Phytoalexin Production in Five Cultivars of Peas Differentially Resistant to Three Races of *Pseudomonas syringae* pv. *pisi*

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ABSTRACT

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Five cultivars of peas are differentially susceptible to three races of *Pseudomonas syringae* pv. *pisi*. Within a given cultivar, the host-pathogen combination in which resistance was expressed in seedling assays resulted in high levels of pisatin and yellow-green pigmentation in epidermal cells in endocarp tissue. These responses were either absent or lower in susceptible interactions. Pisatin at 68 μg/ml was inhibitory to in vitro growth of all three races of *P. syringae* pv. *pisi*.

Additional key words: *Pisum sativum*.

Disease resistance in plants has been categorized as nonhost resistance (5) and race-specific resistance. The technology is now available to distinguish if the resistance responses have common biochemical features. The nonhost response of pea tissue to *Fusarium solani* f. sp. *pisi*, a pathogen of peas, and *F. solani* f. sp. *phaseoli*, a pathogen of beans, has been characterized in terms of the mRNA and protein species that selectively accumulate or whose synthesis is enhanced following inoculation (4,12). The incompatible (resistance) reaction of pea against *F. solani* f. sp. *phaseoli* is associated with the enhanced synthesis of 20 major proteins. The mRNAs that code for these proteins remain prevalent in the pea tissue 96 hr after inoculation. However, in the compatible reaction against *F. solani* f. sp. *pisi*, the 20 major proteins increase initially but the levels of mRNAs coding for some of these proteins decline markedly starting ~24 hr after inoculation.

It is of importance for further biochemical definition of resistance responses to obtain a pea system in which race-specific resistance responses could be compared with those of nonhost resistance. This paper characterizes interactions between five lines of peas that react differentially to three races of *Pseudomonas syringae* pv. *pisi* (PSP). The accumulation of phytoalexins and yellow-green pigmentation adjacent to the inoculum have been associated with a hypersensitive type of resistance (6). Both of these changes are most intense in cultivars of pea resistant to the nonpathogenic fungi and avirulent races of the pathogen.

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MATERIALS AND METHODS

Inoculation of pea seedlings. Cultures of races 2 and 3 of PSP were obtained from J. D. Taylor of the National Vegetable Research Station at Wellesbourne, England (10). The race 1 culture was isolated from infected pea plants grown in a field near Twin Falls, ID. Cultures were stored on slants of nutrient agar (Difco) amended with 2% glycerol (NAG) at 3 C. Inoculum was produced by streaking from stock cultures onto plates of NAG, incubating the plates for 1 day at room temperature, and then suspending the resulting growth of bacteria in sterile deionized water. Inoculum density was adjusted to 5×10^7 colony-forming units (cfu) per milliliter according to the optical density measured with a colorimeter. Seeds of the differential pea cultivars Partridge and Early Onward also were obtained from J. D. Taylor. Seeds of cultivars Abador, Ceras, Mars, Mini, and Spring were obtained from Asgrow Seed Co., Kalamazoo, MI. Disease resistance was evaluated in cultivars maintained at 18 ± 4 C without supplemental lighting in a greenhouse at the Asgrow Western Breeding Station in Twin Falls, ID. Plants for inoculation were grown in 15-cm-diameter plastic pots in a soil mix of sand, peat moss, and perlite (1:2:1, v/v). They were thinned to two uniform seedlings per pot after emergence and fertilized weekly with Hoagland's solution. Plants were ready for inoculation 3 wk after the day of sowing, when they were ~10 cm tall. A drop of inoculum from a syringe fitted with a 0.89-mm (20-gauge) needle was applied to each plant at

the middle of the internode between the first two leaves, and the stem was pierced with the needle at this site.

Elongate, water-soaked stem lesions, typical of bacterial blight, developed on certain cultivar/pathogen combinations, and lesion lengths were measured 10 days after inoculation. For other combinations, the only symptom was a necrotic fleck (NF) ~1 mm in diameter at the site of inoculation. Evaluations were made on two plants in each of two pots for each host/pathogen combination.

Inoculation of the endocarp surface of immature pea pods. Peas were grown in a greenhouse until pods were 1.5 cm in length. Following excision and two washes in deionized water, 0.5-g lots of immature pods were gently separated in halves and placed in petri plates with the endocarp surface up. Bacterial cells were grown for 3 days on King's B agar medium. *F. solani* f. sp. *phaseoli* was grown on pea seedling-supplemented potato-dextrose agar (PDA) for 2–3 wk. Cells or spores were harvested with a sterile spatula and dispersed in 100 ml of sterile H_2O. Bacterial cell suspensions were adjusted to $OD_{420\ nm} = 1.0$. Viable bacterial cell counts for each race were derived by dilution plating and were 1.5×10^8, 3.4×10^8, and 8.1×10^8 for races 1, 2, and 3, respectively. Spore counts of *F. solani* were 7×10^5 macroconidia per milliliter. Macroconidial suspensions (500 μl) or bacterial cell suspensions (500 μl) were applied to the endocarp surfaces and were held at 22 C under fluorescent light. Due to the large numbers of treatments and multiple time points, we chose to repeat the entire experiment four times in lieu of multiple replications within an experiment.

