of host specificity in powdery mildew in view of Cruickshank's implication that the difference of sensitivity to phytoalexin and the rate of phytoalexin production are important factors for determining the host specificity (6). This could further be extended to the notion that phytoalexin exhibit a dual function in the determination of specificity. In the case where specificity is determined at the level of cultivar-race interaction, there is no difference among races in the sensitivity to phytoalexins. Races of *Phytophthora infestans* were similarly sensitive to rishitin (18), and races of *E. graminis* were nearly as sensitive to phytoalexins detected in powdery mildewed barley leaves (14), suggesting that a phytoalexin-tolerant or degrading mechanism is not involved in these interactions. The only difference among these races is the ability to induce phytoalexin in the host tissue. In these diseases, therefore, the determination of specificity depends solely upon the rate of phytoalexin production, because phytoalexin synthesis appears to be host specific.

On the contrary, specificity in species-species interaction is determined not only by the rate of phytoalexin production, but also by the sensitivity of pathogens to the phytoalexin. The pea pathogen, *E. pisi*, is characterized by delayed production of and a tolerance to pisatin, while barley powdery mildew fungus elicited pisatin production at an earlier stage and was sensitive to it. In view of the obscurity of the role of pisatin in disease resistance (3, 16), however, the above inferences require further investigation to test their validity.

Unlike other diseases caused by perthophytic, facultative or obligate parasite (1, 9, 10, 17, 19), a quantity of phytoalexin was produced in powdery mildew-inoculated tissues of pea plants prior to any perceptible cell collapse or hypersensitive necrosis. That this is true was confirmed from microscopic observations that the inoculated pea leaves did not show any visible change when pisatin is detectable. This finding extends previous information on the significance of hypersensitive necrosis in plants' resistance. It not only supports the notion that hypersensitive necrosis is the consequence but not the cause of resistance (10), but also presents an explicit evidence for a new concept that phytoalexin induction and/or plant resistance is not necessarily accompanied by the necrotic response of the tissue.

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