Pisatin extraction. Pod lots were placed directly into 5 ml of hexane and placed in the dark at 22 C overnight. The hexane was decanted, and evaporated to dryness in a dark hood. The residue was immediately redissolved in 1 ml 95% ethanol and the $A_{309\ nm}$ determined. The ethanolic solutions were then subjected to silica gel thin-layer chromatography with chloroform as the mobile phase to determine if proportions of pure pisatin corresponded to optical density readings of the ethanol extract. We have observed that estimates of pisatin read directly are more representative because there is a partial loss of pisatin from light degradation during TLC and recovery. Pisatin was specifically identified (11) on silica gel thin-layer chromatographic plates by conversion to anhydro-pisatin (which fluoresces under long-wave UV irradiation) catalyzed by HCl fumes, and by its absorbance spectrum.

Evaluation of the yellow-green pigment that accumulates in the pea endocarp. Thin, handcut sections of the endocarp surface were periodically examined every 24 hr with a light microscope from 20

TABLE 1. Average length (mm) of water-soaked lesions on pea cultivars following stem inoculation with races of *Pseudomonas syringae* pv. *pisi*

Pea cultivars	*Pseudomonas syringae* pv. *pisi*		
	Race 1	Race 2	Race 3
Partridge	NF[a]	3.0	4.0
Early Onward	5.0[b]	NF	3.0
Mars	16.0	NF	20.2
Spring	13.0	NF	NF
Ceras	NF	3.8	8.5
Mini	NF	2.8	7.7
Abador	NF	NF	10.8

[a] All four plants responded with a necrotic fleck (NF) ~1 mm in diameter at the site of inoculation.
[b] Average lesion length from a total of four plants, read 10 days after inoculation.

TABLE 2. Disease reaction and phytoalexin production by differential cultivars inoculated with *Pseudomonas syringae* pv. *pisi* races 1, 2, and 3 and *Fusarium solani* f. sp. *phaseoli*

Cultivar	Incubation time (hr)	Pisatin accumulation (μg/g fresh weight) in pea pods inoculated with:			
		Race 1	Race 2	Race 3	*F. solani*
Mars	24	0[a]	33	0	35
	48	0⁻	213 †††[b]	0⁻	191 †††
Spring	24	0	27	31	55
	48	0	296 †††	89 ††	22 ††
Ceras	24	117	0	7	69
	48	276 †††	0 †	0⁻	168 †††
Mini	24	216	7	62	72
	48	476 †††	57 †	87 †	95 †††
Abador	24	51	116	24	48
	48	406 ††	400 ††	136⁻	382 †

[a] Pisatin values from one of four separate experiments.
[b] Cytological observation of hypersensitivity coloration developing on pea endocarp within 48–96 hr after inoculation. ††† = An intense yellow-green pigmentation of at least 1 of 10 surface endocarp cells; †† = general yellow-green coloration of all surface cells; † = light yellow-green pigmentation of <10% of the surface cells. − = No significant pigmentation even at 96 hr.

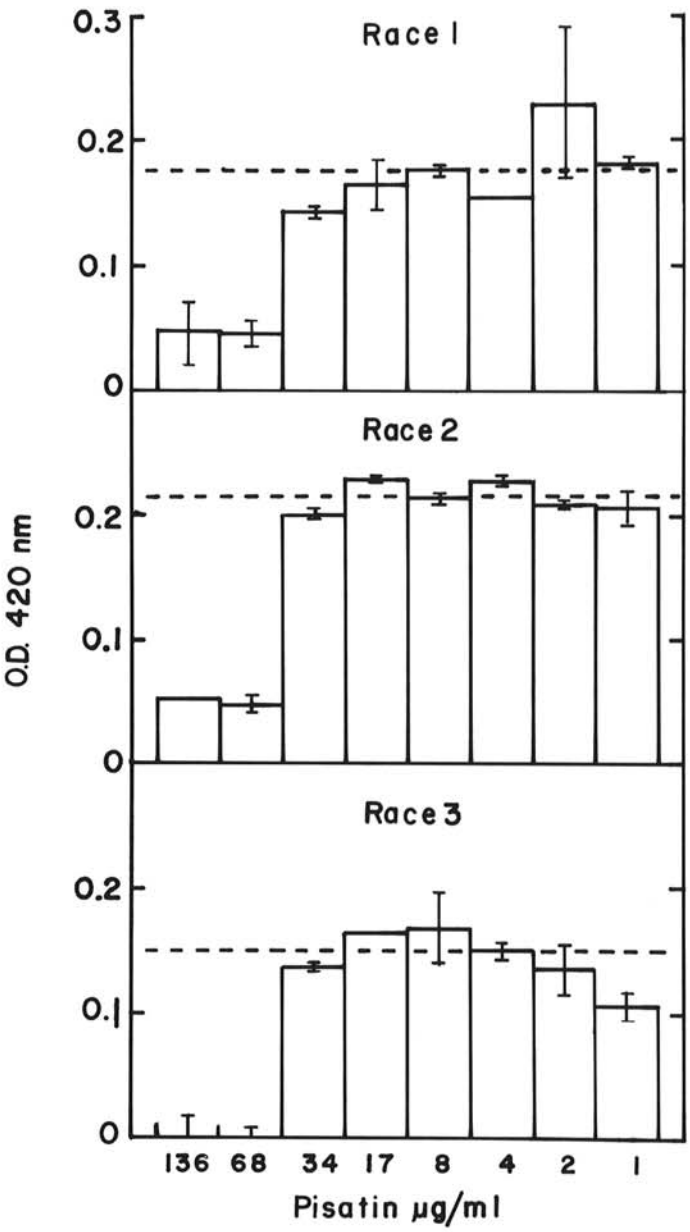

Fig. 1. The effect of pisatin on the growth of *Pseudomonas syringae* pv. *pisi*, races 1, 2, and 3. Dotted line represents $A_{420\ nm}$ in control treatment after 24 hr.

through 96 hr after inoculation to check for the localized yellow-green pigmentation in inoculated tissue which often accumulates in an incompatible reaction.

Bioassay of antibacterial effect of pisatin on the growth of PSP in shake culture. Bacterial cells from actively growing cultures on King's medium B agar were assayed in liquid King's B medium in the wells of microdilution plates. Pisatin was added to the treatment wells in an ethanol solution. The ethanol was allowed to evaporate prior to the addition of King's B medium (50 μl) and inoculum ($\sim 10^3$ cells). The dilution plate was aseptically covered and placed on a shaker at 23 C. Bacterial cells were removed from the wells and diluted 10× for spectrophotometric analysis. Growth was observed as increased turbidity at $A_{420\ nm}$ after 24 or 72 hr.

RESULTS AND DISCUSSION

Seedlings of seven cultivars of peas expressed differential reactions to races 1, 2, and 3 of PSP (Table 1). Five of these differential cultivars were subsequently utilized to evaluate the accumulation of pisatin and yellow-green pigmentation in pea endocarp tissue.

The correlation between resistance in stem and pod evaluations was good (Tables 1 and 2). Combinations that had a necrotic fleck reaction in the stem inoculation accumulated intense yellow-green pigmentation in 10% or more of the endocarp epidermal cells or a general yellow-green coloration of all pod surface cells. Any combination that produced a stem lesion of >10 mm in length did not exhibit any significant yellow-green coloration in pea endocarp cells. Three of four combinations resulting in shorter stem lesions gave a weak coloration reaction in endocarp cells (Ceras and Mini with races 2 and 3). Pisatin accumulation was only partially correlated with expression of resistance. Ceras and Mini exhibited intermediate resistance to race 2, but these combinations produced little pisatin. Abador was susceptible to race 3, but this combination eventually produced more pisatin than the resistant combination of Spring with race 3. Mini, at best intermediate in resistance to race 3, still produced as much or more pisatin than did Spring inoculated with the same race. Even though there were these cultivar differences in pisatin yield, within a given cultivar the resistant reaction always yielded greater accumulations of pisatin.

Pisatin inhibited growth of all three races of PSP at levels comparable to those extractable from resistant pod reactions (Fig. 1). We do not presently know if there is a selective localization of phytoalexin in cells adjacent to the inoculum that would show phytoalexin accumulations radically different from the pisatin values obtained from total pod extracts. Taken together, the data do not prove or disprove that phytoalexin is a decisive factor in disease resistance; however, the relative accumulation of pisatin within a given cultivar appeared to be indicative of the intensity of the incompatible response. Also, many of the other considerations that are typically involved in more conclusive analyses of resistance, such as pisatin degradation potential of the races, are presently untested. Reportedly, *P. pisi* is unable to degrade pisatin (9).

Pisatin accumulation elicited by bacterial cells appeared to develop slower than that induced by fungal pathogens. No pisatin accumulation was detected up to 9 hr after inoculation; however, moderate levels of pisatin accumulated in the resistance reactions of cultivars Abador, Ceras, and Mini at 20 hr. Pisatin did not accumulate in cultivars Spring and Mars until 27 hr after inoculation. Pisatin accumulation in response to inoculation by *F. solani* f. sp. *pisi* was detectable at 6 hr (11).

Phytoalexins have been implicated in resistance of peas and other plants to pathovars of *Pseudomonas syringae* (1,7,8,13); however, the precise role for phytoalexins in resistance of plants in general to bacterial pathogens is not clear (2,3,6,7,13). The reaction of host plants to incompatible races of *Pseudomonas* has previously been shown to be associated with phytoalexin accumulation (2,6,14). The accumulation of pisatin in pea pod endocarp following inoculation with either incompatible races of PSP or *F. solani* f. sp. *phaseoli* suggests that both organisms induce activation of the phenylpropanoid pathway. Also, the incompatible response of pods of a given pea cultivar based on symptomology and accumulation of pisatin is greater than in the compatible responses. It appears that the disease reaction of pea pod tissue generally resembles that of pea stems against these fungal and bacterial pathogens. Thus, a common host tissue is now available to compare, at the molecular level, nonhost resistance to fungal pathogens with race-specific resistance to a bacterial pathogen.

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